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The Role of Apoptosis in Muscle Remodeling

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Dissertation submitted to the
School of Medicine at West Virginia University
in partial fulfillment of the requirements
for the degree of

Doctor of Philosophy
in
Exercise Physiology

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ABSTRACT

The Role of Apoptosis in Muscle Remodeling

Parco M. Siu

Apoptosis has been implicated in mediating the process of muscle loss during muscle disuse and with aging. However, the physiologic role of apoptosis in muscle remodeling is largely unknown. The purposes of this dissertation were to examine the role of apoptosis in different muscle remodeling conditions including muscle denervation, hindlimb suspension, muscle unloading following overload, endurance treadmill training, and muscle overload in mice, rats, or quails. Apoptotic signaling components and cellular stress markers including Bax, Bcl-2, Apaf-1, cytochrome c, caspases, Smac/DIABLO, XIAP, ARC, FLIP, AIF, p53, Id2, p21, c-Myc, PARP, HSP70, HSP27, HSP60, MnSOD, CuZnSOD, catalase, H₂O₂, MDA/4-HAE, nitrotyrosine or 8-OHdG were assessed. The incidence of apoptosis, activation of the pro-apoptotic signaling, and elevation of oxidative stress were generally evident in the skeletal muscles following denervation, hindlimb suspension, and unloading following overload in mice, rats, or quails. Moreover, p53 and Id2 were responsive to muscle unloading or overload in a subcellular compartmentalized manner. Furthermore, anti-apoptotic alterations were found in the muscles following endurance training or muscle overloading. These findings are consistent with the hypotheses that apoptosis has a role in regulating muscle loss and exercise training is able to alter the apoptotic signaling in skeletal muscle.

DEDICATIONS

This is dedicated to my mother Lam Hau Kwan and my late father Siu Chun Leung. I love and miss you very so much.

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SPECIFIC AIMS

Aging is a naturally occurring biological process, which is characterized by progressive deterioration in physiologic processes and therefore functional capacity. Among a variety of age-associated changes, significant losses of skeletal muscles mass and strength called sarcopenia is one of the unavoidable consequences with aging. Although the exact mechanism(s) that contribute to sarcopenia is/are not fully understood, there has been evidence showing that sarcopenia is mainly attributed to the loss of α -motoneurons and the subsequent denervation. Moreover, muscle disuse (e.g., bed rest and space flight) has been demonstrated to induce considerable muscle wasting and may further exacerbate sarcopenia. Since upregulation of apoptosis in skeletal muscles has been demonstrated under various physiologic and pathophysiologic atrophic situations, it has been reasonably hypothesized that apoptosis plays an important role in promoting muscle wasting. Although the apoptotic cell death with muscle atrophy has been preliminarily examined in the experimental models of muscle denervation and unloading, the regulatory mechanism(s) of apoptosis in muscle is still largely unknown. To date, it is unclear which cellular or molecular pathway present in the skeletal muscles might be potentially treated pharmacologically, genetically, or by certain therapeutic/preventive strategy to maintain the muscle mass with aging or during disuse, and this will not be known until the regulatory mechanism(s) of apoptosis is fully revealed. The objective of this dissertation is to understand the cellular and molecular regulatory mechanisms of apoptosis during denervation- and unloading-induced muscle losses. Furthermore, the prospective effect of endurance training and muscle hypertrophy on apoptotic signaling will be investigated with the aim of exploring the opportunity in adopting exercise training to offset the apoptosis-associated muscle wasting.

CENTRAL HYPOTHESIS: The central hypotheses of this dissertation are (1) that the activation of apoptosis in skeletal muscle is evident during denervation- or unloading-induced muscle wasting and that this would be associated with the elevation of oxidative stress, and (2) that exercise or overload will reduce apoptotic signaling in skeletal muscle. In this work we tested the hypothesis that the extent of denervation-induced muscle loss would be attenuated in *Bax*-knockout mice. We also hypothesize that p53 and Id2 are involved in the apoptosis-associated muscle loss. Moreover, we hypothesize that endurance training and muscle hypertrophy result in suppressed apoptotic signaling in skeletal muscles. We will test our central hypothesis by pursuing the following specific aims:

Specific Aim 1: To determine if apoptosis is associated with skeletal muscle wasting during muscle denervation or unloading.

Hypothesis 1.1: Apoptosis is activated during denervation-induced muscle loss in rat (addressed in Chapter 1).

Hypothesis 1.2: Hindlimb suspension induces skeletal muscle loss together with the activation of an apoptotic program in rat muscle (addressed in Chapter 2).

Hypothesis 1.3: An apoptotic program is activated in atrophied quail skeletal muscle during unloading following hypertrophy (addressed in Chapter 3 and 4).

Hypothesis 1.4: Denervation induces muscle loss in company with the activation of apoptosis in mice (addressed in Chapter 5).

Hypothesis 1.5: The extent of denervation-induced muscle loss is decreased concomitant with suppressed apoptotic signaling in mice that are genetically deficient of pro-apoptotic *Bax* gene (addressed in Chapter 5).

Specific Aim 2: To determine if oxidative stress is involved in apoptosis-associated skeletal muscle wasting during muscle denervation or unloading.

Hypothesis 2.1: The level of oxidative stress is elevated in mice atrophied muscle during muscle denervation (addressed in Chapter 5).

Hypothesis 2.2: Hindlimb suspension induces apoptosis-associated muscle loss together with increased oxidative stress (addressed in Chapter 6).

Hypothesis 2.3: Oxidative stress induces apoptosis in skeletal muscle cell culture (addressed in Chapter 7).

Specific Aim 3: To determine if apoptosis-associated proteins including tumor suppressor p53 and inhibitor of DNA-binding/differentiation Id2 are responsive to skeletal muscle wasting and/or muscle hypertrophy during muscle denervation or unloading and overload, respectively.

Hypothesis 3.1: p53 and Id2 are responsive to unloading following hypertrophy in quail muscle (addressed in Chapter 8).

Hypothesis 3.2: p53 and Id2 are responsive to disuse atrophy induced by hindlimb suspension-induced muscle unloading in rats (addressed in Chapter 9).

Hypothesis 3.3: p53 and Id2 are responsive to overload in quail muscle (addressed in Chapter 10).

Specific Aim 4: To determine if endurance and hypertrophy exercise trainings have an effect on the apoptotic signaling.

Hypothesis 4.1: Endurance training decreases the level of apoptosis in rat skeletal muscle (addressed in Chapter 11 and 12).

Hypothesis 4.2: The apoptotic signaling is suppressed during stretch-induced muscle hypertrophy in quail fast-twitched skeletal muscle (addressed in Chapter 13).

Hypothesis 4.3: The apoptotic signaling is suppressed during stretch-induced muscle hypertrophy in quail slow-twitched skeletal muscle (addressed in Chapter 14).

BACKGROUND AND SIGNIFICANCE

Programmed cell death or apoptosis is a fundamental biological process essentially conserved from invertebrates to mammals (25, 80, 81). It is an internal-encoded suicide program requiring coordinated regulation of specific genes that tightly regulates the process of apoptosis and has been widely accepted to be crucial in coordinating the balance between cell survival and death in various cell types (16, 65). This specific form of suicidal cell death has been documented under several physiological and pathophysiological conditions in skeletal and cardiac muscles as well as in the other tissues. For example, apoptosis has been observed in muscle dystrophy (59, 60), muscle unloading (2, 38), intense physical exercise (58, 61), ischemia-reperfusion (30, 68), chronic heart failure (1, 63), and pathogenesis of several severe diseases (e.g., cancers, acquired immune deficiency syndrome, autoimmune diseases, viral infections, and neurodegenerative diseases) (24, 71, 75, 83). Since apoptosis research has been expanded rapidly in the past two decades, recent works have suggested that apoptotic cell death has an essential role in the process of normal aging. There are studies demonstrated that significant loss of postmitotic tissues during aging is associated with the accelerated activation of apoptotic machinery (3, 4, 19, 20, 37, 49, 52, 53, 62, 64, 66, 73, 77). Intriguingly, apoptosis has also been shown to be involved in the regulation of muscle atrophy during muscle denervation (3, 8, 32, 33, 40, 48, 69, 70, 72, 79) which is an event that typically occurs in aged muscles resulting from the loss of peripheral α -motoneurons (10, 17, 35, 74). Taken together, the development of sarcopenia could be practically attributed to an aberrant upregulation of apoptosis which directs the catabolic events in denervated aged muscles. Furthermore, muscle disuse (e.g., bed rest and space flight) may exacerbate sarcopenia in which apoptotic machinery may also be involved (1, 38). Given that the sarcopenic and disuse-induced muscle losses are related to apoptotic events, it is surprising that there is not a clear understanding of the cellular and molecular regulatory mechanisms of apoptosis in muscle. Thorough understanding the regulation of apoptosis and the underlying regulatory mechanisms are believed to be useful in developing new strategies to offset the deteriorating effects with apoptosis-related muscle wasting (e.g., sarcopenia, bed rest, and space flight).

Cellular regulation of apoptosis. Apoptotic cell death program is initiated and activated as a result of diverse internal or external signals. Reactive oxygen species (ROS), intracellular Ca^{2+} disturbance, ceramide, DNA damage, tumor necrosis factor (TNF), and Fas ligand (FasL) have been studied and shown to be the potential stimuli for apoptosis (29, 34, 39, 45, 82). After a cell is committed to apoptosis, it will destroy and eliminate itself by activating the apoptotic cascade without causing any inflammatory response and disturbance to the surrounding cells. Several apoptotic pathways have been shown to be involved in the apoptotic cascades, depending on the stimulus

characteristic. These include mitochondrial-dependent pathway (28, 49), ligand-mediated death receptor pathway (39), and endoplasmic reticulum stress-induced pathway (45). Furthermore, the apoptotic signaling cascades usually involve expression of genes that regulate the execution of apoptosis (e.g. B-cell leukemia/lymphoma-2 or BCL-2 family proteins). A family of protease proteins called caspase (cysteine-dependent aspartate protease) has been found to be important in executing apoptosis. Specific initiator caspases (e.g. caspase-8, -9, and -12) are activated when the cell is exposed to the corresponding apoptotic stimuli. For example, TNF or FasL can initiate apoptosis through the death receptor pathway by activating caspase-8 (39, 67). Apoptosis stimulated by endoplasmic reticulum stress and intracellular Ca^{2+} disturbance has been reported to be related to the activation of caspase-12 (44, 45). In addition, caspase-9 has been shown to mediate the mitochondrial-dependent apoptosis through the interaction of procaspase-9 with apoptotic protease activating factor-1 (APAF-1), dATP, and mitochondrial released-cytochrome *c* (49). Although different initiator caspases are recruited in different apoptotic pathways, it appears that the apoptotic signals finally converge on the activation of the common effector caspases (e.g. caspase-3, -6, and -7), which cause eventual destruction of the cell. However, it has been demonstrated that apoptosis can also be executed through the caspase-independent manner which involves the proteins with endonuclease activity (e.g. apoptosis inducing factor or AIF, and endonuclease G) (11, 12, 78).

BCL-2 family proteins. BCL-2 family proteins act as the key upstream regulators of apoptotic cell death. It has been suggested that BCL-2 family constitutes a crucial intracellular checkpoint in the apoptotic signaling pathway by responding to the apoptotic stimuli (16). Generally, mammalian cells possess an entire family of BCL-2 proteins, which can be divided into three subclasses (i.e., anti-apoptotic, multidomain pro-apoptotic, and BH3-only pro-apoptotic) defined by the homology shared within four conserved sequence motifs namely BCL-2 homology (BH1-4) domains. The anti-apoptotic BCL-2 subclass, including BCL-2, BCL-X_L, BCL-W, A1, and MCL-1, displays conservation in all four BH1-4 domains. They function to oppose the effects of the pro-apoptotic BCL-2 members and prevent apoptosis from execution. For the pro-apoptotic BCL-2 subclasses, the multidomain pro-apoptotic subclass containing BAX, BAK, and BOK shares the BH1, BH2, and BH3 domains, while the BH3-only pro-apoptotic subclass shares only the BH3 sequence motif and includes BAD, BID, BIM, Dp5/Hrk, NOXA, and PUMA. Among the BCL-2 members, the BH3 domain is thought to be vital for the pro-apoptotic interactions since all pro-apoptotic members contain a BH3 domain, although some anti-apoptotic members also contain the BH3 domain (14). Provided that the BH3 sequence motif refers to a hydrophobic α -helix which is favourable for protein interaction, it has been hypothesized that this is the region for the association among the BCL-2 family proteins via homo- or hetero-oligomerization (14, 41). Pieces of evidence have suggested that the relative concentrations of pro- and anti-apoptotic BCL-2 family members provide a tight control

over the balance of cell survival and apoptotic cell death (14-16). Among the entire BCL-2 family, BAX and BCL-2 have been designated to be the main protagonists in the regulation of apoptotic machinery as they bear the ion channel forming activities. There is evidence showing that BAX can translocate to the mitochondria and expose its N-terminus via a conformational change upon induction of apoptosis (7, 13, 18, 31, 76). This conformational change has been suggested to allow the BAX-BAX-oligomerization and insertion of BAX into the outer mitochondrial membrane (84), which is followed rapidly by the release of the apoptogenic factors (e.g., cytochrome c and AIF) from the mitochondrial intermembrane space. Overall, BAX oligomerization is thought to be critical for mitochondrial membrane permeabilization whereas BCL-2 opposes the pro-apoptotic activity of BAX by preventing the process of BAX-BAX-oligomerization (6). Although the precise mechanism of BAX-mediated apoptogenic factors release from the mitochondrial intermembrane space is still under active investigation, it is indisputable that BAX plays an indispensable role in promoting the activation of apoptotic signaling cascades. Intuitively, the aging-induced detrimental effects on muscles would be offset if certain strategies can delay or slow down the accelerated apoptosis by suppressing the pro-apoptotic factor BAX in the aged muscles. Nevertheless, those strategies could only be developed after the regulatory role of BAX during denervation-induced apoptosis in aged muscles is fully understood.

Muscle loss with aging. A significant reduction of skeletal muscle mass and muscular strength, which is termed sarcopenia, has been well documented to occur progressively throughout the life from maturity to senescence in humans and animals. Epidemiological data indicate that sarcopenia is an inevitable consequence with aging which affects all people, even healthy individuals (54-56). Based on the fact that sarcopenia contributes to overall muscle atrophy and weakness, it is generally believed that sarcopenia plays a key role in the pathogenesis of impaired functional mobility and frailty which results in disability and loss of independence in the geriatric population (54, 56). In fact, sarcopenia becomes one of the most serious social and health care problems due to the heavy health care burden, which exceeds \$80 billion/year, caused by the deleterious functional outcomes of sarcopenia (e.g., frailty). This problem is anticipated to worsen as the average lifespan is prolonged by improved medicine where the elderly population is estimated to grow from 13% to 20% by the year 2030. Approximately, the potential is to double the health care costs associated with elderly frailty (i.e., \$160 billion/year) within 40 years (9, 27, 42, 43, 46, 47). Although researchers have been actively investigating the causes of sarcopenia aimed to develop the potential interventions or strategies that can encounter the detriments of sarcopenia, the exact causes of sarcopenia are still largely unknown, possibly due to the complexity of the processes with aging. Up to now, several potential mechanisms have been proposed to explain why the aged muscles become unfavorable to

accumulate or maintain the proteins of contractile components and consequently develop into the sarcopenic state. These mechanisms depict that the pathogenesis of sarcopenia is attributed to withdrawal of anabolic stimuli (loss of motor neurons, decreased levels of anabolic hormones/growth factors including growth hormone, testosterone, estrogen, and insulin-like growth factor or IGF), involvement of the catabolic factors (increased endogenous productions of cytokines including tumor necrosis factor-alpha or TNF- α , interleukin-1beta or IL-1 β , and IL-6), physical inactivity, aging-associated anorexia, and inadequate dietary protein consumption (21, 55, 56, 74). Providing these proposed mechanisms contribute to the development of sarcopenia in greater or lesser extent, it has been suggested that the loss of α -motoneurons is the most important single cause of sarcopenia (21, 56). Since nerve innervation is crucial to the growth and maintenance of myofibers, it is certain that loss of α -motoneurons with aging could lead to their innervated myofibers suffer muscle atrophy and even myocyte death due to the absence of anabolic stimuli originated from the motoneurons. This suggestion is supported by histochemical evidence, including increased fiber number per motor unit, fiber type grouping, disproportionate atrophy of type IIa muscle fibers, and increased coexpression of myosin heavy chain isoforms in the same fiber (10, 21-23, 26, 35, 36), showing that a progressive denervation and reinnervation process continuously occurs in the skeletal muscles with aging.

Increased apoptosis with aging. In the past two decades, apoptosis research has been dramatically expanded in the literature. Although most apoptosis studies focus on the actively dividing or mitotic cell lineages (e.g., lymphocytes), recently, there are novel data demonstrating that post-mitotic cells (e.g., skeletal myocytes) also exhibit activation of apoptosis under certain physiological and pathophysiological conditions including muscle dystrophy, muscle unloading and disuse, intense physical stress, ischemia-reperfusion, and chronic heart failure (2, 5, 8, 32, 33, 40, 48, 50, 51, 53, 57-59, 61, 68, 70, 72, 77). Moreover, there is evidence indicating that the significant loss of post-mitotic myocytes with aging is possibly attributed to the accelerated activation of apoptotic machinery (3, 4, 19, 20, 37, 49, 52, 53, 62, 64, 66, 73, 77). For instance, Strasser and colleagues (66) clearly demonstrated that the number of apoptotic rhabdosphincter striated muscle fibers detected by TUNEL staining increases in an age-dependent manner. They reported that the decreased volume densities of the striated muscle cells is linearly correlated with the age of the specimens and this possibly leads to a dramatic decrease in the number of rhabdosphincter myocytes which results in a high incidence of stress incontinence in the elderly populations. Consistent with the findings in humans, a greater level of apoptosis has also been observed in rodent skeletal muscles with aging (3, 4, 19, 20). These findings bring about the suggestion that the age-related decline in muscle mass is closely associated with the accelerated apoptotic events in the skeletal muscles, although the exact mechanism(s) for the apoptosis-induced loss of muscles is still largely unclear.

Apoptosis during denervation- and unloading-induced muscle atrophy. Motivated by the fact that muscle denervation resulting from the loss of α -motoneurons largely accounts for the age-associated decrease in muscle mass, a number of studies have been conducted to examine the regulation of muscle loss during muscle denervation. Moreover, attention has been put to the regulation of disuse-induced muscle loss with the aim of understanding the process of muscle wasting during bed rest and space flight. Since apoptotic cell death has been demonstrated to be an active regulatory process to degenerate or remove unwanted or damaged tissues, it is not inconceivable that there has been interest in investigating apoptosis in the experimental model of muscle denervation and disuse. Indeed, there is strong evidence showing that activation of apoptotic machinery occurs during muscle denervation (3, 8, 32, 33, 40) and unloading (1, 38). Although the precise physiologic role of apoptosis in muscle has yet to be identified, these consistent reports of activated apoptotic machinery during denervation and unloading suggest that apoptosis possibly plays an important role in regulating the process of muscle loss during muscle disuse or inactivity (2, 3, 5, 8, 32, 33, 40, 59-61, 70, 72). Furthermore, Allen and colleagues (2) have proposed that execution of apoptosis can be restricted to the individual myonuclei within the multinucleated skeletal myofibers and so called "programmed nuclear death". Since myonuclear domain (i.e., cytoplasmic volume per myonucleus) has been suggested to be a cellular tool to control the size of a myofiber, one of the possible physiologic functions of apoptosis could be responsible for the elimination of myonuclei and/or satellite cells from the atrophying fibers thereby monitoring the process of muscle atrophy. Indisputably, more research is needed to completely reveal the physiologic role and the regulatory mechanisms of apoptosis during denervation- and disuse-induced muscle loss in order to extend our knowledge to fully understand the mechanisms of sarcopenic and disuse-induced muscle wasting.

Importance of the dissertation and potential benefits from understanding the role of apoptosis in muscle. As apoptosis is highly regulated and partially reversible in nature, detrimental effects on the physiologic function from the significant loss of muscles with aging and during disuse are expected to be retarded or decreased if accelerated apoptosis can be attenuated by certain strategies (e.g., exercise training). According to the existing evidence showing that apoptosis is related to muscle loss, understanding the regulatory mechanisms of the denervation- and disuse-induced apoptotic cell death is urgently needed for exploiting the possible strategies to offset muscle wasting with aging and during disuse. Results from the proposed studies are expected to provide novel scientific data in understanding the regulatory mechanism(s) of apoptosis and the apoptotic adaptation from exercise training in muscle, and this information is critical for designing the appropriate preventive strategies, therapeutic interventions, or exercise regimens to improve the muscle function in the populations including elderly, long term bed rest patients, and astronauts.

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CHAPTER 1

Mitochondria-associated apoptotic signalling in denervated rat skeletal muscle

ABSTRACT

Apoptosis has been implicated in the regulation of denervation-induced muscle atrophy. However, the activation of apoptotic signal transduction during muscle denervation has not been fully elucidated. The present study examined the apoptotic responses to denervation in rat gastrocnemius muscle. Following 14 days of denervation, the extent of apoptotic DNA fragmentation as determined by a cytosolic nucleosome ELISA was increased by 100% in the gastrocnemius muscle. RT-PCR and immunoblot analyses indicated that Bax was dramatically up regulated while Bcl-2 was modestly increased; however, the Bax/Bcl-2 ratio was significantly increased in denervated muscles relative to control muscles. Analyses of ELISA and immunoblots from mitochondria-free cytosol extracts showed a significance increase in mitochondria-associated apoptotic factors including cytochrome c, Smac/DIABLO, and AIF. In addition to the upregulation of caspase-3 and -9 mRNA, pro-/cleaved caspase protein, and proteolytic activity levels, the XIAP protein level was down regulated. The cleaved product of PARP was detected in muscle samples following denervation. Although we did not find a difference in Id2 and c-Myc protein contents between the denervated and control muscles, the protein content of tumour suppressor p53 was significantly increased in both the nuclear and the cytosolic fractions with denervation. Moreover, denervation increased the protein content of HSP70 whereas the MnSOD protein content was diminished which indicated that denervation might have induced cellular and/or oxidative stress. Our data show that mitochondria-associated apoptotic signalling is up regulated during muscle denervation. We interpret these findings to indicate that apoptosis has a physiologically important role in regulating denervation-induced muscle atrophy.

Key words: Muscle; muscle atrophy; BCL-2, apoptosis

INTRODUCTION

Apoptosis is an active, internal-encoded biological process that requires regulation of specific genes to tightly coordinate the corresponding apoptotic events (Steller, 1995, Yuan, 1995, Danial & Korsmeyer, 2004). It has been widely accepted that apoptotic cell death is crucial in monitoring the balance between cell survival and death in mitotic cell lineages and it has been recognized to be an active regulatory process to degenerate or remove abnormal or damaged tissues (Ellis *et al.*, 1991, Thompson, 1995). The role of apoptosis in health and disease has been established by demonstrating that the pathogenesis of several severe diseases including various cancers, acquired immune deficiency syndrome, autoimmune diseases, viral infections, and neurodegenerative diseases are attributed to the aberrant regulation of apoptosis (Williams, 1991, Thompson, 1995, Duke *et al.*, 1996, Yuan & Yankner, 2000). Recently, apoptosis has also been reported in postmitotic skeletal muscle under certain physiological and pathophysiological conditions (e.g., muscle denervation, muscle dystrophy, neuromuscular disorders, hindlimb unweighting, muscle unloading, strenuous physical exercise, and aging-associated sarcopenia) (Sandri *et al.*, 1995, Allen *et al.*, 1997, Sandri *et al.*, 1997, Sandri *et al.*, 1998, Sandri *et al.*, 2001, Tews, 2002, Dirks & Leeuwenburgh, 2002, Pollack *et al.*, 2002, Alway *et al.*, 2003, Leeuwenburgh, 2003, Dirks & Leeuwenburgh, 2004) and these consistent observations of activated apoptotic machinery under muscle atrophic conditions call attention to the existence of a physiologic role of apoptosis in regulating the atrophic process of muscle remodelling during disuse or inactivity.

Among various experimental models of muscle atrophy, skeletal muscle denervation has been actively adopted to investigate the regulation of disuse-induced atrophy including denervation resulting from the degeneration of motor neurons which is closely associated with the pathogenesis of severe neurological diseases such as amyotrophic lateral sclerosis (Brown, Jr., 1997). Moreover, it has been suggested that muscle denervation may be involved in significant aging-associated loss of muscle (i.e., sarcopenia). This is based on the histochemical evidence in aged muscles including increased fibre number per motor unit, fibre type grouping, disproportionate atrophy of type IIa muscle fibres, and increased co-expression of myosin heavy chain isoforms in the same fibre showing that a progressive denervation and reinnervation process continuously occurs in the aged skeletal muscle (Brown, 1972, Essen-Gustavsson & Borges, 1986, Doherty *et al.*, 1993, Larsson, 1995, Larsson, 1998). Providing that nerve innervation is essential to the growth and maintenance of skeletal myofibres (Hughes, 1998, Trachtenberg, 1998, Pette, 2001), it is certain that loss and/or dysfunction of motoneurons with neurological diseases and aging leads to muscle atrophy and myocyte death.

There is evidence that apoptosis may regulate at least in part denervation-induced muscle atrophy (Migheli *et al.*, 1997, Tews *et al.*, 1997, Yoshimura & Harii, 1999, Borisov & Carlson, 2000,

Olive & Ferrer, 2000, Tang *et al.*, 2000, Jin *et al.*, 2001, Jejuri *et al.*, 2002, Alway *et al.*, 2003). Nevertheless, the underlying mechanism(s) accounting for the activation of apoptosis and its physiologic role during denervation remain largely unknown. Therefore, the purpose of the present study was to examine apoptotic signalling and the apoptosis-associated cellular responses during denervation in skeletal muscle. We hypothesized that apoptosis is activated as a result of the promotion of mitochondria-associated pro-apoptotic factors whereas the anti-apoptotic factors are suppressed in denervated muscle.

METHODS

Animals. Experiments were conducted on eight 6-mo old adult Fischer₃₄₄ x Brown Norway rats that were obtained from the NIA colony. The rats were housed in pathogen-free conditions at ~20°C. They were exposed to a reverse light condition of 12:12 h of light: darkness each day and they were fed rat chow and water ad libitum throughout the study period.

Denervation procedure. The animals were placed under a general anaesthesia using 2% isoflurane. After reflex activity had disappeared, an incision was made from the calcaneus to just proximal to the popliteal fossa. The tibial nerve was then dissected proximal to the cranial border of the gastrocnemius muscle. Care was taken to avoid any damage to the nerves, blood vessels, and connective tissues. The medial and lateral branches of the tibial nerve that innervate the plantar flexor muscles (i.e., gastrocnemius and soleus) were transected close to their neuromuscular junction (Degens *et al.*, 1995). The cut nerve ends were sutured into the biceps femoris muscle to ensure that the nerve stumps did not reinnervate the gastrocnemius muscle. Innervation to the plantaris and the deep toe flexor muscles were left intact so that the animals ambulated normally around the cage after the surgical denervation. Following the surgery, the hamstring muscle layers were closed with reabsorbable suture and the skin incision were closed with wound clips. The incision sites were covered with an antibacterial cream to prevent infection. The contralateral limb served as the intra-animal control. We have observed that the animals recovered quickly and were able to walk around within ~45 minutes post-surgery (Alway *et al.*, 2002, Alway *et al.*, 2003).

Fourteen days after the surgical denervation, animals were anaesthetised (ketamine hydrochloride, 9 mg.100⁻¹ g body weight and xylazine hydrochloride 1 mg.100⁻¹ g body weight, i.p.) then the gastrocnemius muscles from each limb were dissected from the surrounding connective tissues, removed, and stored at -80°C. Animals were subsequently euthanised with an overdose of pentobarbital sodium (5 mg . g⁻¹ body weight, i.p.). The institutional animal use and care committee from West Virginia University School of Medicine approved all experiments. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as

advocated by the American Association for Accreditation of Laboratory Animal Care and fully conformed with the Animal Welfare Act of the U.S. Department of Health and Human Services.

RT-PCR. Total RNA was extracted from the gastrocnemius muscle of both denervated and control muscles with TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Frozen muscle was mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA was solubilized in RNase-free H₂O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assured by examining OD₂₆₀/OD₂₈₀ ratio. Five micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 20 µl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). Control RT reaction was done in which the RT enzyme was omitted. The control RT reaction was PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of complementary DNA (cDNA) was then amplified by PCR using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, TX), 250 µM deoxyribonucleotide triphosphates (dNTPs), 1 x PCR buffer, and 2.5 units *Taq* DNA polymerase (USB Corp., Cleveland, OH) in a final volume of 50 µl. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). The primer pairs were designed from sequences published in GenBank (Table 1) and PCR products were verified by restriction digestions. Preliminary experiments were conducted with each gene to assure that the number of cycles represented a linear portion for the PCR optical density curve for the muscle samples. The cDNA from all muscle samples were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 30 µl of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide. Images were captured and the signals were quantified in arbitrary units as optical density (OD) x band area using Kodak image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. Ribosomal 18S primers were used as internal controls while all RT-PCR signals were normalized to the 18S signal of the corresponding RT product to eliminate the measurement error from uneven samples loading and provide a semi-quantitative measure of the relative changes in gene expression.

Subcellular protein fractionation. The fractionation method described by Rothermel et al. (Rothermel *et al.*, 2000) was adopted with minor modification to extract the cytosolic and nuclear protein fractions from the gastrocnemius muscle. We have previously obtained the fractionated cytosolic and nuclear proteins from skeletal and heart muscles using this modified protocol (Siu & Alway, 2004, Siu *et al.*, 2004a, Siu *et al.*, 2005). Briefly, after removal of connective tissues, muscle was homogenized on ice in ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT, pH 7.4). Following centrifuging at 3,000 rpm for 3 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and

these supernatants were further centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual nuclei and stored as nuclei-free total cytosolic protein fraction. A portion of the cytosolic extract (without addition of protease inhibitors) was stored and used for fluorometric caspase protease activity assay while a protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO) was added to the remaining portion. The cytosolic protein fraction with the addition of protease inhibitors was later used for cell death ELISA and Western immunoblots. The remaining nuclear pellets were washed 3 times with ice-cold lysis buffer, resuspended in 300 µl of lysis buffer in the presence of 41.5 µl of 5 M NaCl and protease inhibitor cocktail, and rotated for 1 h at 4°C to lyse the nuclei. Following a spin at 15,000 rpm for 15 min at 4°C, the supernatants were collected and stored as a cytosol-free nuclear protein fraction.

Furthermore, with the intention of estimating the release of mitochondria-residing apoptotic factors including cytochrome c, AIF, and Smac/DIABLO into the cytosol, a nuclei-free, mitochondria-free cytosolic protein fraction was prepared as described by Rokhlin *et al.* (Rokhlin *et al.*, 2002) and the protein contents of these mitochondrial apoptotic factors were then measured in this mitochondria-free cytosolic fraction as described in the later sections. For the extraction, muscle was dissected from the connective tissues and minced in ice-cold extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) in the presence of the protease inhibitor cocktail. Following a gentle homogenisation with a Teflon pestle motorized with an electronic stirrer, homogenates were centrifuged at 800 × g for 10 min at 4°C to pellet the nuclei and cell debris. The supernatants were then spun twice at 16,000 × g for 20 min at 4°C to pellet the mitochondria and the final supernatants were collected as nuclei-free, mitochondria-free cytosolic protein fractions. The purity of the extracted fractions was examined by immunoblotting the extracted fractions with an anti-histone H2B (a nuclear protein) rabbit polyclonal antibody (1:2000 dilution, 07371, Upstate, Lake Placid, NY), an anti-CuZnSOD (a cytosolic isoform of superoxide dismutase) polyclonal rabbit antibody (1:500 dilution, sc-11407, Santa Cruz Biotechnology, Santa Cruz, CA), and an anti-MnSOD (a mitochondrial isoform of superoxide dismutase) goat antibody (1:2000 dilution, A300449A, Bethyl Lab, Montgomery, TX) (Figure 1). In order to further verify the purity, especially the mitochondria-free cytosolic fraction, a kinetic enzyme activity measurement of citrate synthase (a mitochondrial oxidative enzyme) was performed in the extracted fractions as described (Figure 1) (Siu *et al.*, 2003, Siu *et al.*, 2004b).

The protein contents of the muscle extracts were quantified in duplicate by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of

cuprous cation (Smith *et al.*, 1985). As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion by DC Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay (Lowry *et al.*, 1951).

Insert Figure 1 —————> Verification of the extracted protein fractions

Apoptotic cell death ELISA. Cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligo-nucleosomes. Briefly, the nuclei-free cytosolic fraction of gastrocnemius muscle was used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated to the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate for 10 min at 20°C. The change in colour was measured at a wavelength of 405 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). Measurements were performed in duplicate, with denervated and contralateral control samples analysed on the same microtiter plate in the same setting. The OD₄₀₅ reading was then normalized to the mg of protein used in the assay.

Fluorometric caspase activity assay. Previously, caspase-3,-6,-7 and caspase-8 protease activities in the denervated muscle have been assessed by a colorimetric method (Alway *et al.*, 2003). In the present study, we used a more sensitive fluorometric assay to examine the protease activities of caspase-3 and -9 in the denervated gastrocnemius muscle. In brief, 50 µl of the total cytosolic protein fraction without protease inhibitor of the muscles was incubated in 50 µl of assay buffer (50 mM PIPES, 0.1 mM EDTA, 10% glycerol, 10 mM DTT, pH 7.2) with 100 µM of the fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated substrate (Ac-DEVD-AFC for caspase-3, Ac-LEHD-AFC for caspase-9, Alexis Corp., San Diego, CA) at 37°C for 2 h. Caspase specific inhibitor, Z-VAD-FMK (Calbiochem, La Jolla, CA) was used as a control to validate the specificity of caspase. The change in fluorescence was measured on a spectrofluorometer with an excitation wavelength of 390/20 nm and an emission wavelength of 530/25 nm (CytoFluor, Applied Biosystems, Foster City, CA) before and after the 2 h incubation. Caspase activity was estimated as the change in arbitrary fluorescence units normalized to milligram protein. Measurements were performed in duplicate while denervated and intra-animal control samples were run on the same microplate in the same setting.

Western immunoblot analyses. The protein expression of Bcl-2, Bax, Bcl-2-related ovarian killer (Bok), apoptosis protease activating factor-1 (Apaf-1), X-linked inhibitor of apoptosis (XIAP), caspase-3 and -9, heat shock protein-70 (HSP70), and superoxide dismutases (CuZn-SOD and Mn-SOD) was determined in the total cytosolic protein fraction while apoptosis inducing factor (AIF) and c-Myc was measured in the nuclear fraction. Both the total cytosolic and nuclear fractions were used to measure the protein content of cleaved poly(ADP-ribose) polymerase (PARP), p53, and inhibitor of DNA binding/differentiation-2 (Id2).

Forty micrograms of protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE at room temperature. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and probed with the following primary antibodies diluted in PBS-T with 2% BSA: anti-Bcl-2 mouse monoclonal antibody (1:100 dilution, sc-7382), anti-Bcl-2 mouse monoclonal antibody (1:100 dilution, 610538) anti-Bax rabbit polyclonal antibody (1:200 dilution, sc-493), anti-Bok rabbit polyclonal antibody (1:100 dilution, ab2304, Abcam, Cambridge, MA), anti-Apaf-1 rabbit polyclonal antibody (1:200 dilution, 3018, BioVision, Mountain View, CA), anti-hILP/XIAP mouse monoclonal antibody (1:250 dilution, 610762), anti-pro-/cleaved caspase-3 rabbit polyclonal antibody (1:2000 dilution, 552037), anti-cleaved caspase-9 rabbit polyclonal antibody (1:100 dilutions, sc-22182), anti-HSP70 rabbit polyclonal antibody (1:2000 dilution, SPA812, StressGen, Victoria, BC, Canada), anti-SOD-1 rabbit polyclonal antibody (1:500 dilution, sc-11407), anti-MnSOD goat antibody (1:2000 dilution, A300449A, Bethyl Lab, Montgomery, TX), anti-AIF mouse monoclonal antibody (1:800 dilution, sc-13116HRP), anti-cleaved PARP rabbit polyclonal antibody (1:1000 dilution, G734, Promega, Madison, WI), anti-c-Myc mouse monoclonal antibody (1:200 dilution, 3800-1), anti-Id2 rabbit polyclonal antibody (1:100 dilution, sc-489), anti-p53 mouse monoclonal antibody (1:100 dilution, sc-99). Bcl-2 (sc-7382), Bax, cleaved caspase-9, SOD-1, AIF, Id2, and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while Bcl-2 (610538), XIAP, caspase-3, and c-Myc antibodies were purchased from BD Biosciences (San Jose, CA). With the intention of examining the protein content of pro-caspase-9, immunoblots were performed with anti-pro-caspase-9 rabbit polyclonal antibodies purchased from different sources (sc-8355, Santa Cruz, CA; 3016, BioVision, Mountain View, CA), but we failed to detect an ~46 kDa immunoreactive corresponding to the predicted molecular mass of pro-caspase-9. All primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon

International, Temecula, CA), and signals were developed by West Pico chemiluminescent substrate (Pierce, Rockford, IL). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Estimation of mitochondrial cytochrome c, Smac/DIABLO, and AIF release.

Cytochrome c, AIF, and Smac/DIABLO (second mitochondria-derived activator of caspase) are apoptotic factors normally confined to mitochondria and their release into the cytosol has been demonstrated during the activation of apoptosis (Susin *et al.*, 1999). In the present study, the release of Smac/DIABLO and AIF into the cytosol was estimated by measuring their protein contents in the extracted mitochondria-free cytosolic protein fraction by immunoblotting with an anti-Smac/DIABLO mouse monoclonal antibody (1:500 dilution, 612244, BD Biosciences, San Jose, CA) and an anti-AIF monoclonal mouse antibody. Moreover, a cytochrome c ELISA kit (MBL International, Woburn, MA) was used to assess the protein content of cytochrome c in the mitochondria-free cytosol fraction to evaluate the release of the mitochondrial cytochrome c into the cytosol. By following the manufacturer's protocol, 60 μ l the extracted mitochondria-free cytosolic fraction was used as an antigen source in a sandwich ELISA with a horseradish peroxidase-conjugated anti-cytochrome c polyclonal antibody in microwell strips coated with an anti-cytochrome c antibody. After washing, the peroxidase retained in the immunocomplex was detected by incubating with a chromogenic substrate, tetramethylbenzidine/hydrogen peroxide (TMB/H₂O₂) followed by adding an acid solution to terminate the enzyme reaction and to stabilize the developed colour. The change in colour was monitored at a wavelength of 450 nm using a Dynex MRX plate reader. Measurements were performed in duplicate with the denervated and contralateral control samples analysed on the same microplate and the cytochrome c content was expressed as OD₄₅₀ per mg protein.

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package. Student's *t*-test for paired data was used to examine differences between the denervated and contralateral control muscles. Statistical significance was accepted at $P < 0.05$. Data are expressed as mean of percent relative to the contralateral control muscle (i.e., control refers to 100%).

RESULTS

Apoptotic DNA fragmentation. The cell death ELISA analysis indicated that the extent of the apoptotic DNA fragmentation in the denervated muscle was 100% ($P < 0.05$, **Figure 2**) higher than that in the control muscle.

Insert Figure 2 ———→ Apoptotic DNA fragmentation

BCL-2 family: Bcl-2, Bax, and Bok. As estimated by RT-PCR, denervated muscle had a 177% increase in Bcl-2 mRNA and a 482% increase in Bax mRNA when compared to the control muscle ($P < 0.05$, **Figure 3**). The ratio of Bax/Bcl-2 mRNA content was further evaluated and was found to be increased by 131% in the denervated muscle relative to the control muscle ($P < 0.05$, **Figure 3**).

In our Western blot analyses, we detected immunoreactive bands corresponding to the Bcl-2 and Bax proteins. Consistent with the mRNA data, both Bcl-2 and Bax protein content increased in the muscle following denervation relative to the control muscle. We found that the Bcl-2 and Bax protein content in denervated muscle was 295% ($P < 0.05$) and 785% ($P < 0.01$) higher than that of control muscle, respectively (**Figure 3**). It is noted that the increased Bcl-2 protein content was verified by using anti-Bcl-2 mouse monoclonal antibodies obtained from two different sources (sc-7382 Santa Cruz Biotechnology and 610538 BD Biosciences). As a result of a relatively greater increase in the Bax protein content comparing to Bcl-2, the ratio of Bax/Bcl-2 was increased by 263% in the denervated muscle when compared to the control muscle ($P < 0.05$, **Figure 3**). In our immunoblots, although an ~18 kDa immunoreactive band corresponding to Bok protein (a pro-apoptotic member of BCL-2 family) was not detected in most of the muscle samples, we observed that it was present weakly in two muscle samples following denervation (**Figure 3**).

Insert Figure 3 ———→ Bcl-2, Bax, and Bok

Mitochondria-mediated apoptotic factors: mitochondrial cytochrome c release and Apaf-1 content. The ELISA analyses on the extracted mitochondria-free cytosolic fraction showed that the protein content of cytosolic cytochrome c in the denervated muscle increased by 40% when compared to the control muscle ($P < 0.05$, **Figure 4**). Given that cytochrome c is primarily housed in the mitochondria under normal condition, this indicated that cytochrome c was relocated to the cytosol during denervation. In contrast, we did not find any difference in the mRNA and the cytosolic protein content of Apaf-1 between the denervated and control samples as measured by RT-PCR and Western immunoblotting, respectively ($P > 0.05$, **Figure 4**).

Insert Figure 4 ———→ Cytochrome c and Apaf-1

Caspase-3 and -9 mRNA, protein content, and protease activity. The responses of caspase-3 and -9 to muscle denervation were evaluated by examining caspase mRNA, protein,

and protease activity. As estimated by RT-PCR, the mRNA content of caspase-3 and -9 in the denervated muscle increased by 125% and 300% relative to the control muscle, respectively ($P < 0.05$, **Figure 5**). In our immunoblots, we detected immunoreactive bands corresponding to the predicted positions for pro-caspase-3 and cleaved caspase-3. There was a 649% increase ($P < 0.01$) in the protein content of pro-caspase-3 and the cleaved caspase-3 protein content increased by 500% ($P < 0.05$) in the muscle following denervation when compared to the control muscle (**Figure 5**). In addition, an immunoreactive band corresponding to the predicted molecular weight of cleaved caspase-9 was detected and there was a 519% increase in the denervated muscle compared to the control muscle ($P < 0.01$, **Figure 5**). The activation of caspase-3 and -9 was further confirmed by a fluorometric caspase enzymatic activity analysis showing that there was a 394% ($P < 0.01$) and 69% ($P < 0.05$) increase in the protease activity of caspase-3 and -9, respectively (**Figure 5**).

Insert Figure 5 ———→ Caspase-3 and -9

Modulators of caspases: XIAP protein content and mitochondrial Smac/DIABLO release. An immunoreactive band that corresponded to the predicted position for XIAP protein, an inhibitor of caspase-3 and -9, was detected in the immunoblots of the total cytosolic fractions. In the muscle following denervation, the protein content of XIAP was 39% lower than that in the control muscle ($P < 0.01$, **Figure 6**). For the immunoblots of the extracted mitochondria-free cytosolic fractions, we detected an immunoreactive band that corresponded to the predicted position of the Smac/DIABLO protein, an upstream inhibitor of XIAP. We found that the Smac/DIABLO protein content increased by 81% ($P < 0.05$, **Figure 6**) in the cytosolic fraction (in the absence of mitochondria) of the denervated muscle relative to the control muscle signifying that mitochondrial Smac/DIABLO was released into the cytosol during denervation.

Insert Figure 6 ———→ XIAP and Smac/DIABLO

Caspase-independent mitochondrial AIF release and nuclear translocation. Translocation of mitochondrial AIF to the nuclei has been suggested to be a caspase-independent apoptogenic event (Joza *et al.*, 2001, Cande *et al.*, 2002). In the present study, we evaluated the mRNA, nuclear and mitochondria-free cytosolic protein contents of AIF in the denervated and control muscles. As assessed by RT-PCR and immunoblots of the nuclear protein fraction, we found that the mRNA and the nuclear AIF were not different between the denervated and control muscles ($P > 0.05$, **Figure 7**). However, as an estimate of the mitochondrial AIF release to the cytosol, the AIF protein content measured in the mitochondria-free cytosolic fraction of the denervated muscle was 1029% higher than

the control muscle ($P < 0.05$, **Figure 7**) indicating that AIF was significantly released from mitochondria into the cytosol during denervation.

Insert Figure 7 —————→ AIF

Cleaved PARP protein content. PARP plays an important role in the process of DNA repairing and it has been demonstrated to be a cleavage target for the effector caspases (e.g., caspase-3) during the execution of apoptosis (Lazebnik *et al.*, 1994). Although PARP is a nuclear protein, there have been reports illustrating the presence of cleaved PARP protein in the cytosol (Rosenthal *et al.*, 1997, Cookson *et al.*, 1999). Thus, by using immunoblotting, we examined the protein content of cleaved PARP in both the nuclear and total cytosolic fractions. For the nuclear fraction, an immunoreactive band corresponding to the cleaved PARP protein was not detected in any of the control muscles but it was found in the denervated muscle samples of five animals (**Figure 8**). We found that the cleaved PARP protein content was increased by 275% in the cytosolic fraction of denervated muscle when compared to the control muscle ($P < 0.05$, **Figure 8**).

Insert Figure 8 —————→ Cleaved PARP

Cellular regulatory factors: p53, Id2, and c-Myc protein contents. In our immunoblot analyses, an immunoreactive band corresponding to the predicted position of the p53 protein was barely detected in the nuclear and the cytosolic protein fraction of all the control muscles, while the p53 protein content was markedly elevated in both the nuclear (~10-fold increase, $P < 0.05$) and the cytosolic fraction (~5-fold increase, $P < 0.05$, **Figure 9**) of the muscle following denervation. In contrast, we found that the nuclear and cytosolic Id2 and nuclear c-Myc protein content was not different between the denervated and control muscles ($P > 0.05$, **Figure 9**).

Insert Figure 9 —————→ p53, Id2, and c-Myc

Stress protein and antioxidant enzyme: HSP70, MnSOD, and CuZnSOD protein contents. According to our immunoblot analyses, the protein content of CuZnSOD in the denervated muscle was similar to the control muscle ($P > 0.05$, **Figure 10**). However, we found that the HSP70 protein content was elevated by 22% ($P < 0.05$) while the protein content of MnSOD was diminished by 36% ($P < 0.01$, **Figure 10**) in the muscle after denervation relative to the control muscle, and these denoted that the denervated muscle examined in the current study may have endured some degree of cellular and/or oxidative stress.

Insert Figure 10 ———→ HSP70, MnSOD, and CuZnSOD

DISCUSSION

Apoptosis, is a cellular process that is conserved from worms to humans. It has been extensively studied, but primarily in mitotic cells/tissues. Apoptosis plays a crucial role in a variety of biological events, including embryonic development, tissue turnover, and immunological defence (Thompson, 1995). Recently, there has been several studies suggesting that apoptosis may be involved in the loss of postmitotic skeletal muscle during denervation (Migheli *et al.*, 1997, Tews *et al.*, 1997, Yoshimura & Harii, 1999, Borisov & Carlson, 2000, Olive & Ferrer, 2000, Tang *et al.*, 2000, Jin *et al.*, 2001, Jejuri *et al.*, 2002, Alway *et al.*, 2003). These studies have demonstrated increases in TUNEL positive cells and BCL-2 family expression to denervation. In the present study, we have provided novel data that shows activation of mitochondria-associated apoptotic signalling in post mitotic skeletal muscle tissue. We found apoptotic DNA fragmentation, increase in Bax/Bcl-2 relative ratio, mitochondrial release of cytochrome c, Smac/DIABLO and AIF, increase in caspase-3 and -9 mRNA, active protein and proteolytic activity, decrease in XIAP, elevation of cleaved PARP protein, and upregulation of p53 in the 14 days denervated gastrocnemius muscle. Our data extend previous indications of apoptosis during denervation by clearly showing that mitochondria associated apoptosis pathways are activated by skeletal muscle denervation.

BCL-2 family and mitochondria-mediated apoptosis. Mitochondrion, an essential organelle, has been generally implicated to have an important apoptotic role in acting as a central modulator for the activation of the apoptotic machinery in response to a variety of apoptogenic stimuli (Desagher & Martinou, 2000, Kuwana & Newmeyer, 2003). Among the known apoptotic regulators in the mitochondria-mediated apoptosis, the BCL-2 protein family has been suggested to be a pivotal upstream regulator and constitutes a key intracellular checkpoint in the corresponding apoptotic signal transduction (Burlacu, 2003, Danial & Korsmeyer, 2004, Sharpe *et al.*, 2004). In particular, among the entire BCL-2 family, Bax, a pro-apoptotic member and Bcl-2, an anti-apoptotic member have been relatively well examined and have been designated to be the main protagonists in the regulation of apoptotic machinery. This designation is principally originated from the existing evidence showing that Bax can translocate to the mitochondria and expose its N-terminus via a conformational change upon induction of apoptosis (Wolter *et al.*, 1997, Desagher & Martinou, 2000). This conformational change allows the Bax/Bax-homo-oligomerization and therefore insertion of Bax into the outer mitochondrial membrane (Zha *et al.*, 1996). This is followed rapidly by the formation of a channel and subsequent release of the mitochondria-resided apoptogenic factors (e.g., cytochrome c, AIF, and Smac/DIABLO) into the cytosol. Conversely, the anti-apoptotic role of Bcl-2 has been established by showing that Bcl-2 is capable of forming Bcl-2/Bax-heterodimers which oppose the pro-apoptotic

activity of Bax by preventing the process of Bax/Bax-homo-oligomerization (Antonsson *et al.*, 2000, Burlacu, 2003, Danial & Korsmeyer, 2004, Sharpe *et al.*, 2004). Although the precise mechanism of Bax/Bcl-2-mediated apoptogenic factors release from the mitochondrial intermembrane space is still under active investigation, it is generally acknowledged that the relative content of pro-/anti-apoptotic BCL-2 family members provides a tight control in promoting the execution of apoptotic cascades (Desagher & Martinou, 2000, Burlacu, 2003, Kuwana & Newmeyer, 2003, Danial & Korsmeyer, 2004, Sharpe *et al.*, 2004). In accordance with this idea we have demonstrated that following denervation, the relative Bax/Bcl-2 ratio (both the mRNA and protein) are significantly elevated concomitant with the increased release of mitochondrial apoptogenic factors including cytochrome c, AIF, and Smac/DIABLO. Notably, we found that the anti-apoptotic Bcl-2 mRNA and protein content increased, rather than decreased as anticipated, in the denervated muscle and this observation appeared to be in contradiction to the overall pro-apoptotic changes that we have demonstrated in the denervated muscle as well as our previously reported Bcl-2 response to unloading in the quail hypertrophied muscle (Siu *et al.*, 2005). However, we interpret these observations of increase in Bcl-2 as an adaptive response of the skeletal muscle as an attempt to offset the pro-apoptotic stimulus provoked from denervation. In support to this interpretation, there have been studies reporting that the level of Bcl-2 in skeletal muscle increased concurrent with the elevation of Bax in response to denervation (Tews *et al.*, 1997, Jejurikar *et al.*, 2002). It is unclear whether our observed differences in the response of Bcl-2 between the denervated rat muscle and the unloaded quail hypertrophied muscle are attributed to the different natures of the experimental models (e.g., differences in innervation or the starting muscle level prior to the onset of the atrophy process) and it warrants further investigation. Nonetheless, our findings suggested that the relative content of the pro-/anti-apoptotic members (i.e., the ratio of Bax/Bcl-2) is a better predictor of apoptotic events than solely assessing the expression of individual levels of BCL-2 family members in denervated muscles.

Tumour suppressor p53 has been implicated as one of the regulators in apoptotic signal transduction, although it is unclear if this is by a transcription-dependent and/or -independent mechanism because both of these mechanisms are related to Bax (Schuler & Green, 2001, Chipuk *et al.*, 2004). Recently, we have shown that upregulation of p53 possibly contributes to part of the apoptotic signalling during unloading-induced muscle atrophy in quail hypertrophied muscle (Siu & Alway, 2004, Siu *et al.*, 2005). Here, we found that both the nuclear and cytosolic p53 protein contents are markedly elevated in parallel with the upregulation of Bax in the denervated muscle. These findings suggest that p53 may also be involved in the denervation-induced apoptotic signalling transduction in skeletal muscle. Nevertheless, more research is needed to reveal the possible regulatory role of p53 in the apoptosis-associated muscle atrophy during denervation. Interestingly, several regulatory factors shown to have roles in apoptosis in other models including Id2, c-Myc, and

CuZnSOD were not altered by denervation. These results are in accordance with our previous findings indicating that Id2 and c-Myc may not be directly involved in regulating apoptosis during denervation and unloading following hypertrophy, respectively (Alway *et al.*, 2003, Siu & Alway, 2004). Furthermore, we observed that variations in the response of MnSOD protein expression exist between the denervated rat muscle studied in here, and the quail unloaded muscle following hypertrophy studied previously (Siu & Alway, 2004). Since oxidative stress has been shown to be elevated during muscle disuse (Lawler *et al.*, 2003) and it may have an important role in regulating muscle atrophy (Powers *et al.*, 2005), it is reasonable to interpret our findings as due at least in part to a disturbance of redox balance in both the denervated rat muscle and the unloaded quail hypertrophied muscle (Siu & Alway, 2004).

Apoptotic cascade of caspases. A specific family of broadly conserved proteases named caspase (cysteine-dependent aspartate protease) has also been extensively investigated and advocated to be another important component in mediating the apoptotic signal transduction (Earnshaw *et al.*, 1999, Grutter, 2000, Chang & Yang, 2000, Degterev *et al.*, 2003). With the exception of caspase-9 (Stennicke *et al.*, 1999), caspases are normally synthesized and present in the cell as inactive zymogens termed procaspases. Structural and biochemical analyses revealed that the activation of procaspases requires self-/auto-activation through oligomerization (for some initiator caspases), scaffold-mediated transactivation, or limited proteolytic cleavage by upstream proteases for the acquisition of the protease activity once upon triggered by an apoptotic signal (Earnshaw *et al.*, 1999, Chang & Yang, 2000). As categorized as an initiator caspase, caspase-9 has been demonstrated to mediate the mitochondrial apoptosis by assembling an apoptosome complex through the interaction of procaspase-9 with Apaf-1, dATP/ATP, and mitochondrial released-cytochrome c, which subsequently recruits procaspase-3 to the complex and efficiently activates caspase-3, a common effector caspase, by the caspase-9-mediated proteolytic cleavage (Chang & Yang, 2000, Acehan *et al.*, 2002, Shi, 2002a). A variety of cellular molecules including proteins for DNA metabolism and repair (e.g., PARP and ICAD or inhibitor of caspase-activated DNase), signalling transduction (e.g., Akt/PkB), cell cycle regulation (e.g., p21^{Cip1/Waf1}), cytoskeletal scaffold (e.g., gelsolin), and cell death (e.g., Bcl-2 and IAP) are the potential cleavage substrates for caspase-3 or other effector caspases (Chang & Yang, 2000). In the current study, we have demonstrated that cytochrome c was significantly released into the cytosol while both caspase-3 and -9 increased based upon the levels of mRNA, active protein, as well as enzyme activity in the denervated muscle. These changes indicate that the mitochondria-mediated apoptotic caspase cascade was activated in the muscle following denervation. In agreement with this observation we have also observed that the cleaved protein content of PARP (a cleavage substrate for caspase-3) elevated in company with the increase in the extent of apoptotic DNA fragmentation as assessed by a cytosolic nucleosome ELISA in the denervated muscle.

As a modulating mechanism for the cascade of caspases, the proteolytic activity of both initiator and effector caspases is subject to the suppression by an anti-apoptotic protein family called IAP (Deveraux *et al.*, 1998, Earnshaw *et al.*, 1999, Chang & Yang, 2000). This IAP-mediated inhibition of caspases is repressed by Smac/DIABLO, a mitochondria-resided apoptotic protein which is released into the cytosol to promote apoptosis by neutralizing the inhibitory effect of multiple IAPs (Shi, 2002b). XIAP is one of the potent IAP members in inhibiting caspase-3, -7, and -9 (Deveraux *et al.*, 1998, Earnshaw *et al.*, 1999, Chang & Yang, 2000, Shi, 2002b). We have shown that the protein content of XIAP was down regulated while the release of mitochondrial Smac/DIABLO increased in concomitant with the elevation of the caspase-3 and -9 protease activity in the muscle following denervation. These data demonstrate that the cascade of caspases and the XIAP-Smac/DIABLO-mediated modulating mechanism are involved in the activated apoptotic signal transduction in the denervated muscle.

While our data shown that the caspase cascade indisputably plays an important role in the apoptotic signal transduction in denervated muscle, it is possible that there has been suggested that apoptosis can be executed without the involvement of caspase in certain experimental model (Cande *et al.*, 2002, Jaattela, 2002). Since caspase-independent apoptotic signalling is primarily driven by specific mitochondria-housed apoptotic factors (e.g., AIF and endonuclease G) that are released into the cytosol during apoptosis (Cande *et al.*, 2002, Jaattela, 2002), the striking elevation of the AIF protein content in the mitochondria-free cytosol of our examined denervated muscle prompted a reasonable suspicion that the caspase-independent machinery may have contributed to the activated apoptotic signalling during denervation. Although we failed to acquire evidence showing nuclear translocation of AIF, it is unclear that the increase in cytosolic AIF may have functioned to the apoptotic signal transduction in the absence of nuclear translocation during muscle denervation. Nonetheless, additional research is required to inspect the suspicious role of AIF in promoting the denervation-induced apoptosis in skeletal muscle.

Inflammation caused by surgical trauma has been implicated as a confounding factor in several experimental muscle models (e.g., tenotomy and muscle ablation) (Armstrong *et al.*, 1979, Lowe & Alway, 2002). However, the present muscle denervation model induces relatively lower levels of trauma, because there is less bleeding during surgery resulting in less post-surgery inflammation when compare with other models (Lowe & Alway, 2002, Alway *et al.*, 2005). In addition, the inflammatory response has been shown to peak at 1-5 d post-surgery in the muscle ablation model, a model causes severe inflammation, and the response subsides to control level by 16 d after surgery (Armstrong *et al.*, 1979). Taken together, it is unlikely that our results are masked by the post-surgery inflammation.

Some non-muscle cell populations (e.g., endothelia and fibroblasts) are included in the muscle tissue homogenates that we used to study apoptosis in this study. Nevertheless, it is not likely that the marked changes in our study are the results of major contributions by these non-muscle cells due to their small proportion relative to the skeletal myocytes in our homogenates. Furthermore, most models of decreased neuromuscular activity and possibly denervation are known to produce a slow to fast fibre transformation (Hamalainen & Pette, 2001, Geiger *et al.*, 2003). However, the short-term denervation used in this study is unlikely to determine a large change in fibre type distribution, and therefore our results are not likely to be significantly affected by the factor of fibre transformation.

In conclusion, we have identified mitochondrial-apoptotic signalling in denervated skeletal muscle (Summarized in **Figure 11**). Our data demonstrate increases in BCL-2 family proteins, mitochondria-mediated apoptosis regulators, caspase family proteases, caspase modulators, caspase-independent apoptogenic factor, and apoptosis-associated cellular regulatory factors to muscle denervation. We show that the pro-apoptotic increases in DNA fragmentation, and increases in Bax/Bcl-2 ratio, mitochondrial cytochrome c, Smac/DIABLO, AIF release, and upregulation of caspase-3 and -9 mRNA, active protein and protease activity, diminished XIAP, and increased cleaved PARP following 14 days of denervation. In addition to the signs of cellular stress, including increased HSP70 and diminished MnSOD, we found that p53 was upregulated concomitant with the pro-apoptotic signalling during denervation. These observations raise the possibility that p53 may contribute to denervation-induced muscle remodelling. These findings support the hypothesis that apoptosis has a physiologic role in the regulation of the muscle loss during skeletal muscle denervation. It has been proposed that apoptosis may occur at the level of nuclei in addition to the death of whole multinucleated myofibres. This so called “nuclear apoptosis” is hypothesized to have a role in the regulation of muscle remodelling based on the concept of myonuclear domain (Allen *et al.*, 1997, Leeuwenburgh *et al.*, 2005). In the present study, although the extent of myofiber death or the number of apoptotic nuclei were not determined, our cell death ELISA data indicate a significant increase in apoptosis DNA fragmentation in muscle following 14 d of denervation.

It has been shown that skeletal muscle may exhibit distinct ultrastructural apoptotic characteristics following long term denervation (Borisov & Carlson, 2000). These findings raise the possibility that multinucleated myocytes may execute the apoptotic program differently from classical apoptosis. Although we did not use electron microscopy to examine classical apoptotic features in the denervated myocytes of the current study, our data show increased DNA laddering, and direct evidence of mitochondrial apoptotic signalling in denervated skeletal muscle. Collectively, our data indicate that “classical” mitochondria-associated apoptosis is well conserved in mature denervated skeletal muscle.

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Figure 1 Verification of protein extract purity

Legend for Figure 1 Verification of purity of protein extraction. The purity of extracted protein fractions was verified by immunoblotting the extracted fractions with an anti-histone H2B, an anti-CuZnSOD, or an anti-MnSOD antibody, and assessing the citrate synthase activity. NP, nuclear protein fraction; Total CP, total cytosolic protein fraction; Mito-free CP, mitochondria-free cytosolic protein fraction.

Figure 2 Apoptotic DNA fragmentation

Legend for Figure 2 Apoptotic DNA fragmentation. The extent of apoptotic DNA fragmentation was determined by measuring the cytosolic mono- and oligo-nucleosomes. The data are presented as means $\pm$ standard error of mean (SE) of percent relative to CON (i.e., CON refers to 100%). * $P < 0.05$, data are significantly different from CON. CON, control muscle; DEN, denervated muscle.

Figure 3 Bcl-2, Bax, and Bok

Legend for Figure 3 Bcl-2, Bax, and Bok. The content of mRNA and protein was examined by RT-PCR and Western immunoblot, respectively, in the CON and DEN muscles. The insets show representative results for the mRNA and protein. The data are presented as means $\pm$ SE of percent relative to CON (i.e., CON refers to 100%). *P < 0.05, **P < 0.01, data are significantly different from CON.

Figure 4 Cytochrome c and Apaf-1

Legend for Figure 4 Cytochrome c and Apaf-1. The release of mitochondrial cytochrome c was estimated by measuring the protein content of cytochrome c in the extracted mitochondria-free cytosolic fraction using an ELISA. The mRNA and total cytosolic protein content of Apaf-1 was determined by RT-PCR and Western immunoblot, respectively. The insets show representative results for the mRNA and protein of Apaf-1. The data are presented as means $\pm$ SE of percent relative to CON (i.e., CON refers to 100%). * $P < 0.05$, data are significantly different from CON. Mito-free cytosolic protein, mitochondria-free cytosolic protein.

Figure 5 Caspase-3 and -9

Legend for Figure 5 Caspase-3 and -9. The mRNA and protein content of caspase-3 and -9 was determined by RT-PCR and Western immunoblot, respectively. The enzyme activity was assessed by a fluorometric caspase activity assay. The insets show representative results for the mRNA and protein. The data are presented as means $\pm$ SE of percent relative to CON (i.e., CON refers to 100%). * $P < 0.05$, ** $P < 0.01$, data are significantly different from CON.

Figure 6 **XIAP and Smac/DIABLO protein**

Legend for Figure 6 XIAP and Smac/DIABLO protein. The protein content of XIAP and Smac/DIABLO was measured by Western immunoblot in the total cytosolic and mitochondria-free cytosolic fraction, respectively. The insets show representative blots. The data are presented as means $\pm$ SE of percent relative to CON (i.e., CON refers to 100%). *P < 0.05, **P < 0.01, data are significantly different from CON.

Figure 7 **AIF**

Legend for Figure 7 **AIF**. The mRNA and protein content of AIF was evaluated by RT-PCR and Western immunoblot, respectively. The mitochondria-free cytosolic and nuclear AIF protein content indicated the extent of mitochondrial release and nuclear translocation of AIF, correspondingly. The insets show representative results for the mRNA and protein. The data are presented as means $\pm$ SE of percent relative to CON (i.e., CON refers to 100%). *P < 0.05, data are significantly different from CON.

Figure 8 **Cleaved PARP**

Legend for Figure 8 Cleaved PARP. The cleaved PARP protein content was determined by Western immunoblot in the CON and DEN muscles. The insets show representative blots for the cleaved PARP protein. The data are presented as means $\pm$ SE of percent relative to CON (i.e., CON refers to 100%). *P < 0.05, data are significantly different from CON.

Figure 9 p53, Id2, and c-Myc protein

Legend for Figure 9 p53, Id2, and c-Myc protein. The protein content of p53, Id2, and c-Myc was determined by Western immunoblot. The p53 and Id2 were measured in both the nuclear and total cytosolic fraction while c-Myc was determined in the nuclear fraction. The insets show representative blots. The data are presented as means $\pm$ SE of percent relative to CON (i.e., CON refers to 100%). *P < 0.05, data are significantly different from CON.

Figure 10 HSP70, MnSOD, and CuZnSOD protein

Legend for Figure 10 HSP70, MnSOD, and CuZnSOD protein. The protein content of HSP70, MnSOD, and CuZnSOD was determined in the total cytosolic fraction by Western immunoblot. The insets show representative blots. The data are presented as means $\pm$ SE of percent relative to CON (i.e., CON refers to 100%). * $P < 0.05$, ** $P < 0.01$, data are significantly different from CON.

Figure 11

Legend for Figure 11 Proposed activated mitochondrial apoptotic signal transduction in denervated skeletal muscle. Following 14-days of denervation, apoptosis is evident based on the elevation of apoptotic DNA fragmentation and PARP cleavage. Regarding the mitochondrial apoptotic signalling, pro-apoptotic Bax dramatically increases while anti-apoptotic Bcl-2 is modestly upregulated resulting in an increase in Bax/Bcl-2 ratio. Cytosolic release of mitochondria-resided apoptotic factors including cytochrome c (Cyto c) and Smac/DIABLO are found concomitant with the activation of caspases (Casp)-9 and -3. XIAP, a potent inhibitor on caspases is down regulated whereas p53 markedly elevates in both nuclei and cytosol. Moreover, mitochondrial AIF is found to release to cytosol in the absence of nuclear translocation. ICAD, inhibitor of caspase-activated DNase, CAD, caspase-activated DNase.

Table 1. Primers used for PCR amplification of cDNA

Product	Accession No.	Sequence	Position	T _A , °C	Cycles	Product length, bp	Restriction enzyme	Restriction products, bp
Bcl-2	NM_016993	F: 5'-CCGGGACGCGAAGTGCTATTGGTAC-3' R: 5'-AGCTGATTGACCAATTTGCCTGAA-3'	187-211 1113-1136	55	39	950	<i>Bam</i> HI <i>Bgl</i> II <i>Bst</i> XI <i>Dra</i> I	602, 348 686, 264 572, 438 865, 85
Bax	AF235993	F: 5'-GCACCCCTTTCCTCCTCTCTCCACCAG-3' R: 5'-TGCCTTTCCCGTTCCCCATTTCATC-3'	462-488 1091-1115	55	39	654	<i>Bam</i> HI <i>Pst</i> I <i>Bst</i> XI	445, 209 431, 223 521, 133
AIF	AF375656	F: 5'-CCGGCTTCCAGGCAACTTGTTC-3' R: 5'-CCCGGATGGATCTAGCTGCTGCA-3'	93-115 429-451	58.1	33	359	<i>Kpn</i> I <i>Pst</i> I	290, 69 259, 100
Apaf-1	AF218388	F: 5'-CGGCCCTGCGCATCTGATTTCAT-3' R: 5'-GGGCGAACGACTAAGCGGGACAG-3'	1623-1644 1888-1910	57.8	36	288	<i>Alu</i> I <i>Pst</i> I	193, 95 198, 90
Caspase-3	NM_012922	F: 5'-TGGCCCTGAAATACGAAGTC-3' R: 5'-CGGCCTCCACTGGTATCT-3'	262-281 583-600	53.3	36	339	<i>Rsa</i> I <i>Spe</i> I <i>Alu</i> I	260, 79 183, 156 266, 73
Caspase-9	NM_031632	F: 5'-GGCCGGTGGACATTGGTTCTGG-3' R: 5'-CCATGAAGCGCAGCCAGCAGAA-3'	543-564 743-764	55	36	222	<i>Bam</i> HI <i>Hind</i> III	130, 92 186, 36

T_A, annealing temperature; Accession No., GenBank accession number; F, forward primer; R, reverse primer.

CHAPTER 2

Apoptotic responses to hindlimb suspension in gastrocnemius muscles from young adult and aged rats

ABSTRACT

Hindlimb suspension-induces extensive muscle atrophy in plantar flexor muscles that contain predominantly slow-twitch fibers (e.g., soleus). However, plantar flexor muscles containing a high percentage of fast twitch fibers (e.g., gastrocnemius) also atrophy. Although apoptosis has been demonstrated in soleus during hindlimb suspension, it is not known whether apoptosis is also involved in the loss of muscles dominated by mixed fibers. Moreover, the effect of aging on suspension-induced apoptosis is unclear. Therefore, we examined the apoptotic responses in gastrocnemius muscles of young adult and aged Fischer₃₄₄×Brown Norway rats after acute hindlimb suspension. Following 14 days of suspension, medial gastrocnemius muscle wet weight significantly decreased by 30% and 32%, and muscle wet weight normalized to the animal bodyweight decreased by 11% and 15% in young adult and aged animals, respectively. As determined by a cytosolic nucleosome ELISA, the extent of apoptotic DNA fragmentation increased by 119% and 61% in suspended gastrocnemius muscles from young and aged rats, respectively. RT-PCR analysis indicated that the transcriptional expression of Bax was elevated by 73% in young muscle following suspension. According to our immunoblot analyses, Bax and Bcl-2 protein levels were greater in the suspended muscle relative to the control sample in both young and aged animals. As demonstrated by the analyses of ELISA and immunoblots the level of cytosolic mitochondria-resided apoptotic factor cytochrome c was significantly increased in the mitochondria-free cytosol of suspended muscles from both young and aged rats. In contrast, the release of AIF, a caspase-independent apoptogenic factor, was exclusively expressed in the suspended muscles from aged rats. Our data also show that aging favors the pro-apoptotic tendencies in skeletal muscle by altering the contents of Bax, Bcl-2, Apaf-1, AIF, caspases, XIAP, Smac/DIABLO, and cytochrome c. These results further clarify the response of apoptosis to hindlimb suspension-induced muscle disuse by demonstrating that apoptosis is activated not only in slow-twitch soleus muscle, but also in the mixed-fibered (predominately fast fibered) gastrocnemius muscle. Moreover, our data are consistent with the hypothesis that different apoptotic mechanisms may be responsible for the activation of apoptosis in young adult and aged gastrocnemius muscles during hindlimb suspension.

Key words: Muscle disuse; sarcopenia; programmed cell death

INTRODUCTION

Skeletal muscle is a highly adaptable tissue, which undergoes hypertrophy and atrophy in response to overload and disuse, respectively. An inevitable consequence of normal aging is a significant loss of muscle mass and strength (i.e., sarcopenia). Sarcopenia impairs musculoskeletal function and decreases independence in elderly populations (18). Although the etiology of sarcopenia is not fully understood, it is known that considerable muscle fiber atrophy and decrease in total fiber number contribute to the sarcopenic muscle loss (12). Aging diminishes muscle function and also attenuates the hypertrophic capability to overload (6,8). Furthermore, during muscle disuse, it has been demonstrated that the extent of losses in muscle mass and performance may be exacerbated in muscles from aged animals when compared to muscles from young animals (4,9). Since aging is an inevitable process it is important to unravel the underlying mechanisms for the development of sarcopenia if we are to eventually develop strategies that will slow or prevent muscle loss and thereby improve the quality of living in old age.

Apoptosis is an essential biological process which functions in maintaining the homeostasis of cell survival/death, tissue turnover, and other fundamental physiological events (15,36). Recently, there has been consistent evidence showing that apoptosis is activated or accelerated in skeletal muscle during disuse and with normal aging (1-3,5,10,16,17,26,28,29,31,35). Although the importance of apoptosis in muscle atrophy has yet to be resolved, these findings support the hypothesis that apoptosis may have a physiologic role in regulating the process of muscle loss in response to muscle disuse. Indeed, it has been suggested that apoptosis can occur within a particular myonucleus without influencing the entire myofiber and this may function to mediate the elimination of individual myonuclei during muscle disuse in order to coordinate the size of myonuclear pool (perhaps maintaining the nuclei to cytoplasm relationship) and therefore muscle atrophy (1,17,31). However, there are also findings showing that myonuclear number does not change with hindlimb suspension-induced atrophy in muscle of aged animal and this suggests that the homeostatic balance of myonuclear domain may be perturbed in aged skeletal muscle (13,17). Furthermore, it has been exhibited that aging may influence the apoptotic response to unloading-induced muscle atrophy (17,31). Altogether, aging presumably complicates a number of events (e.g., myonuclei homeostasis and apoptotic regulation) that have been proposed to be involved in mediating the process of atrophy.

Although the activation of apoptosis has been demonstrated in hindlimb suspended soleus muscle (1,17), it is unclear whether apoptosis is also involved in muscles composed of primarily relatively mixed fiber types (e.g., gastrocnemius) as these mixed-fibers muscles also atrophy during hindlimb suspension. Moreover, there is a lack of data examining the influence of aging in suspension unloading-induced apoptosis. Therefore, this study examined the responses of apoptosis and apoptotic

regulatory factors to 14 days of hindlimb suspension in young adult and aged gastrocnemius muscles. We tested the hypotheses that apoptosis is associated with the hindlimb suspension-induced mixed fiber-muscle loss and those apoptotic responses to suspension are age-dependent.

METHODS

Animals. Experiments were conducted on ~6-mo old young adult and 30-mo old aged Fischer₃₄₄×Brown Norway rats (Harlan, Indianapolis, IN). The rats were housed in pathogen-free conditions at ~20°C and were exposed to a reverse light condition of 12:12 h of light:darkness each day. They were fed rat chow and water ad libitum throughout the study period.

Hindlimb suspension procedure. The animals were randomly assigned to suspension group (Young n=10, Aged n=10) or control group (Young n=10, Aged n=8). The procedure of hindlimb suspension described by Morey-Holton and Globus (20) was adopted in the present study. In brief, an adhesive (tincture of benzoin) was applied to the tail, air-dried, and an orthopedic tape was put along the proximal one-third of the tail. This practice distributed the load evenly and avoided excessive tension on a small area to the tail. The tape was then placed through a wire harness that was attached to a fishline swivel at the top of a specially designed N.A.S.A.-approved hindlimb suspension cage. This provided the rats with 360° of movement around the cage and the forelimbs maintained contact with a grid floor allowed the animals to move and assess food and water freely. Sterile gauze was wrapped around the orthopedic tape and was subsequently covered with a thermoplastic material, which formed a hardened cast (Vet-Lite, Veterinary Specialty Products, Boca Raton, FL). The distal tip of the tail was examined to verify that the procedure did not occlude the blood flow to the tail (i.e., tail remained pink). The suspension height was adjusted to prevent the animal's hindlimb from touching any supportive surface, with care taken to maintain a suspension angle of approximately 30° (14). The suspension height and animal behavior were monitored daily. Control animals were allowed to move unconstrained around the cages. Following 14 days of suspension, animals were killed with an overdose of xylazine. Medial gastrocnemius muscles from the hindlimbs were excised, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and stored at -80°C until used for analyses.

All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and following the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals as published by the U.S. Department of Health and Human Services and proclaimed in the Animal Welfare Act (PL89-544, PL91-979, and PL94-279).

RT-PCR. Total RNA was extracted from the medial gastrocnemius muscle of both suspended and control animals with TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Frozen muscle was mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA were solubilized in RNase-free H₂O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assured by examining OD₂₆₀/OD₂₈₀ ratio. Ten micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 30 µl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). Control RT reaction was done in which the RT enzyme was omitted. The control RT reaction was PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of complementary DNA (cDNA) was then amplified by PCR using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, TX), 250 µM deoxyribonucleotide triphosphates (dNTPs), 1 x PCR buffer, and 2.5 units *Taq* DNA polymerase (USB Corp., Cleveland, OH) in a final volume of 50 µl. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). The primer pairs were designed from sequences published in GenBank (**Table 1**) and PCR products were verified by restriction digestions. Preliminary experiments were conducted with each gene to assure that the number of cycles represented a linear portion for the PCR optical density curve for the muscle samples. The cDNA from all muscle samples were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 30 µl of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide. Images were captured and the signals were quantified in arbitrary units as optical density (OD) x band area using Kodak image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. Ribosomal 18S primers were used as internal controls while all RT-PCR signals were normalized to the 18S signal of the corresponding RT product to eliminate the measurement error from uneven samples loading and provide a semi-quantitative measure of the relative changes in gene expression.

Subcellular protein fractionation. The fractionation method described by Rothermel et al. was adopted with minor modification to extract the cytosolic and nuclear protein fractions from the gastrocnemius muscle. We have previously obtained the fractionated cytosolic and nuclear proteins from skeletal or heart muscles using this modified protocol (28-31). Briefly, after removal of connective tissues, 400 mg of muscle was homogenized on ice in 1.5 ml lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT). Following centrifuging at 5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and these supernatants were further centrifuged three times at 6,000 rpm

for 5 min at 4°C to remove residual nuclei. The final collected supernatants were stored as nuclei-free total cytosolic protein fraction. A portion of this collected cytosolic extract (without addition of protease inhibitors) was stored and used for fluorometric caspase protease activity assay. A protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO) was added to the remaining portion. The cytosolic protein fraction with the addition of protease inhibitors was used for cell death ELISA and Western immunoblots. The remaining nuclear pellets were washed 3 times with ice-cold lysis buffer, resuspended in 420 µl of lysis buffer in the presence of 58.1 µl of 5 M NaCl and protease inhibitor cocktail, and rotated for 2 h at 4°C to lyse the nuclei. Following a spin at 15,000 rpm for 15 min at 4°C, the supernatants were collected and stored as cytosol-free nuclear protein fraction.

Furthermore, in order to estimate the release of mitochondria-housed apoptotic factors including cytochrome c, AIF, and Smac/DIABLO to the cytosol, a nuclei-free, mitochondria-free cytosolic protein fraction was prepared as described by Rokhlin et al (23) and the protein contents of these mitochondrial apoptotic factors were measured in this mitochondria-free cytosolic fraction as described in the later sections. Muscle was dissected from the connective tissues and minced in ice-cold extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF, pH 7.4) in the presence of protease inhibitor cocktail. Following a gentle homogenization with a Teflon pestle motorized with an electronic stirrer, homogenates were centrifuged at 800 × g for 10 min at 4°C to pellet the nuclei and cell debris. The supernatants were then spun twice at 16,000 × g for 20 min at 4°C to pellet the mitochondria and the final supernatants were collected as nuclei-free, mitochondria-free cytosolic protein fractions. The above described subcellular protein fractionation procedures have been routinely used in our laboratory to obtain high purity protein fractions as assessed by immunoblotting the fractions with an anti-histone H2B (a nuclear protein), an anti-CuZnSOD (a cytosolic isoform of superoxide dismutase), and an anti-MnSOD (a mitochondrial isoform of superoxide dismutase) antibody (29,31).

The protein contents of the protein extracts were quantified in duplicate by *DC* Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay (19). As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation (32).

Apoptotic cell death ELISA. Cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligo-nucleosomes. Briefly, the extracted nuclei-free cytosolic fraction of gastrocnemius muscle was used as an antigen source in a sandwich

ELISA with a primary anti-histone mouse monoclonal antibody coated to the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate for 20 min at 20°C. The change in color was measured at a wavelength of 405 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). Measurements were performed with control and suspended samples analyzed on the same microtiter plate in the same setting. The OD₄₀₅ reading was then normalized to the mg of protein used in the assay and presented as an apoptotic index.

Fluorometric caspase activity assay. 50 µl of the total cytosolic protein fraction without protease inhibitor of the muscles was incubated in 50 µl of assay buffer (50 mM PIPES, 0.1 mM EDTA, 10% glycerol, 10 mM DTT, pH 7.2) with 50 µM of the fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated substrate (Ac-DEVD-AFC for caspase-3, Ac-LEHD-AFC for caspase-9, Alexis Corp., San Diego, CA) at 37°C for 2 h. Caspase specific inhibitor, Z-VAD-FMK (Calbiochem, La Jolla, CA) was used as a control to validate the specificity of caspase. The change in fluorescence was measured on a spectrofluorometer (CytoFluor, Applied Biosystems, Foster City, CA) with an excitation wavelength of 390/20 nm and an emission wavelength of 530/25 nm before and after the 2 h incubation. Caspase activity was estimated as the change in arbitrary fluorescence units normalized to milligram protein used in the assay. Control and suspended samples were run on the same microplate in the same setting.

Western immunoblot analyses. Protein expression of Bcl-2 associated X protein (Bax), B-cell leukemia/lymphoma-2 (Bcl-2), X-linked inhibitor of apoptosis (XIAP), X-link inhibitor of apoptosis protein (XIAP), apoptosis repressor with caspases recruitment domain (ARC), and [Fas-associated death domain protein-like interleukin-1β-converting enzyme]-like inhibitory protein (FLIP) was determined in the total cytosolic protein fraction while apoptosis inducing factor (AIF) was measured in the nuclear fraction.

Sixty micrograms of protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and probed with the following primary antibodies diluted in PBS-T with 2% BSA: anti-Bcl-2 mouse monoclonal antibody (1:100 dilution, sc-7382), anti-Bax rabbit polyclonal antibody (1:200 dilution, sc-6236), anti-hILP/XIAP mouse monoclonal antibody (1:250 dilution, 610762, BD Biosciences, San

Jose, CA), anti-ARC rabbit polyclonal antibody (1:200 dilution, sc-11435), anti-FLIP α rabbit polyclonal antibody (1:500 dilution, ab6144, Abcam, Cambridge, MA) or anti-AIF mouse monoclonal antibody (1:500 dilution, sc-13116HRP). Bcl-2, Bax, ARC, and AIF antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed by West Pico chemiluminescent substrate (Pierce, Rockford, IL) or ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Estimation of mitochondrial cytochrome c, Smac/DIABLO, and AIF release.

Cytochrome c, AIF, and Smac/DIABLO (second mitochondria-derived activator of caspase) are apoptotic factors normally confined to mitochondria and their release into the cytosol has been demonstrated during the activation of apoptosis (34). In the present study, the release of Smac/DIABLO and AIF into the cytosol was estimated by measuring their protein contents in the extracted mitochondria-free cytosolic protein fraction by immunoblotting with an anti-Smac/DIABLO mouse monoclonal antibody (1:500 dilution, 612244, BD Biosciences, San Jose, CA) and an anti-AIF monoclonal mouse antibody. Moreover, a cytochrome c ELISA kit (MBL International, Woburn, MA) was used to assess the protein content of cytochrome c in the mitochondria-free cytosol fraction to evaluate the release of the mitochondrial cytochrome c into the cytosol. By following the manufacturer's protocol, 60 μ l the extracted mitochondria-free cytosolic fraction was used as an antigen source in a sandwich ELISA with a horseradish peroxidase-conjugated anti-cytochrome c polyclonal antibody in microwell strips coated with an anti-cytochrome c antibody. After washing, the peroxidase retained in the immunocomplex was detected by incubating with a chromogenic substrate, tetramethylbenzidine/hydrogen peroxide (TMB/H₂O₂) followed by adding an acid solution to terminate the enzyme reaction and to stabilize the developed color. The change in color was monitored at a wavelength of 450 nm using a Dynex MRX plate reader. Measurements were performed with the control and suspended samples analyzed on the same microplate and the cytochrome c content was expressed as OD₄₅₀ per mg protein.

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package. ANOVA was performed to examine the main effects of suspension, age, and interaction

(suspension $\times$ age) on the measured variables. ANOVA followed by Tukey HSD post hoc analysis was used to examine differences between groups. All data are given as means $\pm$ standard error of mean (SE). Statistical significance was accepted at $P < 0.05$.

RESULTS

Muscle Mass. The degree of sarcopenic muscle loss was estimated by comparing the medial gastrocnemius muscle wet weight between the young adult and aged control animals. The muscle wet weight of control aged rats was 23% lower and the muscle weight normalized to the animal bodyweight was 48% lower than that of young adult rats (muscle wet weight: 993 vs. 761 mg; normalized muscle weight: 2.7 vs. 1.4 mg/g) (**Table 2**). Following 14 days of hindlimb suspension, the medial gastrocnemius muscle wet weight was decreased by $\sim 30\%$ in both young adult and aged rats (Young: 993 vs. 699 mg; Aged: 761 vs. 520 mg) while the muscle weight normalized to the animal bodyweight was reduced by 11% and 19% in young adult and aged rats, respectively (Young: 2.7 vs. 2.4 mg/g; Aged: 1.4 vs. 1.1 mg/g) (**Table 2**).

Insert Table 2 $\longrightarrow$ Muscle Mass

Apoptotic DNA fragmentation. Consistent with the previous findings showing that apoptosis is accelerated in skeletal muscle with aging (10,17), our quantitative cell death ELISA analysis indicated that there was a ~ 4 -fold elevation in the extent of apoptotic DNA fragmentation in the aged control gastrocnemius muscle relative to the young adult muscle (**Figure 1**). As a result of 14 days hindlimb suspension, the extent of the apoptotic DNA fragmentation in the suspended muscle was 119% and 61% higher than that in the control muscle of the young and aged animals, respectively (**Figure 1**).

Insert Figure 1 $\longrightarrow$ Apoptotic DNA fragmentation

BCL-2 family: Bax and Bcl-2. As estimated by the RT-PCR and western immunoblot, the mRNA and protein contents of pro-apoptotic Bax and anti-apoptotic Bcl-2 in the aged muscle were generally greater than the young adult muscle (**Figure 2**). In the young animals, the suspended muscles had a 73% greater Bax mRNA content when compared to the control muscle (**Figure 2A**). The Bax protein content increased by 43% and 22% following suspension in the young and aged muscle, respectively (**Figure 2B**). The Bcl-2 protein content in the muscle following suspension was 55% and 33% higher than the control muscle of the young and aged animals, respectively (**Figure 2D**).

Insert Figure 2 ———→ Bax and Bcl-2

Mitochondria-mediated apoptotic factors: mitochondrial cytochrome c release and Apaf-1 mRNA content. The ELISA analysis on the mitochondria-free cytosolic fraction indicated that the protein content of cytosolic cytochrome c in the suspended muscle was elevated by 41% and 31% when compared to the control muscle from the young and aged animals, respectively (**Figure 3**). Given that cytochrome c mainly resides in the mitochondria under normal condition, this indicated that cytochrome c was relocated to the cytosol in response to suspension. By comparing the cytochrome c content in the mitochondria-free cytosolic lysate between young and aged muscles, we found that aging resulted in a greater mitochondrial release of cytochrome c into the cytosol in gastrocnemius muscle (**Figure 3A**). Although we did not find any difference in the mRNA content of Apaf-1 between the suspended and control samples in the young and aged muscles, there was a greater content of Apaf-1 mRNA in the aged muscle when compared to the young muscle (**Figure 3B**).

Insert Figure 3 ———→ Cytochrome c release and Apaf-1 mRNA

Caspase-3 and -9: mRNA content and protease activity. Hindlimb suspension did not cause any changes in the mRNA content or protease activity of caspase-3 and -9 in both young and aged gastrocnemius muscles ($P > 0.05$, **Figure 4**). However, the mRNA content and protease activity of caspase-9 in the aged muscle was higher than the young muscle (**Figure 4C and 4D**) while the protease activity of caspase-3 appeared to be elevated in the aged muscle relative to the young muscle (**Figure 4B**).

Insert Figure 4 ———→ Caspase-3 and -9

Mitochondrial apoptogenic factors Smac/DIABLO and AIF. The release of mitochondrial Smac/DIABLO and AIF was estimated by using immunoblot analyses on the mitochondria-free cytosolic fraction. Although we did not find changes in the release of Smac/DIABLO with suspension ($P > 0.05$, **Figure 5A**), we observed that the protein content of cytosolic Smac/DIABLO in the aged muscle was higher than the young muscle (**Figure 5A**). While the AIF mRNA content was greater in the aged muscle when compared to the young muscle (**Figure 5B**), we found that, in the aged animals, the mitochondria-free cytosolic AIF protein content in the suspended muscle was 45% greater than the control muscle and this suspension-induced increase in the mitochondrial AIF release was not observed in the young muscle (**Figure 5C**). The AIF protein content of the nuclear fraction was not

different between the suspended and control samples of young and aged animals (**Figure 5D**).

Insert Figure 5 ———→ Smac/DIABLO and AIF

Apoptotic suppressor XIAP, ARC, and FLIP. No difference in the mRNA and protein content of XIAP, ARC, and FLIP was found between the suspended and control muscles in both the young and aged animals ($P > 0.05$, **Figure 6**). The mRNA and protein content XIAP in the aged muscle was higher than the young muscle (**Figure 6A and 6B**).

Insert Figure 6 ———→ XIAP, ARC, and FLIP

DISCUSSION

Age-related apoptotic issues during acute (e.g., 14 days) hindlimb suspension have been recently examined in the rodent soleus muscle. This muscle predominately contains slow-twitch fibers and it undergoes severe atrophy during hindlimb suspension (17). Terminal deoxynucleotidyl transferase (TdT) histochemical findings first suggested that the activation of the apoptotic program may explain in part, soleus muscle loss in young rats in response to 14 days of hindlimb suspension (1). Furthermore, Leeuwenburgh and colleagues showed that apoptosis, as estimated by TUNEL staining and apoptotic DNA fragmentation, is also elevated in the soleus muscles from aged rats following 14 days of hindlimb suspension (17). More importantly, they demonstrated that the suspension-induced activation of apoptosis maybe mediated through different apoptotic mechanisms (i.e., caspase-dependent or -independent pathways) in the young and aged soleus muscles (17). Our study has extended these findings by showing that increased apoptosis is also evident in gastrocnemius muscle (a muscle that is predominately, but not exclusively composed of fast fibers and undergoes moderate muscle loss during hindlimb suspension) of both young and aged rats after 14 days of hindlimb suspension. Also, we found that the release of mitochondrial AIF, a caspase-independent apoptogenic factor, is evident exclusively in the suspended aged muscles while this is not found in the suspended muscle of young rats. These novel observations support the hypotheses that aging complicates the apoptotic regulation with suspension-induced muscle atrophy and different apoptotic mechanisms may be involved in activating apoptosis in young and aged skeletal muscles during hindlimb suspension. Additionally, our data further clarify the components responsible for the activation of apoptosis with sarcopenic muscle loss in the aged rodent gastrocnemius muscle.

Age-specific apoptotic responses to hindlimb suspension in gastrocnemius muscle.

Apoptosis has been implicated to have a physiologic role in regulating the process of skeletal muscle wasting. This idea was primarily based on the consistent observations that apoptosis is activated or

accelerated under various muscle atrophic conditions including hindlimb suspension-mediated hypokinesia, muscle unloading, muscle denervation, muscle dystrophy, neuromuscular disorders, strenuous physical exercise, and aging-associated sarcopenia (1,2,10,17,22,24-27,29,31,35). With a focus on the muscle disuse mediated by hindlimb suspension, Allen and colleagues (1) first demonstrated that the number of myonuclei exhibiting double-stranded DNA fragmentation and/or morphologically abnormal myonuclei was significantly increased in the soleus muscles of young rats after 14 days of suspension. Accordingly, apoptosis was suggested to have a role in the elimination of myonuclei during hindlimb suspension-induced muscle atrophy (1). Recently, the findings of apoptosis in hindlimb suspension-mediated muscle disuse have been expanded by Leeuwenburgh and coworkers who showed that soleus muscle from aged animals also demonstrates activation of apoptosis in response to hindlimb suspension (17). Furthermore, by using the technique of immunohistochemistry, they demonstrated that apoptosis in soleus muscle is partly mediated by the subsarcolemmal mitochondria through endonuclease G (a mitochondrial caspase-independent apoptogenic factor) translocation to the myonucleus in response to hindlimb suspension and these results suggest that the underlying pathways involved in apoptosis may be distinct in young and old muscles (17). Here we extend these observations by showing that in addition to slow-twitch plantar flexor soleus muscle, the mixed-fibered (but primarily fast-twitch containing) plantar flexor gastrocnemius muscle also demonstrates activation of apoptosis following acute hindlimb suspension. This finding shows that apoptosis in muscle loss induced by hindlimb suspension in muscle groups is not limited to only muscles containing only slow myosin heavy chains. Moreover, we found that the release of mitochondrial AIF (another caspase-independent apoptogenic factor) into the cytosol is evident exclusively in the aged muscle after hindlimb unloading but this change was not observed in the young muscle. These AIF findings are in accordance with the endonuclease G data that have been recently reported in suspended soleus muscles of aged rats (17). Together these data support the concept that different apoptotic mechanisms are responsible for the activation of apoptosis in young and aged skeletal muscles. In line with these age-related findings, previously we have also observed that the apoptotic signaling components (e.g., Bax, Bcl-2, AIF, Id2, and p53) respond to unloading differently in the hypertrophied muscles from young and aged birds (28,31). Aging has also been demonstrated to affect the homeostatic regulation of myonuclear domain (13,17). Nevertheless it remains largely unclear if the age-dependent alteration of myonuclear domain regulation or the age-specific apoptotic signaling has a physiologically important role during muscle disuse. Nevertheless, future research is needed to resolve these novel but relatively unresolved age-related responses.

Clarification of the changes in apoptotic signaling accompany with the aging-associated sarcopenic muscle loss. There has been recent evidence indicating that apoptosis may play a considerable role in mediating the progression of sarcopenia (2,3,10,11,16,17,21,22,33). Given that

the etiology of sarcopenia is still largely unknown, active investigation has been conducting with the aim of fully understanding this unavoidable and detrimental process with aging. Although there has been evidence showing apoptosis in sarcopenic skeletal muscle, the apoptotic signaling pathways responsible for the activation of apoptosis and the importance of apoptotic contributions to sarcopenia has not been fully unraveled. Several pieces of data indicate that certain BCL-2 family proteins, caspases, and other apoptotic regulators may be responsive to aging (2,3,10,11,17). Previous investigations conducted in our laboratory have shown that pro-apoptotic Bax and selective caspases activities are upregulated in the gastrocnemius and plantaris muscles of ~30 mo old Fischer₃₄₄×Brown Norway rats while anti-apoptotic Bcl-2 is down-regulated in aged plantaris muscle when compared to the young adult muscles (2,3). It is noted that these age-related apoptotic changes, to some extent, are not in agreement with the aging data reported by Leeuwenburgh's group in which they did not find any age-related changes in Bax, Bcl-2, and caspase-3 activity (10,11). However, these discrepancies are reasonably explained by the differences in the age and the strain of the animals being examined in the different studies and the approach of measurement being adopted. In both of the studies reporting unchanged Bax, Bcl-2 or caspase activity (10,11), ~24 mo old Fischer₃₄₄ rats were used as the aged model. It is likely that the different responses between our study using Fischer₃₄₄×Brown Norway rats and that of other groups who examined Fischer₃₄₄ rats (10,11), can be accounted for by the differences in the ages and species of rats used in these studies. Data from Leeuwenburgh's group showing that caspase-3 activity tends to increase in the soleus muscle of 32 mo old Fischer₃₄₄×Brown Norway rats relative to young animals ($P = 0.052$) further supports the above explanation (17). Moreover, unchanged Bax and Bcl-2 levels were reported according to the results of an ELISA analysis performed on the mitochondrial fraction of muscle lysates. As compared to the total protein lysate, this may also contribute to the contradictory findings on Bax and Bcl-2 as these BCL-2 family proteins have been demonstrated to localize also in the subcellular compartments other than the mitochondria (e.g., endoplasmic reticulum) (7,37).

In the present study, we provide additional information showing that aging favors the pro-apoptotic tendencies in the gastrocnemius muscle by upregulating the content of Bax mRNA and protein, Apaf-1 mRNA, AIF mRNA, caspase-3 activity, caspase-9 mRNA and activity, XIAP mRNA and protein and the releases of mitochondrial cytochrome c and Smac/DIABLO. Notably, we found that the Bcl-2 content is upregulated with aging in gastrocnemius muscles. The reported down-regulated Bcl-2 in the plantaris muscle from ~30 mo old Fischer₃₄₄×Brown Norway rats (3) and unchanged Bcl-2 in the gastrocnemius muscle from ~24 mo old Fischer₃₄₄ rats (10), in conjunction with our current Bcl-2 observation suggests that muscle group (gastrocnemius vs. plantaris) and species and/or age of rats may be important factors that influence the response of anti-apoptotic Bcl-2 to aging.

In conclusion, we have demonstrated that the activation of apoptosis is evident in a mixed-fibered (gastrocnemius) muscle from both young adult and aged rats in response to acute hindlimb suspension. We report increases in the protein expression of pro-apoptotic Bax, release of mitochondrial apoptogenic factor cytochrome c, and the extent of apoptotic DNA fragmentation in young adult and aged gastrocnemius muscles following 14 days of hindlimb suspension. These findings further clarify the role of apoptosis in skeletal muscle atrophy during hindlimb suspension by showing that the apoptotic program is activated not only in slow-twitch plantar flexor muscle (e.g., soleus) but also in mixed-fibers plantar flexor muscle (e.g., gastrocnemius muscle). Another notable finding in the present study is that only the suspended aged gastrocnemius muscle exhibits the release of mitochondrial AIF (a caspase-independent apoptogenic factor) into the cytosol but this is not observed in the suspended young adult muscle. Along with the recent findings demonstrating that endonuclease G (another mitochondrial caspase-independent apoptogenic factor) is involved in hindlimb suspension-induced muscle atrophy exclusively in the aged soleus muscle (17), our AIF findings support the hypothesis that the mechanisms responsible for the activation of apoptosis during hindlimb suspension may be age-dependent. Nonetheless, more investigations are required to fully understand the complicated influence of aging in the apoptotic regulation under the situation of muscle disuse.

Remarks: The findings presented in this chapter have been submitted to the *Am. J. Physiol. Regul. Integ. Comp. Physiol.* at the time of dissertation submission.

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Table 1. Primers used for PCR amplification of cDNA

Product	Accession No.	Sequence	Position	T _A , °C	Cycles	Product length, bp	Restriction enzyme	Restriction products, bp
Bcl-2	U34964	F: 5'-CGGGCTGGGGATGACTTCTCTC-3' R: 5'-GCCGGTTCAGTACTCAGTCAT-3'	286-307 520-541	59.1	36	256	<i>AluI</i>	196, 60
Bax	AF235993	F: 5'-GCACCCCTTTCCTCTCTCTCCACCAG-3' R: 5'-TGCCTTTCCCGTTCCCAATTCATC-3'	462-488 1091-1115	55	36	654	<i>BstXI</i> <i>BamHI</i>	151, 105 445, 209
AIF	AF375656	F: 5'-CCGGCTTCCAGGCAACTTGTTC-3' R: 5'-CCCGGATGGATCTAGCTGCTGCA-3'	93-115 429-451	58.1	33	359	<i>KpnI</i> <i>PstI</i>	290, 69 259, 100
Apaf-1	AF218388	F: 5'-CGGCCCTGCGCATCTGATTTCAT-3' R: 5'-GGGCGAAGCACTAAGCGGGACAG-3'	1623-1644 1888-1910	57.8	36	288	<i>AluI</i> <i>PstI</i>	193, 95 198, 90
Caspase -3	NM_012922	F: 5'-TGGCCCTGAAATACGAAGTC-3' R: 5'-CGGCCTCCACTGGTATCT-3'	262-281 583-600	53.3	36	339	<i>RsaI</i> <i>SpeI</i>	260, 79 183, 156
Caspase -9	NM_031632	F: 5'-GGCCGGTGGACATTGGTTCTGG-3' R: 5'-CCATGAAGCGCAGCCAGCAGAA-3'	543-564 743-764	55	36	222	<i>BamHI</i> <i>HindIII</i>	130, 92 186, 36
XIAP	AF183429	F: 5'-GCGGCAGTAGATAGATGGCAGTA-3' R: 5'-TGGGGCTTAAATGGGCATAGT-3'	202-224 524-544	54.9	36	343	<i>AluI</i> <i>RsaI</i>	309, 34 201, 142
ARC	NM_053516	F: 5'-CCCCCCCACCAACAGTATTC-3' R: 5'-TGCGTGTGGCTTCTGTCTACTC-3'	1407-1426 1672-1693	56.2	36	287	<i>NcoI</i> <i>XbaI</i>	148, 139 239, 48
FLIP	NM_057138	F: 5'-GGCCCTGAGCACTGTGTCTG-3' R: 5'-TCGGCCTGTGTAATCCTTTGTAA-3'	56-75 412-434	56.4	36	379	<i>RsaI</i> <i>AluI</i>	191, 188 195, 160, 19, 5

T_A, annealing temperature; Accession No., GenBank accession number; F, forward primer; R, reverse primer.

Table 2. Muscle weight

	Young			Aged		
	Control	Suspended	% Change	Control	Suspended	% Change
Animal bodyweight (g)	375 ± 23	295 ± 13*	- 21%	539 ± 16 [#]	457 ± 11* [#]	- 15%
Muscle wet weight (mg)	993 ± 45	699 ± 31*	- 30%	761 ± 22 [#]	520 ± 27* [#]	- 32%
Muscle wet weight normalized to bodyweight (mg/g)	2.7 ± 0.1	2.4 ± 0.1*	- 11%	1.4 ± 0.0 [#]	1.1 ± 0.0* [#]	- 19%

Muscle mass loss during hindlimb suspension was monitored by examining the change in muscle wet weight and the muscle weight normalized to the animal bodyweight. The data are presented as means ± SE.

*P < 0.05, Suspended group was significant different from the Control group under the same age group;

[#]P < 0.05, Aged animals were significant different from the Young animals under the same experimental condition.

% Change, the percent change of the measured variables in Suspended group relative to the Control group.

2×2 ANOVA indicated that main effects of age and suspension existed in animal bodyweight [Age: F(1,34)=96.7, P=0.0001; Suspension: F(1,34)=23.7, P=0.0001], muscle wet weight [Age: F(1,34)=38.3, P=0.0001; Suspension: F(1,34)=64.5, P=0.0001], and normalized muscle weight [Age: F(1,34)=659.9, P=0.0001; Suspension: F(1,34)=32.7, P=0.0001].

Figure 1 Apoptotic DNA fragmentation

Legend for Figure 1. Apoptotic DNA fragmentation. The extent of apoptotic DNA fragmentation was estimated by measuring the cytosolic mono- and oligo-nucleosomes. The OD₄₀₅ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ standard error of mean (SE). * $P < 0.05$, Suspended group was significant different from the Control group under the same age group; # $P < 0.05$, Muscles from aged animals were significant different from the young adult animals under the same experimental condition. The main effects of age, suspension, and interaction (age $\times$ suspension) on apoptotic index in these animals were analyzed by a 2×2 ANOVA.

Figure 2 Bax and Bcl-2

Legend for Figure 2 Bax and Bcl-2. The Bax and Bcl-2 mRNA and protein content was examined by RT-PCR and Western immunoblot, respectively. The insets show representative blots for the mRNA and protein. The data are presented as means $\pm$ SE. *P < 0.05, Suspended group was significant different from the Control group under the same age group; #P < 0.05, Muscles from aged animals were significant different from the young adult animals under the same experimental condition. The main effects of age, suspension, and interaction (age $\times$ suspension) in these animals were analyzed by a 2 $\times$ 2 ANOVA.

Figure 3 **Cytochrome c and Apaf-1**

A. Cytochrome c release

B. Apaf-1 mRNA

Legend for Figure 3 Cytochrome c and Apaf-1 mRNA. The release of mitochondrial cytochrome c was estimated by measuring the protein content of cytochrome c in the mitochondria-free cytosolic fraction using an ELISA. The mRNA content of Apaf-1 was determined by RT-PCR. The insets show representative results for the Apaf-1 mRNA. The data are presented as means $\pm$ SE. * $P < 0.05$, Suspended group was significant different from the Control group under the same age group; # $P < 0.05$, Muscles from aged animals were significant different from the young adult animals under the same experimental condition. The main effect of age and suspension were analyzed by a 2×2 ANOVA.

Figure 4 Caspase-3 and -9

Legend for Figure 4 Caspase-3 and -9. The caspase-3 and -9 mRNA content and protease activity were determined by RT-PCR and fluorometric caspase assay, respectively. The insets show representative results for the caspases mRNA. The data are presented as means $\pm$ SE. [#]P < 0.05, Muscles from aged animals were significant different from the young adult animals under the same experimental condition. The main effect of age was analyzed by a 2×2 ANOVA.

Figure 5 Smac/DIABLO and AIF**A. Smac/DIABLO release** **B. AIF mRNA** **C. Mitochondrial AIF release** **D. Nuclear AIF protein**

Legend for Figure 5 Smac/DIABLO and AIF. The release of mitochondrial Smac/DIABLO and AIF was estimated by immunoblotting analysis on the mitochondria-free cytosolic fraction. The mRNA and nuclear AIF protein content was evaluated by RT-PCR and immunoblot of nuclear fraction, respectively. The data are presented as mean $\pm$ SE. The insets show representative blots. *P < 0.05, Suspended group was significant different from the Control group under the same age group; #P < 0.05, Muscles from aged animals were significant different from the young adult animals under the same experimental condition. The main effects of age, suspension, and interaction (age $\times$ suspension) in these animals were analyzed by a 2 $\times$ 2 ANOVA.

Figure 6 XIAP, ARC, and FLIP**A. XIAP mRNA** **B. XIAP protein** **C. ARC mRNA** **D. ARC protein** **E. FLIP mRNA** **F. FLIP α protein**

Legend for Figure 6 XIAP, ARC, and FLIP. The mRNA and protein content of XIAP, ARC, and FLIP was examined by RT-PCR and Western immunoblot, respectively. The insets show representative blots for the mRNA and protein. The data are presented as means $\pm$ SE. [#]P < 0.05, Muscles from aged animals were significant different from the young adult animals under the same experimental condition. The main effect of age on XIAP in these animals was analyzed by a 2 $\times$ 2 ANOVA.

CHAPTER 3

Aging influences the cellular and molecular responses of apoptosis to skeletal muscle unloading

ABSTRACT

The influence of aging on skeletal myocyte apoptosis is not well understood. In this study we examined apoptosis and apoptotic regulatory factor responses to muscle atrophy induced via limb unloading following loading-induced hypertrophy. Muscle hypertrophy was induced by attaching a weight to one wing of young and aged Japanese quails for 14 days. Removing the weight for 7 or 14 days following the initial 14 days of loading induced muscle atrophy. The contralateral wing served as the intra-animal control. A time-released bromodeoxyuridine (BrdU) pellet was implanted subcutaneously with wing weighting to identify activated satellite cells/muscle precursor cells throughout the experimental period. Bcl-2 mRNA and protein levels decreased after 7-days of unloading but they were unchanged after 14-days of unloading in young muscles. Bcl-2 protein but not mRNA level decreased after 7-days of unloading in muscles of aged birds. 7-days of unloading increased the mRNA level of Bax in muscles from both young and aged birds. 14-days of unloading increased mRNA and protein levels of Bcl-2, decreased mRNA and protein levels of Bax, and decreased nuclear AIF protein level in muscles of old birds. BrdU-positive nuclei were found in all unloaded muscles from both age groups but the number of BrdU-positive nuclei relative to the total nuclei decreased after 14-days of unloading as compared with 7-days of unloading. The TUNEL index was higher after 7-days of unloading in both young and aged muscles and after 14-days of unloading in aged muscles. Immunofluorescent staining revealed that almost all of the TUNEL-positive nuclei were also BrdU-immunopositive suggesting, that activated satellite cell nuclei (both fused and unfused) underwent nuclear-apoptosis during unloading. There were significant correlations among Bcl-2, Bax, AIF, and TUNEL index. Our data are consistent with the hypothesis that apoptosis regulates, at least in part, unloading-induced muscle atrophy and loss of activated satellite cell nuclei in previously loaded muscles. Moreover, these data suggest that aging influences the apoptotic responses to prolonged unloading following hypertrophy in skeletal myocytes.

Key words: Muscle atrophy; satellite cells; Bcl-2 protein family

INTRODUCTION

Aging is characterized by a significant loss of skeletal muscle mass (i.e., sarcopenia) which is a result of muscle fiber atrophy and decrease in total fiber number (23). This loss contributes to the functional impairment and declined quality of life in elderly populations (31). Although the muscle's hypertrophic response to functional overload is retained in aged skeletal muscle, it has been shown that the plasticity and function of muscles are diminished with aging (16,20). Moreover, a greater detriment of muscle mass and function during muscle disuse was observed in aged muscles when compared with young muscles (11,21). Nonetheless, the mechanism accounting for the differences in muscle adaptations between aged and young muscles is unclear.

Apoptosis is an internal-encoded suicide program and has been widely accepted to be crucial in coordinating the balance between cell survival and death in various cell types. Although most apoptosis studies have been conducted in actively dividing or mitotic cells (e.g., lymphocytes), it has been recently demonstrated that postmitotic cells (e.g., skeletal myocytes) also exhibit apoptosis under various conditions. For instance, apoptosis has been documented in skeletal myocytes under disuse including unloading and denervation (1,8,12,27). Although the significance of apoptosis in muscle remodeling has yet to be determined, these findings suggest that apoptosis may have a physiologic role in coordinating muscle remodeling in response to unloading. Moreover, several studies imply that normal physiological aging *per se* influences apoptosis in skeletal muscles and other post-mitotic tissues (22,30,42-44). It is possible that the apoptotic response to unloading-induced muscle atrophy in aged muscles is different from young muscles. It is likely that aging-related differences in apoptotic responses to muscle unloading could be part of the mechanisms that regulate the differential adaptations to unloading in skeletal myocytes between aged and young individuals.

Although apoptosis has been demonstrated during unloading-induced muscle atrophy that reduces muscle mass below the control muscle mass level, only a few studies have been conducted to examine the apoptotic responses to unloading-induced muscle atrophy following hypertrophy, which returns muscle mass back to the control muscle mass level. Moreover, the influence of aging in unloading-induced apoptosis in skeletal myocytes has not been well investigated. Therefore, the purpose of this study was to examine the responses of apoptosis and apoptotic regulatory factors to unloading-induced muscle atrophy following hypertrophy in young adult and aged quails. We hypothesized that 1) apoptosis is associated with the unloading-induced muscle atrophy following hypertrophy and this is independent of aging, and 2) apoptotic gene responses to prolonged unloading following muscle hypertrophy are aging dependent.

METHODS

Animals. Japanese Coturnix quails were hatched and raised in pathogen-free conditions in the central animal care center at West Virginia University School of Medicine. The birds were housed at a room temperature of 22°C with a 12:12-h light:dark cycle and were provided with food and water *ad libitum*. Sixteen young adult birds (2 mo old) and sixteen aged birds (24 mo old) were examined in the present study. The average lifespan of Japanese quails is ~26-28 mo. They are both physically and sexually mature by 1.5 mo of age and do not grow thereafter (35,41). All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and fully conformed with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings".

Muscle Loading and Unloading Protocol. The patagialis (PAT) muscle is flexed with the wing on the birds' back at rest, but it is stretched when the wing is extended. In our experimental stretch-overloading model, we placed a tube containing 10-12% of the bird's bodyweight over the left humeral-ulnar joint (13). This maintains the joint in extension throughout the period of stretch and induces stretch at the origin of the PAT muscle. Previous studies have shown this stretch-loading protocol results in moderate hypertrophy of the PAT muscles (7,12,50). The unstretched right PAT muscle served as the intra-animal control muscle for each bird. Consistent with the fact that Japanese quails show no maturational changes in bodyweight and carcass composition beyond ~1.5-2 mo after hatching (33,34,56), it has been demonstrated that the bodyweights of Japanese quails do not change throughout stretch overloading and do not differ between adult and aged quails (6,13-15,19,32). Therefore, the responses to the same absolute and relative loads could be compared in muscles from young adult and aged quails.

The left wing was loaded for 14 days and then the weight was removed. Seven days after the removal of weight, eight young and eight aged quails were killed by an overdose of pentobarbital sodium. The remaining young and aged quails were sacrificed 14 days after the weight removal. The whole PAT muscles were dissected from the surrounding connective tissue, removed, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and then stored at -80°C until used for analyses.

BrdU Administration. A subcutaneous time-released bromodeoxyuridine (BrdU) pellet (21-day release, 0.22 µg BrdU·g body mass⁻¹·day⁻¹, Innovative Research, Sarasota, FL) was implanted in each quail while anesthetized with 2% isoflurane, at the point when the wing was weighted. It was used to identify activated satellite cells/muscle precursor cells during 14 days period of muscle loading-induced hypertrophy as BrdU is a thymidine analog and is incorporated in nuclei during DNA

synthesis. BrdU was not provided during the final 7 days of unloading, because unloading following hypertrophy does not produce a stimulus for satellite cell activation and proliferation (12).

RT-PCR. Total RNA was extracted from PAT muscles with TriReagent (Molecular Research Center, Cincinnati, OH, USA), which is based on the guanidine thiocyanate method. Frozen muscles were mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA were solubilized in RNase-free H₂O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assured by obtaining an OD₂₆₀/OD₂₈₀ ratio of ~2.0. Two micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 20 µl according to standard methods (Invitrogen Life Technologies, Bethesda, MD, USA). Control RT reactions were done in which the RT enzyme was omitted. The control RT reactions were PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of complementary DNA (cDNA) was then amplified by PCR using 100 ng of each primer, ribosomal 18S primer pairs (Ambion, TX, USA), 250 µM deoxyribonucleotide triphosphates (dNTPs), 1 x PCR buffer, and 2 units *Taq* DNA polymerase (Sigma Chemical Co, St Louis, MO, USA) in a final volume of 50 µl. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). A primer pair was designed against the chicken B-cell lymphoma-2 (Bcl-2) gene (forward primer 5'GCCCCCGCCTCACCATG3'; reverse primer 5'CCCGGGGTGAGCCATGGTTTC3', GenBank D11381). The Bcl-2-associated X (Bax) gene of quail or chicken has not been previously reported. As the mouse Bax sequence (GenBank NM_007527) is ~57% homologous to a cloned chicken activated T cell cDNA (GenBank AI981354), and 59% homologous to the human Bax gene (GenBank BC014175) which is putatively suggested to be similar to the chicken Bax sequence (55), we designed a primer pair against the mouse Bax gene (forward primer 5'ACAGGGTTTCATCCAGGATCGAGCA3'; reverse primer 5'ATGGTGAGCGAGGCGGTGAGGA3', GenBank NM_007527). The resulting PCR product was the correct molecular weight from the predicted Bax sequence. The primers for Bcl-2 and Bax were designed with an annealing temperature of 55.5°C. All PCR products were verified by restriction digestion. Preliminary experiments were conducted with each gene to assure that the number of cycles (36 cycles) represented a linear portion for the PCR optical density curve for the muscle samples. The cDNA from all muscle samples were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 20 µl of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide. Images were captured and the signals were quantified in arbitrary units as optical density (OD) x band area using Kodak one-dimensional (1-D) image analysis system (Eastman Kodak, Rochester, NY, USA). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. All RT-PCR signals were normalized to the

ribosomal 18S mRNA signal of the corresponding RT product. This eliminated the measurement error from uneven sample loading and provided a semi-quantitative measure of the relative changes in gene expression.

Protein Extraction and Fractionation. The method described by Rothermel et al (45) was used to obtain the cytoplasmic and nuclear protein extracts of the PAT muscles. Briefly, 50 mg of PAT muscles were homogenized on ice in 1 ml of ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol). The homogenates were centrifuged at 1,000 rpm for 1 min at 4°C. The supernatants contained the cytoplasmic protein fraction and were collected. The remaining nuclear pellet was washed and resuspended in 360 µl of lysis buffer, and 49.8 µl of 5 M NaCl was added to lyse the nuclei. The mixture was then rotated for 1 h at 4°C, and centrifuged at 14,000 rpm for 15 min at 4°C. The supernatants contained the nuclear protein fraction and were collected. Purity of the extracted fractions was confirmed by performing immunoblots on the extracted proteins using an anti-histone H2B (a nuclear protein) rabbit polyclonal antibody (1:2000 dilution, 07371, Upstate, Lake Placid, NY) and an anti-CuZn-superoxide dismutase (a cytoplasmic antioxidant enzyme) rabbit polyclonal antibody (1:500 dilution, sc-11407, Santa Cruz Biotechnology, Santa Cruz, CA, USA) (Figure 1). A protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (P8340, Sigma-Aldrich, St Louis, MO, USA) was added to the collected cytoplasmic and nuclear protein fractions. The total protein contents of the cytoplasmic and nuclear extracts were quantified in duplicate by using bicinchoninic acid reagents (Pierce, Rockford, IL, USA) and bovine serum albumin (BSA) standards. The cytoplasmic fraction protein was then used for immunoblotting of Bcl-2, Bax, and apoptotic protease-activating factor-1 (Apaf-1) while apoptosis-inducing factor (AIF) protein content was measured in both the cytoplasmic and nuclear fraction protein.

Insert Figure 1 ———→ Verification of the extracted protein fraction purity

Western Immunoblots. Protein expression of apoptotic regulatory factors including Bcl-2, Bax, Apaf-1, and AIF was determined in the PAT muscles of experimental (left) and intra-animals control (right) wings. Forty micrograms of soluble protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE for 1.5 hours at 20°C. The gels were blotted to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO, USA) to confirm equal loading and transferring of proteins to the membrane in each lane. As another approach to verify similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue to further

confirm it. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) and probed with anti-Bcl-2 mouse monoclonal antibody (1:200 dilution, sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax rabbit polyclonal antibody (1:200 dilution, sc-6236, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Apaf-1 rabbit polyclonal antibody (1:500 dilution, AB16503, Chemicon International, Temecula, CA), or anti-AIF mouse monoclonal antibody (1:500 dilution, sc-13116HRP, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS-T with 2% BSA. Secondary antibodies were conjugated to horseradish peroxidase (HRP) (Chemicon, CA), and the signals were developed by West Pico Chemiluminescent Substrate (34080, Pierce, Rockford, IL). The signals were visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY, USA), and digital records of the films were captured with a Kodak 290 camera. The resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional (1-D) image analysis system (Eastman Kodak, Rochester, NY, USA) and expressed in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Immunofluorescent Staining. Activated, proliferated satellite cells/muscle precursor cells were identified by double immunofluorescence staining with BrdU and laminin. Frozen 10 μ m thick muscle cross-sections from the experimental and control PAT muscles were cut in a freezing cryostat at -20°C and were placed on the same glass slide to control for processing differences (e.g., incubation time or temperature, etc.). The sections were air-dried at room temperature, fixed in ice-cold methanol:acetone 1:1 for 10 min, permeabilized with 0.2% Triton X-100 in 0.1% sodium citrate at 4°C for 5 min, and blocked in 1.5% goat serum in PBS. All incubations were performed at room temperature for 30 min. After washes in PBS, sections were incubated with an anti-BrdU mouse monoclonal antibody (1:20 dilution, 555627, BD Pharmingen, San Diego, CA) followed by an anti-mouse IgG Cy3 conjugate F(ab')₂ fragment incubation (dilution 1:200, C2181, Sigma Chemical Co, St Louis, MO). Negative control experiments were done by omitting the BrdU antibody on the tissue sections. In order to visualize the basal lamina of the PAT muscles and therefore identify whether the BrdU-positive nuclei were muscle-originated nuclei (e.g., muscle satellite cell nuclei), the tissue sections were then incubated with an anti-chick laminin mouse monoclonal antibody (20 μ g·mL⁻¹, clone 31-2, D.M. Fambrough, The Johns Hopkins University, Baltimore, MD) followed by an anti-mouse IgG biotin-conjugated antibody (Vector Laboratories, Burlingame, CA) and then fluorescein avidin DCS incubation (1:200 dilution, A2011, Vector Laboratories, CA). The sections were finally mounted with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Vectashield mounting medium, Vector Laboratories, CA). BrdU- and DAPI-positive nuclei and laminin staining were examined under a fluorescence microscope with excitation wavelengths of 330-380 nm for DAPI blue

fluorescence, 485-585 nm for Cy3 red fluorescence, and 450-490 nm for fluorescein green fluorescence (Biological Research Microscope Eclipse E800, Nikon Inc., Melville NY). Images were obtained using a SPOT RT camera (Diagnostic Instruments Inc., Sterling Heights, MI) and SPOT RT software (Universal Imaging Corporation, Downingtown, PA) was used to stack the images of BrdU- and DAPI-positive nuclei and laminin staining. The numbers of BrdU- and DAPI-positive nuclei were counted from six random non-overlapping fields at an objective magnification of 40 $\times$. Only the labeled nuclei that were under the laminin staining were counted in order to exclude any non-muscle nuclei in the sections. Data were expressed as BrdU index which was calculated from the number of BrdU-positive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei) $\times$ 100.

***In situ* TUNEL Staining.** The nuclei with DNA strand breaks were assessed using a fluorometric terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) detection kit according to manufacturer's instructions (1684795, Roche Applied Science, Indianapolis, IN). In brief, 10 μ m thick frozen muscle cross-sections from muscles were cut in a freezing cryostat at -20°C. Tissue sections were air-dried at room temperature, fixed in 4% paraformaldehyde in PBS, pH 7.4 at room temperature for 20 min, permeabilized with 0.2% Triton X-100 in 0.1% sodium citrate at 4°C for 2 min, and incubated with fluorescein-conjugated TUNEL reaction mixture in a humidified chamber at 37°C for 1 h at dark. Negative control experiments were done by omitting the TdT enzyme in the TUNEL reaction mixture on the tissue sections. The sections following TUNEL were then labeled by laminin or dystrophin in order to visualize the basal lamina or the sarcolemma, respectively. After TUNEL labeling, the muscle sections were incubated with an anti-chick laminin mouse monoclonal antibody (for visualizing the basal lamina, 20 μ g $\cdot$ ml⁻¹, clone 31-2) or with an anti-dystrophin mouse monoclonal antibody (for visualizing the sarcolemma, 1:2 dilution, D505, Vector Laboratories, Burlingame, CA) followed by an anti-mouse IgG Cy3 conjugate F(ab')₂ fragment incubation (dilution 1:200, C2181) and mounted with DAPI Vectashield mounting medium. TUNEL- and DAPI-positive nuclei and laminin or dystrophin staining were examined under a fluorescence microscope and the captured images were stacked using SPOT RT software as described above. The numbers of TUNEL- and DAPI-positive nuclei were counted and only the labeled nuclei that were under the laminin staining were counted in order to include solely the muscle-originated nuclei. Data were expressed as TUNEL index which was calculated by counting the number of TUNEL-positive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei) $\times$ 100. The TUNEL index for each muscle was calculated from six random non-overlapping fields at an objective magnification of 40 $\times$.

In a separate set of measurements, we performed the BrdU labeling on the muscle sections following the TUNEL labeling procedure in order to determine whether the TUNEL-positively

labeled nuclei under the laminin staining had undergone proliferation. After labeling the tissue sections using the TUNEL protocol, they were incubated with an anti-BrdU mouse monoclonal antibody (1:20 dilution, 555627) followed by an anti-mouse IgG Cy3 conjugate F(ab')₂ fragment incubation (dilution 1:200, C2181) and then mounted with DAPI Vectashield mounting medium. TUNEL-, BrdU-, and DAPI-positive nuclei were examined under a fluorescence microscope and the captured images were stacked using SPOT RT software as described above. BrdU-positive nuclei that were under the basal lamina were taken to be proliferated satellite cell nuclei, which could consist of nuclei that were proliferated but did not fuse with the adjacent fiber (i.e., fused nuclei) or nuclei that had proliferated but did not fuse to reside inside the adjacent muscle fiber (non-fused nuclei). BrdU negative nuclei under the basal lamina were taken to be the post-mitotic, myocyte nuclei.

Statistical Analyses. Statistical analyses were performed using the SPSS 10.0 software package. Student's *t*-test for paired data was used to examine differences between the experimental and contralateral control muscles. Relationships between given variables were examined by computing the Pearson product-moment correlation coefficient (*r*). Multivariate analysis of variance (MANOVA) was performed on the percent changes in all measured variables including muscle mass, BrdU index, and all apoptotic measurements in order to examine the main effects of time (7- and 14-days of unloading), age (young and aged muscles), and interaction (time × age). Estimate of the effect size by computing Eta squared (η^2) was included in the MANOVA with the aim of indicating how much of the total variance in the present experiment was explained by the main effect or interaction. Statistical significance was accepted at $P < 0.05$. All data are given as means ± standard error of the mean (SE).

RESULTS

Change in Muscle Mass. The percent differences in the muscle weight between the experimental and the contralateral control muscles were estimated from the whole PAT muscle wet weight. Previous studies have consistently shown that the extent of muscle hypertrophy relative to the contralateral control muscle following 14-days of stretch-overloading is ~35% and ~15% in young adult and aged quails, respectively (7,13,50), by adopting the same stretch-loading procedure in the present study. The degree of hypertrophy decreased to 15% and 12% after loading followed by 7-days of unloading. After 14-days of unloading, no difference in the muscle weight was found between the experimental and control muscles in young adult quails ($P > 0.05$) whereas the weight of the experimental muscle was still 6% greater than the control muscle in aged quails (Figure 2). In agreement with the finding that edema did not contribute to the increased muscle wet weight of stretch-enlarged muscle (10), we did not observe any histological abnormality that indicated severe inflammation or edema in all the examined muscle sections in the present study.

Insert Figure 2 ———→ Muscle mass change

Bcl-2 mRNA and Protein Levels. As estimated by RT-PCR, we found that Bcl-2 mRNA level of the experimental muscle was $15 \pm 5\%$ ($P < 0.05$) lower than the control muscle after 7-days of unloading while no difference was found after 14-days of unloading in young adult quails (Figure 3A). Furthermore, in aged quails, Bcl-2 mRNA level of the experimental muscle is similar to the control muscle after 7-days of unloading whereas Bcl-2 mRNA level of the experimental muscle was $18 \pm 7\%$ ($P < 0.05$) higher than the control muscle after 14-days of unloading (Figure 3A).

In our Western blot analyses, we identified an immunoreactive band of ~25 kDa corresponding to the predicted molecular mass of Bcl-2 protein. Bcl-2 protein level of the experimental muscle was $15 \pm 5\%$ ($P < 0.05$) lower than the control muscle after 7-days of unloading but no difference was found between the experimental and control muscles after 14-days of unloading in young adult quails (Figure 3B). In aged quails, the Bcl-2 protein level of the experimental muscle was $37 \pm 6\%$ ($P < 0.01$) lower and $29 \pm 11\%$ ($P < 0.05$) higher than the control muscle after 7-days and 14-days of unloading, respectively (Figure 3B).

Insert Figure 3A ———→ Bcl-2 mRNA

Insert Figure 3B ———→ Bcl-2 protein

Bax mRNA and Protein Levels. The Bax mRNA level of the experimental muscle was $23 \pm 7\%$ ($P < 0.01$) greater than the control muscle after 7-days of unloading while a similar Bax mRNA level was found between the experimental and control muscles after 14-days of unloading in young adult quails (Figure 4A). In aged quails, the Bax mRNA level of the experimental muscle was $12 \pm 4\%$ ($P < 0.01$) greater than the control muscle after 7-days of unloading but similar Bax mRNA level was found between the experimental and control muscles after 14-days of unloading (Figure 4A).

We detected a ~21 kDa immunoreactive band corresponding to the predicted molecular mass of Bax protein in the immunoblots. There was no difference in Bax protein level between the experimental and control muscles after both 7-days and 14-days of unloading in young adult quails (Figure 4B). In aged quails, although Bax protein level of the experimental muscle appeared to increase by $34 \pm 25\%$ when compared with the control muscle after 7-days of unloading, it did not reach the statistical significant level ($P = 0.312$). However, Bax protein level of the experimental muscle was $25 \pm 7\%$ lower than the control muscle after 14-days of unloading (Figure 4B).

Insert Figure 4A ———→ Bax mRNA

Insert Figure 4B ———→ Bax protein

Apaf-1 and AIF Protein Levels. In our Western blots, we detected a ~130 kDa immunoreactive band corresponding to Apaf-1 protein. However, there was no difference in Apaf-1 protein level between the experimental and control muscles in all groups of quails (Figure 5). An immunoreactive band of ~67 kDa corresponding to the predicted molecular mass of AIF protein was detected in both the cytoplasmic and nuclear protein fraction of all muscle samples. No difference in cytoplasmic AIF protein level was found between the experimental and control muscles after both 7-days and 14-days of unloading in both young adult and aged quails (Figure 6A). The nuclear AIF protein level was similar between the experimental and control muscles after 7-days of unloading in young adult and aged quails as well as after 7-days of unloading in aged quails. However, we found that protein level of nuclear AIF of the experimental muscle was $16 \pm 5\%$ ($P < 0.01$) lower than the control muscle after 14-days of unloading in aged quail (Figure 6B).

Insert Figure 5 ———→ Apaf-1 protein

Insert Figure 6A ———→ Cytoplasmic AIF protein

Insert Figure 6B ———→ Nuclear AIF protein

TUNEL index. Nuclear DNA breaks in skeletal muscles were detected by TUNEL staining and expressed as TUNEL index to estimate the extent of apoptosis. Figure 7A shows an example of a TUNEL-positively labeled nucleus that is located under the basal lamina in an experimental PAT muscle from an aged quail after 7-days of unloading. Although TUNEL-positive nuclei were rarely detected in the young control muscles, we found that the TUNEL index of the experimental muscle was $246 \pm 157\%$ ($P < 0.01$) greater than the control muscle after 7-days of unloading but no difference was found between the experimental and control muscles after 14-days of unloading in young quails (Figure 7B). In aged quails, the TUNEL index of the experimental muscle was $91 \pm 13\%$ ($P < 0.001$) and $44 \pm 12\%$ ($P < 0.01$) greater than the control muscle after 7-days and 14-days of unloading, respectively (Figure 7B).

Insert Figure 7A ———→ TUNEL under laminin staining

Insert Figure 7B ———→ TUNEL index

Our double immunofluorescent analyses on TUNEL and BrdU from a separate set of measurements showed that almost all of the TUNEL-positive nuclei were also labeled with BrdU

(Figure 8). This indicates that the TUNEL-positively labeled nuclei under the basal lamina staining, as illustrated in Figure 7A, were proliferated satellite cell/muscle precursor cell nuclei. Additionally, the double immunocytochemical staining with TUNEL and dystrophin revealed that the TUNEL-positive nuclei were present both under (Figure 9A) and on (Figure 9B) the dystrophin staining. This finding suggests that the TUNEL-positively labeled nuclei included both the fused (under dystrophin staining) and unfused (on dystrophin-stained sarcolemma) proliferated satellite cell/muscle precursor cell nuclei.

Insert Figure 8 ———→ TUNEL and BrdU double staining

Insert Figure 9A-B ———→ TUNEL under/on dystrophin staining

BrdU index. Previously, we have demonstrated that the number of activated satellite cells/muscle precursor cells following 14-days of stretch-overloading in young adult PAT muscle is ~7% according to the muscle-related BrdU-labeled nuclei relative to the total nuclei population (12). BrdU-immunopositive nuclei were rarely found in the control muscles of all groups. Figure 10A shows an example of a BrdU-immunopositive nucleus under the laminin staining in the experimental PAT muscle from a young quail after 14-days of unloading. As an estimate of the number of activated satellite cells/muscle precursor cells, the BrdU index was 2.7 and 1.3% of the total nuclei population in the experimental muscles of young quails after 7-days and 14-days of unloading, respectively. In the aged quails, the BrdU index was 2.6 and 1.8% in the experimental muscles after 7-days and 14-days of unloading, respectively (Figure 10B). It is noted that these BrdU indices were generally lower than our previous reported BrdU indices following 7- and 14-days of unloading (12) because only the BrdU-immunopositive nuclei under the laminin staining were counted in the present study.

Insert Figure 10A ———→ BrdU under laminin staining

Insert Figure 10B ———→ BrdU index

Relationships among apoptotic regulatory factors and apoptosis. The relationships among Bcl-2, Bax, AIF, and TUNEL index were analyzed by examining the corresponding Pearson's correlation coefficient (r). When the experimental and control PAT muscles of all groups were collapsed and treated as a single group, Bax protein content was positively correlated with the apoptotic index ($r = 0.393$, $P < 0.001$, $N = 64$, Figure 11A) while Bcl-2 protein content was negatively correlated with the apoptotic index ($r = -0.577$, $P < 0.0001$, $N = 64$, Figure 11B).

When the muscle samples were collapsed and analyzed as different sub-groups (e.g., all control muscle or all aged muscles), we found that, generally, Bax protein and mRNA levels were

positively correlated with TUNEL index and cytoplasmic AIF, respectively whereas Bcl-2 protein and mRNA were negatively correlated with TUNEL index and nuclear AIF, respectively ($P < 0.05$, Table 1).

Insert Figure 11A-B ———→ Correlations among TUNEL index, Bcl-2, and Bax

Insert Table 1 ———→ Correlation Table

Main effects of age, time, and age $\times$ time. The outcomes of the multivariate tests in MANOVA indicated that there were significant main effects of age [$F(10, 19) = 2.393$, $P < 0.05$, $\eta^2 = 0.557$] and time [$F(10, 19) = 4.808$, $P < 0.01$, $\eta^2 = 0.717$]. Moreover, we found that a significant main effect of interaction (time $\times$ age) existed in explaining the variances of the present findings [$F(10, 19) = 2.872$, $P < 0.05$, $\eta^2 = 0.602$].

DISCUSSION

The present study delineates the responses of apoptosis as estimated by TUNEL and apoptotic regulatory factors including Bcl-2, Bax, AIF, and Apaf-1 to unloading-induced muscle atrophy following stretch-induced muscle hypertrophy in young adult and aged quails. Previous findings have shown that a role for apoptosis in muscle atrophy below the control muscle mass level induced by hindlimb suspension/unweighting, muscle denervation, and limb immobilization (1,8,17,27,28,37,48,54). In this study, we demonstrated that apoptosis is also involved in unloading-induced muscle atrophy, where muscle mass is reduced from a hypertrophied state to control levels. Our data indicate that elimination of the activated satellite cell nuclei (both fused and un-fused activated nuclei) via apoptotic mechanisms occurs during unloading-induced muscle atrophy following muscle hypertrophy. Moreover, we provide evidence that suggests differential apoptotic responses exists to prolonged unloading-induced muscle atrophy after muscle hypertrophy between young adult and aged animals. Conclusively, our findings support the hypotheses that 1) apoptosis plays a physiologic role in unloading-induced muscle atrophy following hypertrophy and 2) aging suppresses the apoptotic responses to muscle atrophy induced by muscle unloading (i.e., 14-days of unloading in the present study) following hypertrophy.

Apoptosis during unloading-induced muscle atrophy following hypertrophy. In the present study, the decreased degree of muscle hypertrophy due to unloading following stretch-induced hypertrophy in both young adult and aged quails denoted muscle atrophy with unloading. It has been commonly shown that muscle mass loss during disuse is associated with a decreased number of muscle nuclei (3-5,25,38), therefore, it is reasonable to suggest that muscle mass loss during muscle

disuse is mediated by decreasing the nuclei number and therefore maintaining a homeostatic balance in the myonuclear domain (i.e., the cytoplasmic volume per myonuclei) in the multinucleated skeletal myocytes (3-5,25,38-40). Based on the fact that apoptosis is consistently demonstrated during hindlimb suspension/unweighting, muscle denervation, and limb immobilization (1,8,17,27,28,37,48,54), it has been proposed that the loss in nuclei number could possibly be achieved through the activation of pathways resulting in apoptosis in the skeletal myocytes. Indeed, Allen and colleagues (1) have demonstrated that apoptosis is associated with the loss of muscle mass and myonuclei during hindlimb unweighting as they found that TUNEL-positive nuclei increased in muscles following 14-days of unweighting. Their findings suggested that the decreased myonuclei number during hindlimb unweighting-induced muscle atrophy is accounted for by elimination of the existing myonuclei by apoptotic mechanisms.

Recently, we have demonstrated that unloading-induced muscle atrophy following hypertrophy in young adult quails is also related to the activation of apoptosis as indicated by increased caspase protease activities and poly(ADP-ribose) polymerase-positive nuclei (12). Consistent with these findings, in the present study, we have demonstrated pro-apoptotic changes including increased TUNEL index, increased Bax content, and decreased Bcl-2 content in the experimental muscle after 7-days of unloading following muscle hypertrophy in both the young adult and aged quails. Furthermore, we have shown that significant relationships exist among TUNEL index, Bax, Bcl-2, and AIF in our quail muscles. Taken together, we have provided evidence confirming that apoptosis occurs during muscle remodeling induced by atrophy following hypertrophy (i.e., muscle loss after hypertrophy that returns to control muscle mass level) and we have extended our findings by showing that apoptosis is associated with unloading-induced muscle atrophy following hypertrophy not only in young adult quails, but also in the aged quails.

Notably, our double immunofluorescent staining revealed that almost all the TUNEL-positively labeled nuclei in the unloaded muscles were BrdU-immunopositive indicating that the TUNEL-positive nuclei originated from the proliferating cell populations. As we have already excluded the non-muscle cells (e.g., fibroblasts) and included only the TUNEL-positive nuclei that were under the basal lamina according to the immunolabeling of laminin, it is most likely that the TUNEL-positively nuclei under the laminin staining were nuclei originating from activated satellite cells or other myogenic precursor cell populations. Moreover, our double staining on TUNEL and dystrophin demonstrated that the TUNEL-positive nuclei were found both under and on the dystrophin staining. Taken together, these data suggest that unloading-induced muscle atrophy following stretch-induced hypertrophy eliminates both the fused and un-fused activated satellite cell nuclei via apoptotic pathways in young adult and aged quails, and this is further supported by the finding of a decreased BrdU index along with unloading following hypertrophy. Our findings are in

agreement with the suggestion that muscle remodeling is mediated by regulating the number of muscle nuclei so as to maintain the homeostatic myonuclear domain in multinucleated skeletal myocytes (2-5,25,36,38-40,46). Since it has been shown that satellite cells are more susceptible to apoptosis during chronic denervation (26,27), it is not surprising that the activated satellite cell nuclei, rather than the existing myonuclei, were eliminated during unloading following hypertrophy in order to attain muscle atrophy. Additionally, although “nuclear” apoptosis has been reported in skeletal myocytes during muscle atrophy below the control muscle mass level (e.g., by hindlimb unweighting), it is expected that those observed apoptotic nuclei belonged to the existing myonuclei population as it has been shown that the mitotic activity of satellite cells is significantly suppressed during the degenerative muscle states including hindlimb unweighting and muscle denervation (24,47).

Aging influences the apoptotic responses to prolonged unloading following hypertrophy.

Another notable finding in this study is that the rate of muscle mass change and the responses of apoptotic regulatory factors after 14-days of unloading in the experimental muscles of aged quails were different from young adult quails. After 14-days of unloading following hypertrophy, we found that the muscle mass of the experimental muscles was not different from the control muscles in young quails (i.e., the experimental muscles have returned to the control muscle mass level from the hypertrophic level), but the muscle mass of the experimental muscles were still ~6% greater than the control muscles in aged quails. Intriguingly, although the TUNEL index after 14-days of unloading was higher, we found some anti-apoptotic changes including generally increased Bcl-2 and decreased Bax level in the experimental muscles of aged quails even with the existence of a few inconsistent alterations in protein versus mRNA levels which was possibly because the measurements were limited to the selected time points. Moreover, given that translocation of AIF from mitochondria to nuclei has been suggested to be an apoptogenic event (18,29), we found a decreased level of nuclear AIF protein in the 14-days unloaded muscles of aged quails. These data suggest that the apoptosis-unfavorable changes in the apoptotic regulatory factors may provide an “apoptosis-braking” machinery to decelerate the apoptotic elimination of the muscle nuclei and therefore slow down the muscle mass loss during unloading in the aged quails. However, the reason for the decelerating loss in muscle mass and the apoptosis-unfavorable changes after 14-days of unloading following stretch-induced hypertrophy in the aged quails is unknown. Nonetheless, we interpret this observation as an anti-apoptotic adaptation to prolonged unloading (i.e., 14-days of unloading in this study) following muscle hypertrophy in aged quails, although this anti-apoptotic response did not appear to be successful (based on the TUNEL data).

Recently, apoptosis has been suggested to have an important role in the development of aging-associated sarcopenia as aged skeletal muscles have been characterized by an increased level of

apoptosis (9,22,30,43,44,49). Furthermore, the detrimental effects of muscle disuse on muscle mass and function have been shown to be greater in aged muscles (11,21). From the point of view of survival, it is not known whether the aged skeletal muscles could have developed an adaptive anti-apoptotic response to prolonged disuse following muscle hypertrophy aimed to preserve the hypertrophied muscle status in the frail aged muscles. We speculate that this age-related adaptive response may function as an attempt to favor the preservation of the enlarged muscle masses of old animals during prolonged muscle disuse following hypertrophy. Nevertheless, it is noted that this anti-apoptotic adaptation is assumed to decelerate the hypertrophied muscle mass loss during prolonged unloading, but this is partially in contradiction to the suggestion that aged skeletal muscles are subjected to a greater muscle mass loss during muscle disuse. Further research is required to verify that this anti-apoptotic adaptation also occurs during prolonged disuse using other experimental models (e.g., muscle denervation) following muscle hypertrophy.

In the present study, the apoptotic responses to unloading following hypertrophy have been discussed according to the findings obtained from a unilateral wing weighting/unloading model with the contralateral wing acting as the intra-animal control in these quails. Because we could not rule out the possibility that loading of one wing may have a slight effect on the mechanical environment or the central environmental milieu (circulating stress hormones etc.) that would affect the contralateral control muscle, we conducted subsequent studies to address this issue. First, we have closely monitored the daily living activities and biomechanics of the animals (e.g., eating habits, body center of gravity, body balancing ability, and movement and locomotion patterns) throughout the study period and we did not observe any apparent abnormality in our studied animals or differences when comparing the experimental animals (with one wing weighted and one non-weighted wing, or one control wing and one wing that was unloaded following stretch-loading) to the normal untreated animals that did not receive a stretch load/unload. Moreover, by using immunoblotting, we found that Bcl-2, Bax, Apaf-1, and AIF protein contents of the contralateral control muscles from the experimental animals were not different from muscles of normal (non-experimental) animals that have not experienced any stretch/unload (data not shown). These observations suggest that the influence of wing weighting/unloading of the experimental limb did not alter the normal activities of the animal or the muscle environment sufficient to alter markers for apoptosis in the contralateral control muscles. Thus, we conclude that the contralateral control muscles were not different from the “normal non-experimental control” muscles in the present study.

The exact identity of the mitotic myogenic contributors that have been involved in the loading-unloading process has not been comprehensively evaluated in this study. Thus, in addition to the muscle satellite cell population, we cannot rule out the possibility that some other mitotic cell populations (e.g., invading bone marrow, blood mesenchymal cells, and/or resident interstitial

myogenic cells) may also have contributed nuclei to the overloaded quail muscles during muscle hypertrophy, as is the case in mammalian muscles (51-53).

In summary, we have demonstrated pro-apoptotic changes including decreased Bcl-2 content, increased Bax content, and increased nuclei with DNA breaks as estimated by TUNEL in the experimental muscles after 7-days of unloading following hypertrophy in both young adult and aged quails. We have also shown that moderate correlational relationships exist among TUNEL index, Bcl-2, Bax, and AIF contents in the quail muscles. These findings support the hypothesis that apoptosis may have a physiologic role in mediating muscle remodeling during unloading-induced muscle atrophy following muscle hypertrophy. Our findings indicate that one of the possible physiologic roles of apoptosis during unloading-induced muscle atrophy following hypertrophy could be the elimination of excessive activated satellite cells (both fused and un-fused) in order to maintain the myonuclear domain. Additionally, although the TUNEL index of the experimental muscles was still higher than the control muscles after 14-days of unloading in aged quails, we have found some anti-apoptotic changes including increased mRNA and protein levels of Bcl-2, decreased mRNA and protein levels of Bax, and decreased nuclear AIF protein level in the 14-days unloaded aged muscles. This supports the hypothesis that aged muscles may respond differently from the young muscles during unloading-induced muscle atrophy following muscle hypertrophy. Additional research is needed to further confirm this anti-apoptotic adaptation and its physiologic role during prolonged unloading in aged muscles.

Although we and other groups have provided evidence suggesting the physiologic role of apoptosis during muscle remodeling and normal aging (1,8,9,12,22,27,28,30,43,44), further research is required to clarify the cellular and molecular upstream-regulatory mechanism(s) contributing to the activation of the apoptotic signaling pathway which results in subsequent apoptosis. Understanding the upstream-regulatory pathways leading to apoptosis during muscle remodeling and aging will provide insight into developing the novel preventive or therapeutic regimens to alleviate or delay the loss of post-mitotic myocytes with muscle disuse (e.g., bed rest, space flight) and aging.

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Figure 1 **Verification of extracted protein fraction purity**

Legend for Figure 1 **Verification of the extracted protein fraction purity.** The purity of the extracted protein fractions was determined by immunoblotting different fractions probed with anti-histone H2B and anti-CuZnSOD antibodies. The nuclear and cytoplasmic protein fractions were extracted from a same piece of young or aged muscle tissue and equal amount of protein was employed in the immunoblot. Young, young adult quail muscle; Aged, aged quail muscle; NP, nuclear protein fraction; CP, cytoplasmic protein fraction.

Figure 2 Muscle mass change

Legend for Figure 2 Muscle mass change. The muscle mass loss during unloading following stretch-induced hypertrophy was monitored by examining the decrease in the extent of muscle hypertrophy. The extent of muscle hypertrophy was simply estimated by examining the percent difference in the muscle weight between the experimental and the contralateral control whole PAT muscle wet weight. The data are presented as means $\pm$ SE. *P < 0.05, **P < 0.01, data are significantly different from control muscles. Young, young adult quails; Aged, aged quails; 7d unloading, 7-days of unloading following 14-days of stretch-overloading; 14d unloading, 14-days of unloading following 14-days of stretch-overloading.

Figure 3A Bcl-2 mRNA

Legend for Figure 3A Bcl-2 mRNA. The level of Bcl-2 mRNA was estimated by RT-PCR. The insert shows a representative result of PCR in control and experimental PAT muscles. PCR products were visualized with ethidium bromide staining. Quantification of PCR signals was obtained by densitometric analysis of the signal product optical density (OD) x resulting band area. Gene expression is normalized to the ribosomal 18S signal from the same RT product. The normalized data are presented as means $\pm$ SE. Data were run in duplicate on different gels for each gene. *P < 0.05, data are significantly different from control muscles.

Figure 3B Bcl-2 protein

Legend for Figure 3B Bcl-2 protein. The protein level of Bcl-2 was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁸. The insert shows a representative blot for Bcl-2 in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, **P < 0.01, data are significantly different from control muscles.

Legend for Figure 4A Bax mRNA. The level of Bax mRNA was estimated by RT-PCR. The insert shows a representative result of PCR in control and experimental PAT muscles. PCR products were visualized with ethidium bromide staining. Quantification of PCR signals was obtained by densitometric analysis of the signal product optical density (OD) x resulting band area. Gene expression is normalized to the ribosomal 18S signal from the same RT product. The normalized data are presented as means $\pm$ SE. Data were run in duplicate on different gels for each gene. ** $P < 0.01$, data are significantly different from control muscles.

Figure 4B Bax protein

Legend for Figure 4B Bax protein. The protein level of Bax was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁷. The insert shows a representative blot for Bax in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from control muscles.

Figure 5 Apaf-1 protein

Legend for Figure 5 Apaf-1 protein. The protein level of Apaf-1 was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁴. The insert shows a representative blot for Apaf-1 in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

Figure 6A Cytoplasmic AIF protein

Legend for Figure 6A Cytoplasmic AIF protein. The cytoplasmic protein level of AIF was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁸. The insert shows a representative blot for AIF in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

Figure 6B Nuclear AIF protein

Legend for Figure 6B Nuclear AIF protein. The nuclear protein level of AIF was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁸. The insert shows a representative blot for AIF in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. **P < 0.01, data are significantly different from control muscles.

Figure 7A TUNEL under laminin staining

Legend for Figure 7A TUNEL under laminin staining. Immunofluorescent labeling of laminin was performed following the TUNEL staining in order to identify the TUNEL-positively labeled nuclei under the laminin staining. (Red) laminin stained with secondary Cy3 labeling, (Green) fluorescein-mediated TUNEL staining, and (Blue) nuclei labeled by DAPI staining. This illustration was obtained at an objective magnification of 100 $\times$. The enlarged box exemplifies a TUNEL-positive nucleus under the laminin staining. Bar 10 μ m.

Figure 7B TUNEL Index

Legend for Figure 7B TUNEL index. The number of TUNEL-positively labeled nuclei under laminin staining relative to the total number of nuclei estimated by fluorometric TUNEL staining was expressed as TUNEL index. The data are presented as means $\pm$ SE. **P < 0.01, data are significantly different from control muscles.

Figure 8 TUNEL and BrdU double staining

Legend for Figure 8 TUNEL and BrdU double staining. Immunofluorescent labeling of BrdU was performed following the TUNEL staining in order to identify the TUNEL-positively labeled nuclei were mitotic or postmitotic in nature. Almost all the TUNEL-positively labeled nuclei were BrdU-immunopositive. (A) nuclei labeled by DAPI staining, (B) fluorescein-mediated TUNEL staining, (C) BrdU-immunopositive nuclei with secondary labeling by Cy3, and (D) pictures of DAPI, TUNEL, and BrdU were merged. The arrow indicated a TUNEL- and BrdU-positively labeled nucleus. These illustrations were obtained at an objective magnification of 100 $\times$. Bar 10 μ m.

Figure 9 TUNEL and dystrophin staining

Legend for Figure 9 TUNEL under/on dystrophin staining. Immunofluorescent labeling of dystrophin was performed following the TUNEL staining in order to identify the localization of the TUNEL-positive nuclei regarding to the sarcolemma. TUNEL-positive nuclei were found both under (A) and on (B) the dystrophin staining. (Red) dystrophin stained with secondary Cy3 labeling, (Green) fluorescein-mediated TUNEL staining, and (Blue) nuclei labeled by DAPI staining. The enlarged boxes exemplify TUNEL-positive nuclei under and on the dystrophin staining. These illustrations were obtained at an objective magnification of 100 $\times$. Bar 10 μ m.

Figure 10A BrdU under laminin staining

Legend for Figure 10A BrdU under laminin staining. Immunofluorescent labeling on BrdU, laminin, and DAPI was used to identify the BrdU-positively labeled nuclei under the laminin staining as an estimate of the activated muscle satellite cell nuclei. (Red) BrdU stained with secondary Cy3 labeling, (Green) laminin stained with secondary fluorescein labeling, and (Blue) nuclei labeled by DAPI staining. This illustration was obtained at an objective magnification of 100 $\times$. The enlarged box exemplifies a BrdU-positive nucleus under the laminin staining. Bar 10 μ m.

Figure 10B BrdU Index

Legend for Figure 10B BrdU index. The number of BrdU-positively labeled nuclei under the laminin staining relative to the total number of nuclei estimated by immunofluorescent staining was expressed as BrdU index. The data are presented as means $\pm$ SE. ** $P < 0.01$, data are significantly different from control muscles.

Figure 11 Correlation Analysis

Legend for Figure 11 The relationships between the TUNEL index and protein levels of Bax as well as Bcl-2 were investigated by examining the Pearson product-moment correlation coefficient, r . The control and experimental muscles from both young adult and aged quails of all groups were collapsed and treated as a single pooled group ($N=64$). Y7, young adult quails after 7-days of unloading; Y14, young adult quails after 14-days of unloading; A7, aged quails after 7-days of unloading; A14, aged quails after 14-days of unloading.

Table 1

Correlation existed between	<i>Muscle samples examined</i>	N	r	P
Bax protein and TUNEL index	All control muscles	32	0.476	0.006
	All unloaded muscles	32	0.393	0.026
	Young 14-days control and unloaded muscles	16	0.521	0.039
Bax mRNA and TUNEL index	All young muscles	32	0.377	0.033
	Young 7-days control and unloaded muscles	16	0.581	0.018
Bax mRNA and Cytoplasmic AIF	Young 14-days unloaded muscles	8	0.741	0.035
Bcl-2 protein and TUNEL index	All control muscles	32	-0.693	0.0001
	All unloaded muscles	32	-0.529	0.002
Bcl-2 mRNA and Nuclear AIF	Young 7-days and 14-days unloaded muscles	16	-0.521	0.038
	Young 14-days unloaded muscles	8	-0.723	0.043

Table 1 The relationships among the TUNEL index, Bcl-2, Bax, and AIF levels. The control and experimental muscles from the young adult and aged quails of all groups were collapsed and analyzed as different sub-groups. r, the Pearson product-moment correlation coefficient.

CHAPTER 4

Aging sustains the hypertrophy-associated elevation of apoptotic suppressor XIAP in skeletal muscle during unloading

ABSTRACT

This study tested the hypotheses that apoptotic suppressors: (i) increase during muscle overload, (ii) decrease in response to unloading following hypertrophy and (iii) respond to unloading in an aging-dependent fashion. Following 14 days of stretch-induced overloading, the XIAP protein was elevated by 140% and 116% in patagialis (PAT) muscles of young and old quail respectively, when compared to the contralateral control side. XIAP mRNA or protein was not different in experimental and control muscles of young birds after 7 or 14 days of unloading. In old birds, PAT XIAP mRNA and protein were 47% and 67% greater in experimental than control muscles, respectively after 7-days of unloading. Furthermore, XIAP mRNA had returned to control level by 14 days of unloading, but XIAP protein content was 57% greater than control muscles. Higher levels of XIAP during unloading in old than young muscles may be an attempt to counterbalance apoptosis-induced muscle atrophy.

Key words: Muscle atrophy; XIAP; ARC; FLIP

INTRODUCTION

Apoptosis has been suggested to have a physiologic role in regulating muscle atrophy including muscle denervation, muscle unloading, hindlimb unweighting, neuromuscular disorders, muscle dystrophy, and aging-associated sarcopenia (1-14). It has been shown that aging reduces the ability of skeletal muscle to achieve hypertrophy in response to overload (15-17). Moreover, there is evidence suggesting that aging may exacerbate the extent of muscle loss during muscle disuse (18,19), although a few studies have shown a similar muscle loss between young and aged animals following hindlimb unloading (20,21). Nevertheless, it has been demonstrated that muscle atrophy in the models of unloading where muscle mass is reduced below control muscle level (e.g., hindlimb suspension) is associated with apoptosis (1,22). We have also found that apoptosis is evident in muscles from old and young quails that are first hypertrophied by loading, and then atrophied by unloading. However, regulation of apoptosis was aging dependent. For example, we have recently shown a reduced pro-apoptotic tendency including decreases in Bax and AIF, and increase in Bcl-2 in muscles of aged birds that were loaded to induce hypertrophy then unloaded for 14-days (12,13). These changes appear to be an attempt by aging muscles to offset the apoptotic induced losses occurring during unloading.

Apoptosis is controlled in a highly coordinated manner. The cellular decision for the execution of the apoptotic program is driven by simultaneous influences of both pro- and anti- apoptotic signaling which are orchestrated by a specific cluster of apoptotic proteins (e.g., BCL-2 family) (23,24). Among a number of apoptotic proteins that have been identified, X-linked inhibitor of apoptosis protein (XIAP), apoptosis repressor with caspase recruitment domain protein (ARC), and [Fas-associated death domain protein-like interleukin-1 β -converting enzyme]-like inhibitory protein (FLIP) establish a group of endogenous apoptotic suppressors which primarily function in modulating the anti-apoptotic signaling (25-30). Although we have found that aging affects the response of apoptotic regulatory factors to unloading-induced atrophy in previously hypertrophied quail muscles (13), it is not known if apoptotic suppressor proteins participate in regulating apoptosis in this situation. Furthermore, it is unclear whether muscle overloading has an effect on the expression of the apoptotic suppressors and aging muscle may influence the apoptotic suppressor proteins during unloading, which helps to reduce the rate of loss of muscle mass during unloading following a period of loading-induced hypertrophy as compared to muscles of young birds. Hence, this study examined the response of apoptotic suppressors XIAP, ARC, and FLIP to overloading and subsequent unloading in young adult and aged quail muscles. It is noted that the present study was designed to investigate the process of muscle loss from the “overloaded state” to the “normally loaded state” of the muscle. The term “unloading” used in this study refers to the removal of the load from the hypertrophied muscle and this induces the atrophy of the hypertrophied muscle. We tested the hypotheses that 1) apoptotic

suppressors are increased in response to muscle hypertrophy and are down-regulated during subsequent unloading, and 2) apoptotic suppressors respond to unloading following muscle hypertrophy differently in young adult and aged muscles.

METHODS

Animals. Japanese Coturnix quails were hatched and raised in pathogen-free conditions in the central animal care center at West Virginia University School of Medicine. The birds were housed at a room temperature of 22°C with a 12:12-h light:dark cycle and were provided with food and water *ad libitum*. Sixteen young adult birds aged 2 mo and sixteen 24 mo old aged birds were examined in the present study. The lifespan of Japanese quails is ~26–28 mo and they are both physically and sexually mature by 1.5 mo of age (31,32).

Stretch-induced Overloading and Unloading Protocol. The patagialis (PAT) muscle is flexed with the wing on the bird's back at rest, but it is stretched when the wing is extended. In our experimental stretch-overloading model, a tube containing ~12% of the bird's body weight was placed over the left humeral-ulnar joint (33). This maintains the joint in extension throughout the period of stretch and induces stretch at the origin of the PAT muscle. Previous studies have shown this stretch-overloading protocol results in moderate hypertrophy of the PAT muscles (i.e., 14-day stretch-loading induces ~35% and ~15% increases in muscle mass of young adult and aged birds, respectively) (3,34,35). The left wing of animals was overloaded for 14 days and then the load was removed. Seven days after the removal of weight, eight young and eight aged birds were killed by an overdose of pentobarbital sodium. The remaining young and aged animals were sacrificed 14 days after the weight removal. The unstretched right PAT muscle served as the intra-animal control muscle for each bird. PAT muscles were dissected from the surrounding connective tissue, removed, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and then stored at -80°C until used for analyses.

All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and following the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals as published by the U.S. Dept. of Health and Human Services and proclaimed in the Animals Welfare Act.

RT-PCR. Total RNA was obtained from PAT muscles with TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Briefly, frozen muscle following mincing was mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA

was solubilized in RNase-free H₂O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was determined by examining OD₂₆₀/OD₂₈₀ ratio. Two micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 20 µl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). Control RT reaction was done in which the RT enzyme was omitted. The control RT reaction was PCR amplified to ensure that DNA did not contaminate the RNA. One µl of complementary DNA (cDNA) was then amplified by PCR using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, TX), 250 µM deoxyribonucleotide triphosphates (dNTPs), 1 x PCR buffer, and 2.5 units *Taq* DNA polymerase (USB Corp., Cleveland, OH) in a final volume of 50 µl. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). The primer pairs were designed from sequences published in GenBank (**Table 2**) and PCR products were verified by restriction digestions. Preliminary experiments were conducted with each gene to assure that the number of cycles represented a linear portion for the PCR optical density curve for the muscle samples. It is noted that the mRNA expression of ARC was not examined in this study because the sequence of the ARC gene of quail or chicken has not been reported previously. The cDNA from all muscle samples were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 30 µl of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide. Images were captured and the signals were quantified in arbitrary units as optical density (OD) x band area using Kodak image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. Ribosomal 18S primers (Ambion, TX) were used as internal controls while all RT-PCR signals were normalized to the 18S signal of the corresponding RT product to eliminate measurement error from uneven samples loading and provide a semi-quantitative measure of the relative changes in gene expression.

Protein Extraction and Immunoblot Analysis. Total cytosolic protein was extracted from PAT muscles according to the procedure originally described by Rothermel et al (36) with modifications as previously reported (12,13,37). In brief, muscles were homogenized in ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol). The cytoplasmic protein fraction-containing supernatant was obtained after centrifugation. A protease inhibitor cocktail (P8340, Sigma-Aldrich, St Louis, MO) was added to the protein extracts. The protein concentration of the protein extract was quantified in duplicate by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation. As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion by DC Protein Assay (BioRad, Hercules, CA) based on

the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay.

Forty micrograms of protein was boiled in Laemmli buffer (161-0737, Bio-Rad, Hercules, CA) in the presence of 2-mercaptoethanol and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE at room temperature. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk and probed with an anti-hILP/XIAP mouse monoclonal antibody (1:250 dilution, 610762, BD Biosciences, San Jose, CA) or an anti-ARC rabbit polyclonal antibody (1:200 dilution, sc-11435, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in phosphate buffered saline with 0.5% Tween 20 (PBS-T) with 2% BSA at 4°C for overnight. Whole cell lysate of actively dividing human 293T/17 cells was included as a positive control for probing XIAP and ARC. Secondary antibodies were conjugated to horseradish peroxidase (1:3000 dilution, Chemicon International, Temecula, CA), and signals were developed by ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The predicted molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD). It is noted that we attempted to measure the protein content of long and short isoforms of FLIP (FLIP_L and FLIP_S) in the PAT muscles by using an anti-FLIP_{S/L} mouse monoclonal antibody (1:100 dilution, sc-5276, Santa Cruz Biotechnology, Santa Cruz, CA) and an anti-FLIP _{α} rabbit polyclonal antibody (1:500 dilution, ab6144, Abcam, Cambridge, MA) as the primary antibody in the immunoblots. However, we did not detect any immunoreactive band corresponding to the correct molecular size of FLIP_L or FLIP_S.

Statistics. Statistical analyses were performed using the SPSS 10.0 software package. 2 $\times$ 2 ANOVA was performed to examine the main effects of time (7- and 14-days of unloading), age (young adult and aged), and interaction (time $\times$ age) on the measured variables in all unloaded groups of animals. ANOVA followed by Student-Newman-Keuls post hoc analysis was used to examine differences between the experimental and intra-animal contralateral control PAT muscles. Relationships between given variables were examined by computing the Pearson product-moment correlation coefficient, *r*. All data are given as means $\pm$ standard error of mean (SE). Statistical significance was accepted at *P* < 0.05.

RESULTS

Muscle mass change. The present 14-days stretch-loading procedure has been consistently shown to result in a moderate extent of muscle hypertrophy relative to the contralateral control muscle (35% and 15% in young adult and aged birds, respectively) (33-35). We have previously reported a reduced degree of muscle hypertrophy in the experimental muscle that was unloaded following loading-induced hypertrophy relative to the contralateral control muscle (13). Following 7-days of unloading, the muscle mass of the experimental muscles was 15% and 12% greater than the contralateral control muscle in the young adult and aged birds, respectively (**Table 1**). After 14-days of unloading, in the young adult birds the muscle mass of the experimental side had returned to the contralateral control level, whereas in the aged birds there was still a 6% hypertrophy in the experimental muscle when compared to the contralateral control muscle (**Table 1**).

Table 1 —————→ Muscle mass change

XIAP and FLIP mRNA level. As estimated by RT-PCR with 18S RNA as an internal control, following 14-days of overloading, the XIAP and FLIP mRNA content was not different between the experimental and control muscles in both the young adult and aged birds (**Figure 1**). During the subsequent unloading, in young adult birds, both the XIAP and FLIP mRNA contents of the experimental muscles were not different ($P > 0.05$) when compared to the contralateral control after either 7- or 14-days of unloading (**Figure 1**). However, in the aged birds, the XIAP mRNA content was 47% greater ($P < 0.05$) in the experimental muscle relative to the contralateral control side after 7-days of unloading, but by 14-days of unloading, the XIAP mRNA content was not different between the experiment and control muscles (**Figure 1A**). In contrast, we did not find any significant difference in the mRNA content of FLIP between the experimental and control muscles from the aged birds that were unloaded for 7- or 14-days (**Figure 1B**).

Insert Figure 1A —————→ XIAP mRNA

Insert Figure 1B —————→ FLIP mRNA

XIAP and ARC protein level. In our Western analyses, we detected an immunoreactive band of ~57 kDa and ~25 kDa corresponding to the predicted molecular mass of XIAP and ARC protein, respectively. Following 14-days of overloading, the protein content of ARC was not different between the experimental and control muscles in both the young adult and aged birds, whereas the XIAP protein content in the experimental muscle was 140% and 116% higher than the contralateral side in young adult and aged birds, respectively (**Figure 2**). After 7- or 14-days of the removal of overload, in

the young adult birds, we did not find any differences in the XIAP and ARC protein content between the experimental and contralateral control muscles ($P > 0.05$, **Figure 2**). However, in the aged birds, XIAP protein content in the experimental muscle following 7-days of unloading was 67% higher ($P < 0.05$) relative to the contralateral control side while it was still 57% higher than the contralateral control level ($P < 0.05$) after 14-days of unloading (**Figure 2A**). The outcomes of the 2×2 ANOVA indicated main effects of age [$F(1,60)=48.96$, $P=0.0001$] on the XIAP protein content in the unloaded animals (**Figure 2A**). For the protein content of ARC in the aged muscles, no significant differences were found between the experimental and contralateral control sides following 7- or 14-days of unloading ($P > 0.05$, **Figure 2B**).

Insert Figure 2A ———→ XIAP protein

Insert Figure 2B ———→ ARC protein

Relationship between XIAP protein content and TUNEL index. The relationship of XIAP protein content and TUNEL index was analyzed by examining the corresponding Pearson's correlation coefficient (r). We have previously shown apoptosis was evident as indicated by the increase in TUNEL-positive nuclei (expressed as apoptotic index) and the changes in apoptosis regulatory factors including Bcl-2, Bax, and AIF which were consistent with elevated apoptosis (13). In the current study, when the unloaded and control PAT muscles of all groups were collapsed and treated as a single group, a positive relationship was found between XIAP protein content and apoptotic index ($r = 0.506$, $P = 0.001$, $N = 64$, **Figure 3**).

DISCUSSION

By using the present hypertrophied-muscle unloading model, we have previously demonstrated apoptosis is involved in unloading-induced muscle atrophy where muscle mass is reduced from a hypertrophied state toward basal level (13). We described the pro-apoptotic changes including decreased Bcl-2 and increased Bax, p53, cytosolic Id2, and TUNEL-positive nuclei in the experimental muscles after unloading in young adult or aged quails (12,13). These previous findings also indicated that apoptosis is responsible for eliminating the excessive activated/proliferated muscle precursor cell nuclei (e.g., satellite cell nuclei) during unloading in hypertrophied muscles. Moreover, our previous data showed that aging influences the apoptotic regulation during muscle unloading based on the anti-apoptotic observations of increase in Bcl-2, decreases in Bax and nuclear AIF but concomitant with increased TUNEL-positive nuclei in hypertrophied muscle from aged birds after 14-days of unloading following hypertrophy (13). Here, we extend our previous findings by demonstrating that XIAP protein expression is increased in response to muscle overload in both

young adult and aged muscles while this elevated XIAP protein level returns to the basal level after 7- or 14-days of subsequent unloading exclusively in the young adult muscle. On the contrary, this hypertrophy-associated elevation of XIAP is partially sustained in the muscle from aged birds, even though the TUNEL-indicated apoptosis was evident in these unloading aged muscle (13). This distinct aging-associated response of apoptotic suppressor XIAP further suggests that aging complicates the regulation of apoptotic machinery and a more complex mechanism may be involved in mediating the apoptotic signaling in the aged skeletal muscle.

XIAP expression is elevated in response to overload and is declined during subsequent unloading in young adult muscle. A considerable amount of research in mitotic cells/tissues have demonstrated apoptosis is a conserved cellular process as significant as the vital proliferating events such as mitosis (24,38-40). This notion was fundamentally established by the exhibited importance of apoptosis in maintaining the cell survival/death homeostasis and the fact that a number of severe diseases (e.g., Alzheimer's disease and cancers) are attributed, at least in part, to aberrant regulation of apoptosis (24,38-40). Lately, there have been novel findings showing that apoptosis is consistently evident under various muscle atrophic situations (e.g., muscle unloading), and thereby implicated that, in post-mitotic skeletal muscle, apoptosis may further have a role in the regulation of muscle loss (1-12,14,41). Recently, the apoptotic events have been investigated in our laboratory by using a quail hypertrophied-muscle unloading model which permits us to examine apoptosis and the regulatory mechanisms during muscle atrophy where the reducing muscle mass was still above the basal level (3,12,13). Data from our laboratory have shown apoptotic changes in unloaded muscle that was first hypertrophied including increases in caspase protease activities, cleaved poly(ADP-ribose) polymerase (PARP)-positive nuclei, pro-apoptotic Bax, p53, cytosolic Id2, and TUNEL-positive nuclei as well as decrease in anti-apoptotic Bcl-2 (3,12,13). We have also demonstrated that apoptosis functions to eliminate excessive activated/proliferated muscle precursor cell nuclei (e.g., satellite cell nuclei) during muscle regression from a state of hypertrophy towards control levels (13). In the current study, we further demonstrated that the protein expression of apoptotic suppressor XIAP is elevated during muscle overloading in both young adult and aged muscles and this hypertrophy-associated elevation of XIAP is then down-regulated during subsequent removal of the overload in the young adult muscle. It is noted that the findings of XIAP are in accordance to our previous observations of increased apoptotic markers (e.g., TUNEL nuclei) in these unloaded muscles (12,13) and the identified anti-apoptotic properties of the apoptotic suppressors (25-27). These findings indicate that down-regulation of the elevated XIAP expression during previous hypertrophy is required for mediating the activation of apoptosis during unloading in young adult muscle.

Aging alters the response of XIAP to unloading in previously hypertrophied muscle. The response of apoptotic suppressor XIAP to unloading following loading-induced hypertrophy differed

between young adult and aged muscles. We have recently demonstrated reduced pro-apoptotic tendencies including decreases in Bax and AIF, and an increase in Bcl-2 in hypertrophied muscles of aged birds that were unloaded for 14-days (12,13). Although the reason for this decreased pro-apoptotic tendency in unloaded aged muscle was not resolved, it appeared that the aged hypertrophied muscle attempted to initiate pathways that would attenuate the promotion of pro-apoptotic signaling during muscle unloading. But clearly, these changes did not prevent the aged muscle from increases in TUNEL-indicated apoptosis or muscle loss during unloading (12,13). In the present study, although all the measurements of apoptotic suppressors did not show any changes with unloading in young adult hypertrophied muscle, we found that the mRNA and protein contents of XIAP were higher in the 7- or 14-days unloaded muscles relative to the contralateral control muscles from the aged animals. It is noted that these XIAP findings agreed with the reduced pro-apoptotic tendency that we have previously observed in these aged hypertrophied muscle during unloading (12,13). Furthermore, after all the control and unloaded muscle samples were pooled as a single group, we found that XIAP protein content was positively correlated with the TUNEL index (13) where this relationship was mainly attributed to the elevation of XIAP in the aged muscles following 7- or 14-days of unloading (**Figure 3**). We interpret our data to indicate that the increased XIAP levels represents a compensatory response to unloading in aged hypertrophied muscles. Indeed, it has been demonstrated that XIAP was elevated under certain age- or disuse-associated atrophic situations (6,42,43). By comparing 26-month (aged) to 12-month (adult) old Fischer 344 rats, Dirks and Leeuwenburgh (6) reported that XIAP was upregulated concomitant with the elevation of pro-/cleaved caspase-3 protein content but unaltered caspase-3 protease activity and increased apoptotic DNA fragmentation in gastrocnemius muscles from the aged animals. It is noted that we also found that a main effect of age exists in influencing the XIAP protein content in the birds examined in this study. This suggests that muscles from aged birds had a greater content of XIAP protein relative to muscles in the young birds. Providing that the anti-apoptotic effect of XIAP is likely related to its suppressive influence on caspase-3 and/or -9 (26,44), findings by Dirks and Leeuwenburgh denoted that an increase in XIAP with aging might be an adaptive response for the aged muscle to overcome the increasing tendency of the caspase activation, but obviously these did not preclude the aged muscle from elevation of apoptosis as well as incidence of sarcopenic muscle loss (6). Dirks and Leeuwenburgh also demonstrated that life-long caloric restriction, an anti-apoptotic intervention, reduced the XIAP content in the aged gastrocnemius muscle (6). In human osteoblastic cells, the compensatory response of XIAP was observed following gravity unloading, where the ratio of Bax/Bcl-2 increased concurrently with the upregulation of XIAP and resulted in unchanged apoptosis following gravity unloading (43). Moreover, under the pathophysiologic condition in human skeletal muscle, it has been demonstrated that sarcoplasmic expression of XIAP, as determined by immunohistochemical and

immunoblot analyses, was evident in patients with mitochondrial encephalomyopathies, and accordingly the authors suggested that sarcoplasmic XIAP expression might be involved in suspending the apoptotic process in mitochondrial encephalomyopathies (42). Furthermore, increase in XIAP has also been documented in cerebral tissues after ischemia-reperfusion injury or with aging (45,46). Taken together, it is not unreasonable that our observed sustained increase in XIAP during unloading following hypertrophy might have a compensatory regulatory role in the aged muscle. Nonetheless, we are aware that the present data did not allow us to elucidate the precise mechanisms in explaining the physiologic function of XIAP in the present experimental situation. Further investigation is needed to fully understand this distinct aging-associated response of XIAP to unloading in hypertrophied muscle.

According to the existing evidence, it has been proposed that the regulatory process of skeletal muscle hypertrophy and atrophy is related to the alteration of the myonuclear number. While this idea originates from the “myonuclear domain hypothesis”, which suggests that the cytoplasmic volume per myonuclei is strictly maintained in a homeostatic manner in the multinucleated skeletal myocytes (47-49), there also has been data suggesting that aging may influence the homeostatic balance of myonuclear domain under atrophic condition in a rodent hindlimb unweighting model (50). Nonetheless, by using the technique of BrdU incorporation, previous investigations have demonstrated the number of muscle-related BrdU-positive nuclei increase with stretch-overload in young and aged quails (3,51). Moreover, BrdU positive nuclei are eliminated during unloading because the number of BrdU-positive nuclei decreases back toward the basal level after subsequent removal of the load in the hypertrophied muscles. The decline of these muscle-related BrdU-positive nuclei is associated with apoptosis as demonstrated by the concomitant labeling of TUNEL and BrdU (3,13). Taken together, these data show that myogenic precursor cell populations (e.g., muscle satellite cells) are activated and proliferate, and they contribute to the increase in myonuclear number in response to stretch-overload. The newly incorporated (i.e., BrdU positive) myonuclei gained during overload are eliminated possibly through apoptosis when the load is subsequently removed in the quail hypertrophied-muscle unloading model.

Overall, our findings are in accordance with the hypothesis that elimination of the myonuclei gained during previous hypertrophy (possibly through apoptotic machinery) may be an important factor in mediating the process of muscle loss from hypertrophic state to normally loaded state in skeletal muscle of young adult bird (12,13). However, as indicated by the present XIAP data and our previous findings, the apoptotic events that occur in the aged hypertrophied muscle during unloading are evidently different from the muscle of young adult animals. It is not completely clear why the aged quail muscle exhibits up-regulation of the anti-apoptotic machinery during unloading following hypertrophy, as the losses of muscle mass and BrdU-positive muscle-related nuclei are still apparent

with unloading (13). Based on the fact that aged skeletal muscle shows evidence of accelerated apoptosis (5,7,22), it is noted that our interpretations are limited by speculating that this age-related decreased pro-apoptotic tendency maybe a compensatory response to unloading following hypertrophy. We speculate that the increase in anti-apoptotic proteins may be an adaptive event, which is designed to partially offset the increase in apoptosis. This could have a functional effect by preserving muscle mass and therefore muscle force to a greater extent in old animals. Although muscle force increases with loading (52,53), we have not evaluated the functional components of the unloaded quail muscles.

In conclusion, we have demonstrated XIAP protein expression is elevated in response to muscle overload in both young adult and aged muscles while this elevated XIAP protein level returns to the basal level after 7- or 14-days of subsequent unloading in the muscles from young adult birds. We have provided evidence demonstrating that the response of apoptotic suppressor XIAP to unloading following hypertrophy is different between young adult and aged muscles. We have shown that the mRNA and protein contents of XIAP in the 7- or 14-days unloaded muscles were higher than that in the contralateral control muscles in the aged birds, but these changes were not found in young adult birds. Also, the XIAP protein content was positively correlated with the TUNEL index in all the muscle samples. We speculated that the sustained increase in XIAP in aged hypertrophied muscle maybe a compensatory response to unloading. Although the exact reason for this aging-related distinct response is unknown, these findings agree that the apoptotic regulation is complicated in aged muscle compared to the young muscle. More research is warranted in investigating the influence of aging in the apoptotic regulation with the aim of fully understanding the complexity of the apoptotic mechanisms in the aged skeletal muscle.

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Figure 1A XIAP mRNA

Legend for Figure 1A XIAP mRNA. The mRNA content of XIAP was examined by RT-PCR in the PAT muscles from young adult (Young) and old quails (Old) following 14-days of overloading (14d loading), 7-days (7d unloading) or 14-days of unloading after stretch-overloaded hypertrophy (14d unloading). The insets show representative inverted images for the mRNA results. PCR products were visualized with ethidium bromide staining. Quantification of PCR signals was obtained by densitometric analysis of the signal product optical density (OD) x resulting band area. Gene expression is normalized to the ribosomal 18S signal from the same RT product. The normalized data are presented as means $\pm$ standard error of mean (SE). * $P < 0.05$, data are significantly different from the contralateral control.

Figure 1B FLIP mRNA

Legend for Figure 1B FLIP mRNA. The mRNA content of FLIP was examined by RT-PCR. The insets show representative inverted images for the mRNA results. PCR products were visualized with ethidium bromide staining. Quantification of PCR signals was obtained by densitometric analysis of the signal product optical density (OD) x resulting band area. Gene expression is normalized to the ribosomal 18S signal from the same RT product. The normalized data are presented as means $\pm$ standard error of mean (SE).

Figure 2A XIAP protein

Legend for Figure 2A XIAP protein. The protein content of XIAP was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10^6 . The insets show representative blots for XIAP in control and experimental muscles isolated from young adult and old animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from contralateral control muscles. #P < 0.05, data are significantly different from 14-days hypertrophied muscle of the same age group. According to the results of 2×2 ANOVA, a main effect of age [$F(1,60)=48.96$, $P=0.0001$] was found in muscles of the unloaded animals.

Figure 2B ARC protein

Legend for Figure 2B ARC protein. The protein content of ARC was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁶. The insets show representative blots for ARC. The data are presented as means ± SE.

Figure 3 Correlation between XIAP protein and TUNEL index

Legend for Figure 3 Correlation analysis of XIAP protein and TUNEL index. The relationship between XIAP protein content and TUNEL index was determined by examining the Pearson product-moment correlation coefficient, r . The control and experimental muscles from both young adult and old quails of all groups were collapsed and treated as a single pooled group ($N = 64$). Y7, young adult quails after 7-days of unloading; Y14, young adult quails after 14-days of unloading; O7, old quails after 7-days of unloading; O14, old quails after 14-days of unloading.

Table 1. Muscle mass change

	14d stretch-overloading	7d unloading	14d unloading
Young adult quail	~35%	$15 \pm 3\%$ *	$2 \pm 1\%$
Old quail	~15%	$12 \pm 2\%$ *	$6 \pm 2\%$ *

Muscle mass loss during unloading following stretch-induced hypertrophy was monitored by examining the decrease in the extent of muscle hypertrophy. The extent of muscle hypertrophy was determined by examining the % difference in the muscle wet weight between the experimental and the contralateral control whole PAT muscles. The data are presented as means $\pm$ SE. *P < 0.05, significant % difference in muscle wet weight relative to control muscle. 7d unloading, 7-days of unloading following 14-days of stretch-overloading; 14d unloading, 14-days of unloading following 14-days of stretch-overloading.

Table 2. Primers used for PCR amplification of cDNA

Product	Accession No.	Sequence	Position	T _A , °C	Cycles	Product length, bp	Restriction enzyme	Restriction products, bp
XIAP	AF451854	F: 5'-CCCCCTATGGCCTATTGAC-3' R: 5'-CCGCCTCTCATATTCTGCCAT-3'	584-603 851-871	54.9	32	288	<i>NcoI</i> <i>AluI</i>	266, 22 93, 92, 58, 34, 11
FLIP	XM_421935	F: 5'-GCCAGAAGATGAGCAAGAATATACTA-3' R: 5'-TGGCTTGGGTCAGTTGTCTTATTC-3'	1161-1186 1434-1457	52.7	36	297	<i>AluI</i> <i>EcoRV</i>	196, 101 129, 109, 59

T_A, annealing temperature; Accession No., GenBank accession number; F, forward primer; R, reverse primer.

CHAPTER 5

Deficiency of the *Bax* gene attenuates denervation-induced muscle wasting

ABSTRACT

Apoptosis has been implicated in mediating denervation-induced muscle wasting. Here we determined the effect of interference of apoptosis on muscle wasting during denervation by using animals genetically deficient of pro-apoptotic Bax. After denervation, muscle wasting was evident in both wild-type and Bax^{-/-} muscles but reduction of muscle weight was attenuated in Bax^{-/-} mice. Apoptotic DNA fragmentation increased in wild-type denervated muscle whereas the increase in Bax^{-/-} mice did not reach significant level. Mitochondrial AIF and Smac/DIABLO releases and Bcl-2, p53 and HSP27 increased whereas XIAP and MnSOD decreased to a similar extent in wild-type and Bax^{-/-} muscles following denervation. Mitochondrial cytochrome c release was elevated in wild-type denervated muscle but the increase was suppressed in Bax^{-/-} mice. Increases in caspase-3 and -9 activities and oxidative stress markers H₂O₂, MDA/4-HAE and nitrotyrosine were all evident in denervated wild-type muscle but these changes were absent in Bax^{-/-} mice. Moreover, ARC increased exclusively in denervated Bax^{-/-} muscle. Our data demonstrated the attenuation of muscle wasting in accompanied by suppressed pro-apoptotic signaling in skeletal muscle that is deficient of Bax during denervation. These findings suggest that interventions targeting apoptosis may be valuable in ameliorating denervation-associated pathologic muscle wasting in certain neuromuscular disorders.

Key words: muscle atrophy; apoptosis; BCL-2; neuromuscular disease

INTRODUCTION

Innervation of nerves is known to be an essential constituent in the development and survival of mature skeletal muscle carrying customary physiologic functions. It has been also exhibited that nerve innervation has a significant role in determining and maintaining muscle fiber volume as well as phenotype (1, 2). In regards to the clinical situations, nerve innervation removal or denervation is generally involved in the etiology and progression of certain neuromuscular diseases which comprise degeneration and withdrawal of motoneurons, for instance, spinal muscular atrophy, peripheral neuropathies, and amyotrophic lateral sclerosis (3). Intriguingly, there is a growing body of evidence indicating that an active controlled cell death mechanism, apoptosis, may have a regulatory role in the denervation-associated pathologic muscle wasting (4-8). Nevertheless, the importance of apoptosis in mediating muscle wasting in response to the removal of nerve innervation (i.e., denervation) remains to be fully elucidated.

A cluster of upstream regulators of apoptosis, termed BCL-2 family, has been suggested to be a crucial intracellular checkpoint in the apoptotic signaling pathway (9). In general, mammalian cells possess an entire family of BCL-2 proteins. As defined by the homology shared within four conserved sequence motifs namely BCL-2 homology (BH1-4) domains, BCL-2 family is divided into three subclasses including anti-apoptotic (e.g., Bcl-2, Bcl-X_L, Bcl-W, A1, and Mcl-1), multidomain pro-apoptotic (Bax, Bak, and Bok), and BH3-only pro-apoptotic (Bad, Bid, Bim, Bik, Dp5/Hrk, Noxa, and Puma) (9-11). The BH3 domain is thought to be fundamental for the pro-apoptotic interactions since all pro-apoptotic members contain a BH3 domain, although some anti-apoptotic members also contain the BH3 domain (10). Provided that the BH3 sequence motif refers to a hydrophobic α -helix which is favorable for protein interaction, it has been hypothesized that this is the region for the association among the BCL-2 family proteins via homo- or hetero-oligomerization (10, 11). Additionally, there have been evidences suggesting that the relative concentrations of pro- and anti-apoptotic BCL-2 members provide a tight control over the balance of cell survival and apoptotic cell death (9, 10, 12).

Among the entire BCL-2 family, Bax and Bcl-2 have been designated to be the main protagonists in the regulation of apoptotic machinery as they bear the ion channel forming activities. Bax has been demonstrated to translocate to the mitochondria and expose its N-terminus via a conformational change upon induction of apoptosis (13-17). This conformational change has been suggested to allow the Bax-Bax-oligomerization and insertion of Bax into the outer mitochondrial membrane (18), which is followed rapidly by the release of the apoptogenic factors (e.g., cytochrome c and AIF) from the mitochondrial intermembrane space. Collectively, Bax oligomerization is thought to be critical for mitochondrial membrane permeabilization whereas Bcl-2 opposes the pro-apoptotic activity of Bax by preventing the process of Bax-Bax-oligomerization (19). Although the precise

mechanism of Bax-mediated apoptogenic factors release from the mitochondrial intermembrane space is still under active investigation, it is indisputable that Bax plays an important role in promoting the activation of apoptotic signaling cascades.

Previously, the activation of mitochondria-associated apoptosis has been apparently exhibited in skeletal muscle during denervation which suffers a considerable loss of muscle mass (20). Since pro-apoptotic Bax is markedly upregulated and is possibly an important mediator for the activation of apoptosis in response to muscle denervation (20-22), here we tested the hypotheses that deficiency of the pro-apoptotic *Bax* gene would suppress the pro-apoptotic signaling in the atrophying skeletal muscle during denervation and attenuate the extent of denervation-induced muscle loss. The results of this study are of use to determine the feasibility of adopting interventions that target the regulation of apoptosis in offsetting the denervation-associated muscle wasting pathologies in the future.

METHODS

Animals. Experiments were conducted on ~3-mo old adult C57BL/6 wild-type mice (N=8) and homozygous B6.129X1-*Bax*^{tm1Sjk}/J the *Bax* gene-deficient mice (N=6) that were obtained from the Jackson Laboratory JAX® Mice Colony (Bar Harbor, Maine). The deficiency of Bax in the knockout mice was confirmed by immunoblotting on two affinity-purified rabbit polyclonal antibodies raised against peptide mapping at the N-terminus or amino acids 1-171 of Bax (sc-4936 and sc-236, Santa Cruz Biotechnology, Santa Cruz, CA). The mice were housed in pathogen-free conditions at ~20°C after arrival. They were exposed to a reverse light condition of 12:12 h of light: darkness each day and were fed chow and water ad libitum for a familiarization period of at least 5 days before the denervation surgery.

Muscle denervation procedure. The mice were placed under a general anaesthesia using 2% isoflurane. After reflex activity had disappeared, an incision was made from the calcaneus to just proximal to the popliteal fossa. The tibial nerve was then dissected proximal to the cranial border of the gastrocnemius muscle. Care was taken to avoid any damage to the nerves, blood vessels, and connective tissues. The medial and lateral branches of the tibial nerve that innervate the plantar flexor muscles (i.e., gastrocnemius and soleus) were transected close to their neuromuscular junction (23) and ~1-2 mm of the transected nerve was removed. Innervation to the plantaris and the deep toe flexor muscles were left intact so that the animals ambulated normally around the cage after the surgical denervation. Following the surgery, the hamstring muscle layers were closed with reabsorbable suture and the skin incision were closed with wound clips. The incision sites were covered with an antibacterial cream to prevent infection. The denervation procedure was performed in one limb and sham procedure (whole procedure except the transection of the nerve segment) was performed in the contralateral limb of the mice which served as the intra-animal control. We have observed that the

mice recovered quickly and were able to walk around within minutes post-surgery as observed in rats after denervation surgery (21, 24).

Fourteen days after the surgical denervation, mice were euthanized with an overdose of pentobarbital sodium while the gastrocnemius muscle from each limb were dissected from the surrounding connective tissues, removed, and stored at -80°C. The institutional animal use and care committee from West Virginia University School of Medicine approved all experiments. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care and fully conformed with the Animal Welfare Act of the U.S. Department of Health and Human Services.

Subcellular protein fractionation. The fractionation method described by Rothermel et al. (25) was adopted with minor modification to extract the cytosolic and nuclear protein fractions from the gastrocnemius muscle. We have previously obtained the fractionated cytosolic and nuclear proteins from skeletal and heart muscles using this modified protocol (20, 26-29). Briefly, after removal of connective tissues, muscle was homogenized on ice in ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT, pH 7.4). Following centrifuging at 3,000 rpm for 3 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and these supernatants were further centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual a nuclei and stored as nuclei-free total cytosolic protein fraction. A portion of the cytosolic extract (without addition of protease inhibitors) was stored and used for fluorometric caspase protease activity assay while a protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO) was added to the remaining portion. The cytosolic protein fraction with the addition of protease inhibitors was later used for cell death ELISA, Western immunoblots, H₂O₂ assay, MDA/4-HAE assay, and nitrotyrosine dot blot. The remaining nuclear pellets were washed 3 times with ice-cold lysis buffer, resuspended in 300 µl of lysis buffer in the presence of 41.5 µl of 5 M NaCl and protease inhibitor cocktail, and rotated for 1 h at 4°C to lyse the nuclei. Following a spin at 15,000 rpm for 15 min at 4°C, the supernatants were collected and stored as a cytosol-free nuclear protein fraction.

Furthermore, with the intention of estimating the release of mitochondria-residing apoptotic factors including cytochrome c, AIF, and Smac/DIABLO into the cytosol, a nuclei-free, mitochondria-free cytosolic protein fraction was prepared as described by Rokhlin et al. (30) and the protein contents of these mitochondrial apoptotic factors were then measured in this mitochondria-free cytosolic fraction as described in the later sections. For the extraction, muscle was dissected from the connective tissues and minced in ice-cold extraction buffer (250 mM sucrose, 20 mM HEPES, 10

mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) in the presence of the protease inhibitor cocktail. Following a gentle homogenization with a Teflon pestle motorized with an electronic stirrer, homogenates were centrifuged at $800 \times g$ for 10 min at 4°C to pellet the nuclei and cell debris. The supernatants were then spun twice at $16,000 \times g$ for 20 min at 4°C to pellet the mitochondria and the final supernatants were collected as nuclei-free, mitochondria-free cytosolic protein fractions. These subcellular protein fractionation procedures have been routinely used in our laboratory to obtain high purity protein fractions as assessed by immunoblotting the fractions with an anti-histone H2B (a nuclear protein) (07371, Upstate, Lake Placid, NY), an anti-CuZnSOD (a cytosolic isoform of superoxide dismutase) (sc-11407, Santa Cruz Biotechnology, Santa Cruz, CA), and an anti-MnSOD (a mitochondrial isoform of superoxide dismutase) antibody (A300449A, Bethyl Lab, Montgomery, TX) (20, 27, 28).

The protein contents of the muscle extracts were quantified in duplicate by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation (31). As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion by DC Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay (32).

Apoptotic cell death ELISA. Cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligo-nucleosomes. Briefly, the nuclei-free cytosolic fraction of gastrocnemius muscle was used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated to the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate for 10 min at 20°C. The change in color was measured at a wavelength of 405 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). Measurements were performed in duplicate, with denervated and contralateral control samples analyzed on the same microtiter plate in the same setting. The OD₄₀₅ reading was then normalized to the mg of protein used in the assay.

Fluorometric caspase activity assay. A fluorometric assay was used to examine the protease activities of caspase-3 and -9 in the denervated gastrocnemius muscle. In brief, 50 µl of the total cytosolic protein fraction without protease inhibitor of the muscles was incubated in 50 µl of assay buffer (50 mM PIPES, 0.1 mM EDTA, 10% glycerol, 10 mM DTT, pH 7.2) with 100 µM of the fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated substrate (Ac-DEVD-AFC for

caspase-3, Ac-LEHD-AFC for caspase-9, Alexis Corp., San Diego, CA) at 37°C for 2 h. Caspase specific inhibitor, Z-VAD-FMK (Calbiochem, La Jolla, CA) was used as a control to validate the specificity of caspase. The change in fluorescence was measured on a spectrofluorometer with an excitation wavelength of 390/20 nm and an emission wavelength of 530/25 nm (CytoFluor, Applied Biosystems, Foster City, CA) before and after the 2 h incubation. Caspase activity was estimated as the change in arbitrary fluorescence units normalized to milligram protein. Measurements were performed in duplicate while denervated and intra-animal control samples were run on the same microplate in the same setting.

Western immunoblot analyses. The protein expressions of Bcl-2 associated X protein (Bax), B-cell lymphoma/leukemia-2 (Bcl-2), X-linked inhibitor of apoptosis (XIAP), apoptosis repressor with caspase recruitment domain (ARC), heat shock protein-70 (HSP70), HSP27, HSP60, and superoxide dismutases (CuZn-SOD and Mn-SOD) was determined in the total cytosolic protein fraction while apoptosis inducing factor (AIF) was measured in the nuclear fraction. Both the total cytosolic and nuclear fractions were used to measure the protein content of p53.

Forty micrograms of protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE at room temperature. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and probed with the following primary antibodies diluted in PBS-T with 2% BSA: anti-Bcl-2 mouse monoclonal antibody (1:100 dilution, sc-7382), anti-Bax rabbit polyclonal antibody (1:200 dilution, sc-493), anti-hILP/XIAP mouse monoclonal antibody (1:250 dilution, 610762, San Jose, CA), anti-ARC rabbit polyclonal antibody (1:200 dilution, sc-11435), anti-AIF mouse monoclonal antibody (1:800 dilution, sc-13116HRP), anti-p53 mouse monoclonal antibody (1:100 dilution, sc-99), anti-HSP70 mouse monoclonal antibody (1:2000 dilution, SPA810B), anti-HSP27 rabbit polyclonal antibody (1:2000 dilution, SPA801), anti-HSP60 rabbit polyclonal antibody (1:2000 dilution, SPA804), anti-SOD-1 rabbit polyclonal antibody (1:500 dilution, sc-11407), or anti-MnSOD goat antibody (1:2000 dilution, A300449A, Bethyl Lab, Montgomery, TX). Bcl-2, Bax, ARC, SOD-1, AIF, and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while HSP70, HSP27, and HSP60 antibodies were purchased from StressGen (Victoria, BC, Canada). All primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed by West Pico chemiluminescent substrate (Pierce, Rockford, IL). The signals were then

visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Estimation of mitochondrial cytochrome c, Smac/DIABLO, and AIF release.

Cytochrome c, AIF, and Smac/DIABLO (second mitochondria-derived activator of caspase) are apoptotic factors normally confined to mitochondria and their release into the cytosol has been demonstrated during the activation of apoptosis (33). In the present study, the release of Smac/DIABLO and AIF into the cytosol was estimated by measuring their protein contents in the extracted mitochondria-free cytosolic protein fraction by immunoblotting with an anti-Smac/DIABLO mouse monoclonal antibody (1:500 dilution, 612244, BD Biosciences, San Jose, CA) and an anti-AIF monoclonal mouse antibody. Moreover, a cytochrome c ELISA kit (MBL International, Woburn, MA) was used to assess the protein content of cytochrome c in the mitochondria-free cytosol fraction to evaluate the release of the mitochondrial cytochrome c into the cytosol. By following the manufacturer's protocol, 60 μ l the extracted mitochondria-free cytosolic fraction was used as an antigen source in a sandwich ELISA with a horseradish peroxidase-conjugated anti-cytochrome c polyclonal antibody in microwell strips coated with an anti-cytochrome c antibody. After washing, the peroxidase retained in the immunocomplex was detected by incubating with a chromogenic substrate, tetramethylbenzidine/hydrogen peroxide (TMB/H₂O₂) followed by adding an acid solution to terminate the enzyme reaction and to stabilize the developed color. The change in color was monitored at a wavelength of 450 nm using a Dynex MRX plate reader. Measurements were performed in duplicate with the denervated and contralateral control samples analyzed on the same microplate and the cytochrome c content was expressed as OD₄₅₀ per mg protein.

MDA/4-HAE Assay. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (4-HAE) upon decomposition (34). In the present study, the content of MDA/4-HAE was measured in cytosolic fraction as an indicator of lipid peroxidation using a commercial colorimetric assay kit (21012, Oxis International, Portland, OR). Briefly, 50 μ l of the cytosolic protein fraction or standards with known concentrations was incubated with a chromogenic reagent, N-methyl-2-phenylindole, at 45°C for 1 h and the generated chromophore after incubation was detected by spectrophotometry at 586 nm. The MDA/4-HAE concentration of samples was determined according to the standard curve generated in the same setting. Results were presented as OD₅₈₆ normalized to milligram protein used in the assay. Control and suspended samples were run in the same setting to eliminate any assay-to-assay variations.

H₂O₂ Fluorometric Assay. The content of hydrogen peroxide (H₂O₂) in the muscle homogenate was measured in the cytosolic fraction using a fluorometric H₂O₂ detection kit (FLOH 100-3, Cell Technology Inc., Mountain View, CA). By following the manufacturer's instruction, 50 μ l of the total cytosolic fraction protein of the gastrocnemius muscle was incubated in 50 μ l of reaction cocktail containing horseradish peroxidase and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP, a non-fluorescent substrate turns fluorescent after oxidized by H₂O₂) in sodium phosphate buffer at room temperature in dark for 10 min. The fluorescence was measured on a spectrofluorometer with an excitation wavelength of 530/25 nm and an emission wavelength of 590/35 nm (CytoFluor, Applied Biosystems, Foster City, CA) after the incubation. H₂O₂ content was estimated as the arbitrary fluorescence units normalized to milligram protein used in the assay. Measurements were performed with the suspended and control samples run on the same microplate in the same setting.

Nitrotyrosine Dot Blot. As an index of the production of reactive nitrogen species, an immuno-dot blot was carried out to estimate the total nitrotyrosine content in the cytosolic fraction of the gastrocnemius muscles from suspended and control animals. In brief, 4 μ g of cytosolic protein was dotted on a nitrocellulose membrane (VWR, West Chester, PA). After air dry, the membrane was blocked in 5% non-fat milk in PBS-T at room temperature for 1 h and then probed with an anti-nitrotyrosine mouse monoclonal antibody (1:1000 dilution, MAB5404, Chemicon International, Temecula, CA) diluted in PBS-T with 2% BSA incubated at 4°C for overnight. The membrane was incubated with a HRP-conjugated anti-mouse IgG secondary antibody (AP124P, Chemicon International, Temecula, CA) at room temperature for 1 h, and then signals were developed by chemiluminescence (Pierce Biotechnology, Rockford, IL). Negative control experiments were performed by omitting either the anti-nitrotyrosine or the secondary antibody. The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting dot signals were quantified in arbitrary units as optical density (OD) $\times$ dot area using Kodak one-dimensional (1-D) image analysis system (Eastman Kodak, Rochester, NY).

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package. ANOVA followed by Tukey HSD post hoc analysis was used to examine differences between groups. Independent t-test was performed to examine the differences of the variable percent change between wild-type and Bax^{-/-} animals. All data are given as means $\pm$ standard error of mean (SE). Statistical significance was accepted at $P < 0.05$.

RESULTS

Muscle weight change. The denervation-induced muscle loss was monitored by examining the gastrocnemius muscle wet weight normalized to the animal's bodyweight between the denervated

and intra-animal contralateral control limbs in wild-type and Bax^{-/-} mice. Following 14 days of denervation, the normalized muscle wet weight was decreased by 42% and 33% in wild-type and Bax^{-/-} mice, respectively (wild-type: 4.5 vs. 2.6 mg/g; Bax^{-/-}: 4.2 vs. 2.8 mg/g) (**Figure 1A**). The muscle weight percent decline in the Bax^{-/-} mice was significantly lower than the wild-type mice (wild-type vs. Bax^{-/-}: -42% vs. -33%) (**Figure 1B**).

Insert Figure 1A ———→ Normalized muscle weight

Insert Figure 1B ———→ Normalized muscle weight percent change

Apoptotic DNA fragmentation. The cell death ELISA analysis indicated that the extent of apoptotic DNA fragmentation in the denervated muscle was 161% greater than the control muscle in the wild-type mice whereas, in the Bax^{-/-} mice, the increase in the apoptotic DNA fragmentation in the denervated muscle relative to the control muscle of was not statistically significant (74%, $P > 0.05$) (**Figure 2A**). Significant difference was found in the percent change of apoptotic DNA fragmentation between the wild-type and Bax^{-/-} mice (wild-type vs. Bax^{-/-}: +161% vs. +74%) (**Figure 2B**).

Insert Figure 2A ———→ Apoptotic DNA fragmentation

Insert Figure 2B ———→ Apoptotic DNA fragmentation percent change

BCL-2 family: Bax and Bcl-2 protein contents. According to our immunoblot analysis, we did not detect the presence of Bax protein in all samples from the Bax^{-/-} animals (**Figure 3A**). For the wild-type mice, the Bax protein content in the denervated muscle was 415% higher than the contralateral control muscle (**Figure 3A and 3B**). The protein content of Bcl-2 in the muscle following denervation was elevated by 353% and 307% relative to the contralateral control muscle in the wild-type and Bax^{-/-} mice, respectively (**Figure 3C**) while the percent change of Bcl-2 protein content was not different in the wild-type and Bax^{-/-} mice (**Figure 3D**).

Insert Figure 3A ———→ Bax protein

Insert Figure 3B ———→ Bax protein percent change

Insert Figure 3C ———→ Bcl-2 protein

Insert Figure 3D ———→ Bcl-2 protein percent change

Mitochondrial cytochrome c release. The ELISA analysis on the mitochondria-free cytosolic fraction demonstrated that the protein content of cytosolic cytochrome c in the denervated

muscle increased by 98% and 46% when compared to the control muscle in the wild-type and Bax^{-/-} mice, respectively (**Figure 4A**). As cytochrome c is primarily resided in the mitochondria under normal condition, this indicated that cytochrome c was relocated to the cytosol during denervation in both wild-type and Bax^{-/-} muscles. Moreover, the percent increase in the release of mitochondrial cytochrome c in the Bax^{-/-} mice was significantly lower than the wild-type animals (wild-type vs. Bax^{-/-}: +98% vs. +46%) (**Figure 4B**).

Insert Figure 4A ———→ Mitochondrial cytochrome c release

Insert Figure 4B ———→ Mitochondrial cytochrome c release percent change

Caspase-3 and -9 protease activities. The activation of caspase-3 and -9 was determined by measuring the specific protease activity in the muscle samples using a fluorometric caspase enzymatic activity analysis. There was a 150% increase in the protease activity of caspase-3 in the denervated muscle when compared to the control muscle in the wild-type mice but the increase (55%, $P > 0.05$) in the caspase-3 activity in the denervated muscle relative to the control muscle of the Bax^{-/-} mice did not reach the significant level (**Figure 5A**). A significant difference was found in the percent change in caspase-3 activity following denervation in the wild-type and Bax^{-/-} mice (wild-type vs. Bax^{-/-}: +150% vs. +55%) (**Figure 5B**). The protease activity of caspase-9 was 81% higher in the denervated muscle when compared to the contralateral control muscle in the wild-type animals (**Figure 5C**). In contrast, no difference in the caspase-9 activity was found between the denervated and control sides in the Bax^{-/-} mice (**Figure 5C**). Furthermore, the percent change in the caspase-9 activity in response to denervation was significantly different in the wild-type and Bax^{-/-} mice (wild-type vs. Bax^{-/-}: +81% vs. -5%) (**Figure 5D**).

Insert Figure 5A ———→ Caspase-3 protease activity

Insert Figure 5B ———→ Caspase-3 protease activity percent change

Insert Figure 5C ———→ Caspase-9 protease activity

Insert Figure 5D ———→ Caspase-9 protease activity percent change

Mitochondrial Smac/DIABLO release, XIAP and ARC protein contents. As indicated by our immunoblot analysis on the mitochondria-free cytosolic fraction, the protein content of Smac/DIABLO, an upstream inhibitor of XIAP, increased by 213% and 252% in the cytosolic fraction (in the absence of mitochondria) of the denervated muscle relative to the control muscle in the wild-type and Bax^{-/-} mice, respectively (**Figure 6A**). These denoted that mitochondrial Smac/DIABLO was

released into the cytosol during denervation in both wild-type and $Bax^{-/-}$ mice. No significant difference was found in the percent change of mitochondrial Smac/DIABLO release in the wild-type and $Bax^{-/-}$ mice (**Figure 6B**). The protein content of XIAP, an inhibitor of caspases, as detected by immunoblotting on the total cytosolic fraction was 41% and 39% lower in the muscle following denervation when compared to the contralateral control muscle in the wild-type and $Bax^{-/-}$ mice, respectively (**Figure 6C**). But, no difference was found in the denervation-induced decrease in the XIAP protein content between the wild-type and $Bax^{-/-}$ mice (**Figure 6D**). ARC, an apoptotic suppressor, protein content was not altered with denervation in the wild-type animals ($P > 0.05$) whereas the protein content of ARC increased by 37% in the denervated muscle relative to the contralateral control muscle in the $Bax^{-/-}$ mice (**Figure 6E**). Furthermore, the percent change of ARC protein content was significantly different between the wild-type and $Bax^{-/-}$ mice (wild-type vs. $Bax^{-/-}$: -11% vs. +37%, **Figure 6F**).

Insert Figure 6A ———→ Mitochondrial Smac/DIABLO release

Insert Figure 6B ———→ Mitochondrial Smac/DIABLO release percent change

Insert Figure 6C ———→ XIAP protein

Insert Figure 6D ———→ XIAP protein percent change

Insert Figure 6E ———→ ARC protein

Insert Figure 6F ———→ ARC protein percent change

Mitochondrial AIF release and nuclear translocation. The mitochondrial release and nuclear translocation of AIF were estimated by immunoblotting on the mitochondria-free cytosolic and nuclear fractions, respectively. The AIF protein content measured in the mitochondria-free cytosolic fraction of the denervated muscle was 80% and 74% higher than the intra-animal control muscle in the wild-type and $Bax^{-/-}$ mice, respectively (**Figure 7A**). These indicated that AIF was released from mitochondria into the cytosol during denervation in both the wild-type and $Bax^{-/-}$ animals. No significant difference was found in the percent change of mitochondrial AIF release between the wild-type and $Bax^{-/-}$ mice (**Figure 7B**). In contrast, we did not find any difference in the nuclear AIF protein content between the denervated and control muscles in both the wild-type and $Bax^{-/-}$ mice (**Figure 7C and 7D**).

Insert Figure 7A ———→ Mitochondrial AIF release

Insert Figure 7B ———→ Mitochondrial AIF release percent change

Insert Figure 7C ———→ AIF nuclear translocation

Insert Figure 7D ———→ AIF nuclear translocation percent change

Tumor suppressor p53 protein content. Our immunoblot analysis indicated that the p53 protein content increased by 175% and 167% in the nuclear fraction of the muscle following denervation relative to the contralateral control muscle in wild-type and *Bax*^{-/-} mice, respectively (**Figure 8A**). No significant difference was found in the percent change of nuclear p53 protein content between the wild-type and *Bax*^{-/-} mice (**Figure 8B**). In the cytosolic fraction, there was a 353% and 362% increase in the p53 protein content in the denervated muscle when compared to the control muscle in the wild-type and *Bax*^{-/-} mice, respectively (**Figure 8C**). Also, no difference was found in the percent change of cytosolic p53 protein content between the wild-type and *Bax*^{-/-} mice (**Figure 8D**).

Insert Figure 8A ———→ Nuclear p53 protein

Insert Figure 8B ———→ Nuclear p53 protein percent change

Insert Figure 8C ———→ Cytosolic p53 protein

Insert Figure 8D ———→ Cytosolic p53 protein percent change

Stress proteins: HSP70, HSP27, and HSP60 protein contents. A large inter-animals variability in the response of HSP70 protein content to denervation was observed and we did not find any statistical significant difference between the denervated and contralateral control muscles in both the wild-type and *Bax*^{-/-} mice (**Figure 9A and 9B**). In contrast, the protein content of HSP27 was elevated by 82% and 106% in the muscle after denervation relative to the control muscle in the wild-type and *Bax*^{-/-} mice, respectively (**Figure 9C**) but the magnitude of the change of HSP27 protein content was not significantly different between the wild-type and *Bax*^{-/-} mice (**Figure 9D**). Although the protein content of HSP60 in the denervated muscle appeared to decrease by ~20% when compared to the control muscles in both the wild-type and *Bax*^{-/-} mice, the change did not reach the significant level (**Figure 9E and 9F**).

Insert Figure 9A ———→ HSP70 protein

Insert Figure 9B ———→ HSP70 protein percent change

Insert Figure 9C ———→ HSP27 protein

Insert Figure 9D ———→ HSP27 protein percent change

Insert Figure 9E ———→ HSP60 protein

Insert Figure 9F ———→ HSP60 protein percent change

Oxidative stress markers: H₂O₂, MDA/4-HAE, and nitrotyrosine contents. In the wild-type animals, the contents of H₂O₂, MDA/4-HAE, and nitrotyrosine were elevated by 59%, 28%, and 114%, respectively, in the denervated muscle relative to the contralateral control muscle but these changes were not found in the Bax^{-/-} animals (**Figure 10A, 10C, and 10E**). Moreover, the magnitudes of the percent changes of H₂O₂, MDA/4-HAE, and nitrotyrosine contents were significantly greater in the wild-type mice when compared to the Bax^{-/-} mice (wild-type vs. Bax^{-/-} mice, H₂O₂: +59% vs. -15%, MDA/4-HAE: +28% vs. +1%, nitrotyrosine: +114% vs. -2%) (**Figure 10B, 10D, 10F**).

Insert Figure 10A —→ H₂O₂ content

Insert Figure 10B —→ H₂O₂ content percent change

Insert Figure 10C —→ MDA/4-HAE content

Insert Figure 10D —→ MDA/4-HAE content percent change

Insert Figure 10E —→ Nitrotyrosine content

Insert Figure 10F —→ Nitrotyrosine content percent change

Antioxidant enzyme: MnSOD and CuZnSOD protein contents. Our immunoblot analysis indicated that MnSOD protein content was diminished by 23% and 21% in the denervated muscle when compared to the contralateral control muscle in the wild-type and Bax^{-/-} mice, respectively (**Figure 11A**) but no significant difference was found in the percent change of MnSOD protein content between the wild-type and Bax^{-/-} mice (**Figure 11B**). On the contrary, we did not find any difference in the CuZnSOD protein content between the denervated and control muscles in both the wild-type and Bax^{-/-} mice (**Figure 11C and 11D**).

Insert Figure 11A —→ MnSOD protein

Insert Figure 11B —→ MnSOD protein percent change

Insert Figure 11C —→ CuZnSOD protein

Insert Figure 11D —→ CuZnSOD protein percent change

DISCUSSION

In addition to the transferase-mediated dUTP biotin nick-end labeling or TUNEL (an indicative for DNA breaks) and BCL-2 findings in skeletal muscle following denervation (5, 21, 22, 35-40), the activation of apoptotic signaling has been clearly illustrated in the denervated rat muscle by depicting the augmentation of apoptotic DNA fragmentation, increase in Bax/Bcl-2 relative ratio, mitochondrial release of cytochrome c, Smac/DIABLO and AIF, increase in caspase-3 and -9 mRNA,

active protein and proteolytic activity, decrease in XIAP and MnSOD, elevation of cleaved PARP protein, p53 and HSP70 (20). In the present study, by using the genetically knockout mice that are deficient of the *Bax* gene, an upstream essential pro-apoptotic mediator, we provide further evidence supporting that apoptotic mechanism has a significant role in mediating muscle wasting during denervation. We demonstrated that the extent of muscle loss following denervation is attenuated in the skeletal muscle of *Bax*^{-/-} mice compared to wild-type animals. Increases in apoptotic DNA fragmentation, caspase-3 and -9 protease activities and oxidative stress markers including H₂O₂, MDA/4-HAE and nitrotyrosine contents were evident in the wild-type muscle after denervation but these changes were not found in the *Bax*^{-/-} mice. The magnitude of mitochondrial cytochrome c release in the denervated muscle of the *Bax*^{-/-} mice was also lower than the wild-type mice. Moreover, ARC protein content increased exclusively in the *Bax*^{-/-} muscle but not the wild-type muscle following denervation. Overall, these findings suggest that interference of the pro-apoptotic signaling (i.e., lacking the *Bax* gene) during denervation attenuates the resulting extent of muscle loss. Providing that muscle denervation resulting from degeneration and retraction of motoneurons is a general process contributing to the disease progression of some neuromuscular disorders, these findings suggest that interventions targeting apoptosis may be a valuable therapeutic strategy in ameliorating denervation-associated pathologic muscle wasting in certain neuromuscular disorders.

Although the majority of apoptotic investigations were emphasized on mitotic cell populations, there has been a rapidly growing body of data demonstrating that apoptosis may have an important regulatory role in mediating the process of postmitotic skeletal muscle wasting under both physiologic and pathophysiologic conditions (3, 41). A satisfactory amount of clinical reports has been documented and exhibited that apoptosis is possibly involved in a variety of muscle disorders including spinal muscular myopathies, peripheral neuropathies, amyotrophic lateral sclerosis, muscle dystrophies, metabolic/mitochondrial myopathies, inflammatory myopathies, congenital myopathies, distal myopathies, thyroid-associated ophthalmomyopathies, chronic heart failure-associated myopathies, critical ill myopathies, and burn injury-associated muscle wasting (3, 4, 7, 8, 42-50). To date, mounting researches are undertaking with the aim to explore the biologic role of apoptosis in the pathogenesis of various myopathies characterizing by considerable muscle wasting. It is of the essence to fully understand the signaling mechanisms responsible for the activation of apoptotic machinery in myopathies. Remarkably, there was a recent study clearly demonstrated that inhibition of apoptosis improves the outcome of skeletal muscle wasting pathology in the genetically knockout mice that were deficient of the *laminin-α2* gene, an animal model of congenital muscular dystrophy (51). By adopting the genetic intervention by crossbreeding the laminin-α2-deficient mice with pro-apoptotic Bax-deficient mice or with mice carrying the muscle-specific anti-apoptotic Bcl-2 transgene, Girgenrath and colleagues (51) have shown that the extent of muscle pathology is

ameliorated by either inactivation of the pro-apoptotic protein Bax or overexpression of the anti-apoptotic protein Bcl-2. It is worth citing that, particularly by inactivating Bax, both the postnatal growth rate and muscle fiber histology are improved while the amount of fixed muscular contractures is reduced in the skeletal muscle taken from the laminin- α 2/Bax-deficient animals, and these findings denote that pro-apoptotic Bax plays an important role in the progression of muscular pathologies (51). Consistent with the exhibited importance of the pro-apoptotic Bax in the congenital muscular dystrophy (51), the present findings further support the significance of the Bax-associated pro-apoptotic signaling in mediating muscle wasting pathologies particularly induced by muscle denervation. Our results indicate that, in response to the removal of nerve innervation, the extent of muscle loss is attenuated in accompanied by the suppression of pro-apoptotic signaling in skeletal muscle that is deficient of the *Bax* gene.

Differences in the activation of the apoptotic signaling in response to muscle denervation was apparently observed when comparing the skeletal muscles taken from the mice that were deficient of the *Bax* gene and the wild-type mice in this study. In the wild-type mouse muscle, we found changes in the apoptotic components including apoptotic DNA fragmentation, Bax, cytochrome c, Smac/DIABLO, AIF, caspases, XIAP, and p53 which indicated the activation of pro-apoptotic signaling and these findings were consistent with the previously reported denervation-induced apoptotic responses in the rat skeletal muscle (20). However, the denervation-induced activation of the pro-apoptotic components was generally suppressed by lacking the pro-apoptotic Bax protein. The significant elevations of apoptotic DNA fragmentation, caspase-3 and -9 activities as found in the wild-type muscle following denervation were missing in the Bax-deficient denervated muscle. The denervation-induced mitochondrial cytochrome c release was also suppressed in the Bax-deficient muscle relative to the wild-type muscle. In addition, the increase in protein abundance of ARC, an endogenous apoptotic suppressor protein, was evident only in Bax-deficient muscle but not the wild-type muscle after denervation. Intriguingly, providing that ARC was considered to interact with selective caspases when it was first identified as an apoptotic inhibitor which is expressed in skeletal muscle and heart (52), it is worth mentioning that the anti-apoptotic function of ARC may also be mediated by interacting with the activation of pro-apoptotic Bax (53). According to the possibly existing relationship between ARC and Bax, it is not unreasonable to observe that ARC responds to muscle denervation differently in Bax-deficient and wild-type muscles. However, it is unclear that the observed increase in ARC abundance may have contributed to counteract or suppress the activation of the pro-apoptotic signaling during denervation in the Bax-deficient muscle. Nevertheless, the exact reason for the denervation-induced elevation of ARC in muscle that was deficient of the *Bax* gene is not known and it warrants further investigations to understand this phenomenon.

Another notable finding in this study is that the oxidative stress markers including H₂O₂,

MDA/4-HAE, nitrotyrosine contents were all elevated following denervation in the wild-type muscle but none of these markers increased in the Bax-deficient denervated muscle. Lately, the involvement of oxidative stress in the mechanisms regulating muscle wasting has been rationally proposed (54). Evidences have been exemplified in the models of disuse muscle wasting (e.g., limb immobilization and hindlimb suspension) suggesting that elevated oxidative stress possibly contributes to the process of muscle loss as induced by muscle disuse (55-61). Furthermore, the proposition for the role of oxidative stress in muscle wasting is persuasively strengthened by the documented data showing the existences of possible mechanisms responsible for the production of reactive oxidants in inactive skeletal muscle (xanthine oxidase, reactive iron, NAD(P)H oxidase, nitric oxide/peroxynitrite, and/or mitochondrial reactive oxygen species) as well as the potential signaling pathways that could link oxidative stress to the proteolytic processes mediating muscle loss (e.g., signaling of caspase-3, calpain-mediated and proteasome-mediated proteolyses) (54). In regards to the denervation-induced muscle wasting, the involvement of oxidative stress has also been implicated together with the down regulation of NOS/NO in response to denervation (62). It is noted that the present wild-type muscle findings were in agreement with the proposition that oxidative stress may have a role in mediating the muscle loss. Moreover, the absence of the increases in the markers of oxidative stress in the Bax-deficient denervated muscle raised a sensible speculation that Bax protein may be directly or indirectly related to the reduction-oxidation or redox events during muscle denervation. Since Bcl-2 is an intimating working partner of Bax (63) and Bcl-2 has been suggested to have an antioxidant-promoting ability which provides resistance in oxidative stress-induced apoptosis (64), a speculative supposition in explaining this observation may be designated to Bcl-2. Nonetheless, additional research is required to reveal this novel finding regarding the relationship between Bax and oxidative stress.

In conclusion, the present data provide evidence demonstrating that interference of pro-apoptotic signaling intervened by knockout of the pro-apoptotic *Bax* gene attenuates the extent of muscle wasting in response to skeletal muscle denervation. These results implicate that apoptotic regulation is involved in mediating the process of muscle loss during muscle denervation and suggest that strategies targeting apoptosis may be of clinical importance in offsetting muscle wasting pathologies which are related to muscle denervation. Further investigation is required in exploring the likelihood of the other potential apoptotic interventions (e.g., overexpression of the anti-apoptotic factors including Bcl-2, IAP, and ARC) in inhibiting the denervation-associated muscle wasting, and therefore ameliorating muscle wasting in certain neuromuscular diseases.

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Figure 1 Muscle Weight Data

Legend for Figure 1A Muscle weight. The extent of muscle loss following denervation was estimated by examining the gastrocnemius muscle wet weight loss normalized to the animal's bodyweight between the denervated and the contralateral control sides. The data are presented as means $\pm$ standard error of mean (SE). * $P < 0.05$, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 1B Muscle weight percent change. The percent change of muscle weight in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. * $P < 0.05$, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different; # $P < 0.05$, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Figure 2 Apoptotic DNA Fragmentation

Legend for Figure 2A Apoptotic DNA fragmentation. The extent of apoptotic DNA fragmentation was estimated by measuring the cytosolic mono- and oligo-nucleosomes. The OD₄₀₅ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain; **P < 0.05, Bax^{-/-} mice were significant different from the wild-type mice under the same experimental condition.

Legend for Figure 2B Apoptotic DNA fragmentation percent change. The percent change of apoptotic DNA fragmentation in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different; #P < 0.05, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Figure 3 Bax and Bcl-2 Protein Content

Legend for Figure 3A Bax protein content. The Bax protein content was examined by Western immunoblot. The insets show representative blots for the Bax protein. The data are presented as means $\pm$ SE of the arbitrary unit. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 3B Bax protein content percent change. The percent change of Bax protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Legend for Figure 3C Bcl-2 protein content. The Bcl-2 protein content was examined by Western immunoblot. The insets show representative blots for the Bcl-2 protein. The data are presented as means $\pm$ SE of the arbitrary unit. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 3D Bcl-2 protein content percent change. The percent change of Bcl-2 protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Figure 4 Mitochondrial Cytochrome c Release

Legend for Figure 4A Mitochondrial cytochrome c release. The release of mitochondrial cytochrome c was estimated by measuring the protein content of cytochrome c in the mitochondria-free cytosolic fraction using an ELISA. The OD₄₅₀ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain; **P < 0.05, Bax^{-/-} mice were significant different from the wild-type mice under the same experimental condition.

Legend for Figure 4B Mitochondrial cytochrome c release percent change. The percent change of mitochondrial cytochrome c release in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different; #P < 0.05, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Figure 5 Caspase-3 and -9 Protease Activities**A. Caspase-3 activity** **B. Caspase-3 activity percent change** **C. Caspase-9 activity** **D. Caspase-9 activity percent change**

Legend for Figure 5A Caspase-3 protease activity. The caspase-3 activity was assessed by a fluorometric caspase activity assay. The fluorescence unit (FU) is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain; **P < 0.05, Bax^{-/-} mice were significant different from the wild-type mice under the same experimental condition.

Legend for Figure 5B Caspase-3 protease activity percent change. The percent change of caspase-3 protease activity in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different; #P < 0.05, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Legend for Figure 5C Caspase-9 protease activity. The caspase-9 activity was assessed by a fluorometric caspase activity assay. The fluorescence unit (FU) is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain; **P < 0.05, Bax^{-/-} mice were significant different from the wild-type mice under the same experimental condition.

Legend for Figure 5D Caspase-9 protease activity percent change. The percent change of caspase-9 protease activity in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different; #P < 0.05, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Figure 6 Mitochondrial Smac/DIABLO Release and XIAP and ARC Protein Content**A. Smac/DIABLO** **B. Smac/DIABLO percent change** **C. XIAP** **D. XIAP percent change** **E. ARC** **F. ARC percent change**

Legend for Figure 6A Mitochondrial Smac/DIABLO release. The protein content of Smac/DIABLO was measured by Western immunoblot in the mitochondria-free cytosolic fraction in order to estimate the mitochondrial Smac/DIABLO release. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit. * $P < 0.05$, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 6B Mitochondrial Smac/DIABLO release percent change. The percent change of mitochondrial Smac/DIABLO release in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. * $P < 0.05$, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Legend for Figure 6C XIAP protein content. The protein content of XIAP was measured by Western immunoblot in the total cytosolic fraction. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit. * $P < 0.05$, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 6D XIAP protein content percent change. The percent change of XIAP protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. * $P < 0.05$, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Legend for Figure 6E ARC protein content. The protein content of ARC was measured by Western immunoblot in the total cytosolic fraction. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit. * $P < 0.05$, denervated muscle was significant different from the contralateral control muscle under the same animal strain; ** $P < 0.05$, Bax^{-/-} mice were significant different from the wild-type mice under the same experimental condition.

Legend for Figure 6F ARC protein content percent change. The percent change of ARC protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. * $P < 0.05$, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different; # $P < 0.05$, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Figure 7 Mitochondrial AIF Release and Nuclear AIF Translocation

Legend for Figure 7A Mitochondrial AIF release. The protein content of AIF was measured by Western immunoblot in the mitochondria-free cytosolic fraction in order to estimate the mitochondrial AIF release. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit. * $P < 0.05$, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 7B Mitochondrial AIF release percent change. The percent change of mitochondrial AIF release in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. * $P < 0.05$, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Legend for Figure 7C AIF nuclear translocation. The protein content of AIF was measured by Western immunoblot in the nuclear fraction in order to estimate the nuclear translocation of AIF. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit.

Legend for Figure 7D AIF nuclear translocation percent change. The percent change of AIF nuclear translocation in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE.

Figure 8 Cytosolic and Nuclear p53 Protein Content

A. Nuclear p53

B. Nuclear p53 percent change

C. Cytosolic p53

D. Cytosolic p53 percent change

Legend for Figure 8A Nuclear p53 protein content. The protein content of p53 was measured by Western immunoblot in the nuclear fraction. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 8B Nuclear p53 protein content percent change. The percent change of nuclear p53 protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Legend for Figure 8C Cytosolic p53 protein content. The protein content of p53 was measured by Western immunoblot in the total cytosolic fraction. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 8D Cytosolic p53 protein content percent change. The percent change of cytosolic p53 protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Figure 9 HSP70, HSP27, and HSP60 Protein Content

Legend for Figure 9A HSP70 protein content. The protein content of HSP70 was measured by Western immunoblot in the total cytosolic fraction. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit.

Legend for Figure 9B HSP70 protein content percent change. The percent change of HSP70 protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE.

Legend for Figure 9C HSP27 protein content. The protein content of HSP27 was measured by Western immunoblot in the total cytosolic fraction. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 9D HSP27 protein content percent change. The percent change of HSP27 protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Legend for Figure 9E HSP60 protein content. The protein content of HSP60 was measured by Western immunoblot in the total cytosolic fraction. The insets show representative blots. The data are presented as means $\pm$ SE of the arbitrary unit.

Legend for Figure 9F HSP60 protein content percent change. The percent change of HSP60 protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE.

Figure 10 H₂O₂, MDA/4-HAE, and Nitrotyrosine**A. H₂O₂** **B. H₂O₂ percent change** **C. MDA/4-HAE** **D. MDA/4-HAE percent change** **E. Nitrotyrosine** **F. Nitrotyrosine percent change**

Legend for Figure 10A H₂O₂ content. The H₂O₂ content was determined by a fluorometric assay. The fluorescence unit (FU) is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain; **P < 0.05, Bax^{-/-} mice were significant different from the wild-type mice under the same experimental condition.

Legend for Figure 10B H₂O₂ content percent change. The percent change of H₂O₂ content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different. #P < 0.05, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Legend for Figure 10C MDA/4-HAE content. The level of lipid peroxidation was estimated by measuring the content of MDA/4-HAE. The OD₅₈₆ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain; **P < 0.05, Bax^{-/-} mice were significant different from the wild-type mice under the same experimental condition.

Legend for Figure 10D MDA/4-HAE content percent change. The percent change of MDA/4-HAE content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different; #P < 0.05, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Legend for Figure 10E Nitrotyrosine content. The content of nitrotyrosine was measured in the cytosolic fraction by an immuno-dot blot. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for nitrotyrosine. The data are presented as means $\pm$ SE. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain; **P < 0.05, Bax^{-/-} mice were significant different from the wild-type mice under the same experimental condition.

Legend for Figure 10F Nitrotyrosine content percent change. The percent change of nitrotyrosine content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different; #P < 0.05, percent change in Bax^{-/-} mice is significantly different from the wild-type mice.

Figure 11 MnSOD and CuZnSOD Protein Content

Legend for Figure 11A MnSOD protein content. The protein content of MnSOD was assessed in cytosolic fraction by immunoblot analysis. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for MnSOD. The data are presented as means $\pm$ SE. *P < 0.05, denervated muscle was significant different from the contralateral control muscle under the same animal strain.

Legend for Figure 11B MnSOD protein content percent change. The percent change of MnSOD protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE. *P < 0.05, percent change of denervated muscle relative to the contralateral control muscle under the same animal strain was significantly different.

Legend for Figure 11C CuZnSOD protein content. The protein content of CuZnSOD was assessed in cytosolic fraction by immunoblot analysis. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for MnSOD. The data are presented as means $\pm$ SE.

Legend for Figure 11D CuZnSOD protein content percent change. The percent change of CuZnSOD protein content in the denervated limb relative to the contralateral control limb was compared between the wild-type and Bax^{-/-} mice. The percent change are presented as means $\pm$ SE.

CHAPTER 6

Age-related increase in oxidative stress in skeletal muscle during hindlimb unloading

ABSTRACT

Oxidative stress has been demonstrated to be elevated during hindlimb suspension in muscle from young adult animals. However, it has not been investigated whether oxidative stress is also increased in aged skeletal muscle in response to unloading. Therefore, the present study examined the markers of oxidative stress and antioxidant enzymes gene and protein expressions in gastrocnemius muscles of young adult and aged Fischer₃₄₄×Brown Norway rats after 14 days of hindlimb suspension. Age-related sarcopenic muscle mass decline was observed when comparing the muscle weights between young adult and aged animals. Following suspension, decreases in medial gastrocnemius muscle weight and muscle weight normalized to the animal bodyweight were evident in both young adult and aged animals. In the suspended muscle, there were increases in the contents of MDA/4-HAE and nitrotyrosine relative to the control muscle in both young adult and aged animals. H₂O₂ content was elevated while MnSOD protein content was reduced in the suspended muscle when compared to the control muscle exclusively in the aged animals. Among these changes of oxidative stress markers, MDA/4-HAE, H₂O₂ and MnSOD protein contents responded to hindlimb unloading in an age-dependent manner. By comparing the aged to young adult animals, the age-related decrease in muscle mass was accompanied by greater contents of H₂O₂, 8-OHdG and nitrotyrosine but lower abundances of MnSOD mRNA and protein and catalase mRNA. These findings are consistent with the hypotheses that oxidative stress may have a role in mediating disuse-induced and age-related sarcopenic muscle losses. Moreover, our data agree with the proposition that aging may predispose skeletal muscle to an advanced level of oxidative stress at rest and under atrophic situation.

Key words: muscle disuse; sarcopenia; antioxidant

INTRODUCTION

Generation of free radicals and reactive oxygen and nitrogen species (ROS/RNS) is a normal continuous process in the life of aerobic living organisms. When production of ROS/RNS exceeds the endogenous antioxidant buffering capacity, oxidative stress is provoked. There have been extensive data indicating that oxidative stress plays a central role in regulating a variety of cellular events (e.g., cell division, adhesion, differentiation, and death) [3,9,24,30,36]. Moreover, substantial investigations have shown that the level of oxidative stress is elevated with diseases as well as aging, and accordingly the pathogenesis of cardiovascular diseases, diabetes mellitus, cancer, neurodegenerative diseases, and other chronic diseases are thought to be attributed to the deleterious effects of the increased oxidative damage [4,7,8,11,43]. With aging, it has also been demonstrated an accretion of oxidative stress and increased incidence of oxidative injury in skeletal muscle [2,5,13,15,28,29]. Consequently, elevated oxidative stress has been rationally suggested to be involved in the development of sarcopenic muscle wasting [6,12].

With a focus in skeletal muscle, it is well established that free radicals together with ROS/RNS are produced in large quantities in contracting muscle but the production of ROS/RNS is still present in muscle under inactive state [25,27,32,33]. Recently, it has been proposed that oxidative stress may have an important role in mediating the process of muscle atrophy during disuse [31]. In support of this proposition, several studies have demonstrated the incidence of oxidative injury in skeletal muscle under atrophic situations during limb immobilization or hindlimb suspension [16-22]. Nevertheless, although it has been demonstrated that unloading increases oxidative stress and disrupts antioxidant capacity in young adult skeletal muscle [22], the hypothesis that oxidative stress is also elevated in aged muscle during unloading has not been investigated. Moreover, it is unclear whether aging may exacerbate the oxidative challenge when exposed to muscle disuse as aging has been suggested to accumulate oxidative damage in skeletal muscle [6,12]. Thus, this study examined the markers of oxidative stress (MDA/4-HAE, H_2O_2 , 8-OHdG, and nitrotyrosine) and antioxidant enzymes (CuZnSOD, MnSOD, and catalase) gene and protein expressions in gastrocnemius muscles of young adult and aged rats after 14 days of hindlimb suspension. We tested the hypotheses that oxidative stress is increased in both young adult and aged muscles in response to hindlimb unloading and those changes of oxidative stress markers during unloading are age-dependent.

METHODS

Animals. Experiments were conducted on ~6-mo old young adult and 30-mo old aged Fischer₃₄₄×Brown Norway rats (Harlan, Indianapolis, IN). The rats were housed in pathogen-free conditions at ~20°C and were exposed to a reverse light condition of 12:12 h of light:darkness each day. They were fed rat chow and water ad libitum throughout the study period.

Hindlimb suspension procedure. The animals were randomly assigned to suspension group (Young n=10, Aged n=10) or control group (Young n=10, Aged n=8). The procedure of hindlimb suspension described by Morey-Holton and Globus [26] was adopted in the present study. In brief, an adhesive (tincture of benzoin) was applied to the tail, air-dried, and an orthopedic tape was put along the proximal one-third of the tail. This practice distributed the load evenly and avoided excessive tension on a small area to the tail. The tape was then placed through a wire harness that was attached to a fishline swivel at the top of a specially designed N.A.S.A.-approved hindlimb suspension cage. This provided the rats with 360° of movement around the cage and the forelimbs maintained contact with a grid floor allowed the animals to move and assess food and water freely. Sterile gauze was wrapped around the orthopedic tape and was subsequently covered with a thermoplastic material, which formed a hardened cast (Vet-Lite, Veterinary Specialty Products, Boca Raton, FL). The distal tip of the tail was examined to verify that the procedure did not occlude the blood flow to the tail (i.e., tail remained pink). The suspension height was adjusted to prevent the animal's hindlimb from touching any supportive surface, with care taken to maintain a suspension angle of approximately 30° [14]. The suspension height and animal behavior were monitored daily. Control animals were allowed to move unconstrained around the cages. Following 14 days of suspension, animals were killed with an overdose of xylazine. Medial gastrocnemius muscles from the hindlimbs were excised, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and stored at -80°C until used for analyses.

All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and following the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals as published by the U.S. Dept. of Health and Human Services and proclaimed in the Animals Welfare Act (PL89-544, PL91-979, and PL94-279).

RT-PCR analyses of CuZnSOD, MnSOD, and catalase. Total RNA was extracted from the medial gastrocnemius muscle of both suspended and control animals with TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Frozen muscle was mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA were solubilized in RNase-free H₂O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assured by examining OD₂₆₀/OD₂₈₀ ratio. Ten micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 30 µl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). Control RT reaction was done in which the RT enzyme was omitted. The control RT reaction was

PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of complementary DNA (cDNA) was then amplified by PCR using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, TX), 250 μ M deoxyribonucleotide triphosphates (dNTPs), 1 x PCR buffer, and 2.5 units *Taq* DNA polymerase (USB Corp., Cleveland, OH) in a final volume of 50 μ l. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). The primer pairs were designed from sequences published in GenBank (**Table 1**) and PCR products were verified by restriction digestions. Preliminary experiments were conducted with each gene to assure that the number of cycles represented a linear portion for the PCR optical density curve for the muscle samples. The cDNA from all muscle samples were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 30 μ l of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide. Images were captured and the signals were quantified in arbitrary units as optical density (OD) x band area using Kodak image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. Ribosomal 18S primers were used as internal controls while all RT-PCR signals were normalized to the 18S signal of the corresponding RT product to eliminate the measurement error from uneven samples loading and provide a semi-quantitative measure of the relative changes in gene expression.

Protein preparation. The fractionation method described by Rothermel et al. [34] was used with minor modification to extract the cytosolic protein fraction from the gastrocnemius muscle. We have previously obtained the fractionated cytosolic and nuclear proteins from skeletal and heart muscles using this modified protocol [37-40]. In brief, after removal of connective tissues, muscle was homogenized on ice in lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT) supplemented with a protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO). Following centrifuging at 5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and these supernatants were further centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual nuclei. The final collected supernatants were stored as total cytosolic protein fraction. The above described protein fractionation procedure has been routinely used in our laboratory to obtain high purity cytosolic fraction protein as assessed by immunoblotting the fractions with an anti-histone H2B (a nuclear protein) and an anti-CuZnSOD (a cytosolic isoform of superoxide dismutase) antibody [37,38,40]. The protein content of the extract was then measured in duplicate by DC Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay [23]. As a further measure to assure the protein content, all the protein samples were quantified in duplicate on a different occasion by BCA Protein

Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation [41].

Western immunoblot analyses of CuZnSOD and MnSOD. Fixed micrograms of cytosolic protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and then probed with an anti-SOD-1 rabbit polyclonal antibody (1:500 dilution, sc-11407, Santa Cruz Biotechnology, Santa Cruz, CA) or an anti-Mn-SOD goat antibody (1:2000 dilution, A300449A, Bethyl Lab Inc., Montgomery, TX) diluted in PBS-T with 2% BSA incubated at 4°C for overnight. Secondary antibodies were conjugated to horseradish peroxidase (HRP) (Chemicon International, Temecula, CA), and signals were developed by chemiluminescence (Pierce Biotechnology, Rockford, IL). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

MDA/4-HAE Assay. Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxyalkenals (4-HAE) upon decomposition [10]. In the present study, the content of MDA/4-HAE was measured in cytosolic fraction as an indicator of lipid peroxidation using a commercial colorimetric assay kit (21012, Oxis International, Portland, OR). Briefly, 50 μ l of the cytosolic protein fraction or standards with known concentrations was incubated with a chromogenic reagent, N-methyl-2-phenylindole, at 45°C for 1 h and the generated chromophore after incubation was detected by spectrophotometry at 586 nm. The MDA/4-HAE concentration of samples was determined according to the standard curve generated in the same setting. Results were presented as μ M MDA/4-HAE normalized to milligram protein used in the assay. Control and suspended samples were run in the same setting to eliminate any assay-by-assay variations.

H₂O₂ Fluorometric Assay. The content of hydrogen peroxide (H₂O₂) in the muscle homogenate was measured in the cytosolic fraction using a fluorometric H₂O₂ detection kit (FLOH 100-3, Cell Technology Inc., Mountain View, CA). By following the manufacturer's instruction, 50 μ l of the total cytosolic fraction protein of the gastrocnemius muscle was incubated in 50 μ l of reaction cocktail containing horseradish peroxidase and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP,

a non-fluorescent substrate turns fluorescent after oxidized by H_2O_2) in sodium phosphate buffer at room temperature in dark for 10 min. The fluorescence was measured on a spectrofluorometer with an excitation wavelength of 530/25 nm and an emission wavelength of 590/35 nm (CytoFluor, Applied Biosystems, Foster City, CA) after the incubation. H_2O_2 content was estimated as the arbitrary fluorescence units normalized to milligram protein used in the assay. Measurements were performed with the suspended and control samples run on the same microplate in the same setting.

8-OHdG ELISA. 8-hydroxy-2'-deoxyguanosine (8-OHdG) is produced during DNA repair under oxidative damage and has been commonly used as a marker of oxidative damage [35]. A competitive enzyme-linked immunosorbent assay (ELISA) (21026, Oxis International, Portland, OR) was used to measure the 8-OHdG content in the cytosolic fraction of suspended and control muscles. By following the manufacturer's instruction, 50 μ l of the cytosolic fraction of gastrocnemius muscles or 8-OHdG standards with known concentrations was incubated with 8-OHdG monoclonal antibody in a 8-OHdG-precoated 96-well microtiter plate at 37°C for 1 h. The 8-OHdG in the cytosolic fraction or standard competed with the 8-OHdG bound on the plate for the 8-OHdG monoclonal antibody bindings sites. After the incubation, the 8-OHdG antibodies that were bound to the 8-OHdG in the cytosolic fraction or standard were washed out of the well while those that had bound to the 8-OHdG coated on the plate would remain. The sample after washes was incubated with a HRP-labeled secondary antibody at 37°C for 1 h. After washes, a chromogen was added followed by the addition of stop solution. The resulting color development was measured at a wavelength of 450 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). The concentration of 8-OHdG in the cytosolic fraction samples was estimated according to the generated standard curve in the same setting. Negative experiments were performed in the absence of cytosolic fraction and known standard. Measurements were performed with control and suspended samples analyzed on the same microtiter plate. The concentration of samples was then normalized to the mg of protein used in the assay and presented as ng 8-OHdG·mg protein⁻¹.

Nitrotyrosine Dot Blot. As an index of the production of reactive nitrogen species, an immuno-dot blot was carried out to estimate the total nitrotyrosine content in the cytosolic fraction of the gastrocnemius muscles from suspended and control animals. In brief, 4 μ g of cytosolic protein was dotted on a nitrocellulose membrane (VWR, West Chester, PA). After air dry, the membrane was blocked in 5% non-fat milk in PBS-T at room temperature for 1 h and then probed with an anti-nitrotyrosine mouse monoclonal antibody (1:1000 dilution, MAB5404, Chemicon International, Temecula, CA) diluted in PBS-T with 2% BSA incubated at 4°C for overnight. The membrane was incubated with a HRP-conjugated anti-mouse IgG secondary antibody (AP124P, Chemicon International, Temecula, CA) at room temperature for 1 h, and then signals were developed by chemiluminescence (Pierce Biotechnology, Rockford, IL). Negative control experiments were

performed by omitting either the anti-nitrotyrosine or the secondary antibodies. The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting dot signals were quantified in arbitrary units as optical density (OD) $\times$ dot area using Kodak one-dimensional (1-D) image analysis system (Eastman Kodak, Rochester, NY).

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package. ANOVA was performed to examine the main effects of suspension, age, and interaction (suspension $\times$ age) on the measured variables. ANOVA followed by Tukey HSD post hoc analysis was used to examine differences between groups. All data are given as means $\pm$ standard error of mean (SE). Statistical significance was accepted at $P < 0.05$.

RESULTS

Muscle weight. Following 14 days of hindlimb suspension, the whole medial gastrocnemius muscle wet weight decreased by $\sim 30\%$ in young adult and aged rats (young control, 993 mg; young suspended: 699 mg; aged control, 761 mg; aged suspended: 520 mg) (**Figure 1A**). The muscle weight after normalized to the animal bodyweight also decreased by 11% in young adult rats (young control, 2.7 mg/g; young suspended, 2.4 mg/g) and by 19% in aged rats (aged control, 1.4 mg/g; aged suspended, 1.1 mg/g) after hindlimb unloading (**Figure 1B**). Comparisons between young adult and aged control animals demonstrated an age-related sarcopenic decline in muscle mass. The muscle wet weight in young adult rats was 23% higher than the aged rats (young, 993 mg; aged, 761 mg) (**Figure 1A**) while the muscle weight normalized to the animal bodyweight in young adult rats was 48% higher when compared to the aged rats (young, 2.7 mg/g; aged, 1.4 mg/g) (**Figure 1B**).

Figure 1A $\longrightarrow$ Muscle weight

Figure 1B $\longrightarrow$ Muscle weight normalized to animal's bodyweight

MDA/4-HAE content. As an indicator of the level of lipid peroxidation, suspended muscles had a greater content of MDA/4-HAE with a 29% and 58% increase when compared to the control muscle in young adult and aged animals, respectively (**Figure 2**). The interaction effect of suspension $\times$ age in the ANOVA indicated that the response of MDA/4-HAE content to hindlimb suspension in these animals followed an age-dependent manner (**Figure 2**).

Insert Figure 2 $\longrightarrow$ MDA/4-HAE content

H₂O₂ content. According to our fluorometric assay, no difference was found in H₂O₂ content between the suspended and control muscles in young animals. However, there was a 43% increase in the H₂O₂ content in aged suspended muscle when compared to the aged control muscle (**Figure 3**). The main effect of age in the ANOVA suggested that aged muscle had a higher content of H₂O₂ relative to the young adult muscle (**Figure 3**). Moreover, an interaction (suspension × age) effect was found in explaining the change of H₂O₂ content in these animals indicating that the change of H₂O₂ content during hindlimb suspension was age-dependent (**Figure 3**).

Insert Figure 3 ———→ H₂O₂ content

8-OHdG content. The level of oxidative DNA damage as estimated by the content of 8-OHdG in the suspended muscle was not different from the control muscle in animals of both ages. However, a main effect of age was found in explaining the content of 8-OHdG in these animals suggesting that there was a greater content of 8-OHdG in the aged muscle when compared to the young adult muscle (**Figure 4**).

Insert Figure 4 ———→ 8-OHdG content

Nitrotyrosine content. As a marker of the level of reactive nitrogen species, we found that the content of nitrotyrosine in the cytosolic fraction of suspended muscle was elevated by 76% and 65% relative to the control muscle in young adult and aged animals, respectively (**Figure 5**). Moreover, a main effect of age in the ANOVA suggested that the aged muscle had a higher level of nitrotyrosine content compared to young adult muscle (**Figure 5**).

Insert Figure 5 ———→ Nitrotyrosine content

CuZnSOD mRNA and protein content. As indicated by the RT-PCR and immunoblot analyses, CuZnSOD mRNA and protein contents in the suspended muscle were not different from the control muscle in both young adult and aged animals (**Figure 6A and 6B**). Moreover, no difference was found in both the mRNA and protein contents of CuZnSOD between the young adult and aged control muscles (**Figure 6A and 6B**).

Insert Figure 6A ———→ CuZnSOD mRNA content

Insert Figure 6B ———→ CuZnSOD protein content

MnSOD mRNA and protein content. The mRNA abundance of MnSOD was not different between the suspended and control muscles in both young and aged animals (**Figure 7A**). Although MnSOD protein content of the suspended muscle was not different from the control muscle in young adult animals, in the aged animals, MnSOD protein content in the suspended muscle was 28% lower than the control muscle (**Figure 7B**). An interaction effect of suspension $\times$ age in the ANOVA indicated that the response of MnSOD protein content to hindlimb suspension is dependent on the age of the animals (**Figure 7B**). Furthermore, the main effect of age in the ANOVA suggested that there are lower contents of MnSOD mRNA and protein in the aged muscle relative to the young adult muscle (**Figure 7A and 7B**).

Insert Figure 7A $\longrightarrow$ MnSOD mRNA content

Insert Figure 7B $\longrightarrow$ MnSOD protein content

Catalase mRNA content. No difference was found in the mRNA content of catalase between the suspended and control muscles in both young adult and aged animals (**Figure 8**). However, the main effect of age in the ANOVA suggested that there is a lower mRNA content of catalase in the aged muscle when compared to the young adult muscle (**Figure 8**).

Insert Figure 8 $\longrightarrow$ Catalase mRNA content

DISCUSSION

It has been demonstrated that 28 days of hindlimb unloading increases oxidative stress and disrupts antioxidant capacity in slow soleus muscle taken from young adult Sprague Dawley rats [22]. In the present study, we extend these previous observations by showing that increases in oxidative stress markers are also evident in relatively fast gastrocnemius muscles of both young adult and aged Fischer₃₄₄ $\times$ Brown Norway rats after 14 days of hindlimb suspension. We reported that MDA/4-HAE and nitrotyrosine contents are increased in the suspended muscle when compared to the control muscle in both young adult and aged animals whereas only in the aged suspended muscle, H₂O₂ content was elevated and MnSOD protein content was reduced relative to the control muscle. We also found that the unloading-induced changes of the contents of MDA/4-HAE, H₂O₂ and MnSOD protein follow an age-dependent fashion. Moreover, in addition to an age-related sarcopenic muscle mass decline, aged muscle exhibited greater contents of H₂O₂, 8-OHdG and nitrotyrosine but lower abundances of MnSOD mRNA and protein and catalase mRNA. These findings are consistent with the hypotheses that oxidative stress maybe involved in mediating disuse-induced and age-related sarcopenic muscle losses.

Although the consequence of considerable muscle wasting has been generally exhibited during muscle disuse (or decreased activity/loading), the underlying mechanisms responsible for the disuse-induced muscle loss are still largely unknown. Given that understanding the regulatory mechanisms of disuse-induced muscle loss is imperative to give rise to the development of means or strategies that can encounter or ameliorate the deleterious effects of spaceflight, bed rest, limb casting, and physical inactivity, etc, on musculoskeletal system, active research has been undertaking with the aim to fully reveal the process of muscle wasting during muscle disuse. Recently, it has been rationally proposed that oxidative stress, a process that has been demonstrated to be involved in a variety of cellular events and signalings [9,30], may play an important role in regulating the process of muscle loss during disuse [31]. This proposition is persuasively supported by the existing evidences that have demonstrated the production of reactive oxidants in inactive skeletal muscle through different sources (xanthine oxidase, nitric oxide/peroxynitrite, reactive iron, NAD(P)H oxidase, and/or mitochondrial reactive oxygen species) and the possible signaling linkages between oxidative stress and the proteolytic processes mediating muscle loss (caspase-3, calpain-mediated and proteasome-mediated proteolyses) [31]. By using the experimental models of limb immobilization and hindlimb suspension, there have been studies showing that oxidative stress is possibly involved in disuse muscle wasting [16-22]. Several studies have been conducted by Kondo's group to examine the relationship of oxidative stress and muscle atrophy during limb immobilization [17-21]. With muscle atrophy, as induced by immobilizing one ankle joint of rats in a fully extended position for days, Kondo and colleagues demonstrated increases in iron concentration (especially in microsomal fraction), lipid peroxidation marker TBAR, H_2O_2 content, glutathione disulfide, hydroxyl radical, activities of CuZnSOD, glutathione-S-transferase, and glutathione reductase but decreases in total glutathione and MnSOD activity [17-21]. It has been exhibited that injection or administration of antioxidant vitamin E attenuates the rate of muscle atrophy during immobilization [1,19]. Taken together, these animal data indicated that oxidative stress is elevated and possibly involved in muscle atrophy, at least in immobilization-induced muscle wasting. In addition, oxidative stress has been investigated in atrophied skeletal muscle in response to hindlimb suspension [16,22,42]. By using the microarray technique, Stevenson et al. [42] reported the activation of the oxidative stress-related gene including glutathione-S-transferase, glutathione peroxidase, selenoprotein P and W, peroxiredoxin 5, SOD-1 and thioredoxin interacting factor in rat soleus muscle after days of hindlimb suspension. Koesterer and coworkers also demonstrated that protein carbonyl content, a marker for protein oxidation, is increased, although they did not find change in the content of TBARS in the young hindlimb suspended muscle [16]. In soleus muscle taken from young adult rat following hindlimb unloading, there have also been data showing elevation of oxidative stress (increases in dichlorohydrofluorescein and total hydroperoxides) and disruption of antioxidant capacity (reduction

of glutathione peroxidase, catalase and MnSOD activities and non-enzymatic antioxidant scavenging capacity and increase in CuZnSOD activity) [22]. The present findings of MDA/4-HAE and nitrotyrosine in young adult gastrocnemius muscle are consistent with the previous observations that muscle wasting during muscle disuse is generally accompanied with increased level of oxidative stress. In addition to slow-twitch soleus muscle (a muscle atrophies severely during hindlimb suspension), our results indicated that gastrocnemius muscle (a muscle that is predominately but not exclusively composed of fast fibers), which undergoes moderate muscle loss in response to hindlimb unloading, also exhibits elevation of oxidative stress during muscle disuse. Collectively, it necessitates further investigation in fully understanding the possible role of oxidative stress in regulating the signaling mechanisms that mediate disuse muscle wasting.

Based on the consistent findings indicating that oxidative stress is elevated and the antioxidant capability is impaired in skeletal muscle with aging [2,5,6,12,13,15,28,29], it is reasonable hypothesizing that aging may influence the redox events and predispose muscle to be more susceptible to the oxidative injury during muscle disuse. However, the age-related issue of oxidative stress in atrophied skeletal muscle has not been previously examined. In the present study, we have first provided evidence showing that age-specific changes in oxidative stress markers are evident in rat gastrocnemius muscle during muscle unloading as induced by hindlimb suspension. Our findings of age-dependent increases in MDA/4-HAE and H_2O_2 and decrease in MnSOD protein abundance in response to hindlimb suspension are in agreement with the proposition that aging may prompt an advanced level of oxidative stress in atrophied muscle. These aging findings may have clinical implication as it is unclear whether hypokinesia during certain clinical situations (e.g., prolonged bed rest and limb casting) may exacerbate the extent of muscle wasting pathology in elderly population and this may be related to oxidative stress. Nonetheless, our data provide preliminary evidence suggesting that aging may superimpose the disturbance of redox balance during muscle unloading, and it is worthwhile to have further research on this topic to fully identify the influence of aging in oxidative stress during disuse muscle atrophy in skeletal muscle.

In conclusion, we have demonstrated the elevation of oxidative stress markers and alteration of antioxidant enzymes expression in gastrocnemius muscles from young adult and aged rats in response to muscle unloading as induced by hindlimb suspension. In both young adult and aged muscles following suspension, we showed increases in the MDA/4-HAE and nitrotyrosine contents when compared to the age-matched non-suspended control muscles. Elevation of H_2O_2 content and decrease in MnSOD protein abundance were only found in aged muscle after suspension. Age-related changes in the contents of MDA/4-HAE, H_2O_2 and MnSOD protein during hindlimb unloading were evident. Furthermore, by comparing the aged to young adult animals, the sarcopenic decline in muscle mass was in the company of elevated contents of H_2O_2 , 8-OHdG and nitrotyrosine but lower

abundances of MnSOD mRNA and protein and catalase mRNA. Our data are consistent with the hypothesis that oxidative stress is increased in both young adult and aged muscles in response to hindlimb unloading and the changes of oxidative stress markers during unloading are age-dependent. These findings are in agreement with the proposition that aging may predispose skeletal muscle to an advanced level of oxidative stress at rest and under atrophic situation.

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Table 1. Primers used for PCR amplification of cDNA

Product	Accession No.	Sequence	Position	T _A , °C	Cycles	Product length, bp	Restriction enzyme	Restriction products, bp
CuZnSOD	NM_017050	F: 5'-GGCCGTGTGCGTGCTGAAG-3' R: 5'-CAGCCACATTGCCAGGTCTC-3'	105-123 341-361	58	33	257	<i>Bst</i> XI <i>Rsa</i> I	140, 117 163, 94
MnSOD	NM_017051	F: 5'-GCGGGGGCCATATCAATCACAG-3' R: 5'-GGCGGCAATCTGTAAGCGACCT-3'	358-379 551-572	58.2	33	215	<i>Aha</i> I	135, 80
Catalase	NM_012520	F: 5'-CGGGAACCCAATAGGAGATAAA-3' R: 5'-CCACGAGGGTCACGAAGTGT-3'	176-197 456-475	56.3	33	300	<i>Bgl</i> II <i>Rsa</i> I	251, 49 171, 129

T_A, annealing temperature; Accession No., GenBank accession number; F, forward primer; R, reverse primer.

Figure 1 Muscle mass change during hindlimb suspension**A. Muscle wet weight** **B. Muscle wet weight normalized to bodyweight**

Legend for Figure 1A Muscle weight. The extent of muscle loss following hindlimb suspension was estimated by monitoring the muscle mass loss between the suspended and the control whole gastrocnemius muscle wet weight. The data are presented as means $\pm$ SE. * $P < 0.05$, suspended group was significant different from the control group under the same age group; # $P < 0.05$, aged animals were significant different from the young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2×2 ANOVA.

Legend for Figure 1B Muscle weight after normalized to animal bodyweight. The extent of muscle loss following hindlimb suspension was further examined by examining the decrease in gastrocnemius muscle wet weight after normalized to the animal's bodyweight between the suspended and control groups. The data are presented as means $\pm$ SE. * $P < 0.05$, suspended group was significant different from the control group under the same age group; # $P < 0.05$, aged animals were significant different from the young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2×2 ANOVA.

Figure 2 **MDA/4-HAE content**

Legend for Figure 2 **MDA/4-HAE content.** The level of lipid peroxidation was estimated by measuring the content of MDA/4-HAE. The measured μM MDA/4-HAE is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. * $P < 0.05$, suspended group was significant different from the control group under the same age group; # $P < 0.05$, aged animals were significant different from the young animals under the same experimental condition. The main effects of suspension and interaction (suspension $\times$ age) were analyzed by a 2×2 ANOVA.

Figure 3 **H₂O₂ content**Age: $F(1,34)=32.3$, $P=0.000$ Age x Suspended: $F(1,34)=11.1$, $P=0.002$

Legend for Figure 3 **H₂O₂ content.** The H₂O₂ content was determined by a fluorometric assay. The fluorescence unit is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. * $P < 0.05$, suspended group was significant different from the control group under the same age group; # $P < 0.05$, aged animals were significant different from the young animals under the same experimental condition. The main effects of age and interaction (suspension $\times$ age) were analyzed by a 2×2 ANOVA.

Figure 4 **8-OHdG content**

Legend for Figure 4 **8-OHdG content.** The level of oxidative DNA damage was estimated by measuring the content of 8-OHdG. The measured ng 8-OHdG is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. [#]P < 0.05, aged animals were significant different from the young animals under the same experimental condition. The main effect of age was analyzed by a 2×2 ANOVA.

Figure 5 Nitrotyrosine content

Legend for Figure 5 Nitrotyrosine content. The content of nitrotyrosine was measured in cytosolic fraction by an immuno-dot blot. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for nitrotyrosine in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. * $P < 0.05$, suspended group was significant different from the control group under the same age group; # $P < 0.05$, aged animals were significant different from the young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2×2 ANOVA.

Figure 6 CuZnSOD mRNA and protein content**A. CuZnSOD mRNA content** **B. CuZnSOD protein content**

Legend for Figure 6A **CuZnSOD mRNA content.** The mRNA content of CuZnSOD was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of CuZnSOD/18S. The insets show representative results for the CuZnSOD mRNA. The data are presented as means $\pm$ SE.

Legend for Figure 6B **CuZnSOD protein content.** The protein content of CuZnSOD was assessed in cytosolic fraction by immunoblot analysis. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for CuZnSOD in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

Figure 7 MnSOD mRNA and protein content**A. MnSOD mRNA content** **B. MnSOD protein content**

Legend for Figure 7A MnSOD mRNA content. The mRNA content of MnSOD was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of MnSOD/18S. The insets show representative results for the MnSOD mRNA. The data are presented as means $\pm$ SE. The main effect of age was analyzed by a 2×2 ANOVA.

Legend for Figure 7B MnSOD protein content. The protein content of MnSOD was assessed in cytosolic fraction by immunoblot analysis. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for MnSOD in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. * $P < 0.05$, suspended group was significant different from the control group under the same age group; # $P < 0.05$, aged animals were significant different from the young animals under the same experimental condition. The main effects of age and interaction (suspension $\times$ age) were analyzed by a 2×2 ANOVA.

Figure 8 **Catalase mRNA content**

Legend for Figure 8 **Catalase mRNA content.** The mRNA content of catalase was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of catalase/18S. The insets show representative results for the catalase mRNA. The data are presented as means $\pm$ SE. [#] $P < 0.05$, aged animals were significant different from the young animals under the same experimental condition. The main effect of age was analyzed by a 2×2 ANOVA.

CHAPTER 7

Apoptotic signaling induced by H₂O₂-mediated oxidative stress in differentiated C2C12 myotubes

ABSTRACT

Apoptotic signaling proteins were evaluated in postmitotic skeletal myotubes to test the hypothesis that oxidative stress induced by H₂O₂ treatment would activate caspase-dependent and caspase-independent apoptotic proteins and decrease anti-apoptotic protein levels in differentiated C2C12 myotubes. Apoptotic regulatory factors and apoptosis-associated proteins including Bcl-2, Bax, Apaf-1, XIAP, ARC, cleaved PARP, p53, p21^{Cip1/Waf1}, c-Myc, HSP70, CuZnSOD, and MnSOD protein content were measured by immunoblots. H₂O₂ induced apoptosis in myotubes as shown by DNA laddering and an elevation of apoptotic DNA fragmentation. H₂O₂ caused an increase in Bax, cytochrome c, Smac/DIABLO, caspase-3 protease activity, cytosolic AIF and nuclear and cytosol p53 levels. Nuclear AIF protein content was increased, denoting activation of this caspase-independent apoptotic pathway during H₂O₂ exposure. Bcl-2 declined in H₂O₂ treated myotubes. These findings indicate that both caspase-dependent and caspase-independent mechanisms are involved in coordinating the activation of apoptosis induced by H₂O₂ in differentiated myotubes.

Key words: oxidative stress; programmed cell death; sarcopenia

INTRODUCTION

Reactive oxygen and nitrogen species or intermediates (ROS/RNI) are constantly generated via a variety of intracellular processes throughout the entire lifespan of oxygen-dependent living organisms. Oxidative stress occurs when the production of ROS/RNI overwhelms the cellular antioxidant defense capability. Substantial data have been assembled in exhibiting the essential role of oxidative stress in the regulation of diverse cellular events, which are dependent on the extent of oxidative stress (e.g., proliferation, differentiation, adhesion, oxidative damage and cell death, etc). Most of these events are controlled by signaling mechanisms with redox-sensitive components [1-5]. There is a growing body of evidence demonstrating a role of oxidative stress in the activation of apoptosis, which is an indispensable cellular process involved in governing cell survival/death during embryonic development, immunological defense, and tissue turnover [6-8].

Aging is an unavoidable biological process that is typified by progressive loss of skeletal muscle mass and strength called sarcopenia. While the exact mechanisms that contribute to sarcopenia are largely unclear and still under active investigation, there has been recent evidence showing apoptosis is involved in the aging-associated loss of postmitotic tissues (i.e., skeletal and cardiac muscles and brain) because the occurrence of the pro-apoptotic events increases in aged tissues [9-18]. Given that skeletal muscle from the aged individuals is chronically exposed to elevated levels of oxidative stress [19,20] and concurrently, apoptotic machinery can be initiated under redox control in a variety of cell types [6-8], it is reasonable to hypothesize that accelerated apoptosis resulting from the elevated oxidative stress with aging may have an important role in progressing sarcopenia.

Previously, there have been reports documenting the incidence of apoptosis under oxidative stress induced by H_2O_2 or menadione in proliferating skeletal myoblasts [21-23]. However, the apoptotic consequences and the regulatory mechanisms responsible for the activation of the apoptotic program under oxidative stress are still poorly understood. In particular, differentiated muscle cells which are structurally (multinucleated), biochemically (expression of terminal myogenic proteins, e.g., myosin heavy chain), and functionally (contractile ability) different from proliferating myoblasts. Thus, the aim of this study was to test the hypothesis that oxidative stress induced by H_2O_2 treatment in differentiated C2C12 myotubes would activate pro-apoptotic proteins and decrease anti-apoptotic proteins. Instead of using proliferating myoblasts, we examined differentiated skeletal myotubes as an *in vitro* model for mature developed but non-innervated skeletal muscle. The experiments were designed to examine both the dose- and time-dependent responses of apoptosis to H_2O_2 treatment.

METHODS

Cell culture and treatment. Mouse-derived C2C12 myoblasts obtained from American Type Culture Collection (ATCC, Manassa, VA) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin fungizone, and incubated at 37°C in a water-saturated atmosphere of 95% ambient air and 5% CO₂. Differentiated myotubes were prepared by culturing confluent C2C12 myoblasts in DMEM with 2% heat-inactivated horse serum and antibiotics (differentiation medium) to induce myogenic differentiation. In the present study, myotubes after 6 days of differentiation prepared on 100-mm culture plates were examined as it has been shown that over 95% of C2C12 cultures are multinucleated myotubes following ~6-7 days of incubation in differentiation medium [24,25]. For the dose-dependent experiments, myotubes were treated with freshly prepared dilutions of H₂O₂ in differentiation medium resulting in a final concentration of 1, 2, and 4 mM for 48 h. Myotubes were also exposed to 4 mM of H₂O₂ for 24 and 96 h to examine the time-dependent responses. Myotubes prepared from the same passage of myoblasts were treated with sterile water (i.e., vehicle of H₂O₂ dilution) and were included in the experiments of 24, 48, and 96 h treatment to act as the corresponding controls (i.e., 0 mM of H₂O₂). During the experiments, differentiation medium with H₂O₂ or sterile water was changed daily.

Morphological imaging. Morphological changes and survival of cells were monitored by obtaining photomicrographs under an inverted phase contrast microscope (Olympus America Inc., Melville, NY) with a digital camera.

DNA gel electrophoresis laddering assay. Apoptotic DNA fragmentation was qualitatively analyzed by detecting the laddering pattern of nuclear DNA as described [26]. Briefly, cells were harvested by scrapping, washed in PBS, and lysed in 0.5 ml of DNA extraction buffer (50 mM Tris-HCl, 10 mM EDTA, 0.5% Triton, and 100 µg/ml proteinase K, pH 8.0) for overnight at 37°C. The lysate was then incubated with 100 µg/ml DNase-free RNase A for 2 h at 37°C, followed by three extractions of an equal volume of phenol/chloroform (1:1 v/v) and a subsequent re-extraction with chloroform by centrifuging at 15,000 rpm for 5 min at 4°C. The extracted DNA was precipitated in 2 volume of ice-cold 100% ethanol with 1/10 volume of 3 M sodium acetate, pH 5.2 at -20°C for 1 h, followed by centrifuging at 15,000 rpm for 15 min at 4°C. After washing with 70% ethanol, the DNA pellet was air-dried and dissolved in 10 mM Tris-HCl/1 mM EDTA, pH 8.0. The DNA was then electrophoresed on 1.5% agarose gel and stained with ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris-acetate). A 100-bp DNA ladder (Invitrogen Life Technologies, Bethesda, MD) was included as a molecular size marker and DNA fragments were visualized and photographed by exposing the gels to ultraviolet transillumination.

Subcellular protein fractionation. The fractionation method described by Rothermel et al. [27] was used to extract the cytosolic and nuclear protein fractions from C2C12 muscle cells with minor modification. In brief, cells were harvested by scrapping in ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT). Following centrifuging at 5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and these supernatants were further centrifuged three times at 5,000 rpm for 5 min at 4°C to remove residual nuclei and stored as nuclei-free total cytosolic protein fraction. A portion of this collected cytosolic extract (without addition of protease inhibitors) was stored and used later for fluorometric caspases-3 protease activity assay while a protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO) was added to the remaining portion. The cytosolic protein fraction with the addition of protease inhibitors was then used for cell death ELISA and Western immunoblots. The remaining nuclear pellets were then washed 3 times with ice-cold lysis buffer, resuspended in 240 µl of lysis buffer in the presence of 33.2 µl of 5 M NaCl and protease inhibitor cocktail, and rotated for 1 h at 4°C to lyse the nuclei. Following a spin at 15,000 rpm for 15 min at 4°C, the supernatants were collected and stored as cytosol-free nuclear protein fraction.

In order to estimate the release of mitochondria-resided apoptotic factors including cytochrome c, AIF, and Smac/DIABLO to the cytosol, a mitochondria-free cytosolic protein fraction was prepared as described by Rokhlin et al. [28]. Briefly, cells were scrapped in ice-cold extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4) in the presence of protease inhibitor cocktail. Following a gentle homogenization with a Teflon pestle motorized with an electronic stirrer, homogenates were centrifuged at $800 \times g$ for 10 min at 4°C to pellet the nuclei and cell debris. The supernatants were then spun twice at $16,000 \times g$ for 20 min at 4°C to pellet the mitochondria and the final supernatants were collected as mitochondria-free cytosolic protein fractions. The purity of the protein fractions was confirmed by immunoblotting with an anti-histone H2B (a nuclear protein) rabbit polyclonal antibody (1:2000 dilution, 07371, Upstate, Lake Placid, NY), an anti-β-tubulin (a cytosolic cytoskeleton protein) rabbit polyclonal antibody (1:500 dilution, ab6046, Abcam, Cambridge, MA), an anti-SOD-1 or -CuZnSOD (a cytosolic isoform of superoxide dismutase) rabbit polyclonal antibody (1:500 dilution, sc-11407, Santa Cruz Biotechnology, Santa Cruz, CA), and an anti-MnSOD (a mitochondrial isoform of superoxide dismutase) goat antibody (1:2000 dilution, A300449A, Bethyl Lab, Montgomery, TX) (**Figure 1**). The protein concentration of the protein extracts were quantified in duplicate by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation [29]. As a further means to

confirm the protein content, on a different occasion, all samples were then measured in duplicate by *DC* Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay to confirm the protein contents [30].

Insert Figure 1 ———→ Verification of the extracted protein fractions

Apoptotic cell death ELISA. Cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to quantitatively estimate the apoptotic DNA fragmentation by assessing the cytosolic histone-associated mono- and oligo-nucleosomes. In brief, the extracted nuclei-free cytosolic fraction was used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated to the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate for 10 min at 20°C. The change in color was measured at a wavelength of 405 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). Measurements were performed in duplicate with all samples analyzed on the same microtiter plate in the same setting. The OD₄₀₅ reading was then normalized to the mg of protein used in the assay.

Fluorometric caspases-3 activity assay. Fifty µl of the total cytosolic protein fraction without protease inhibitor was incubated in 50 µl of assay buffer (50 mM PIPES, 0.1 mM EDTA, 10% glycerol, 10 mM DTT, pH 7.2) with 100 µM of the fluorogenic 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated substrate (Ac-DEVD-AFC, Alexis Corp., San Diego, CA) at 37°C for 2 h. Caspase specific inhibitor, Z-VAD-FMK (Calbiochem, La Jolla, CA) was used as a control to validate the specificity of caspase. The change in fluorescence was measured on a spectrofluorometer with an excitation wavelength of 390/20 nm and an emission wavelength of 530/25 nm (CytoFluor, Applied Biosystems, Foster City, CA) before and after the 2 h incubation. Caspase activity was estimated as the change in arbitrary fluorescence units normalized to milligram protein used in the assay. Measurements were performed in duplicate while all the measurements were run on the same microplate in the same setting.

Immunoblot analyses. Protein expression of Bcl-2 (B-cell leukemia/lymphoma-2), Bax (Bcl-2-associated X protein), apoptosis protease activating factor-1 (Apaf-1), X-linked inhibitor of apoptosis (XIAP), apoptosis repressor with caspase recruitment domain (ARC), heat shock protein-70 (HSP70), and superoxide dismutases (CuZn- and Mn-SOD) was determined in the total cytosolic protein fraction. The protein content of apoptosis inducing factor (AIF), p21^{Cip1/Waf1}, c-Myc, and

cleaved poly(ADP-ribose) polymerase (PARP) was measured in the nuclear fraction while p53 protein content was measured in both total cytosolic and nuclear fractions. Fifteen micrograms of protein was boiled at 95°C for 5 min in Laemmli buffer and was separated by SDS-PAGE on 12% polyacrylamide gel. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and probed with the following primary antibodies diluted in PBS-T with 2% BSA: anti-Bcl-2 mouse monoclonal antibody (1:100 dilution, sc-7382), anti-Bax rabbit polyclonal antibody (1:200 dilution, sc-6236), anti-Apaf-1 rabbit polyclonal antibody (1:200 dilution, 3018, BioVision, Mountain View, CA), anti-hILP/XIAP mouse monoclonal antibody (1:250 dilution, 610762), anti-HSP70 mouse monoclonal antibody (1:2000 dilution, SPA810, StressGen, Victoria, BC, Canada), anti-SOD-1 rabbit polyclonal antibody (1:500 dilution, sc-11407), anti-MnSOD goat antibody (1:2000 dilution, A300449A, Bethyl Lab, Montgomery, TX), anti-AIF mouse monoclonal antibody (1:800 dilution, sc-13116HRP), anti-ARC rabbit polyclonal antibody (1:200 dilution, sc-11435), anti-cleaved PARP rabbit polyclonal antibody (1:1000 dilution, G734, Promega, Madison, WI), anti-p53 mouse monoclonal antibody (1:100 dilution, sc-99), anti-p21^{Cip1/Waf1} mouse monoclonal antibody (1:100 dilution, 550827), or anti-c-Myc mouse monoclonal antibody (1:200 dilution, 3800-1). Bcl-2, Bax, SOD-1, AIF, ARC, and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while XIAP, p21^{Cip1/Waf1}, and c-Myc antibodies were purchased from BD Biosciences (San Jose, CA). All primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed by ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) × band area by an image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Estimation of mitochondrial cytochrome c, Smac/DIABLO, and AIF release.

Cytochrome c, AIF, and Smac/DIABLO (second mitochondria-derived activator of caspase) are apoptotic factors normally housed inside the mitochondria and their release to the cytosol has been demonstrated during the activation of apoptosis [31]. In this study, the release of Smac/DIABLO and AIF into the cytosol was estimated in the extracted mitochondria-free cytosolic protein fraction by

immunoblotting with an anti-Smac/DIABLO mouse monoclonal antibody (1:500 dilution, 612244, BD Biosciences, San Jose, CA) and an anti-AIF monoclonal mouse antibody (1:800 dilution, sc-13116HRP). Moreover, a cytochrome c ELISA kit (MBL International, Woburn, MA) was used to assess the protein content of cytochrome c in the mitochondria-free cytosol fraction to evaluate the release of the mitochondrial cytochrome c to the cytosol. According to the manufacturer's protocol, 50 μ l the extracted mitochondria-free cytosolic fraction was used as an antigen source in a sandwich ELISA with a horseradish peroxidase-conjugated anti-cytochrome c polyclonal antibody in microwell strips coated with an anti-cytochrome c antibody. After washing, the peroxidase retained in the immunocomplex was detected by incubating with a chromogenic substrate, tetramethylbenzidine/hydrogen peroxide (TMB/ H_2O_2) followed by adding an acid solution to terminate the enzyme reaction and to stabilize the developed color. The change in color was monitored at a wavelength of 450 nm using a Dynex MRX plate reader. Measurements were performed in duplicate with all the samples analyzed on the same microplate and the cytochrome c content was expressed as OD₄₅₀ per mg protein.

Statistical analyses. All experiments were repeated on four different occasions ($N = 4$) and data are expressed as percent relative to the control of the same occasion (i.e., 0 mM referred to 100%). Results are given as means $\pm$ standard error of mean (SE). Statistical analyses were performed using the SPSS 10.0 software package and one-way ANOVA with Tukey HSD post hoc test was used to examine the percent change in treatment relative to control. Multivariate analysis of variance (MANOVA) was performed on the percent changes in all measured variables in this study in order to examine the main effects of time, dose, and interaction (time $\times$ dose). Estimate of the effect size by computing Eta squared (η^2 , ranges from 0 to 1) was included in the MANOVA to designate how much of the total variance in our experiments was explained by the main effects or interaction. Statistical significance was accepted at $P < 0.05$.

RESULTS

Cell morphology. The morphological changes of C2C12 myotubes following a range of H_2O_2 treatment for 24, 48, or 96 h was monitored by an inverted phase contrast microscope. **Figure 2** shows that the morphological health condition and survival of cells was apparently reduced with increasing dose of H_2O_2 treatment or with longer period of treatment.

Insert Figure 2 ———→ **Microscopic image**

Indicators of apoptosis: DNA laddering and cell death ELISA. DNA laddering gel electrophoresis is a suggested tool in detecting apoptosis in a highly specific manner as

endonucleases cleave genomic DNA between the nucleosomes resulting in mono- and oligonucleosomes of ~200 base pairs or multiples during execution of apoptosis [32]. In the present study, apoptotic DNA fragmentation was qualitatively examined using DNA laddering assay. The laddering pattern was absent in all extracted DNA from the control experiments (i.e., 0mM of H₂O₂). Following 48 h exposure to 2mM and 4mM of H₂O₂ and 96 h exposure to 4 mM of H₂O₂, laddering pattern was clearly present in the extracted DNA (**Figure 3A**). According to the results of our quantitative cell death ELISA, the extent of apoptotic DNA fragmentation following 24 h treatment with 4mM of H₂O₂ was increased by 27% relative to 0mM (**Figure 3B**). After 48 h exposure to 2mM and 4mM of H₂O₂, the apoptotic DNA fragmentation was elevated by 37% and 99%, respectively while it was increased by 135% following 4mM of H₂O₂ treatment for 96 h (**Figure 3B**).

Insert Figure 3 —→ DNA laddering and cell death ELISA

BCL-2 family: Bcl-2 and Bax. In our immunoblots, we detected an immunoreactive band of ~25 kDa corresponding to the predicted molecular mass of Bcl-2 protein and a ~21 kDa immunoreactive band corresponding to Bax protein. We found that there was a 31% and 61% decrease in the Bcl-2 protein content following 48 h treatment with 2mM and 4mM of H₂O₂, respectively (**Figure 4A**). After 96 h exposure to 4mM of H₂O₂, Bcl-2 protein content was reduced by 69% (**Figure 4A**). For the Bax protein content, there was a 56% increase after 24 h treatment with 4mM of H₂O₂ (**Figure 4B**). Following 48 h treatment with 2mM and 4mM of H₂O₂, we found that the Bax protein content was elevated by 108% and 122%, respectively while it was increased by 227% after 96 h treatment with 4mM of H₂O₂ (**Figure 4B**). The ratio of Bax/Bcl-2 protein content was calculated based on the results of Western analyses. There was a 567% and 1142% increase in the ratio of Bax/Bcl-2 following exposure to 4mM of H₂O₂ for 48 h and 96 h, respectively (**Figure 4C**).

Insert Figure 4 —→ Bcl-2 and Bax

Mitochondrial cytochrome c release and Apaf-1 content. The results of ELISA on the extracted mitochondria-free cytosolic fraction demonstrated that the protein content of cytosolic cytochrome c increased by 282% following 24 h treatment with 4mM of H₂O₂ (**Figure 5**). After 48 h treatment with 2mM and 4mM of H₂O₂, the cytochrome c was elevated by 442% and 701%, correspondingly while it was increased by 701% after 96 h treatment with 4mM of H₂O₂ (**Figure 5**). In contrast, we did not find any difference in the cytosolic protein content of Apaf-1 following H₂O₂ exposures when compared to control ($P > 0.05$, data not shown).

Insert Figure 5 ———→ Mitochondrial cytochrome c release

Caspase-3 protease activity. The protease activity of caspase-3, as determined by a fluorometric caspase activity analysis, was increased by 37% and 51% after 48 h exposure to 2mM and 4mM of H₂O₂, respectively (**Figure 6**). There was a 141% elevation of caspase-3 activity following 96 h treatment with 4mM of H₂O₂ (**Figure 6**).

Insert Figure 6 ———→ Caspase-3 protease activity

XIAP and ARC protein contents and mitochondrial Smac/DIABLO release. An immunoreactive band of ~57 and ~25 kDa corresponding to the predicted molecular mass of XIAP and ARC protein, respectively, was detected in the immunoblots of the total cytosolic fractions. The XIAP protein content was increased by 31% following 96 h treatment with 4mM of H₂O₂ (**Figure 7A**). For the ARC protein content, there was a 38% and 42% decrease after exposure to 4mM of H₂O₂ for 48 h and 96 h, correspondingly (**Figure 7B**). For the immunoblots of the extracted mitochondria-free cytosolic fractions, we detected an ~22 kDa immunoreactive band corresponding to the predicted molecular mass of the Smac/DIABLO protein. We found that the Smac/DIABLO protein content was upregulated by 115% after 24 h treatment with 4mM of H₂O₂ (**Figure 7C**). Following 48 h exposure to 1mM, 2mM, and 4mM of H₂O₂, there was a 118%, 117%, and 260% increase in the Smac/DIABLO protein content, respectively (**Figure 7C**). The Smac/DIABLO protein content was elevated by 466% after 96 h treatment with 4mM of H₂O₂ (**Figure 7C**).

Insert Figure 7 ———→ XIAP, ARC, and Smac/DIABLO

Caspase-independent apoptotic events: mitochondrial AIF release and nuclear translocation. Translocation of mitochondrial AIF to the nuclei has been suggested to be a caspase-independent apoptotic event [33,34]. As an estimate of the mitochondrial AIF release to the cytosol, the AIF protein content measured in the mitochondria-free cytosolic fraction was elevated by 65% after 96 h treatment with 4mM of H₂O₂ (**Figure 8A**). As assessed by immunoblots of nuclear protein fraction, we found that the nuclear AIF was increased by 174% and 352% following exposure to 4mM of H₂O₂ for 48 h and 96 h, respectively (**Figure 8B**).

Insert Figure 8 ———→ AIF

Cleavage of protein substrate: cleaved PARP protein. PARP functions in the process of DNA repairing and it has been shown to be a cleavage target for the effector caspase-3 during the execution of apoptosis [35]. According to our Western analysis, a ~85 kDa immunoreactive band corresponding to the cleaved PARP product was detected in the nuclear protein fraction extracted from the cells after 48 h and 96 h treatment with 4mM of H₂O₂ (**Figure 9**).

Insert Figure 9 —————> Cleaved PARP

Cellular regulatory factors: p53, p21^{Cip1/Waf1}, and c-Myc protein contents. In our immunoblot analyses, an immunoreactive band of ~53 kDa corresponding to the predicted molecular mass of p53 protein was detected in the nuclear and the cytosolic protein fraction. There was a 719%, 909%, and 1581% increase in the nuclear p53 protein content after exposure to 4mM of H₂O₂ for 24 h, 48 h, and 96 h, respectively (**Figure 10A**). For the cytosolic p53 protein content, it was elevated by 71%, 93%, and 227% following treatment with 4mM of H₂O₂ for 24 h, 48 h, and 96 h, correspondingly (**Figure 10B**). On the contrary, we did not find any changes in the protein content of p21^{Cip1/Waf1} and c-Myc following the H₂O₂ treatments as defined by a detected ~21 kDa and 62 kDa immunoreactive band, respectively, that was not different between the H₂O₂-treated and control cells ($P > 0.05$, data not shown).

Insert Figure 10 —————> p53

Heat shock stress protein and antioxidant enzyme: HSP70, MnSOD, and CuZnSOD protein contents. According to our immunoblot analyses, the protein content of HSP70, a stress protein, was increased by 131%, 103%, and 392% after exposure to 4mM of H₂O₂ for 24 h, 48 h, and 96 h, respectively (**Figure 11A**). For the antioxidant enzyme SOD, MnSOD protein content was upregulated by 23% following 96 h treatment with 4mM of H₂O₂ (**Figure 11B**) whereas there was no significant change in CuZnSOD protein content following H₂O₂ treatments ($P > 0.05$, **Figure 11C**).

Insert Figure 11 —————> HSP70, MnSOD, and CuZnSOD

Main effect of dose, time, and dose × time. The analysis of the multivariate tests in MANOVA examining all the measured variables showed that there were significant main effects of dose [$F(45, 36) = 13.30$, $P < 0.001$, $\eta^2 = 0.943$], time [$F(30, 22) = 393.58$, $P < 0.001$, $\eta^2 = 0.998$], and interaction (dose × time) [$F(30, 22) = 393.58$, $P < 0.001$, $\eta^2 = 0.998$] in explaining the variances of the present findings.

DISCUSSION

Apoptosis is a programmed, active, and tightly coordinated form of cell death. Based on substantial data obtained in mitotic cells or tissues, apoptosis has been widely recognized to be a vital process in regulating various cellular events (e.g., tissue degeneration) [36,37]. Recently, several findings have supported the possibility that apoptosis might have a regulatory role in aging-associated postmitotic skeletal muscle loss [10-12,15,38,39]. Since oxidative stress is elevated in aging muscle [19,20] and apoptosis can be regulated under redox control [4,5,40,41], oxidative stress-induced apoptosis has been hypothesized to be involved, at least in part, in causing sarcopenic muscle loss. Indeed, the incidence of apoptosis has been previously documented in proliferating skeletal myoblasts in response to H_2O_2 or menadione [21-23]. However, to our knowledge this is the first study to examine if apoptotic signaling proteins contribute to apoptosis in postmitotic/differentiated skeletal muscle cells. In the present study, we have confirmed the execution of apoptosis in postmitotic skeletal muscle cells under H_2O_2 induced oxidative stress and have identified changes in expression levels of several proteins involved in apoptotic signaling. We report apoptotic DNA fragmentation (evidence of apoptosis), elevation in pro-apoptotic Bax, decline in anti-apoptotic Bcl-2, increase in Bax/Bcl-2 relative ratio, mitochondrial release of cytochrome c, Smac/DIABLO and AIF, increase in caspase-3 protease activity, down-regulation of ARC, cleavage of PARP, and upregulation of p53 in differentiated C2C12 myotubes following H_2O_2 exposure.

Execution of apoptosis in postmitotic skeletal muscle cell under oxidative stress. There is a scarcity of data addressing the relationship between oxidative stress and apoptosis in postmitotic cells or tissues. Particularly, in skeletal muscle, the apoptotic consequence resulting from oxidative stress has not been fully defined. Although a few studies have attempted to examine the effect of oxidative stress on apoptosis in skeletal lineage muscle cells, all of these studies were conducted in muscle precursor cells (i.e., proliferating myoblasts) instead of differentiated and more functionally-mature muscle cells (i.e., postmitotic myotubes or myocytes) which are lacking the ability for proliferation after exiting the mitotic cell cycle [21-23]. Stangel and colleagues [23] first investigated the potential of skeletal myoblasts obtained from rat thigh muscle to undergo apoptosis when exposed to H_2O_2 , hypoxanthine/xanthine oxidase (a reactive oxygen intermediates including superoxide anion and H_2O_2 -generating model), and S-nitroso-N-acetylpenicillamine (a nitric oxide or NO-generating agent). By using a combination of different techniques including morphological examination, DNA laddering gel electrophoresis, and fluorescence activated cell sorting (FACS)-mediated molecular labeling for DNA strand breaks (*in situ* tailing and nick translation assay), they showed both H_2O_2 and NO induce apoptosis in a dose-dependent manner. Although the underlying mechanisms responsible for the execution of apoptosis were not examined in this study, more importantly, these findings clearly demonstrated that apoptotic cell death is fully operable in

proliferating cells from myogenic origin (i.e., myoblasts) when exposed to H_2O_2 or NO. Subsequently, the observation of the activation of apoptosis in proliferating skeletal myoblasts under oxidative stress was further established [21,22], in which insights on the signaling mechanisms were preliminarily revealed. Caporossi and coworkers [21] showed that, in L6C5 rat myoblasts, apoptosis was evident when exposed to oxidative stress generated by moderate or high intensity of H_2O_2 based on the finding of increase in fragmented/condensed nuclei. They also found that the protease activity of caspase-3 was elevated, suggesting that caspase-dependent pathway was involved in explaining the activation of apoptosis. By manipulating the technique of flow cytometry, Chiou et al. [22] also demonstrated that caspases-3 was activated concomitant with increased hypodiploid cells and phosphatidylserine translocation in C2C12 mouse myoblasts in response to menadione (a reactive oxygen intermediate-generating chemical). On the contrary, in postmitotic/differentiated muscle, the effect of oxidative stress on apoptosis is relatively unknown. Differentiated muscle cells (i.e., C2C12 myotubes) have been shown to preserve the capability of executing apoptosis when exposed to a classic apoptogenic agent staurosporine (a protein kinase inhibitor known to cause apoptosis in various cell types) [25]. In the current study we extend the knowledge of apoptosis in skeletal muscle by demonstrating oxidative stress, as induced by H_2O_2 , is another potent stimulus in activating apoptosis in postmitotic/differentiated skeletal muscle cells. Our *in vitro* data confirm the causal effect of oxidative stress on apoptosis in postmitotic muscle cells and are consistent with the previous *in vivo* findings, which suggested that oxidative stress may have an important role in accelerating apoptosis in mature skeletal muscles with aging or muscle disuse [10-12,14,15,38,39,42].

Apoptotic signaling in skeletal muscle cell under oxidative stress. Another scope of this study was to understand the apoptotic signaling pathways contributed to the activation of apoptosis induced by oxidative stress in postmitotic skeletal muscle cells. Understanding the cellular regulatory mechanisms of apoptosis is of great consequence in thoroughly exploring its biologic role as well as in developing new preventive or therapeutic strategies to offset the deteriorating effects in skeletal muscle with aging by targeting the accelerated apoptosis. Researches investigating mitotic cells/tissues have identified a variety of elemental apoptotic molecules/proteins (e.g., BCL-2 family) crucial in mediating the signaling events leading to execution of apoptosis [37,43]. Although apoptotic signal transduction in postmitotic skeletal muscle is poorly understood, there have been data showing that most of the apoptotic signaling components reported in single cell lineages (e.g., BCL-2 family and caspases, etc.) are well-conserved in mature multinucleated skeletal muscle [9-11,15,25,37-39,42,44-50].

The roles of members of the BCL-2 family in apoptosis, including the pro-apoptotic member Bax and anti-apoptotic member Bcl-2 have been extensively investigated. It is generally agreed that Bax and Bcl-2 serve to control the release of mitochondria-housed apoptotic factors (e.g.,

cytochrome c, AIF, and Smac/DIABLO) by monitoring the formation of outer mitochondrial membrane channel/pore [43,51,52]. In C2C12 myotubes following H_2O_2 treatment, we found an increase in Bax and a decrease in Bcl-2 concomitant with an elevation of the release of mitochondrial cytochrome c, AIF and Smac/DIABLO. The caspase protease family constitutes another important component of the apoptotic machinery [53]. Caspase-3 has been commonly used as an indicator of caspase-dependent apoptotic signaling because the apoptotic signals generated by initiator caspases (e.g., caspases-8, -9, and -12) all converge on the level of the effector, caspase-3 [53]. Consistent with the caspase-3 data in ROS-treated skeletal myoblasts [21,22], we found that the protease activity of caspase-3 was elevated together with the evidence of cleaved PARP fragment (a cleavage product generated by active caspase-3 proteolytic action) in C2C12 myotubes after exposure to H_2O_2 . In addition to the findings of caspase-3 and cleaved PARP, it is noted that we also found increased AIF protein content in both mitochondria-free cytosolic and nuclear protein fractions suggesting the occurrences of mitochondrial AIF release and AIF nuclear translocation in C2C12 myotubes following H_2O_2 treatment. These findings clearly indicated that both caspase-dependent and -independent mechanisms contribute to the activation of apoptosis in postmitotic muscle cells when exposed to H_2O_2 . In this study, we examined the response of apoptotic suppressors including ARC and XIAP to H_2O_2 exposure. As expected, we found that the ARC protein content was down-regulated in response to H_2O_2 . However, surprisingly, we found an increase, rather than a decrease, in XIAP protein content following 96 h treatment with 4mM of H_2O_2 . We interpret this observation as an adaptive response of postmitotic muscle cells to severe level of oxidative stress perhaps in an attempt to minimize apoptosis. In support to this interpretation, it has been demonstrated that XIAP was upregulated under certain apoptotic conditions (e.g., rat skeletal gastrocnemius muscle with aging, human osteoblastic cell after gravity unloading, and human muscle with mitochondrial encephalomyopathies) [38,54,55] while life-long caloric restriction (an anti-apoptotic intervention) reduces XIAP in rat gastrocnemius muscle [38]. Nonetheless, additional research is required to confirm this adaptive response of XIAP to oxidative stress in skeletal muscle by using other ROS-inducing models (e.g., menadione) or other skeletal muscle cell lines (e.g., rat L6 lineage). Tumor suppressor p53 has been suggested to be an important regulator in the apoptotic signal transduction. Intriguingly, in accordance with the pro-apoptotic properties of p53 which are related to the transcription-dependent and/or -independent mechanism associated with Bax [56,57], we found both the nuclear and cytosolic p53 protein content increased concomitant with the elevation of Bax in the H_2O_2 -treated C2C12 myotubes.

In summary, the present study demonstrated elevation of apoptotic DNA fragmentation accompanied with pro-apoptotic signaling orchestrated by increases in Bax, mitochondrial cytochrome c, Smac/DIABLO and AIF releases, caspases-3 protease activity, as well as decreases in

Bcl-2 and ARC in differentiated skeletal muscle cell following H₂O₂ exposure. Moreover, H₂O₂ - induced an increase in nuclear AIF protein content indicating that nuclear translocation of AIF, a caspase-independent apoptotic process, was involved. These findings together suggested that both caspase-dependent and caspase-independent mechanisms are implicated in coordinating the activation of apoptosis induced by H₂O₂. Additionally, p53 was markedly elevated concomitant with the pro-apoptotic signaling suggesting that p53 may also contribute to H₂O₂-induced apoptosis. Our findings confirm the execution of apoptosis under oxidative stress in postmitotic skeletal muscle cells. Also, these data provide a comprehensive analysis of apoptotic signaling and further clarify the proteins responsible for the activation of apoptotic machinery that is induced by H₂O₂ in differentiated skeletal muscle cells.

Remarks: The findings presented in this chapter have been submitted to the *Free Rad. Biol. Med.* at the time of dissertation submission.

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Figure 1 **Verification of protein extract purity**

Legend for Figure 1 **Verification of protein extract purity.** The purity of protein fractions was verified by immunoblotting with anti-histone H2B, anti-β-tubulin, anti-CuZnSOD, and anti-MnSOD antibodies. An equal amount of total cytosolic (Total CP), mitochondria-free cytosolic (Mito-free CP), and nuclear (NP) protein was used in the immunoblot.

Figure 2 Morphological images of C2C12 muscle cells**24 h** **48 h** **96 h**

Legend for Figure 2 Morphological images of C2C12 muscle cells after H₂O₂ treatment.

The C2C12 myotubes were treated with 0, 1, 2, and 4mM of H₂O₂ for 48h and with 4mM of H₂O₂ for 24h and 96h. The morphological health condition and survival of cells was apparently declined with increasing dose of H₂O₂ treatment or with prolonging period of treatment.

Figure 3 Qualitative and quantitative analyses of apoptosis

A. DNA Laddering

B. Apoptotic DNA fragmentation

Legend for Figure 3 DNA laddering and apoptotic DNA fragmentation. Apoptotic DNA fragmentation was qualitatively analyzed by DNA gel electrophoresis **(A)**. The extracted DNA was loaded on 1.5% agarose gel and was stained with ethidium bromide. The extent of apoptotic DNA fragmentation was quantitatively examined by measuring the cytosolic mono- and oligo-nucleosomes with ELISA **(B)**. The data are presented as means $\pm$ standard error of mean (SE) of percent relative to 0mM H_2O_2 (i.e., 0mM refers to 100%). *P < 0.05, data are significantly different from the corresponding 0mM H_2O_2 ; #P < 0.05, data are significantly different from 1mM H_2O_2 ; +P < 0.05, data are significantly different from 2mM H_2O_2 .

Figure 4 BCL-2 family: Bcl-2, Bax, and Bax/Bcl-2 Ratio

Legend for Figure 4 Bcl-2 and Bax. The protein content of Bcl-2 (**A**) and Bax (**B**) was determined by Western analysis. Insets show representative blots. The ratio of Bax/Bcl-2 protein content (**C**) was estimated according to the data of Western analysis. The data are presented as means $\pm$ SE of percent relative to 0mM H₂O₂ (i.e., 0mM refers to 100%). *P < 0.05, data are significantly different from the corresponding 0mM H₂O₂; #P < 0.05, data are significantly different from 1mM; +P < 0.05, data are significantly different from 2mM H₂O₂.

Figure 5 Mitochondrial cytochrome c release

Legend for Figure 5 Mitochondrial cytochrome c release. The release of mitochondrial cytochrome c was estimated by examining the cytochrome c protein content in the extracted mitochondria-free cytosolic fraction with an ELISA. The data are presented as means $\pm$ SE of percent relative to 0mM H₂O₂ (i.e., 0mM refers to 100%). *P < 0.05, data are significantly different from the corresponding 0mM H₂O₂; #P < 0.05, data are significantly different from 1mM; +P < 0.05, data are significantly different from 2mM H₂O₂.

Figure 6 Caspase-3 protease activity

Legend for Figure 6 Caspase-3 protease activity. The change in fluorescence during a 2h incubation of a fluorometric assay specific for caspase-3 protease activity is normalized to mg protein used in the assay. The normalized data are presented as mean $\pm$ SE of percent relative to 0mM H₂O₂ (i.e., 0mM refers to 100%). *P < 0.05, data are significantly different from the corresponding 0mM H₂O₂; #P < 0.05, data are significantly different from 1mM H₂O₂.

Figure 7 XIAP, ARC, and Smac/DIABLO

Legend for Figure 7 XIAP, ARC, and Smac/DIABLO protein. The protein content of XIAP (A), ARC (B), and Smac/DIABLO (C), was measured by Western immunoblot in the total cytosolic (XIAP and ARC) and mitochondria-free cytosolic fractions (Smac/DIABLO). The insets show representative blots. The data are presented as means $\pm$ SE of percent relative to 0mM H₂O₂ (i.e., 0mM refers to 100%). *P < 0.05, data are significantly different from the corresponding 0mM; #P < 0.05, data are significantly different from 1mM H₂O₂; +P < 0.05, data are significantly different from 2mM H₂O₂.

Figure 8 AIF**A. Mitochondrial AIF release** **B. Nuclear AIF**

Legend for Figure 8 AIF protein. The AIF protein content was determined in mitochondria-free cytosolic (A) and nuclear protein fraction (B) by Western analysis in order to indicate the extent of mitochondrial release and nuclear translocation of AIF, correspondingly. The insets show representative blots. The data are presented as means $\pm$ SE of percent relative to 0mM of H_2O_2 (i.e., 0mM refers to 100%). * $P < 0.05$, data are significantly different from the corresponding 0mM H_2O_2 ; [#] $P < 0.05$, data are significantly different from 1mM; ⁺ $P < 0.05$, data are significantly different from 2mM H_2O_2 .

Figure 9 **Cleaved PARP**

Legend for Figure 9 Cleaved PARP. The p85 cleaved PARP fragment was detected by Western immunoblot in the nuclear protein fraction obtained from cells followed 4mM of H₂O₂ treatment for 48h and 96h.

Figure 10 p53**A. Nuclear p53** **Nuclear p53** **B. Cytosolic p53** **Cytosolic p53**

Legend for Figure 10 p53 protein. The p53 protein content was determined in nuclear (A) and total cytosolic protein fraction (B) by Western analysis. The insets show representative blots. The data are presented as means $\pm$ SE of percent relative to 0mM H₂O₂ (i.e., 0mM refers to 100%). *P < 0.05, data are significantly different from the corresponding 0mM H₂O₂; #P < 0.05, data are significantly different from 1mM H₂O₂; +P < 0.05, data are significantly different from 2mM.

Figure 11 Cellular stress markers: HSP70, MnSOD, and CuZnSOD

A. HSP70

B. MnSOD

C. CuZnSOD

Legend for Figure 11 HSP70, MnSOD, and CuZnSOD protein. The protein content of HSP70 (**A**), MnSOD (**B**), and CuZnSOD (**C**) was determined in the total cytosolic fraction by Western immunoblot. The insets show representative blots. The data are presented as means $\pm$ SE of percent relative to 0mM H_2O_2 (i.e., 0mM refers to 100%). *P < 0.05, data are significantly different from the corresponding 0mM; #P < 0.05, data are significantly different from 1mM H_2O_2 ; +P < 0.05, data are significantly different from 2mM H_2O_2 .

CHAPTER 8

Id2 and p53 participate in apoptosis during unloading-induced muscle atrophy

ABSTRACT

Apoptotic signaling was examined in patagialis (PAT) muscles from young adult and old quail. One wing was loaded for 14 days to induce hypertrophy then unloaded for 7 or 14 days to induce muscle atrophy. Although the nuclear Id2 protein content was not different between unloaded and control muscles in either age group, cytoplasmic Id2 protein content of unloaded muscles was higher than contralateral control muscles after 7-days of unloading in young quails. Nuclear and cytoplasmic p53 contents and the p53 nuclear index of the unloaded muscles were higher than control muscles after 7-days of unloading in young quails, whereas in aged quails the p53 and Id2 contents and p53 nuclear index of the unloaded muscles were not altered by unloading. Immunofluorescent staining indicated that myonuclei and activated satellite cell nuclei contributed to the increased number of p53-positive nuclei. Conversely, unloading in either young adult or aged PAT muscles did not alter c-Myc protein content. Although CuZnSOD content was not different in unloaded and control muscles, MnSOD content increased in PAT muscles after 7-days of unloading in young quails, suggesting that unloading induced an oxidative disturbance in these muscles. Moderate correlational relationships existed among Id2, p53, c-Myc, SOD, apoptosis regulatory factors, and TUNEL index. These data indicate that Id2 and p53 are involved in the apoptotic responses during unloading-induced muscle atrophy following hypertrophy in young adult birds. Furthermore, our data suggest that there is an aging-dependent regulation of Id2 and p53 during unloading of previously hypertrophied muscles.

Key words: Inhibitor of DNA binding/differentiation protein; tumor suppressor gene; programmed cell death; aging

INTRODUCTION

Apoptosis has been documented under the experimental situations of skeletal muscle denervation and unloading (1, 3, 6, 25, 28). It has also been proposed that apoptosis may further play a physiologic role in regulating unloading-induced muscle atrophy especially in aging associated muscle loss or sarcopenia (4, 15, 16, 33, 45, 50). With the aim of identifying the possible role of apoptosis during muscle atrophy, it is imperative to understand the underlying regulatory mechanism(s) contributing to the activation of the apoptotic signaling pathway that results in unloading-induced apoptosis. A variety of cell cycle regulatory proteins and transcription factors (e.g., Id2, p53, and c-Myc) have been demonstrated to have an essential role in regulating growth and apoptosis in different cell types (53, 56). As these proteins could act as the mediators of the apoptotic signaling pathway, they may be involved in the regulation of apoptosis during muscle atrophy.

The inhibitor of DNA binding/differentiation (Id) protein family is structurally characterized by the helix-loop-helix (HLH) motif, but lacking a basic amino acid domain necessary to bind DNA (8-10, 40, 47, 56). It has been suggested that the Id protein is one of the important regulators in cell proliferation, differentiation as well as apoptosis in various cell types (56). Id2 is one of the four members of this family (i.e., Id1-4). It can promote Bax-mediated apoptosis independently of the helix-loop-helix dimerization (17). Previous studies from our laboratory have shown that Id2 is associated with the apoptosis-related skeletal muscle atrophy and age-associated sarcopenia (4-6). Although additional research is required to identify the entire molecular and cellular mechanism(s) by which Id2 may be involved in apoptosis regulation, it is reasonable to hypothesize that Id2 may be involved in the upstream-regulatory mechanism for apoptosis during muscle atrophy based on the findings of the close relationship between Id2 and apoptosis.

Activation of tumor suppressor protein p53 has been shown to initiate cell growth arrest via p21^{Cip1/Waf1} mediation or the execution of Bax-associated apoptotic cell death in various cell types (53). It has been recognized recently that p53 is one of the central modulators in cell cycling and apoptosis (53). Although most research in p53 has been conducted in active dividing cells, a few studies have attempted to investigate the response of p53 during disuse, laser irradiation, and ischemia in postmitotic skeletal myocytes (20, 28, 38, 42, 49). However, the possible role of p53 in regulating the apoptotic signaling pathway during unloading-induced muscle atrophy is still unclear. Additionally, the proto-oncogenic transcription factor c-Myc has also been implicated in the initiation of apoptotic cell death and its expression was found in skeletal myocytes during the postnatal development (52, 53). Nevertheless, whether c-Myc is involved in muscle unloading-induced apoptosis remains unknown. Accordingly, understanding the possible roles of Id2, p53, and c-Myc in mediating the apoptotic signaling pathway during muscle atrophy will provide insight in revealing the mechanisms of the significant myocyte loss during muscle disuse and sarcopenia.

Recently, we have demonstrated activation of apoptosis and apoptosis regulatory factors to unloading-induced muscle atrophy following hypertrophy and we found that differential apoptotic responses to unloading following hypertrophy between young and aged quails existed (50). In this study, we investigated the regulatory mechanism of the muscle atrophy-associated apoptosis during unloading using the same experimental model. We tested the hypothesis that Id2, p53, and c-Myc are activated as part of the apoptotic responses during unloading-induced muscle atrophy following hypertrophy.

METHODS

Animals. Japanese Coturnix quails were hatched and raised in pathogen-free conditions in the central animal care center at West Virginia University School of Medicine. The birds were housed at a room temperature of 22°C with a 12:12-h light:dark cycle and were provided with food and water ad libitum. Sixteen young adult birds aged 2 mo and sixteen 24 mo old aged birds were examined in the present study. The lifespan of Japanese quails is ~26–28 mo and they are both physically and sexually mature by 1.5 mo of age (36, 43). All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and fully conformed with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings".

Muscle Overloading and Unloading Protocol. The patagialis (PAT) muscle is flexed with the wing on the birds' back at rest, but it is stretched when the wing is extended. In our experimental stretch-overloading model, a tube containing ~12% of the bird's body weight was placed over the left humeral-ulnar joint (7). This maintains the joint in extension throughout the period of stretch and induces stretch at the origin of the PAT muscle. Previous studies have shown this stretch-overloading protocol results in moderate hypertrophy of the PAT muscles (i.e., 14-day stretch-loading induces ~35% and ~15% increases in muscle mass of young adult and aged birds, respectively) (2, 51).

The left wing of animals was overloaded for 14 days and then the weight was removed in this study. Seven days after the removal of weight, eight young and eight aged birds were killed by an overdose of pentobarbital sodium. The remaining young and aged animals were killed 14 days after the weight removal. The unstretched right PAT muscle served as the intra-animal control muscle for each bird. PAT muscles were dissected from the surrounding connective tissue, removed, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and then stored at -80°C until used for analyses.

Protein Extraction and Subcellular Fractionation. Cytoplasmic and nuclear protein extracts were obtained from PAT muscles using the method described by Rothermel et al (46). Briefly, 50 mg of muscle was homogenized in ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol). The cytoplasmic protein fraction contained in the supernatants was obtained after centrifugation at 1,000 rpm for 1 min at 4°C. The nuclei in the remaining pellet were resuspended in 360 µl of lysis buffer, and 49.8 µl of 5 M NaCl. The nuclear pellet was incubated on a rocker for 1 h at 4 °C, and centrifuged at 14,000 rpm for 15 min at 4 °C, and the nuclear protein fraction was collected from the supernate. A protease inhibitor cocktail (P8340, Sigma-Aldrich, St Louis, MO) was added to the cytoplasmic and nuclear protein extracts then the total protein contents of the extracts were quantified in duplicate by using bicinchoninic acid reagents (Pierce, Rockford, IL) and bovine serum albumin standards. Purity of the extracted fractions was verified by immunoblotting the extracted fractions with an anti-histone H2B and an anti-CuZn-superoxide dismutase rabbit polyclonal antibody (50). The cytoplasmic fraction protein was later used for immunoblotting of superoxide dismutase (CuZnSOD and MnSOD) while c-Myc was determined in the nuclear fraction protein. Protein contents of Id2 and p53 were measured in both the cytoplasmic and nuclear fraction protein.

Western Immunoblots. Protein expressions of transcription repressor Id2, tumor suppressor p53, proto-oncoprotein c-Myc, and antioxidant enzymes (CuZnSOD and MnSOD) were determined in the PAT muscles of experimental (left) and intra-animals control (right) wings. Forty micrograms of soluble protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE. The gels were blotted to nitrocellulose membranes (Bio-Rad, Hercules, CA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to confirm equal loading and transferring of proteins to the membrane in each lane. As another approach to verify similar loading between the lanes, gels were loaded in duplicate and with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) and probed with anti-Id2 rabbit polyclonal antibody (1:200 dilution, sc-489, Santa Cruz Biotechnology, Santa Cruz, CA), anti-p53 mouse monoclonal antibody (1:200 dilution, sc-99, Santa Cruz Biotechnology, Santa Cruz, CA), anti-c-Myc mouse monoclonal antibody (1:250 dilution, 631206, BD Pharmingen, San Diego, CA), anti-SOD-1 rabbit polyclonal antibody (1:500 dilution, sc-11407, Santa Cruz Biotechnology, Santa Cruz, CA), or anti-MnSOD goat antibody (1:2000 dilution, A300449A, Bethyl Lab Inc., Montgomery, TX) diluted in PBS-T with 2% BSA. Whole cell lysate of actively dividing human 293T cells was included as a positive control for probing Id2 and p53. Primary antibodies incubation was performed at 4°C for overnight. Secondary antibodies were conjugated to horseradish peroxidase (HRP) (Chemicon, CA), and the signals were developed by West Pico Chemiluminescent Substrate (34080, Pierce, Rockford, IL). The signals were

visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. The resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional (1-D) image analysis system (Eastman Kodak, Rochester, NY) and expressed in arbitrary units. The sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Immunofluorescent Cytochemistry. Nuclear protein contents of p53 and c-Myc were further estimated by immunofluorescent staining. In brief, frozen 10 μ m thick muscle cross-sections from the middle belly of the experimental and control PAT muscles were cut in a freezing cryostat at -20°C and were placed on the same glass slide to control for processing differences (e.g., incubation time or temperature, etc.). The mid-region of PAT muscles was used to prepare the muscle section based on the previous finding showing that the hypertrophied effect of stretch-loading is greatest among the proximal and distal regions of quail anterior latissimus dorsi muscle (7). The tissue sections were air-dried at room temperature, fixed in ice-cold methanol:acetone (1:1) for 10 min, permeabilized with 0.2% Triton X-100 in 0.1% sodium citrate at 4°C for 5 min, and blocked in 1.5% goat serum in PBS. All incubations were performed at room temperature for 30 min. After washes in PBS, sections were incubated with anti-p53 mouse monoclonal antibody (1:20 dilution, sc-99, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-c-Myc mouse monoclonal antibody (1:20 dilution, 631206, BD Pharmingen, San Diego, CA) followed by anti-mouse IgG Cy3 conjugate F(ab')₂ fragment incubation (dilution 1:200, C2181, Sigma Chemical Co, St Louis, MO). Negative control experiments were done by omitting the p53 or c-Myc antibodies on the tissue sections. In order to visualize the labeled nuclei under the basal lamina of PAT muscles and therefore identify the muscle-originated nuclei (e.g., myonuclei or satellite cell nuclei), the tissue sections were then incubated with anti-chick laminin mouse monoclonal antibody (20 μ g·ml⁻¹, clone 31-2, D.M. Fambrough, The Johns Hopkins University, Baltimore, MD) followed by anti-mouse IgG biotin-conjugated antibody (Vector Laboratories, Burlingame, CA) and then fluorescein avidin DCS incubation (1:200 dilution, A2011, Vector Laboratories, CA). The sections were finally mounted with 4',6-diamidino-2-phenylindole (DAPI) mounting medium (Vectashield mounting medium, Vector Laboratories, CA). The p53- or c-Myc- and DAPI-positive nuclei and laminin staining were examined under a fluorescence microscope (Biological Research Microscope Eclipse E800, Nikon Inc., NY). Images were obtained using a SPOT RT camera and SPOT RT software (Diagnostic Instruments Inc., Sterling Heights, MI) was used to stack the images of p53- or c-Myc-, DAPI-positive nuclei, and laminin staining. The numbers of p53- or c-Myc-positive nuclei and DAPI-positive nuclei were counted from six random non-overlapping fields at an objective magnification of 40 $\times$. Only the labeled nuclei that were under the laminin staining were counted in order to exclude any non-muscle nuclei (e.g., from fibroblasts). It is

noted that c-Myc-immunopositive nuclei was not detected in the muscle sections from all groups of the birds. The p53-positive nuclei were expressed as a p53 nuclear index which was calculated from the number of immunopositive nuclei divided by the total number of nuclei (i.e., DAPI-positive nuclei) $\times 100$.

In order to determine the mitotic property of the p53-positively labeled nuclei (i.e., mitotic or post-mitotic), BrdU labeling on the muscle sections following the p53 labeling procedure was performed in a separate set of measurements. Briefly, after p53 and Cy3 labeling, the tissue sections were incubated with anti-BrdU mouse monoclonal biotinylated antibody (1:5 dilution, 75512L, BD Pharmingen, San Diego, CA) followed by incubation with fluorescein avidin DCS (1:100 dilution, A2011, Vector Laboratories, CA), and finally mounted with DAPI mounting medium. The p53-, DAPI-, and BrdU-positive nuclei were examined under a fluorescence microscope and the captured images were stacked using SPOT RT software as described above.

Statistical Analyses. Statistical analyses were performed using the SPSS 10.0 software package. A 2×2 ANOVA was performed to examine the main effects of time (7- and 14-days of unloading), age (young and aged), and interaction (time $\times$ age) on the measured variables in all groups of animals. Student-Newman-Keuls post hoc analysis was used to examine differences between experimental and contralateral control PAT muscles. Relationships between given variables were examined by computing the Pearson product-moment correlation coefficient, r . Statistical significance was accepted at $P < 0.05$. All data are given as means $\pm$ standard error of mean (SE).

RESULTS

Muscle mass loss. The declined degree of muscle hypertrophy in the experimental muscle relative to the contralateral control muscle was monitored throughout the experiment to evaluate the unloading-induced muscle loss in these previously hypertrophied muscles (50). Moderate extent of muscle hypertrophy ($\sim 35\%$ in young adult and $\sim 15\%$ in aged quails) has been consistently demonstrated following the current 14-days stretch-loading procedure (2, 7, 51). In the young adult quails, the degree of hypertrophy was 15% after 7-days of unloading and had returned to the contralateral control level after 14-days of unloading. In the aged quails, the extent of hypertrophy was 12% following 7-days of unloading and the muscle mass of the experimental muscle was still 6% higher than the control muscle following 14-days of unloading (50).

TUNEL index. The TUNEL index was used to estimate the extent of apoptosis and this has been reported previously in this group of birds (50). In these animals, the TUNEL index in unloaded muscles was 246% ($P < 0.01$) greater than the control muscle after 7-days of unloading but it had returned to control levels by 14-days of unloading in young quail. The TUNEL index in experimental muscles of aged birds was 91% ($P < 0.001$) and 44% ($P < 0.01$) greater than the control muscle after

7-days and 14-days of unloading, respectively.

Id2 Protein Content. An immunoreactive band of ~15 kDa corresponding to the predicted molecular mass of Id2 protein was detected in both the cytoplasmic and nuclear protein fraction of all muscles. In our immunoblots, nuclear Id2 protein content was not different between the experimental and control muscles after 7-days and 14-days of unloading in both young adult and aged quails (**Figure 1A**).

Furthermore, the cytoplasmic Id2 protein content of the experimental muscle was $150 \pm 81\%$ ($P < 0.05$) higher than the control muscle after 7-days of unloading in young quails (**Figure 1B**). In aged quails, although Id2 protein content of the experimental muscle appeared to be higher than the control muscle after 7-days of unloading and to be lower than the control muscle after 14-days of unloading, these did not reach the statistical significant level ($P > 0.05$). For the cytoplasmic Id2 protein content, ANOVA analysis indicated that the main effects of time [$F(1,56) = 6.132$, $P < 0.05$] and interaction (age $\times$ time) [$F(1,56) = 5.204$, $P < 0.05$] exist in these animals (**Figure 1B**).

Insert **Figure 1A** ———→ Nuclear Id2 protein content

Insert **Figure 1B** ———→ Cytoplasmic Id2 protein content

p53 Protein Content. An immunoreactive band of ~53 kDa corresponding to the predicted molecular mass of p53 protein was detected in both the cytoplasmic and nuclear protein fraction of all muscles. The nuclear p53 protein content of the experimental muscle was $126 \pm 71\%$ ($P < 0.05$) greater than the control muscle after 7-days of unloading but no difference was found between the experimental and control muscles after 14-days of unloading in young quails (**Figure 2A**). In the aged quails, we did not find any significant changes in the nuclear p53 protein content of the experimental muscle when compared to the intra-animal control muscle after 7-days or 14-days of unloading ($P > 0.05$, **Figure 2A**).

A main effect of time [$F(1,56)=4.486$, $P<0.05$] and age [$F(1,56)=12.304$, $P<0.01$] were found in the cytoplasmic p53 protein content (**Figure 2B**). The cytoplasmic p53 protein content of the experimental muscle was $85 \pm 27\%$ ($P < 0.05$) higher than the control muscle after 7-days of unloading but no difference was found between the experimental and control muscles after 14-days of unloading in young quails (**Figure 2B**). In the aged quails, the changes in cytoplasmic p53 protein content was not significantly different between the experimental and intra-animal control muscles after 7-days or 14-days of unloading ($P > 0.05$, **Figure 2B**).

Insert **Figure 2A** ———→ Nuclear p53 protein content

Insert **Figure 2B** ———→ Cytoplasmic p53 protein content

c-Myc Protein Content. An immunoreactive band of ~62 kDa was detected on immunoblots, which, corresponded to the predicted molecular mass after SDS-PAGE fractionation of the c-Myc protein. c-Myc protein content was similar in experimental and intra-animal control muscles in both young and aged quails following 7-days or 14-days of unloading ($P > 0.05$, **Figure 3**).

Insert **Figure 3** ———→ Nuclear c-Myc protein content

CuZnSOD and MnSOD Protein Contents. An immunoreactive band of ~20kDa was detected. This corresponded to the predicted molecular mass of the SOD protein. There was no difference in the protein content of CuZnSOD between the experimental and control muscles in either young or aged quails (**Figure 4A**).

A main effect of age [$F(1,56) = 43.476$, $P < 0.01$] was found in MnSOD protein content (**Figure 4B**). MnSOD protein content of the experimental muscle was $13 \pm 5\%$ ($P < 0.05$) higher than the control muscle after 7-days of unloading but it was not different between the experimental and control muscles after 14-days of unloading in young quails (**Figure 4B**). No significant differences were found in MnSOD protein content between the experimental and intra-animal control muscles from aged quails after 7-days or 14-days of unloading ($P > 0.05$).

Insert **Figure 4A** ———→ CuZnSOD protein content

Insert **Figure 4B** ———→ MnSOD protein content

p53 Positively-labeled Nuclei and p53 Nuclear Index. The number of p53 immunopositive nuclei relative to the total nuclei was expressed as a p53 nuclear index. An example of immunopositive staining on p53 under the laminin staining in an experimental PAT muscle from a young quail after 7-days of unloading is shown in **Figure 5A**. ANOVA analysis showed a main effect of age [$F(1,56) = 4.437$, $P < 0.05$] in the p53 nuclear index. The p53 nuclear index of the experimental muscle was 87% ($P < 0.05$) greater than the control muscle after 7-days of unloading but no difference was found between the experimental and control muscles after 14-days of unloading in young quails ($P > 0.05$, **Figure 5B**). The p53 nuclear indices of the experimental muscles tended to be greater than the control muscles after 7- or 14-days of unloading in aged quails, however, these changes did not reach significant differences.

Insert **Figure 5A** ———→ p53-positive nucleus under the laminin staining

Insert **Figure 5B** ———→ p53 nuclear index

Our double immunofluorescent analysis on p53 and BrdU demonstrated that both the BrdU-positive (**Figure 6A**) and BrdU-negative (**Figure 6B**) labeled p53-positive nuclei existed in the experimental muscles of 7-days unloaded young quail and aged quails after 7-days and 14-days of unloading indicating that both the dividing and non-dividing cell nuclei contributed to the p53-immunopositive nuclei in these experimental muscles. In addition, although the nuclear c-Myc protein was detected in our immunoblot, c-Myc-positively labeled nuclei were not found in the PAT muscles of either age group.

Insert **Figure 6A** ———→ p53-positive and BrdU-positive labeled nucleus

Insert **Figure 6B** ———→ p53-positive and BrdU-negative labeled nucleus

Relationships of Id2, p53, c-Myc, SOD, apoptosis regulatory factors, and apoptosis. The relationships of Id2, p53, c-Myc, SOD, TUNEL index, and apoptosis regulatory factors (Bcl-2, Bax and AIF) were analyzed by examining the corresponding Pearson's correlation coefficient (r). We have previously shown evidence for apoptosis as indicated by the increase in TUNEL-positive nuclei and the changes in apoptosis regulatory factors including Bcl-2, Bax, and AIF which were consistent with apoptosis (50).

When the experimental and control PAT muscles of all groups were collapsed and treated as a single group ($N = 64$), cytoplasmic Id2 protein content was negatively correlated with the Bcl-2 protein content ($r = -0.287$, $P < 0.05$, **Figure 7A**) and was positively correlated with the TUNEL index ($r = 0.426$, $P < 0.001$, **Figure 7B**), Bax protein content ($r = 0.261$, $P < 0.05$, **Figure 7C**) as well as cytoplasmic p53 protein content ($r = 0.289$, $P < 0.05$, **Figure 7D**).

Insert **Figure 7** ———→ Correlations with Cytoplasmic Id2 protein content

The cytoplasmic protein content of p53 was negatively correlated with the Bcl-2 protein content ($r = -0.278$, $P < 0.05$, **Figure 8A**) while it was positively correlated with the TUNEL index ($r = 0.421$, $P < 0.001$, **Figure 8B**), Bax protein ($r = 0.390$, $P < 0.001$, **Figure 8C**) and mRNA contents ($r = 0.273$, $P < 0.05$, **Figure 8D**). The nuclear protein content of p53 was positively correlated with the mRNA content of Bax ($r = 0.393$, $P < 0.001$, **Figure 9A**) and TUNEL index ($r = 0.249$, $P < 0.05$, **Figure 9B**). In agreement with the suggestion that nuclear p53 is related to apoptosis, the p53 nuclear index as estimated by immunocytochemistry was positively correlated with the TUNEL index ($r = 0.501$, $P < 0.001$, **Figure 9C**) and cytoplasmic AIF protein content ($r = 0.280$, $P < 0.05$, **Figure 9D**).

Insert **Figure 8** ———→ Correlations with Cytoplasmic p53 protein content

Insert **Figure 9** ———→ Correlations with Nuclear p53 protein content and p53 nuclear index

c-Myc protein content was negatively correlated with the TUNEL index ($r = -0.309$, $P < 0.05$, **Figure 10A**), nuclear p53 protein content ($r = -0.291$, $P < 0.05$, **Figure 10B**), and the p53 nuclear index ($r = -0.311$, $P < 0.05$, **Figure 10C**).

Insert **Figure 10** ———→ Correlations with nuclear c-Myc protein content

Moreover, a positive correlation between the MnSOD protein content and the Bcl-2 protein content ($r = 0.372$, $P < 0.01$, **Figure 11A**) while negative correlations existed between the protein content of MnSOD and the TUNEL index ($r = -0.438$, $P < 0.001$, **Figure 11B**) as well as Bax protein content ($r = -0.314$, $P < 0.05$, **Figure 11C**). Also, the protein content of CuZnSOD was negatively correlated with the nuclear protein content of AIF ($r = -0.250$, $P < 0.05$, **Figure 11D**).

Insert **Figure 11** ———→ Correlations with SOD protein content

When the muscle samples were collapsed and analyzed as different sub-groups (e.g., all control muscles), in general, the cytoplasmic protein content of Id2 was positively correlated with the TUNEL index, Bax protein content, cytoplasmic and nuclear protein contents of AIF as well as nuclear p53 protein content ($P < 0.05$, **Table 1**) whereas the nuclear protein content of Id2 was negatively correlated with the Bax mRNA content and cytoplasmic p53 protein content ($P < 0.05$, **Table 1**). The cytoplasmic protein content of p53 was positively correlated with the TUNEL index, Bax protein and mRNA contents but was negatively correlated with the protein contents of Bcl-2 and MnSOD ($P < 0.05$, **Table 1**). Positive correlations existed between the nuclear protein content of p53 and TUNEL index, Bax mRNA content, and cytoplasmic protein content of AIF ($P < 0.05$, **Table 1**). Additionally, the p53 nuclear index was positively correlated with the TUNEL index, Bax and cytoplasmic Id2 protein contents whereas negative correlation existed between p53 nuclear index and nuclear c-Myc and CuZnSOD protein contents. The nuclear protein content of c-Myc was negatively correlated with the TUNEL index, cytoplasmic and nuclear protein contents of p53 whereas c-Myc was positively correlated with the nuclear protein content of Id2 and CuZnSOD protein content.

Insert **Table 1** ———→ Correlation table of Id2, p53, and c-Myc

In the sub-groups of muscles samples, the protein content of MnSOD was positively correlated with the Bcl-2 protein and mRNA contents but was negatively correlated with the TUNEL

index, Bax protein and mRNA contents, and nuclear protein content of AIF ($P < 0.05$, **Table 2**). Furthermore, the protein content of CuZnSOD was positively correlated with the Bcl-2 protein and mRNA content whereas it was negatively correlated with the TUNEL index, Bax mRNA content, and nuclear protein content of AIF ($P < 0.05$, **Table 2**).

Insert **Table 2** —————> Correlation table of SOD

DISCUSSION

Apoptosis has been implicating in having a physiologic role in mediating unloading-induced muscle atrophy (1, 6, 50). However, the underlying cellular and molecular regulatory mechanism contributing to the activation of the subsequent apoptotic signaling pathway that results in unloading-induced apoptosis is not currently well understood. Here we have shown that Id2 and p53 may be involved in the regulation of unloading-induced apoptosis following muscle hypertrophy. We demonstrated that the subcellular fractionated protein content of Id2 and p53 are moderately correlated with apoptosis as estimated by TUNEL and apoptosis regulatory factors including Bcl-2, Bax, and AIF. Our immunocytochemical findings indicated that both the mitotic and post-mitotic cell nuclei (presumably activated myogenic satellite cells and existing myocytes) are responsible for the elevated nuclear p53 protein content in the unloaded muscle relative to the control muscle. Nonetheless, we previously have shown that the activated satellite cell nuclei (both fused and unfused satellite cell nuclei), but not the existing myonuclei, are eliminated via apoptotic mechanisms during unloading following hypertrophy based on the observations that almost all the TUNEL-positively labeled nuclei were BrdU-immunopositive (50). In this study we report negative relationships between c-Myc and the apoptotic factors in muscle samples. Additionally, we found an aging-specific response of Id2 and p53 to unloading following hypertrophy. This may partly explain our previous reported age-related differences in muscle adaptations during unloading following hypertrophy (50). Taken together, our findings are consistent with the hypothesis that Id2 and p53 may have a role in mediating the homeostasis of apoptosis regulatory factors and apoptosis during unloading-induced muscle atrophy following hypertrophy in young adult skeletal muscle. Moreover, additional research is needed to further identify the influence of aging on the regulatory mechanisms contributing to the activation of apoptosis during unloading following hypertrophy.

Id2 in muscle remodeling. Id proteins are known to play a role in promoting cell proliferation and act as negative regulators of cell differentiation in numerous cell lineages (8-10, 47, 56). However, there is evidence suggesting that Id proteins may also have a role in modulating apoptosis (4, 6, 17, 19, 40). Florio and colleagues have demonstrated that Id2 can initiate the Bax-associated apoptosis and the apoptogenic properties of Id2 reside in the N-terminal region

independent of the helix-loop-helix dimerization (17). Consistent with these reports, previous studies from our laboratory have illustrated the dual roles of Id2 in promoting cell proliferation and apoptotic cell death during muscle hypertrophy and atrophy, respectively, in skeletal myocytes *in vivo* (4, 6). In the present study, we have extended our previous findings by demonstrating that the cytoplasmic Id2 protein content, but not the nuclear Id2 protein content, was elevated in the young adult unloaded muscle relative to the control muscle after 7-days of unloading following hypertrophy. Additionally, we found that the cytoplasmic protein content of Id2 was positively correlated with the pro-apoptotic markers (e.g., Bax, AIF, p53, and TUNEL index) and negatively correlated with the anti-apoptotic marker (e.g., Bcl-2). Notably, the nuclear protein content of Id2 was unchanged in response to unloading following hypertrophy and it was negatively correlated with the pro-apoptotic factors including Bax and p53. We interpret these findings to indicate that the cytoplasmic Id2 is associated with pro-apoptotic events whereas the nuclear Id2 may be related to the anti-apoptotic affair and probably promotion of cell proliferation. This speculation is supported by the data showing that the apoptogenic ability of Id2 is associated with Bax which is a cytoplasmic protein (17).

In contrast, the proliferating and anti-apoptotic properties of the nuclear Id2 may be due to the indirect effects of Id2 on transcriptional regulation in nuclei. Based on the fact that Id2 can suppress the transcription of genes that regulate cell differentiation by sequestering the E proteins (e.g., E12, E47, E2-2) and preventing the heterodimer formation of E proteins with the basic HLH transcription factors (9, 10, 35, 56), it is reasonable to speculate that Id2 may be capable of influencing the transcription of the genes associated with apoptosis and proliferation. Indeed, Ling and coworkers (34) have demonstrated that Id1 can promote cell proliferation by inducing anti-apoptotic factor expression via the activation of the NF-kappaB pathway in prostate cancer cells (34). In addition, Id2 has been implicated to explain the elevated level of an apoptosis inhibitor, survivin and the deficiency of procaspase-8 in neuroblastoma cells (11). Moreover, it has been shown that Id2 can abrogate the growth-suppressive functions of retinoblastoma protein (pRb), p16, p21, and tumor suppressor proteins by interacting with pRb (29, 30, 37, 40, 47, 56). Nevertheless, we are aware that our data do not permit us to conclude if the nuclear Id2 is directly related to the increased expression of the proliferation-related genes and/or the decreased pro-apoptotic genes expression during muscle remodeling. Although we have demonstrated that the cytoplasmic Id2 is associated with apoptosis during unloading following hypertrophy, additional research is required to examine whether the nuclear Id2 is directly related to the cell proliferation in response to the hypertrophic stimulus. Nevertheless, the data from our study suggest that the subcellular fractionated protein expression (cytoplasmic vs. nuclear protein content) maybe an important factor in determining the physiologic roles of Id2.

p53 during unloading-induced apoptosis following hypertrophy. It has been commonly recognized that p53 plays a critical role in regulating cell cycling, survival, and programmed cell death in various mitotic cell lineages (53). However, there is a relative paucity of data on the role of p53 in skeletal myocytes. Although a skeletal myocyte is biologically different from other mitotic cell types as it is terminally differentiated and postmitotic (21), it is reasonable to hypothesize that p53 may have a role in regulating muscle atrophy. In support of this idea, p53 has been shown to be one of the central modulators in apoptosis in tissue non-specific manner (53). Furthermore, p53 protein levels increase in atrophied rat skeletal muscles following a 14-day space flight (42). However, there is still lacking a consensus on the role of p53 in muscle remodeling as p53 has been reported to be unchanged in skeletal muscles during atrophy induced by nerve injury, steroid, or alcohol (28, 32, 38).

In the present study, we have demonstrated that both the nuclear and cytoplasmic fractions of p53 are elevated in the unloaded muscle relative to the control muscle after 7-days of unloading following hypertrophy in young adult animals. We have also shown a positive correlation of p53 protein content and pro-apoptotic markers (Bax, AIF, and TUNEL index) and a negative correlation of p53 with anti-apoptotic marker Bcl-2. Our findings are in accordance with the documented apoptogenic properties of p53 in numerous cell types (13, 14, 44, 53, 54). In the nuclei, p53 has been suggested to mediate apoptotic cell death by elevating the transcriptional expressions of several pro-apoptotic genes (e.g., Bax, PUMA, and Noxa) (24, 41, 48). Thus, it was not surprising that the nuclear fraction of p53 protein content increased concomitant with the TUNEL index and the Bax/Bcl-2 ratio during unloading-induced muscle atrophy following hypertrophy (50). As a further support to the elevation of nuclear p53 protein content measured by immunoblot, we found that the number of p53-positively labeled nuclei assessed by fluorescent immunocytochemistry increased with unloading and these labeled nuclei were muscle-originated because only those under the laminin staining were included. Although we were not able to determine the precise identity of these p53-labeled nuclei (i.e., satellite cells or myocytes nuclei as both of them reside under the basal lamina), our double immunofluorescent staining of p53 and BrdU designated that both the mitotic and post-mitotic cell nuclei were involved in explaining the increased p53-labeled nuclei with unloading. Based on the fact that nearly all the non-muscle cell populations are mitotic (e.g., fibroblasts, pericytes, and other blood vessel associated cells) while the myocyte is the abundant post-mitotic cell type exists in the skeletal muscles, it is reasonable to conclude that our observed p53-immunopositive but BrdU-immunonegatively labeled nuclei had originated from the existing myocyte nuclei. We acknowledge that these labeled p53-positive/BrdU-negative nuclei might, although unlikely, belong to those quiescent mitotic cells that had not been dividing during the whole experimental period of 21-28 days. However, it is unlikely that quiescent cells will express substantially elevated level of p53. Nonetheless, we interpret these findings as indicating that both the activated satellite cell nuclei and

the existing myonuclei contribute to the increased level of p53 during the unloading-induced atrophy. This observation also implied that the cellular changes (e.g., p53) occurring in both satellite cell and myocyte are involved in regulating the apoptosis of activated satellite cell nuclei during unloading following hypertrophy, even though we have previously demonstrated that only the activated satellite cell nuclei (both fused and unfused nuclei), but not the existing myonuclei, undergo apoptosis during unloading following hypertrophy (50). Although the exact mechanisms for the cell-cell interaction (e.g., cell adhesion, cytoskeletal re-organization, and fusion) between myogenic satellite cells and myocytes during muscle remodeling is not completely understood (12), it is reasonable to speculate that the cellular changes occurring in myocytes can be a factor in influencing the response of satellite cells during unloading in previously hypertrophied muscle based on the fact that muscle satellite cells are localized and function intimately with the myofibers.

Recently, it has been suggested that p53 can also directly mediate the apoptotic cascades by translocating to the mitochondria via a transcription-independent mechanism (14, 44). Consistent with this suggestion, we have demonstrated that the cytoplasmic p53 protein content is elevated in young adult muscles that were unloaded for 7-days. Although our data do not allow us to fully identify the physiologic role of p53 during unloading-induced apoptosis, we speculate that the increased cytoplasmic p53 may interact with Bax or other cytosolic apoptogenic factors and activate apoptosis during unloading. Nonetheless, further study is required to reveal other possible p53-related mechanisms involved in regulating the unloading-induced apoptosis in skeletal muscle, perhaps by regulation of apoptosis by p53 phosphorylation (22, 55).

Oxidative stress may be an important factor involved in regulating sarcopenia and increased activity may in part offset oxidative stress (26, 27). On the other hand, mechanical unloading can lead to disruption of antioxidant-oxidant status by increasing CuZnSOD and reactive oxygen species in skeletal muscles (31). Furthermore, p53 can induce an imbalance in antioxidant enzymes and oxidative stress by inappropriately upregulating MnSOD and glutathione peroxidase (23). In this study we observed that both the p53 and MnSOD protein content was increased in young adult muscles unloaded for 7-days. We interpret this finding to indicate that unloading resulted in an oxidative disturbance in the atrophied muscles.

Intriguingly, in the aged muscles, we did not find any significant differences in Id2 and p53 between the experimental and intra-animal control muscles after 7- or 14-days of unloading. Although these proteins appeared to increase with unloading (Figure 2), the magnitude of the responses was more variable in old animals than young animals. If we assume that p53 and the cytoplasmic Id2 are pro-apoptotic, our observations suggest that aging muscle has invoked a strategy that attempts to reduce the tendency towards apoptosis in unloaded muscles as compared to young adult animals. Nevertheless, these findings are consistent with the idea that the regulatory mechanisms contributing

to the activation of apoptosis during unloading may be different between the aged and young adult muscles. Additionally, these data are in agreement with our previous observations of increased mRNA and protein levels of Bcl-2, decreased protein level of Bax, and decreased nuclear AIF protein level in muscles of aged birds that were unloaded for 14-days as compared to the young animals (50). These findings underscore the possibility that the mechanisms regulating apoptotic consequences during unloading-induced muscle loss may be aging specific and this warrants further investigation.

In conclusion, we have provided evidence suggesting that transcriptional repressor protein Id2 and tumor suppressor protein p53 are related to the unloading-induced apoptosis following hypertrophy in young adult quail skeletal muscles. We have demonstrated that both the nuclear and cytoplasmic protein contents of p53 significantly increased in the unloaded muscle relative to the control muscle after 7-days of unloading following hypertrophy. Although the nuclear protein content of Id2 did not respond to unloading, we found that the cytoplasmic protein content of Id2 markedly increased in the unloaded side when compared to the control side after 7-days of unloading following hypertrophy in young adult birds. We have also shown that correlation relationships exist among Id2, p53, TUNEL index, and the apoptosis regulatory factors including Bcl-2, Bax, and AIF. However, our observed correlation relationships among Id2, p53, and apoptosis were moderately varied in strength (r-values of positive correlation ranged from 0.26 to 0.88 and negative correlation ranged from -0.25 to -0.85). Although Id2 and p53 appear to have a role in pathways leading to unloading-induced atrophy, these findings suggest that the unloading-induced apoptosis may not be fully explained by the responses of Id2 and p53. Other cellular modulators (e.g., heat shock proteins) (18) that were not examined in this study may also play a role in regulating apoptosis induced atrophy. Although it has been suggested that c-Myc may have an apoptogenic role, especially during tumor genesis (39), our data do not support the hypothesis that c-Myc has a pro-apoptotic role during unloading-induced apoptosis following muscle hypertrophy. The inverse relationships between c-Myc and pro-apoptotic factors implicate that more complex mechanisms and additional research is needed to understand the physiologic function of c-Myc during muscle remodeling.

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Figure 1A Nuclear Id2 protein content

Legend for Figure 1A Nuclear Id2 protein content. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁷. The insets show representative blots for Id2 in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. Young, young adult quails; Aged, aged quails; 7d unloading, 7-days of unloading following 14-days of muscle loading; 14d unloading, 14-days of unloading following 14-days of muscle loading.

Figure 1B Cytoplasmic Id2 protein content

Legend for Figure 1B Cytoplasmic Id2 protein content. The data are expressed as OD $\times$ resulting band area, and expressed in arbitrary units $\times 10^7$. The insets show representative blots for Id2 in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. * $P < 0.05$, data are significantly different from the corresponding intra-animal control muscles. The main effects of time and interaction (age $\times$ time) on cytoplasmic Id2 protein content in these animals were analyzed by 2×2 ANOVA.

Figure 2A Nuclear p53 protein content **Figure 2B Cytoplasmic p53 protein content**

Legend for Figure 2A Nuclear p53 protein content. The data are expressed as OD x resulting band area, and expressed in arbitrary units x 10^4 . The insets show representative blots for p53 in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Legend for Figure 2B Cytoplasmic p53 protein content. The data are expressed as OD x resulting band area, and expressed in arbitrary units x 10^4 . The insets show representative blots for p53 in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles. The main effects of time and age on cytoplasmic p53 protein content in these animals was analyzed by 2×2 ANOVA.

Figure 3 Nuclear c-Myc protein content

Legend for Figure 3 Nuclear c-Myc protein content. The data are expressed as OD x resulting band area, and expressed in arbitrary units x 10⁶. The insets show representative blots for c-Myc in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

Figure 4A CuZnSOD protein content

Figure 4B MnSOD protein content

Legend for Figure 4A CuZnSOD protein content. The data are expressed as OD x resulting band area, and expressed in arbitrary units $\times 10^8$. The insets show representative blots for CuZnSOD in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

Legend for Figure 4B MnSOD protein content. The data are expressed as OD x resulting band area, and expressed in arbitrary units $\times 10^8$. The insets show representative blots for MnSOD in control and experimental muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. * $P < 0.05$, data are significantly different from the corresponding intra-animal control muscles. The main effect of age on MnSOD protein content in these animals were analyzed by 2×2 ANOVA.

Figure 5A p53-positive nucleus under the laminin staining

Legend for Figure 5A p53-positive nucleus under the laminin staining. Immunofluorescent labeling on p53, laminin, and DAPI was used to identify the p53-positively labeled nuclei under the laminin staining as an estimate of the nuclear p53 index. (Red) p53 stained with secondary Cy3 labeling, (Green) laminin stained with secondary fluorescein labeling, and (Blue) nuclei labeled by DAPI staining. This illustration was obtained at an objective magnification of 100 $\times$. The enlarged box exemplifies a p53-positive nucleus under the laminin staining. Bar 10 μ m.

Figure 5B p53 nuclear index

Legend for Figure 5B p53 nuclear index. The number of p53-positively labeled nuclei relative to the total number of nuclei estimated by immunofluorescent staining was expressed as p53 nuclear index. The data are presented as means $\pm$ SE. * $P < 0.05$ data are significantly different from the corresponding intra-animal control muscles. The main effect of age on p53 nuclear index in these animals were analyzed by 2×2 ANOVA.

Figure 6A p53-positive and BrdU-positive labeled nucleus

Legend for Figure 6A p53-positive and BrdU-positive labeled nucleus. Immunofluorescent labeling of p53, BrdU, and DAPI was used to identify the mitotic property of the p53-positively labeled nuclei. (A) - (D) demonstrated a both p53- and BrdU-positively labeled nucleus. (A) nuclei labeled by DAPI staining, (B) p53-immunopositive nucleus with secondary labeling by Cy3, (C) BrdU-immunopositive nucleus with secondary labeled by fluorescein, and (D) pictures of p53, BrdU, and DAPI were merged. The arrow indicated a p53- and BrdU-positively labeled nucleus. These illustrations were obtained at an objective magnification of 100 $\times$. Bar 10 μ m.

Figure 6B p53-positive but BrdU-negative labeled nucleus

Legend for Figure 6B p53-positive but BrdU-negative labeled nucleus. A BrdU-immunonegative p53-positively labeled nucleus was illustrated. (A) nuclei labeled by DAPI staining, (B) p53-immunopositive nucleus with secondary labeling by Cy3, and (C) pictures of p53 and DAPI were merged. The arrow indicated a p53-positively labeled but BrdU-immunonegative nucleus. These illustrations were obtained at an objective magnification of 100 $\times$. Bar 10 μ m.

Figure 7 Correlations with Cytoplasmic Id2 protein content

Legend for Figure 7 Correlation analyses of cytoplasmic Id2 protein content. The relationships between the cytoplasmic protein content of Id2 and Bcl-2 protein content (A), TUNEL index (B), Bax protein content (C) as well as the cytoplasmic protein content of p53 (D). The control and experimental muscles from both young adult and aged quails of all groups were collapsed and treated as a single pooled group (N=64). r , the Pearson product-moment correlation coefficient; Y7, young adult quails after 7-days of unloading; Y14, young adult quails after 14-days of unloading; A7, aged quails after 7-days of unloading; A14, aged quails after 14-days of unloading.

Figure 8 Correlations with Cytoplasmic p53 protein content

Legend for Figure 8 Correlation analyses of cytoplasmic p53 protein content. The relationships between the cytoplasmic protein content of p53 and Bcl-2 protein content (A), TUNEL index (B) as well as the protein and mRNA content of Bax (C-D). The control and experimental muscles from both young adult and aged quails of all groups were collapsed and treated as a single pooled group (N=64).

Figure 9 Correlations with Nuclear p53 protein content and p53 nuclear index

Legend for Figure 9 Correlation analyses of nuclear p53 protein content and p53 nuclear index. The relationships between the nuclear protein content of p53 and Bax mRNA content (A) as well as the TUNEL index (B). The relationships between the p53 nuclear index and TUNEL index (C) and the cytoplasmic AIF protein content (D). The control and experimental muscles from both young adult and aged quails of all groups were collapsed and treated as a single pooled group (N=64).

Figure 10 Correlations with nuclear c-Myc protein content

Legend for Figure 10 Correlation analyses of nuclear c-Myc protein content. The relationships between the nuclear protein content of c-Myc and TUNEL index (A), nuclear protein content of p53 (B) as well as the p53 nuclear index (C). The control and experimental muscles from both young adult and aged quails of all groups were collapsed and treated as a single pooled group (N=64).

Figure 11 Correlations with SOD protein content

Legend for Figure 11 Correlation analyses of SOD protein content. Figure (A) – (C) demonstrated the relationships between the protein content of MnSOD and Bcl-2 protein content (A), TUNEL index (B) as well as the protein content of Bax (C). Figure (D) demonstrated the relationship between the CuZnSOD protein content and the nuclear protein content of AIF. The control and experimental muscles from both young adult and aged quails of all groups were collapsed and treated as a single pooled group (N=64).

Table 1 Correlation table of Id2, p53, and c-Myc

Correlation existed between	Muscle samples examined	N	r	P
Cytoplasmic Id2 and TUNEL index	All control muscles	32	0.558	0.001
	All aged muscles	32	0.552	0.001
	Aged 7-days control and unloaded muscles	16	0.662	0.005
	Aged 7-days and 14-days unloaded muscles	16	0.638	0.008
	Young 14-days control muscles	8	0.925	0.001
Cytoplasmic Id2 and Bax protein	Young 14-days control and unloaded muscles	16	0.499	0.049
	Young 14-days unloaded muscles	8	0.824	0.012
Cytoplasmic Id2 and Cytoplasmic AIF	Young 7-days control and unloaded muscles	16	0.581	0.018
	Aged 14-days control and unloaded muscles	16	0.518	0.040
	Young 7-days unloaded muscles	8	0.821	0.013
Cytoplasmic Id2 and Nuclear AIF	Aged 14-days unloaded muscles	8	0.773	0.025
Cytoplasmic Id2 and Nuclear p53	All young control muscles	16	0.605	0.013
Nuclear Id2 and Bax mRNA	Young 7-days control and unloaded muscles	16	-0.506	0.046
	Young 7-days control muscles	8	-0.749	0.033
Nuclear Id2 and Cytoplasmic p53	Aged 14-days control muscles	8	-0.759	0.029
Cytoplasmic p53 and TUNEL index	All control muscles	32	0.455	0.009
	All unloaded muscles	32	0.350	0.049
Cytoplasmic p53 and Bax protein	All aged muscles	32	0.423	0.016
	All unloaded muscles	32	0.443	0.011
	Aged 7-days control and unloaded muscles	16	0.529	0.035
Cytoplasmic p53 and Bax mRNA	Aged 7-days and 14-days unloaded muscles	16	0.501	0.048
	All young muscles	32	0.451	0.009
	Young 7-days control and unloaded muscles	16	0.505	0.046
Cytoplasmic p53 and Bcl-2 protein	All control muscles	32	-0.354	0.047
Cytoplasmic p53 and MnSOD	All control muscles	32	-0.564	0.001
Nuclear p53 protein and TUNEL index	All aged muscles	32	0.370	0.037
	Aged 14-days control and unloaded muscles	16	0.624	0.010
Nuclear p53 protein and Bax mRNA	All young muscles	32	0.555	0.001
	All unloaded muscles	32	0.401	0.023
	Young 7-days control and unloaded muscles	16	0.607	0.013
	Young 14-days control and unloaded muscles	16	0.520	0.039
	Young 7-days and 14-days unloaded muscles	16	0.599	0.014
Nuclear p53 protein and Cytoplasmic AIF	Aged 14-days control and unloaded muscles	16	0.518	0.040
	Young 14-days control muscles	8	0.756	0.030
p53 nuclear index and TUNEL index	All aged muscles	32	0.561	0.001
	All unloaded muscles	32	0.557	0.001
	Aged 7-days and 14-days unloaded muscles	16	0.628	0.009
	Aged 7-days control and unloaded muscles	16	0.729	0.001
	Aged 7-days unloaded muscles	8	0.766	0.027
p53 nuclear index and Bax protein	Young 14-days control muscles	8	0.746	0.033
p53 nuclear index and Nuclear c-Myc	All aged muscles	32	-0.360	0.043
	Aged 14-days control muscles	8	-0.786	0.021
p53 nuclear index and Cytoplasmic Id2	Young 7-days control and unloaded muscles	16	0.508	0.045
p53 nuclear index and CuZnSOD	Young 14-days control and unloaded muscles	16	-0.508	0.045
	Young 14-days control muscles	8	-0.724	0.042
c-Myc and TUNEL index	All young muscles	32	-0.471	0.006
	All aged muscles	32	-0.359	0.043
	Young 7-days control and unloaded muscles	16	-0.675	0.004
	Young 7-days control muscles	8	-0.737	0.037
c-Myc and Nuclear Id2	Aged 7-days control muscles	8	0.880	0.014
c-Myc and Cytoplasmic p53	All young muscles	32	-0.412	0.019
	Young 7-days control and unloaded muscles	16	-0.575	0.020
c-Myc and Nuclear p53	All aged muscles	32	-0.398	0.024
	All control muscles	32	-0.355	0.046
	Aged 14-days control and unloaded muscles	16	-0.513	0.042
	Aged 7-days and 14-days control muscles	16	-0.523	0.038
c-Myc and CuZnSOD	Aged 14-days control and unloaded muscles	16	0.538	0.032
	Aged 14-days unloaded muscles	8	0.724	0.042

Table 1 Correlation table of Id2, p53, and c-Myc. The relationships among Id2, p53, c-Myc, TUNEL index, and the apoptosis regulatory factors. The control and experimental muscles from the young adult and aged quails of all groups were collapsed and analyzed as different sub-groups. r , the Pearson product-moment correlation coefficient.

Table 2 **Correlation table of SOD**

Correlation existed between	Muscle samples examined	N	r	P
MnSOD and Bcl-2 protein	All control muscles	32	0.530	0.002
	Aged 14-days control muscles	8	0.744	0.034
MnSOD and Bcl-2 mRNA	All young muscles	32	0.499	0.004
	Young 14-days control and unloaded muscles	16	0.766	0.001
	Young 7-days and 14-days unloaded muscles	16	0.599	0.014
	Young 14-days unloaded muscles	8	0.811	0.015
	All control muscles	32	-0.393	0.026
MnSOD and Bax protein	Aged 7-days and 14-days control muscles	16	-0.570	0.021
MnSOD and Bax mRNA	Aged 14-days control and unloaded muscles	16	-0.699	0.003
MnSOD and Nuclear AIF	Aged 14-days control muscles	8	-0.732	0.039
	Aged 14-days unloaded muscles	8	-0.759	0.029
	All control muscles	32	-0.518	0.002
	All unloaded muscles	32	-0.479	0.006
MnSOD and TUNEL index	Aged 14-days control and unloaded muscles	16	-0.569	0.021
	Aged 14-days unloaded muscles	8	-0.729	0.040
CuZnSOD and Bcl-2 protein	Aged 7-days control muscles	8	0.733	0.039
CuZnSOD and Bcl-2 mRNA	Aged 7-days control and unloaded muscles	16	0.501	0.048
	Aged 7-days unloaded muscles	8	0.723	0.043
CuZnSOD and Bax mRNA	Aged 7-days control and unloaded muscles	16	-0.521	0.039
	Aged 7-days control muscles	8	-0.715	0.046
CuZnSOD and Nuclear AIF	Young 7-days control and unloaded muscles	16	-0.652	0.006

Table 2 **Correlation table of SOD.** The relationships among MnSOD, CuZnSOD, TUNEL index, and the apoptosis regulatory factors. The control and experimental muscles from the young adult and aged quails of all groups were collapsed and analyzed as different sub-groups. r, the Pearson product-moment correlation coefficient.

CHAPTER 9

Gene and subcellular protein expressions of Id2, p53 and HSP in atrophied skeletal muscle

ABSTRACT

Tumor suppressor protein p53 and inhibitor of differentiation-2 (Id2) have been implicated in apoptosis. We studied these proteins following disuse-induced muscle atrophy, which results in part, from apoptosis. Muscle atrophy did not change mRNA levels of Id2 or p53 as compared to control muscles of young adult or aged animals. Id2 and p53 protein contents were elevated in cytosolic protein fractions, but were unchanged in nuclear fractions of atrophic muscles when compared to control muscles in young or aged animals. These data support the hypothesis that cytosolic Id2 and p53 have a role in atrophy and apoptosis during disuse.

Key words: sarcopenia; muscle apoptosis; muscle atrophy

Abbreviations: p53, tumor suppressor protein p53; Id2, inhibitor of differentiation-2; HSP, heat shock protein

1. INTRODUCTION

Activation of apoptosis has been consistently reported under different situations of skeletal muscle loss. Furthermore, it has been hypothesized that apoptosis may have a physiologic role in regulating muscle wasting [1-19]. With an objective of unraveling the possible role of apoptosis in muscle wasting, it is essential to delineate the underlying regulatory mechanism(s) contributing to the promotion of the pro-apoptotic signaling that leads to the activation of apoptosis during muscle loss. A variety of cellular factors (e.g., Id2, p53, and HSP) have been demonstrated to have an important role in growth or apoptosis in response to various stress stimuli [20-23]. As these proteins can act as the mediators of the apoptotic signaling pathway it is possible that, they may also be involved in the regulation of apoptosis-associated muscle wasting.

The inhibitor of DNA binding/differentiation-2 (Id2) protein is a member of the Id family. Id2 has been suggested to be essential in regulating cell proliferation and apoptotic cell death under different circumstances [21,24-27]. These opposite functions of Id2 are presumably related to the structure of DNA-binding domain-negative helix-loop-helix motif which promotes cell division and the apoptotic capability of Id2 mediated by the pro-apoptotic Bax protein [21,24-27]. In agreement with the dual role of Id2 in cell growth and death, our laboratory has provided data demonstrating that Id2 is involved in both overload-induced muscle hypertrophy and apoptosis-related atrophy of hypertrophied muscle during unloading and sarcopenia with aging [2,3,16,28,29]. Although the exact mechanism(s) by which Id2 regulates muscle hypertrophic or atrophic process is largely unknown, we have previously demonstrated that cytosolic but not nuclear Id2 is associated with muscle atrophy during unloading following muscle hypertrophy where the loss of muscle mass is still above the basal level [16]. These observations suggest that the subcellular compartmentalization of protein expression (nuclear vs. cytosolic) maybe of significance in coordinating the physiologic role of Id2. Nonetheless, the role of subcellular Id2 in muscle atrophy has not been verified in muscle wasting where the muscle loss occurs below the basal level (e.g., hindlimb suspension induced muscle atrophy).

Tumor suppressor protein p53 has been demonstrated to be one of the central regulators in cell growth and death by inducing cell growth arrest via p21^{Cip1/Waf1} mediation or apoptosis through the activation of Bax in various mitotic cell lineages [20]. Only a few studies have attempted to investigate the response of p53 in postmitotic skeletal muscle and these have been under conditions of disuse, laser irradiation, and ischemia [16,30-34]. Although a common agreement on the role of p53 in muscle wasting has not been compromised, there has been at least some evidence indicating that p53 is responsive to certain muscle atrophic conditions (i.e., rat muscle during space flight and quail muscle during unloading following hypertrophy) [16,33]. Additionally, heat shock proteins (HSPs) have been implicated as an anti-apoptotic component in determining the prevention or execution of apoptotic cell death in response to a variety of cellular stresses [22,23,35]. Nevertheless, the possible role of HSPs in

apoptosis-associated muscle wasting remains to be fully identified. Collectively, understanding the possible roles of Id2, p53, and HSP in mediating the apoptosis-associated muscle atrophy will provide insight in revealing the mechanisms leading to significant muscle loss during muscle disuse as well as sarcopenia.

Although the activation of apoptosis has been demonstrated in hindlimb suspended skeletal muscle [1,7], it is unclear whether the apoptosis-associated factors including Id2, p53, and HSPs may be involved. These proteins have been previously shown to be involved in apoptosis-related atrophy of hypertrophied muscle during unloading where muscle mass is reduced from a hypertrophy to control levels or in muscle damage [15,16,36]. Nevertheless, it is unknown if they also contribute to muscle wasting as induced by hindlimb suspension where the loss of muscle mass is well below the basal level. Therefore, this study investigated the gene and subcellular protein expressions of these apoptosis-associated factors following 14 days of hindlimb suspension-mediated muscle unloading. We tested the hypothesis that Id2, p53, and HSPs are responsive to suspension-induced muscle atrophy.

2. MATERIALS AND METHODS

2.1 *Animals.*

Experiments were conducted on 6-mo old young adult and 30-mo old aged Fischer₃₄₄×Brown Norway rats (Harlan, Indianapolis, IN). The rats were housed in pathogen-free conditions at ~20°C and were exposed to a reverse light condition of 12:12 h of light:darkness each day. They were fed rat chow and water ad libitum throughout the study period.

2.2 *Hindlimb suspension.*

The animals were randomly assigned to a suspension group (Young n=10, Aged n=10) or a control group (Young n=10, Aged n=8). The procedure of hindlimb suspension described by Morey-Holton and Globus [37] was adopted in the present study. Briefly, an adhesive (tincture of benzoin) was applied to the tail, air-dried, and an orthopedic tape was put along the proximal one-third of the tail. This practice distributed the load evenly and avoided excessive tension on a small area to the tail. The tape was then placed through a wire harness that was attached to a fishline swivel at the top of a specially designed N.A.S.A.-approved hindlimb suspension cage. This provided the rats with 360° of movement around the cage and the forelimbs maintained contact with a grid floor allowed the animals to move and access food and water freely. Sterile gauze was wrapped around the orthopedic tape and was subsequently covered with a thermoplastic material, which formed a hardened cast (Vet-Lite, Veterinary Specialty Products, Boca Raton, FL). The distal tip of the tail was examined to verify that the procedure did not occlude the blood flow to the tail (i.e., tail remained pink). The suspension height was adjusted to prevent the animal's hindlimb from touching any supportive surface, with care

taken to maintain a suspension angle of approximately 30° [38]. The suspension height and animal behavior were monitored daily. Control animals were allowed to move unconstrained around the cages. Following 14 days of suspension, animals were killed with an overdose of pentobarbital. Medial gastrocnemius muscles from the hindlimb were excised, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and stored at -80°C until used for analyses. All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the AAALAC and following the policies in the Animals Welfare Act.

2.3 RT-PCR analyses.

Total RNA was extracted from the medial gastrocnemius muscle of both suspended and control animals with TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Frozen muscle was mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA were solubilized in RNase-free H₂O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assured by examining OD₂₆₀/OD₂₈₀ ratio. Ten micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 30 µl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). Control RT reaction was done in which the RT enzyme was omitted. The control RT reaction was PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of complementary DNA (cDNA) was then amplified by PCR using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, TX), 250 µM deoxyribonucleotide triphosphates, 1 x PCR buffer, and 2.5 units *Taq* DNA polymerase (USB Corp., Cleveland, OH) in a final volume of 50 µl. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). The primer pairs were designed from sequences published in GenBank (**Table 1**) and PCR products were verified by restriction digestions or sequencing. Preliminary experiments were conducted with each gene to assure that the number of cycles represented a linear portion for the PCR optical density curve for the muscle samples. The cDNA from all muscle samples were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 30 µl of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide. Images were captured and the signals were quantified in arbitrary units as optical density (OD) x band area using Kodak image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. Ribosomal 18S primers were used as internal controls while all RT-PCR signals were normalized to the 18S signal of the corresponding

RT product to eliminate the measurement error from uneven samples loading and provide a semi-quantitative measure of the relative changes in gene expression.

2.4 Subcellular protein fractionation.

The fractionation method described by Rothermel et al. [39] was adopted with minor modification to extract the cytosolic and nuclear protein fractions from the gastrocnemius muscles. We have previously obtained the fractionated cytosolic and nuclear proteins from skeletal and heart muscles using this modified protocol [14-16,40]. In brief, after removal of connective tissues, muscle was homogenized on ice in lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT) supplemented with a protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO). Following centrifuging at 5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and these supernatants were further centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual nuclei. The final collected supernatants were stored as nuclei-free total cytosolic protein fraction. The remaining nuclear pellets were washed 3 times with ice-cold lysis buffer, resuspended in 360 µl of lysis buffer in the presence of 49.8 µl of 5 M NaCl and protease inhibitor cocktail, and rotated for 2 h at 4 °C to lyse the nuclei. Following a spin at 15,000 rpm for 15 min at 4 °C, the supernatants were collected and stored as cytosol-free nuclear protein fraction. This protein fractionation procedure has been routinely used in our laboratory to obtain high purity protein fractions as assessed by immunoblotting the fractions with an anti-histone H2B (a nuclear protein) and an anti-CuZnSOD (a cytosolic isoform of superoxide dismutase) antibody [14-16].

The protein contents of the extracts were quantified in duplicate by *DC* Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay [41]. As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation [42].

2.5 Western immunoblot analyses.

Protein expressions of HSP70, HSP27, and HSP60 were examined in the cytosolic fraction while Id2 and p53 were determined in both nuclear and cytosolic fractions. Fixed micrograms of nuclear or cytosolic protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma

Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and then probed with an anti-p53 mouse monoclonal antibody (1:200 dilution, sc-99), anti-Id2 rabbit polyclonal antibody (1:200 dilution, sc-489), anti-HSP70 mouse monoclonal antibody (1:2000 dilution, SPA810B), anti-HSP27 rabbit polyclonal antibody (1:2000 dilution, SPA801), or anti-HSP60 rabbit polyclonal antibody (1:2000 dilution, SPA804) diluted in PBS-T with 2% BSA incubated at 4°C for overnight. An anti-HSP70 rabbit polyclonal antibody (1:2000 dilution, SPA812) was further used to confirm the findings of HSP70 in the present study. Id2 and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while HSP70, HSP27 and HSP60 were purchased from StressGen (Victoria, BC, Canada). Whole cell lysate of actively dividing human 293T cells was included as a positive control. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed by an ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

2.6 Statistical analyses.

Statistical analyses were performed using the SPSS 10.0 software package. ANOVA was performed to examine the main effects of suspension, age, and interaction (suspension $\times$ age) on the measured variables. ANOVA followed by Tukey HSD post hoc analysis was used to examine differences between groups. All data are given as means $\pm$ standard error of mean (SE). Statistical significance was accepted at $P < 0.05$.

3. RESULTS

3.1 Muscle Weight.

Sarcopenic muscle loss was observed by comparing the whole medial gastrocnemius muscle wet weight between young adult and aged control animals. The muscle wet weight of the aged control rats was 23% lower than young control adults (young adult vs. aged: 993 vs. 761 mg, **Figure 1A**). The muscle weight normalized to the animal bodyweight in aged rats was 48% lower than that of the young adult rats (young adult vs. aged: 2.7 vs. 1.4 mg/g, **Figure 1B**). 14 days of unloading reduced

the medial gastrocnemius muscle wet weight by ~30% in both young adult and aged rats (young control vs. suspended: 993 vs. 699 mg; aged control vs. suspended: 761 vs. 520 mg) (**Figure 1A**) while the muscle weight normalized to the animal bodyweight decreased by 11% and 19% in young adult and aged rats, respectively (young control vs. suspended: 2.7 vs. 2.4 mg/g; aged control vs. suspended: 1.4 vs. 1.1 mg/g) (**Figure 1B**).

Figure 1A ———→ Muscle weight

Figure 1B ———→ Muscle weight normalized to animal's bodyweight

3.2 *Id2 mRNA, nuclear and cytosolic protein contents*

As indicated by the RT-PCR analyses, no difference was found in the mRNA content of Id2 between the suspended and control muscles (**Figure 2A**). According to our immunoblot analysis, nuclear Id2 protein content of the suspended muscle was not different from the control muscle (**Figure 2B**) whereas the cytosolic Id2 protein content in the suspended muscle was 1.7-fold and 1.4-fold higher than the control muscle in young adult and aged animals, respectively (**Figure 2C**). The 2×2 (suspension $\times$ age) ANOVA indicated that there is a greater Id2 protein content in the aged muscle when compared to the young adults (**Figure 2B and 2C**).

Insert Figure 2A ———→ Id2 mRNA content

Insert Figure 2B ———→ Nuclear Id2 protein content

Insert Figure 2C ———→ Cytosolic Id2 protein content

3.3 *p53 mRNA, nuclear and cytosolic protein contents*

There was no significant difference in the p53 mRNA content between the suspended and control muscles (**Figure 3A**). The nuclear p53 protein content of the suspended muscle was not different from the control muscle (**Figure 3B**). In contrast, the cytosolic p53 protein content was 44% and 52% greater in the suspended muscle when compared to the control muscle in young adult and aged animals, respectively (**Figure 3C**). The 2×2 (suspension $\times$ age) ANOVA indicated that aged muscle has higher levels of both nuclear and cytosolic p53 protein relative to the young adult muscle (**Figure 3B and 3C**).

Insert Figure 3A ———→ p53 mRNA content

Insert Figure 3B ———→ Nuclear p53 protein content

Insert Figure 3C ———→ Cytosolic p53 protein content

3.4 HSP70 mRNA and protein contents

Transcriptional expressions of both HSP70-1 and HSP70-2 genes that individually encode HSP70 were examined by RT-PCR analyses. We found that the mRNA content of HSP70-1 was 44% and 74% reduced in the suspended muscle relative to the control muscle in young adult and aged animals, respectively (**Figure 4A**). Although no difference was found in the mRNA content of HSP70-2 between the suspended and control muscles of both ages, HSP70-2 mRNA content was greater in the aged muscle when compared to young muscle (**Figure 4B**). The protein content of HSP70 was not different between the suspended and control muscle in both young and aged muscles whereas aged muscle exhibited a higher level of HSP70 protein content relative to the young muscle (**Figure 4C**). The 2×2 (suspension $\times$ age) ANOVA suggested that aged muscle has a lower level of HSP70-1 mRNA but greater level of HSP70-2 mRNA and HSP70 protein relative to the young adult muscle (**Figure 4A, 4B, and 4C**).

Insert Figure 4A ———→ HSP70-1 mRNA content

Insert Figure 4B ———→ HSP70-2 mRNA content

Insert Figure 4C ———→ HSP70 protein content

3.5 HSP27 mRNA and protein contents

No difference was found in the HSP27 mRNA content between the suspended and control muscles in both young and aged animals (**Figure 5A**). Although the protein content of HSP27 did not change with suspension in aged muscle, we found that the HSP27 protein content was increased by 89% in the young suspended muscle relative to the young control muscle (**Figure 5B**). The 2×2 (suspension $\times$ age) ANOVA suggested that aged muscle has a higher levels of HSP27 protein when compared to the young adult muscle (**Figure 5B**).

Insert Figure 5A ———→ HSP27 mRNA content

Insert Figure 5B ———→ HSP27 protein content

3.6 HSP60 protein content

No difference was found in HSP60 protein content between the suspended and control muscles in both young and aged animals (**Figure 6**).

Insert Figure 6 ———→ HSP60 protein content

4. DISCUSSION

Although it has been consistently shown that accelerated apoptosis is evident during unloading-induced muscle atrophy [1,2,7,15], the underlying cellular and molecular mechanisms that contribute to the activation of the apoptotic signaling pathway leading to the unloading-induced apoptosis remain to be fully identified. In this study, we have novel provided evidence showing that Id2 and p53 may be involved in the regulation of hindlimb suspension-induced muscle loss. We demonstrated that the subcellular cytosolic fractions of Id2 and p53 proteins are responsive to hindlimb suspension-mediated muscle unloading. With hindlimb unloading, we observed that cytosolic but not nuclear Id2 and p53 protein contents were elevated in unloaded muscles from both young adult and aged rats. These findings are consistent with the hypothesis that subcellular compartmentalization of accumulated proteins is one factor determining the physiologic role of Id2 and p53 in muscle atrophy. Furthermore, we found that the HSP70-1 mRNA content is lower whereas the mRNA content of HSP70-2 and protein contents of Id2, p53, HSP70 and HSP27 are greater in the control gastrocnemius muscle of aged rats relative to the control muscle in young adult rats. These results indicate that sarcopenic muscle loss is accompanied with the changes of Id2, p53, and HSPs expression in rat gastrocnemius muscle.

In addition to the demonstrated differentiation-inhibitory and proliferating functions of Id2 [21,43,44], it has also been shown that Id2 can initiate apoptosis through the apoptogenic properties resident in the amino-terminal region of Id2 [26]. Consistent with this dual role of Id2, previous studies from our laboratory have demonstrated the possible role of Id2 in mediating cell growth/proliferation and apoptotic cell death in mature skeletal muscle in response to muscle overload and unload, respectively [2,16,28,29]. By using an avian model of unloading following a period of loading, we have previously shown that the atrophic role of Id2 during muscle unloading may be mediated by the subcellular localization of its protein accumulation. Specifically, our data shown that cytosolic but not nuclear Id2 protein abundance is responsive to unloading in birds [16]. However, it is noted that this regulatory role of subcellular Id2 is inferred from the observations during unloading of muscle that was first hypertrophied, and muscle loss returned muscle mass back to the resting control mass level [16]. In the present study, we extend our previous findings in quail muscles, in that, we show that the subcellular location (i.e., cytosolic protein fraction) of Id2 protein expression also responds to hindlimb suspension-mediated unloading where muscle mass is reduced well below control mass levels, in a similar subcellular compartmentalized manner in both young adult and aged gastrocnemius muscles [16]. Collectively, the hypothesis of the physiologic role of cytosolic Id2 in muscle loss is supported by the findings in these two muscle atrophy experimental models where the loss of muscle is at or just above control mass levels, or below the basal muscle mass level.

Tumor suppressor p53 has been demonstrated to be essential in the regulation of cell survival and programmed cell death in mitotic cell lineages [20,45]. Although the expression of p53 is well conserved in mature muscle [14,16,31-33,46], the role of p53 in postmitotic skeletal myocytes is relatively unknown. Based on the pro-apoptotic properties of p53, it has been hypothesized that p53 maybe related to the activation of apoptosis during muscle disuse-induced muscle loss [16,30-34]. In addition, p53 has been demonstrated to be able to mediate the apoptotic cascades by both transcriptional-dependent mechanism through the upregulation of the transcriptional expressions of certain pro-apoptotic genes (e.g., Bax, Noxa, and PUMA) [45,47,48] and/or a transcriptional-independent mechanism via translocating to the mitochondria thereby activating the cytosolic pro-apoptotic factors (e.g., Bax) [45,49,50]. Hence, it is required to test if the role of p53 in muscle loss maybe linked in a nuclear/cytosolic compartment-specific fashion. Previously, results from our laboratory have suggested that p53 maybe involved in mediating the apoptosis-associated muscle regression in hypertrophied muscle during unloading. We have reported that both the nuclear and cytosolic p53 protein abundances are increased concomitant with the elevation of pro-apoptotic markers during unloading in formerly hypertrophied quail muscle [15,16]. Consistent with these findings, here we demonstrated that the protein expression of p53 is also elevated in both young and aged rat muscles after hindlimb suspension but these changes are only found in the cytosolic fraction. The current results are in support of the findings that the total p53 protein content was increased in atrophied rat skeletal muscle following unloading that was induced by 14 days of space flight [33]. It is noted that the increase in nuclear p53 protein that we have observed in the quail hypertrophied muscle following unloading is not evident in the present rat hindlimb suspended muscle, albeit the change in cytosolic p53 is found in both atrophic situations [16]. However, these discrepancies in the change of nuclear p53 protein content may be reasonably attributed to the different nature of muscle loss in these atrophic conditions (i.e., muscle loss that stops above the basal control muscle level in the quail model *vs.* muscle loss below the basal level in the rodent hindlimb suspension model of the current study) and between species (i.e., birds *vs.* rodents). Overall, our findings in both models of quail unloading and rat hindlimb suspension generally agreed with the hypotheses that p53 is associated with muscle atrophy induced by muscle disuse and the atrophic role of p53 maybe related to the subcellular location of the protein accumulation. Nonetheless, future investigation is required to fully identify the biological significance and the underlying mechanism(s) of p53 in mediating muscle atrophy.

Heat shock proteins (HSPs) are commonly believed to function as a protective mechanism in response to various stressful events including elevated temperature, oxidative stress, hypoxia, and other cellular damages [51]. Previously, the response of HSP70 to hindlimb unloading has been examined but the results are rather contradictory. By examining the soleus muscle, it has been shown

that HSP70 protein content decreases after 18 hours, 8 days or 9 weeks of hindlimb suspension in adult rats [52-54]. However, it has also been reported that HSP70 protein content in adult soleus muscle is not altered with 5 days of hindlimb unloading [55]. In addition, Desplanches and colleagues [56] demonstrated that the protein expression of HSP70 remains unaffected in response to 14 days of hindlimb suspension in both slow-fiber predominated muscles (soleus and adductor longus) and fast-twitch muscles (plantaris and extensor digitorum longus) of adult rats. In light of these previous findings, we investigated the gene and protein expressions of HSP70 in gastrocnemius muscle which is a muscle that has not been well examined. Furthermore, in addition to young adult rats, aged rats were also investigated in the present study as the data showing the response of HSP70 to hindlimb unloading in aged muscle is lacking. We report that HSP70-1 mRNA content increases concomitant with unchanged abundances of HSP70-2 mRNA and HSP70 protein in both young adult and aged gastrocnemius muscles following 14 days of hindlimb unloading. Although the protein abundance of HSP70 remains unchanged with hindlimb unloading, our novel mRNA data suggest that the expressions of HSP70-1 and HSP70-2 gene may respond differently, at least in gastrocnemius muscle, to hindlimb suspension-mediated muscle unloading even though these HSP70 genes encode the same peptide sequence for HSP70 protein [57-59]. With unloading, we also found that HSP27 protein content increases exclusively in suspended muscle from young animals, although HSP27 mRNA and HSP60 protein contents are unchanged with hindlimb suspension in muscles of both ages. Notably, our present findings in both mRNA and protein responses of HSP27 in gastrocnemius muscle are consistent with the findings of Kato and coworkers [60] who have demonstrated that following 2-10 days of hindlimb suspension, the protein accumulations as well as the phosphorylations of two α -crystallin small HSPs including α B-crystallin and HSP27 are increased together with elevated α B-crystallin mRNA content but unchanged HSP27 mRNA content as estimated by Northern blot analysis in the soleus muscle. Based on the protective role of HSPs, we speculate that the increase in HSP27 protein content in suspended gastrocnemius muscle might be part of a compensatory adaptation in young adult rats and this potentially protective increase in HSP27 during hindlimb unloading is not present in the aged suspended muscle.

In conclusion, we have provided evidence indicating that transcriptional repressor protein Id2 and tumor suppressor protein p53 are associated with muscle wasting induced by hindlimb suspension in young adult and aged gastrocnemius muscles. We have shown that protein expression of Id2 and p53 increased exclusively in the cytosolic fraction of the suspended muscle relative to the control muscle in both young and aged rat, whereas these changes were not found in nuclear fraction. These observations suggest that the hypothesized role of Id2 and p53 in mediating apoptosis-related muscle loss during hindlimb suspension may be attributed to the compartmental localization of the subcellular protein accumulation. Nevertheless, additional research is needed to fully understand the regulatory

mechanisms of Id2 and p53 in muscle disuse through other possible machineries (e.g., by phosphorylation) [61-63]. Furthermore, we report that the sarcopenic gastrocnemius muscle mass decline is accompanied by decreased HSP70-1 mRNA but increased HSP70-2 mRNA and Id2, p53, HSP70 and HSP27 protein abundances compared to gastrocnemius muscle in young adult rats. Further investigation is warranted to evaluate the role of these alterations of apoptosis-associated factors in sarcopenia.

Remarks: The findings presented in this chapter have been submitted to the *FEBS Lett.* at the time of dissertation submission.

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Table 1. Primers used for PCR amplification of cDNA

Product	Accession No.	Sequence	Position	T _A , °C	Cycles	Product length, bp	Restriction enzyme	Restriction products, bp
Id2	NM_013060	F: 5'-GCGGCGCAGCACCTCAGC-3' R: 5'-TGCAAAAGAAAAAGAGAAAGTAAAAATAA-3'	3-20 508-536	58.4	36	534	<i>EcoRI</i> <i>PstI</i>	416, 118 258, 246, 30
p53	NM_030989	F: 5'-GCCGGCCCATCCTTACCATCA-3' R: 5'-TCGGCAGCACGGGCATCCT-3'	920-940 1229-1247	59.6	33	328	<i>AluI</i>	91, 88, 64, 44, 41
HSP72-1	NM_031971	F: 5'-GGGCCAAGAGGACGCTGT-3' R: 5'-CGCGCCCGTTGAAGAAGT-3'	983-1000 1232-1249	60.4	33	267	<i>PstI</i> <i>AluI</i>	247, 20 107, 69, 63, 28
HSP72-2	X77208	F: 5'-CTCGTCCATGGTGCTGACCAAG-3' R: 5'-CCGCTGCGAGTCGTTGAAGTAG-3'	1604-1625 1691-1712	59.0	33	109	N/A	sequenced
HSP27	NM_031970	F: 5'-CGCCGCGTGCCCTTCTC-3' R: 5'-CGCCTTCCTTGGTCTTCACTGTG-3'	74-90 400-422	62.4	33	349	<i>HindIII</i> <i>RsaI</i>	263, 86 291, 58

T_A, annealing temperature; Accession No., GenBank accession number; F, forward primer; R, reverse primer

Figure 1 Muscle mass change during hindlimb suspension

A. Muscle wet weight

B. Muscle wet weight normalized to bodyweight

Legend for Figure 1A Muscle wet weight. The extent of muscle loss following hindlimb suspension was estimated by examining the muscle mass loss between the suspended and the control whole gastrocnemius muscle wet weight. The data are presented as means $\pm$ SE. * $P < 0.05$, Suspended group was significant different from the Control group under the same age group; ** $P < 0.05$, Aged animals were significant different from the Young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2×2 ANOVA.

Legend for Figure 1B Muscle wet weight normalized to bodyweight. The extent of muscle loss following hindlimb suspension was further determined by examining the decline in gastrocnemius muscle wet weight normalized to the animal's bodyweight between the suspended and control groups. The data are presented as means $\pm$ SE. * $P < 0.05$, Suspended group was significant different from the Control group under the same age group; ** $P < 0.05$, Aged animals were significant different from the Young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2×2 ANOVA.

Figure 2 Id2 mRNA and protein content

Legend for Figure 2A Id2 mRNA content. The mRNA content of Id2 was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of Id2/18S. The insets show representative results for the Id2 mRNA. The data are presented as means $\pm$ SE.

Legend for Figure 2B Nuclear Id2 protein content. The protein content of Id2 was assessed in nuclear fraction by immunoblot analysis. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Id2 in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. $**P < 0.05$, Aged animals were significant different from the Young animals under the same experimental condition. The main effect of age was analyzed by a 2×2 ANOVA.

Legend for Figure 2C Cytosolic Id2 protein content. The protein content of Id2 was measured in cytosolic fraction. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Id2 in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. $*P < 0.05$, Suspended group was significant different from the Control group under the same age group; $**P < 0.05$, Aged animals were significant different from the Young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2×2 ANOVA.

Figure 3 p53 mRNA and protein content

Legend for Figure 3A p53 mRNA content. The mRNA content of p53 was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of p53/18S. The insets show representative results for the p53 mRNA. The data are presented as means $\pm$ SE.

Legend for Figure 3B Nuclear p53 protein content. The protein content of p53 was assessed in nuclear fraction by immunoblot analysis. The data are expressed as OD $\times$ resulting band area, and expressed in arbitrary units. The insets show representative blots for p53 in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. $^{**}P < 0.05$, Aged animals were significantly different from the Young animals under the same experimental condition. The main effect of age was analyzed by a 2×2 ANOVA.

Legend for Figure 3C Cytosolic p53 protein content. The protein content of p53 was determined in cytosolic fraction. The data are expressed as OD $\times$ resulting band area, and expressed in arbitrary units. The insets show representative blots for p53 in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. $^{*}P < 0.05$, Suspended group was significantly different from the Control group under the same age group; $^{**}P < 0.05$, Aged animals were significantly different from the Young animals under the same experimental condition. The main effects of age, suspension, and interaction (age $\times$ suspension) were analyzed by a 2×2 ANOVA.

Figure 4 HSP70 mRNA and protein content

Legend for Figure 4A HSP70-1 mRNA content. The mRNA content of HSP70-1 was estimated by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of HSP70-1/18S. The insets show representative results for the HSP70-1 mRNA. The data are presented as means $\pm$ SE. * $P < 0.05$, Suspended group was significant different from the Control group under the same age group; ** $P < 0.05$, Aged animals were significant different from the Young animals under the same experimental condition. The main effects of age and suspension were analyzed by a 2×2 ANOVA.

Legend for Figure 4B HSP70-2 mRNA content. The mRNA content of HSP70-2 was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of HSP70-2/18S. The insets show representative results for the HSP70-2 mRNA. The data are presented as means $\pm$ SE. ** $P < 0.05$, Aged animals were significant different from the Young animals under the same experimental condition. The main effect of age was analyzed by a 2×2 ANOVA.

Legend for Figure 4C HSP70 protein content. The protein content of HSP70 was assessed by immunoblot analysis. The data are expressed as OD $\times$ resulting band area, and expressed in arbitrary units. The insets show representative blots for HSP70 in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. ** $P < 0.05$, Aged animals were significant different from the Young animals under the same experimental condition. The main effect of age was analyzed by a 2×2 ANOVA.

Figure 5 HSP27 mRNA and protein content

Legend for Figure 5A HSP27 mRNA content. The mRNA content of HSP27 was determined by RT-PCR with 18S rRNA as an internal control. The data are expressed as arbitrary ratio of HSP27/18S. The insets show representative results for the HSP27 mRNA. The data are presented as means $\pm$ SE.

Legend for Figure 5B HSP27 protein content. The protein content of HSP27 was determined by immunoblot. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for HSP27 in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. * $P < 0.05$, Suspended group was significant different from the Control group under the same age group; ** $P < 0.05$, Aged animals were significant different from the Young animals under the same experimental condition. The main effects of age, suspension, and interaction (age $\times$ suspension) were analyzed by a 2×2 ANOVA.

Figure 6 HSP60 protein

Legend for Figure 6 HSP60 protein content. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for HSP60 in control and suspended muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

CHAPTER 10

Subcellular responses of p53 and Id2 in fast and slow skeletal muscle in response to stretch-induced overload

ABSTRACT

The present study examined the subcellular response of tumor suppressor p53 and inhibitor of DNA-binding/differentiation Id2 to muscle overload. Overload was induced by attaching a tube weight corresponding to 12% of the animal's bodyweight to one wing of young adult or old Japanese quails for 7 or 21 days. This approach induces muscle hypertrophy in fast patagialis (PAT) and slow anterior latissimus dorsi (ALD) wing muscles of the loaded side. The contralateral wing served as the intra-animal control. PAT muscle weight increased by 28% in young bird but no significant hypertrophy was found in aged birds following 7 days of loading. After 21 days of loading, PAT muscle weight increased by 49% and 29% in young and aged birds, respectively. ALD muscle weight increased by 96% in young bird whereas the gain in aged birds was not significant after 7 days of loading. Following 21 days of loading, ALD muscle weight increased by 179% and 102% in young and aged birds, respectively. Nuclear and cytosolic subcellular protein fractions were analyzed by Western immunoblots. The nuclear Id2 protein content was greater in PAT muscles from old and young birds that were loaded for 7 days relative to the contralateral control muscles. These changes were not found in PAT muscles after 21 days of loading. In ALD muscles, elevation of nuclear Id2 protein content was evident in both 7 and 21 day-loaded muscles relative to the contralateral control side in both young and aged birds. Nuclear p53 protein content was greater in all loaded PAT or ALD muscles when compared to the contralateral control muscles in all groups of birds. In contrast, no difference was found in either cytosolic Id2 or p53 protein content among all the loaded PAT or ALD muscles and the corresponding contralateral control muscles. These data suggest that nuclear, but not cytosolic Id2 and p53 are responsive to stretch-induced muscle overload. Moreover, the attenuated ability of the aged skeletal muscle to achieve hypertrophy is not explained by the subcellular changes of Id2 and p53 with overload.

Key words: muscle hypertrophy, inhibitor of DNA binding/differentiation, tumor suppressor

INTRODUCTION

Skeletal muscle is a highly adaptable organ system that responds rapidly to overload by increasing the anabolic events via upregulation of protein synthesis and accumulation of proteins (42). As a result, muscle hypertrophy or enlargement ensues if an overload stress is applied in a repeated or sustained pattern for a period of time (40). An appropriate overload (e.g., resistance training) has been suggested to be valuable and practical in offsetting or intervening for sarcopenia- or disease-associated muscle wasting (18,21,28,31,34,49,60). Several studies have been conducted with the aim of identifying the precise cellular and molecular mechanisms that contribute to muscle hypertrophy (22,25). Although potential signaling pathways (e.g., IGF-1, MGF, PI3K, PKB/Akt, mTOR, p70S6K, RhoA, SRF, and FAK) have been preliminarily established in having a role in the hypertrophy-associated cellular changes (20,22,24-26,41,48), the exact underlying mechanisms responsible for the hypertrophic response, especially in the aged skeletal muscle, remain to be fully resolved.

The inhibitor of DNA binding/differentiation-2 (Id2) protein is a member of Id family, which has been demonstrated to have a dual role in regulating cell proliferation and cell death under different situations (10,11,19,45,59). These opposite functions of Id2 are primarily owing to the structure of helix-loop-helix motif in the absence of basic amino acid domain as well as the Bax-mediated apoptotic property of the N-terminal region of Id2 (10,11,19,45,59). Consistent with the suggested dual role of Id2, previous studies from our laboratory has shown Id2 is associated with both skeletal muscle hypertrophy induced by functional or stretch-induced overload and the apoptosis-related muscle atrophy (4-6,52). Although the mechanisms coordinating the differing functions of Id2 in muscle hypertrophy and atrophy is largely unknown, our recent data suggested that subcellular fractionated protein expression (cytosolic vs. nuclear) maybe of importance in determining the physiologic roles of Id2, with high cytosolic levels of Id2 associated with muscle atrophy (52).

Tumor suppressor protein p53 has been widely recognized to be one of the essential regulators in cell cycle and apoptosis in mitotic cell lineages (57). However, it is relatively unknown whether p53 has a role in mediating muscle remodeling. With an emphasis on mature skeletal muscle, several studies have attempted to investigate the response of p53 to laser irradiation, muscle disuse, muscle unloading, and ischemia (27,30,43,46,51,52). Although a consensus on the role of p53 in muscle loss has not been achieved, there have been at least some evidences showing that p53 responds to certain muscle atrophic conditions (46,52). While more work is needed to determine the role of p53 in muscle atrophy, there has been novel data demonstrating that p53 is upregulated after resistance exercise in skeletal muscle (16). Correspondingly, p53 has been proposed to play a role in mediating the biochemical cascades leading to muscle hypertrophy (16). In support of the proposed hypertrophic role of p53, it has also been shown that p53 is related to Ankrd2, a sarcomeric protein found in I-band

which is involved in muscle hypertrophy (32). Nonetheless, mechanisms involved in regulating p53 during muscle hypertrophy are largely unknown.

Taken together, it is unclear whether cytosolic or nuclear subcellular Id2 and p53 fractions are involved in mediating hypertrophy during muscle overload. Therefore, the purpose of this study was to examine the subcellular response of Id2 and p53 to stretch-induced overload in young adult and aged quails. Cytosolic levels of Id2 and p53 increase with unloading induced atrophy and apoptosis (52). Therefore, we hypothesized that the f Id2 and p53 would increase in the nuclear by not cytoplasmic subcellular protein fraction in loaded muscles

METHODS

Animals. Japanese Coturnix quails were hatched and raised in pathogen-free conditions in the central animal care center at West Virginia University School of Medicine. The birds were housed at a room temperature of 22°C with a 12:12-h light:dark cycle and were provided with food and water ad libitum. Sixteen young adult birds (~2 mo old) and sixteen senescent aged birds (~48 mo old) were examined in the present study. The average lifespan of Japanese quails is ~26-28 mo and therefore the birds examined in this study were almost twice the age of the expected life span of these birds. Presumably these extremely old birds represented "successful aging" in this model of aging. We anticipated that if aging-induced changes in hypertrophic responses to Id2 or p53 occurred with aging, this would be readily detectable in these old birds. Japanese quail are both physically and sexually mature by 1.5 mo of age and do not grow thereafter (39,47). All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Stretch-induced Overload. Fast-twitch α -fiber predominated patagialis (PAT) and slow-twitch β -fiber predominated anterior latissimus dorsi (ALD) muscles are flexed with the wing on the birds' back at rest, but they are both stretched when the wing is extended. In our experimental stretch-overload model, we placed a tube containing ~12% of the bird's bodyweight over the left humeral-ulnar joint (7). This maintains the joint in extension throughout the period of stretch and induces stretch at the origin of the PAT and ALD muscles. Previous studies have consistently shown this stretch-overloading protocol results in muscle hypertrophy of the PAT and ALD muscles and the magnitude of increased muscle mass is dependent on the duration of the overload. (2,3,6,12-14). The unstretched right PAT and ALD muscles served as the intra-animal control muscle for each bird. Consistent with the fact that Japanese quails show no maturational changes in bodyweight and carcass composition beyond ~1.5-2 mo after hatching (37,38,58), it has been demonstrated that the

bodyweights of Japanese quails do not change throughout stretch overloading and do not differ between adult and aged quails (1,7-9,14,35). Therefore, the responses to the same absolute and relative loads could be compared in muscles from young adult and aged quails.

The left wing of eight young and eight aged birds was loaded for 7 days and then the birds were killed by an overdose of pentobarbital sodium. The remaining young and aged quails were loaded for 21 days and then sacrificed. The whole PAT and ALD muscles were dissected from the surrounding connective tissue, removed, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and then stored at -80°C until used for analyses.

Subcellular protein fractionation. The fractionation method described by Rothermel et al. (50) was adopted with minor modification to extract the cytosolic and nuclear protein fractions from the PAT and ALD muscles. We have previously obtained the fractionated cytosolic and nuclear proteins from skeletal or heart muscles using this modified protocol (52-55). In brief, after removal of connective tissues, muscle was homogenized on ice in lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT) supplemented with a protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO). Following centrifuging at 5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and these supernatants were further centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual nuclei. The final collected supernatants were stored as nuclei-free total cytosolic protein fraction. The remaining nuclear pellets were washed 3 times with ice-cold lysis buffer, resuspended in 360 µl of lysis buffer in the presence of 49.8 µl of 5 M NaCl and protease inhibitor cocktail, and rotated for 2 h at 4°C to lyse the nuclei. Following a spin at 15,000 rpm for 15 min at 4°C, the supernatants were collected and stored as cytosol-free nuclear protein fraction. This protein fractionation procedure has been routinely used in our laboratory to obtain high purity protein fractions as assessed by immunoblotting the fractions with an anti-histone H2B (a nuclear protein) and an anti-CuZnSOD (a cytosolic isoform of superoxide dismutase) antibody (52,53,55).

The protein contents of the protein extracts were quantified in duplicate by *DC* Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay (36). As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation (56).

Western immunoblot analyses. Id2 and p53 were examined in both nuclear and cytosolic fractions. Fixed micrograms of nuclear or cytosolic protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE. The

gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and probed with anti-p53 mouse monoclonal antibody (1:200 dilution, sc-99, Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Id2 rabbit polyclonal antibody (1:200 dilution, sc-489, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS-T with 2% BSA incubated at 4°C for overnight. Whole cell lysate of actively dividing human 293T cells was included as a positive control for probing Id2 and p53. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed by ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package. ANOVA followed by Tukey HSD post hoc analysis was used to examine differences between groups. Statistical significance was accepted at $P < 0.05$. All data are given as means $\pm$ standard error of mean (SE).

RESULTS

Change in PAT muscle mass following stretch overload. The extent of muscle hypertrophy was estimated by examining the percent difference in the whole PAT or ALD muscle wet weight between the loaded and the intra-animal contralateral control wings. Following 7 days of loading in PAT muscle, there was a 28% of hypertrophy in young adult birds whereas no significant extent of hypertrophy was found in aged birds (**Figure 1A**). After 21 days of loading, there was a 49% and 29% hypertrophy in PAT muscles of young and aged birds, respectively (**Figure 1A**). The percents of hypertrophy in young PAT muscles following 7 and 21 days of loading were both significantly greater than the aged muscles (**Figure 1B**).

Insert Figure 1A $\longrightarrow$ PAT muscle mass

Insert Figure 1B $\longrightarrow$ Percent change of PAT muscle hypertrophy

Change in ALD muscle mass following stretch overload. After 7 days of loading in ALD muscle, there was a 96% of hypertrophy in young adult birds whereas no significant extent of hypertrophy was found in aged birds (**Figure 2A**). Following 21 days of loading, there was a 179% and 102% hypertrophy in ALD muscles of young and aged birds, respectively (**Figure 2A**). The percents of hypertrophy in young ALD muscles following 7 and 21 days of loading were both significantly greater than the aged muscles (**Figure 2B**). Our results confirmed that the extent of hypertrophy, as estimated by increase in whole PAT or ALD muscle wet weight, in response to stretch overload was attenuated in aged muscle when compared to the muscle from young adult birds.

Insert Figure 2A ———→ ALD muscle mass

Insert Figure 2B ———→ Percent change of ALD muscle hypertrophy

Nuclear and cytosolic Id2 protein content in PAT muscle. According to our immunoblot analysis, the nuclear Id2 protein content of the loaded PAT muscle was 103% and 85% ($P < 0.05$) higher than the contralateral control muscle after 7 days of loading in young and aged birds, respectively (**Figure 3A**). Following 21 days of loading, there was no difference between the nuclear Id2 protein content between the loaded and control PAT muscles in the young and aged birds ($P > 0.05$, **Figure 3A**). In contrast, cytosolic Id2 protein content was not different between the loaded and control PAT muscles after 7 days and 21 days of stretch overloading in both young adult and aged birds (**Figure 3B**).

Insert Figure 3A ———→ Nuclear Id2 protein in PAT muscle

Insert Figure 3B ———→ Cytosolic Id2 protein in PAT muscle

Nuclear and cytosolic Id2 protein content in ALD muscle. The nuclear Id2 protein content of the loaded ALD muscle was 227% and 201% ($P < 0.05$) higher than the contralateral control muscle after 7 days of loading in young and aged birds, respectively (**Figure 4A**). Following 21 days of loading, the nuclear Id2 protein content in the loaded ALD muscle was 186% and 188% greater than the control muscles in the young and aged birds (**Figure 4A**). On the contrary, cytosolic Id2 protein content was not different between the loaded and control ALD muscles after 7 days and 21 days of stretch overloading in both young adult and aged birds (**Figure 4B**).

Insert Figure 4A ———→ Nuclear Id2 protein in ALD muscle

Insert Figure 4B ———→ Cytosolic Id2 protein in ALD muscle

Nuclear and cytosolic p53 protein content in PAT muscle. The nuclear p53 protein content of the loaded PAT muscle was significantly higher than the contralateral control muscle in all groups of birds (**Figure 5A**). Following 7 days of loading, nuclear p53 protein content was 182% and 150% greater in the loaded side when comparing to the control side in young adult and aged birds, respectively (**Figure 5A**). The nuclear p53 protein content in the 21 days-loaded PAT muscle was still elevated by 113% and 152% relative to the intra-animal control muscle in young and aged birds, respectively (**Figure 5A**). No difference was found in the cytosolic p53 protein content between the loaded and the contralateral control PAT muscles in all groups of birds ($P > 0.05$, **Figure 5B**).

Insert Figure 5A ———→ Nuclear p53 protein in PAT muscle

Insert Figure 5B ———→ Cytosolic p53 protein in PAT muscle

Nuclear and cytosolic p53 protein content in ALD muscle. The nuclear p53 protein content of the loaded ALD muscle was significantly higher than the contralateral control muscle in all groups of birds (**Figure 6A**). Following 7 days of loading, nuclear p53 protein content was 206% and 194% greater in the loaded side when comparing to the control side in young adult and aged birds, respectively (**Figure 6A**). The nuclear p53 protein content in the 21 days-loaded ALD muscle was still elevated by 140% and 154% relative to the intra-animal control muscle in young and aged birds, respectively (**Figure 6A**). In contrast, we did not find any difference in the cytosolic p53 protein content between the loaded and the contralateral control ALD muscles in all groups of birds ($P > 0.05$, **Figure 6B**).

Insert Figure 6A ———→ Nuclear p53 protein in ALD muscle

Insert Figure 6B ———→ Cytosolic p53 protein in ALD muscle

DISCUSSION

In this study, we have shown Id2 and p53 respond to stretch-induced muscle overload in both quail fast- and slow-twitch skeletal muscles in a specific subcellular compartmentalized fashion. We demonstrate for the first time that nuclear, but not cytosolic, protein contents of Id2 and p53 are elevated in muscle hypertrophy. Both of these proteins increased in the nuclear fraction of loaded fast PAT and slow ALD muscles relative to the intra-animal contralateral control muscles following 7 or 21 days of stretch-induced overload. Moreover, we observed that the subcellular changes of Id2 and p53 occur in a similar extent in the muscles from young adult and aged birds suggesting that the subcellular responses of Id2 and p53 do not account for the age-related difference in the capability of muscle hypertrophy. Collectively, our findings are consistent with the hypothesis that nuclear but not

cytosolic Id2 and p53 have a role in mediating the process of muscle hypertrophy.

Response of Id2 to muscle overload. Id gene expression has been exhibited to be augmented in response to mitogenic stimuli and associated with the stimulation of DNA synthesis (10,17,44). Accordingly, it has been suggested that Id2 may have an important role in the regulation of cell proliferation/growth (10,17,44). Id2 may also have a regulatory role in muscle cell lineage, including proliferation/growth. This proposition is primarily based on the findings demonstrating that overexpression of Id prolongs proliferation and inhibits differentiation in myogenic lineage cells (17,29,33) and *Id2* gene-deficient mice do not survive at birth with a noticeable lack of muscle tissues (33). Consistent with these observations in Id2 *in vitro* and knockout animals, our laboratory has previously provided *in vivo* data suggesting that Id2 maybe involved in the mediation of fiber hypertrophy during overload in mature skeletal muscle (4,6). By examining the functionally overloaded rat plantaris muscle as induced by denervating its agonist plantarflexor muscles (i.e., gastrocnemius and soleus), Alway and colleagues (4) first demonstrated that muscle hypertrophy is accompanied with increased Id2 expression. Moreover, a subsequent study also illustrated the elevation of expression of Id2 in quail patagialis muscle in response to stretch-induced muscle overload (6). While Id2 has been preliminarily demonstrated to have a role in muscle hypertrophy, the involved underlying mechanisms are largely unclear. Here, we extend the findings of Id2 in muscle hypertrophy by showing that the change of Id2 with muscle overload is compartmentalized in a subcellular manner. We demonstrated that nuclear Id2 protein content is increased in the loaded PAT or ALD muscle when compared to the contralateral control muscle in both young adult and aged quails following 7 or 21 days of stretch-induced overload. However, these changes were not observed for the cytosolic Id2 protein content in all groups of birds. Together with our previous data demonstrating that cytosolic but not nuclear Id2 protein content is elevated during unloading-induced muscle atrophy (52), these findings are consistent with the overall hypothesis that Id2 is involved in regulating both hypertrophic and atrophic processes in skeletal muscle (4-6). Our results support the hypothesis that nuclear Id2 is associated with muscle hypertrophy whereas cytosolic Id2 is related to atrophy. Nonetheless, it warrants further research in exploring other possible regulatory mechanisms (e.g., phosphorylation of Id2) that might be comprised in Id2 and muscle remodeling.

p53 and muscle hypertrophy. Motivated by the fact that aberrant regulation of tumor suppressor p53 protein is implicated in the pathogenesis of severe diseases (e.g., carcinogenesis), extensive research have been conducted in examining the cellular functions of p53 in cell division and apoptotic cell death primarily in mitotic cell lineages (57). Conversely, the role of p53 in postmitotic muscle remodeling is relatively unidentified. Indeed, there have been preliminary data suggesting that elevation of p53 maybe involved in muscle wasting under certain atrophic situations (46,52). Ohnishi and coworkers (46) have shown that accumulation of p53 protein is evident in rat muscle following

space flight. In addition, our laboratory has also demonstrated both the nuclear and cytosolic p53 protein contents are increased during unloading in previously hypertrophied quail patagialis muscles (52). While additional research is required to verify the role of p53 in muscle wasting as there is still lacking consent on the role of p53 in other atrophic conditions (27,30,43,51), there have been novel data suggesting that p53 is involved in overload-induced muscle hypertrophy (16). By using the microarray technique, Chen et al. (16) found that three p53-associated growth inhibitory genes including GADD45, TRPM-2, and PC3 are all upregulated in rat skeletal muscle following an acute bout of maximally activated eccentric contraction, and furthermore, they reported an increase in p53 protein content, as estimated by Western immunoblotting, in the nuclear fraction extracted from the exercised muscle (16). The potential role of p53 in muscle hypertrophy is elucidated by the findings illustrating that p53 is associated with a hypertrophy-related sarcomeric protein called Ankrd2 (32). Consistent with these previous findings, we reported that the elevation of nuclear, but not cytosolic p53 protein content is evident in quail muscle after stretch-induced overload. Our data support the hypothesis that p53 is involved in mediating the cellular cascades resulting in muscle hypertrophy or enlargement during muscle overloading. Moreover, our results indicate that the hypertrophic role of p53 is limited to the change of p53 content in nuclei, but not cytosol during overload. Correspondingly, these observations lead to us to speculate that the role of p53 in muscle hypertrophy may be associated with the p53-mediated transcriptional events, which occur in the nuclei. Nonetheless, there is a need to have more investigations to confirm that p53 is directly and/or indirectly involved in the transcriptional control in overloading skeletal muscle.

In conclusion, we have provided evidence showing that subcellular inhibitor of DNA-binding/differentiation protein Id2 and tumor suppressor protein p53 respond to the hypertrophic stimulus initiated from stretch-induced overload in both fast and slow skeletal muscles of young adult and aged quails. We have demonstrated that the nuclear but not cytosolic protein contents of Id2 and p53 significantly increased in the loaded muscle when compared to the contralateral control muscle after 7- or 21-days of loading. Nevertheless, although nuclear Id2 and p53 appear to be involved in pathways leading to stretch overload-induced muscle hypertrophy, our findings suggest that the attenuation of muscle hypertrophy in old age is not explained by the responses of Id2 and p53. Other factors including increases in an apoptotic environment (4,52,55), or inappropriate alterations in gene expression (23,31) or the hormonal milieu (15,26) may explain in part reduced hypertrophic adaptation in muscles of aged animals and humans.

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Figure 1A PAT Muscle Weight

Legend for Figure 1A PAT muscle data. The extent of muscle hypertrophy following stretch-induced overload was estimated by examining the muscle mass gain between the loaded and the contralateral control whole PAT muscle wet weight. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from control muscles. 7d loading, 7 days of loading; 21d loading, 21 days of loading; Young, young adult quails; Aged, aged quails.

Figure 1B Percent of PAT muscle hypertrophy relative to the contralateral side

Legend for Figure 1B Percent of PAT muscle hypertrophy relative to the contralateral side. The extent of muscle hypertrophy was presented as the percent difference in the muscle weight between the loaded and the contralateral control whole PAT muscle wet weight. The data are presented as means $\pm$ SE. * $P < 0.05$, percent change is significantly different from the animals of same age following 7 days of loading. # $P < 0.05$, percent change is significantly different from young animals following the same days of loading.

Figure 2 **ALD Muscle Data****A. ALD muscle weight** **B. Percent of ALD muscle hypertrophy relative to the contralateral control side**

Legend for Figure 2A ALD muscle data. The extent of muscle hypertrophy following stretch-induced overload was estimated by examining the muscle mass gain between the loaded and the contralateral control whole PAT muscle wet weight. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from control muscles. 7d loading, 7 days of loading; 21d loading, 21 days of loading; Young, young adult quails; Aged, aged quails.

Legend for Figure 2B Percent of ALD muscle hypertrophy relative to the contralateral side. The extent of muscle hypertrophy was presented as the percent difference in the muscle weight between the loaded and the contralateral control whole PAT muscle wet weight. The data are presented as means $\pm$ SE. *P < 0.05, percent change is significantly different from the animals of same age following 7 days of loading. #P < 0.05, percent change is significantly different from young animals following the same days of loading.

Figure 3 Id2 protein content in PAT muscle**A. PAT nuclear Id2** **B. PAT cytosolic Id2**

Legend for Figure 3A Nuclear Id2 protein content in PAT muscle. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Id2 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Legend for Figure 3B Cytosolic Id2 protein content in PAT muscle. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Id2 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

Figure 4 Id2 protein content in ALD muscle**A. ALD nuclear Id2** **B. ALD cytosolic Id2**

Legend for Figure 4A Nuclear Id2 protein content in ALD muscle. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Id2 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Legend for Figure 4B Cytosolic Id2 protein content in ALD muscle. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Id2 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

Figure 5 p53 protein content in PAT muscle**A. PAT nuclear p53** **B. PAT cytosolic p53**

Legend for Figure 5A Nuclear p53 protein content in PAT muscle.

The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for p53 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Legend for Figure 5B Cytosolic p53 protein content in PAT muscle. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for p53 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

Figure 6 p53 protein content in ALD muscle**A. ALD nuclear p53** **B. ALD cytosolic p53**

Legend for Figure 6A Nuclear p53 protein content in ALD muscle. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for p53 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Legend for Figure 6B Cytosolic p53 protein content in ALD muscle. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for p53 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE.

CHAPTER 11

Apoptotic adaptations from exercise training in skeletal and cardiac muscles

ABSTRACT

The effect of exercise on apoptosis in postmitotic tissues is not known. In this study, we investigated the effect of regular moderate physical activity (i.e., exercise training) on the extent of apoptosis in rat skeletal and cardiac muscles. Adult Sprague Dawley rats were trained (TR) 5 days weekly for 8 wk on treadmill. Sedentary rats served as controls (CON). An ELISA was used to detect mono- and oligonucleosome fragmentation as an indicator of apoptosis. Bcl-2, Bax, Apaf-1, AIF, cleaved PARP, cleaved caspase-3, cleaved/active caspase-9, heat shock protein (HSP)70, Cu/Zn-SOD, and Mn-SOD protein levels were determined by Western analyses. Bcl-2 and Bax transcript contents were estimated by RT-PCR. A spectrofluorometric assay was used to determine caspase-3 activity. DNA fragmentation in ventricles of the TR group decreased by 15% whereas that in soleus of the TR group tended to decrease ($P=0.058$) when compared with CON group. Protein contents of Bcl-2, HSP70, and Mn-SOD increased in both soleus and ventricle muscles of TR animals when compared with CON animals. Apaf-1 protein content in the soleus of TR animals was lower than that of CON animals. Bcl-2 mRNA levels increased in both ventricle and soleus muscles of TR animals, and Bax mRNA levels decreased in the soleus of TR animals when compared with CON animals. Furthermore, HSP70 protein content was negatively correlated to Bax mRNA content and was positively correlated to Bcl-2 protein and mRNA contents. Mn-SOD protein content was negatively correlated to the apoptotic index, and caspase-3 activity and was positively correlated to Bcl-2 transcript content and HSP70 protein content. These data suggest that exercise training attenuates the extent of apoptosis in cardiac and skeletal muscles.

Key words: programmed cell death • DNA fragmentation • Bcl-2 • Bax • caspase

INTRODUCTION

Apoptosis is a biological process that is fundamentally conserved from worms to mammals (1–3). It is tightly regulated by an internally encoded suicide program that requires coordinated regulation of specific genes (4). Several distinctive morphological characteristics define apoptosis, including cell shrinkage, plasma membrane blebbing, chromatin condensation, degradation of chromosomal DNA at internucleosomal intervals, and formation of apoptotic bodies (5). The necessity for apoptosis in multicellular organisms is widely accepted, because this process allows for the elimination of nonfunctional, misplaced, abnormal, or damaged as well as harmful cells, thereby playing an indispensable role in embryonic development, tissue homeostatic turnover, and immunological defense (6). Moreover, several pathological conditions, including tumorigenesis, autoimmune diseases, neurodegenerative disorders, viral infections, and acquired immune deficiency syndrome have also been demonstrated to be attributable to the aberrant regulation of apoptosis (6–9).

An apoptotic cell death program is initiated and activated as a result of diverse internal or external signals. Reactive oxygen species (ROS), intracellular Ca^{2+} disturbance, tumor necrosis factor (TNF), and Fas ligand (FasL) have been studied and shown to induce apoptosis (10–14). After a cell is committed to apoptosis, it will destroy and eliminate itself by activating the apoptotic cascade without causing any inflammatory response and disturbance to the surrounding cells. Several apoptotic pathways have been shown to be involved in the apoptotic cascades, depending on the stimulus. These include a mitochondrial-dependent pathway (15, 16), a ligand-mediated death receptor pathway (12), and an endoplasmic reticulum stress-induced pathway (13). Furthermore, the apoptotic signaling cascades usually involve expression of genes that regulate the execution of apoptosis (e.g., Bcl-2, Bax, and p53). A family of protease proteins called caspase (cysteine-dependent aspartate protease) has been suggested to be crucial in executing apoptosis. Specific initiator caspases (e.g., caspase-8, -9, and -12) are activated when the cell is exposed to the corresponding apoptotic stimuli. For example, TNF or FasL can initiate apoptosis through the death receptor pathway by activating caspase-8 (12, 17). Apoptosis stimulated by endoplasmic reticulum stress and intracellular Ca^{2+} disturbance has been reported to be related to the activation of caspase-12 (13, 18). In addition, caspase-9 has been shown to mediate the mitochondrial-dependent apoptosis through the interaction of procaspase-9 with apoptotic protease activating factor-1 (Apaf-1), dATP, and mitochondrial-released cytochrome *c* (16). Although different initiator caspases are recruited in different apoptotic pathways, it appears that the apoptotic signals finally converge on the activation of the common effector caspases (e.g., caspase-3, -6, and -7), which cause eventual destruction of the cell. However, it has been demonstrated that apoptosis can also be executed through the caspase-independent manner that involves the proteins with endonuclease activity (e.g., apoptosis inducing factor or AIF, and endonuclease G) (19, 20).

Only a few studies have attempted to investigate the incidence of apoptosis in muscles from adult individuals (21, 22). However, no study has shown conclusive justification for the functional or physiological role of apoptosis in postmitotic muscle cells in young healthy individuals. Therefore, it is still unclear to what extent apoptosis takes place in adult healthy postmitotic tissues. Nonetheless, apoptosis has been documented under extreme physiological and pathophysiological conditions in skeletal and cardiac muscles. For instance, apoptosis has been observed in aging (21, 23), intense physical stress (24), muscle dystrophy (25, 26), denervation (27), muscle unloading (28), ischemia-reperfusion (29, 30), and chronic heart failure (31, 32). However, the influence of regular moderate physical activity (i.e., exercise training) in apoptosis is still unknown. There is evidence showing that oxidative stress and heat shock proteins (HSPs) contribute to the mediation of apoptosis (10, 33–36). Increased oxidative stress has been shown to be related to the activation of apoptosis (10, 23, 36), whereas HSP70 has been suggested to be capable of inhibiting apoptosis (33, 35, 37, 38). Because exercise training has been consistently shown to increase the antioxidant defense capacity and elevate the expression of HSP in skeletal and cardiac muscles (39–44), it seems plausible to hypothesize that exercise training is able to decrease the level of apoptosis. Therefore, in the present study, we examined the effect of exercise training on apoptosis and tested the hypothesis that exercise training attenuates the extent of apoptosis in skeletal and ventricle muscles as measured 48 h after the last exercise training session.

MATERIALS AND METHODS

Animals

Three-month-old adult male Sprague Dawley rats (Harlan, Indianapolis, IN) were used in this study. The rats were housed in pathogen-free conditions at ~20°C. They were exposed to a reverse light condition of 12 h of light and 12 h of darkness each day and were fed rat chow and water ad libitum throughout the study period.

Treadmill exercise

Sixteen rats with similar body weights (range of 210–240 g) were randomly assigned to control (CON, $n=8$) or training (TR, $n=8$) groups. Animals assigned to the TR group were trained by running on a level motorized rodent treadmill (Columbus Instruments, Columbus, OH) 5 days weekly for 8 wk. During the first 4 wk, the speed of the treadmill and duration of the training sessions were gradually increased from a speed of 10 m·min⁻¹ for 10 min, to a running speed of 28 m·min⁻¹ for 55 min by the end of the 4th week. For the next 4 wk, a 5-min warm-up session at a speed of 20 m·min⁻¹ was followed by the 55-min training session at a speed of 28 m·min⁻¹. We have previously shown that

this training protocol stimulates a moderate aerobic endurance training effect, including a ~25% increase in citrate synthase activity in rat skeletal muscles (45).

Animals assigned to the CON group were handled daily and were subjected to the noise of the running treadmill by placing their cages next to the treadmill when their TR-matched partner ran on the treadmill. This procedure was designed to minimize any possible confounding effect of external factors (e.g., handling, treadmill noise) on the dependent variables. TR animals were sacrificed 48 h after the last training session. CON animals were euthanized at the same time as their partner TR animal. All animals were killed via CO₂ inhalation followed by decapitation, at which time the soleus muscle and 4–5 mm of the apex cordis from the heart muscle were quickly removed and frozen immediately in liquid nitrogen and stored at –80°C until further analysis.

All experimental procedures were approved by the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC), and following the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals as published by the U.S. Dept. of Health and Human Services and proclaimed in the Animals Welfare Act (PL89-544, PL91-979, and PL94-279) and fully conformed with the American Physiological Society's "Guiding Principles for Research Involving Animals and Human Beings."

RT-PCR

Total RNA was extracted from rat soleus and both left and right ventricles muscles with TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Frozen muscles were mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA were solubilized in RNase-free H₂O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was assured by obtaining an OD₂₆₀/OD₂₈₀ ratio of ~2.0. Two micrograms of RNA were reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 20 µl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). Control RT reactions were done in which the RT enzyme was omitted. The control RT reactions were PCR amplified to ensure that DNA did not contaminate the RNA. One microliter of complementary DNA (cDNA) was then amplified by PCR using 100 ng of each primer, ribosomal 18S primer pairs (Ambion, TX), 250 µM deoxyribonucleotide triphosphates (dNTPs), 1× PCR buffer, and 2 units *Taq* DNA polymerase (Sigma, St. Louis, MO) in a final volume of 50 µl. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany).

Primer pairs were designed against Bcl-2 (forward 5'CCGGGTGGCAGCTGACATGTTT3'; reverse 5'GCACAGGGCCTTGAGCACC AGTT3', GenBank accession no. U34964) and Bax (forward 5'CGGGCCCCAC CAGCTCTGAACA3'; reverse 5'GGGCGGCTGCTCCAAGGTCA3', GenBank accession no. NM_017059). The primers for Bcl-2 and Bax were designed with an annealing temperature of 55°C. All PCR products were verified by restriction digestion and by sequencing. Preliminary experiments were conducted with each gene to ensure that the number of cycles (36 cycles) represented a linear portion for the PCR OD curve for the muscle samples. The cDNA from all muscle samples was amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 15 µl of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide.

Images were captured, and the signals were quantified in arbitrary units as OD × band area using Kodak one-dimensional (1-D) image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. In the present study, ribosomal 18S primers were selected as internal controls because the level of 18S rRNA is not affected by endurance exercise training (46). All RT-PCR signals were normalized to the 18S signal of the corresponding RT product. This eliminated the measurement error from uneven samples loading and provided a semiquantitative measure of the relative changes in gene expression.

Protein extraction and fractionation

Cytoplasmic and nuclear protein extracts were obtained from soleus and ventricle muscles using the method described by Roethermel et al. (47). Fifty milligrams of soleus and ventricle muscles were homogenized on ice in 1 ml of ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol, pH 7.4). The homogenates were centrifuged at 1000 rpm for 1 min at 4°C. The supernatants contained the cytoplasmic protein fraction and were collected. The collected cytoplasmic extracts were further divided into two halves, and protease inhibitor cocktail (104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64) (P8340, Sigma-Aldrich, St Louis, MO) was added to one of them. The cytoplasmic protein fraction with protease inhibitors was used for Western blot analyses and cell death ELISA while the cytoplasmic fraction without protease inhibitors was used for caspase activity assay. The remaining nuclear pellet was resuspended in 360 µl of lysis buffer, and 39.8 µl of 5 M NaCl was added to lyse the nuclei. The mixture was then rotated for 1 h at 4 °C and centrifuged at 14,000 rpm for 15 min at 4 °C. The supernatants contained the nuclear protein fraction and were collected. Protease inhibitor cocktail (P8340, Sigma-Aldrich) was added to the collected nuclear

protein fraction. The total protein contents of the cytoplasmic and nuclear extracts were quantified in duplicate by using bicinchoninic acid reagents (Pierce, Rockford, IL) and bovine serum albumin (BSA) standards.

Western immunoblots

Protein expressions of Bcl-2, Bax, apoptotic protease activating factor-1 (Apaf-1), apoptosis-inducing factor (AIF), cleaved poly-ADP-ribose polymerase (PARP), cleaved caspase-3, cleaved/active caspase-9, HSP70, copper/zinc-superoxide dismutase (Cu/Zn-SOD), manganese-superoxide dismutase (Mn-SOD), and actin were determined in the soleus and ventricle muscles of CON and TR rats. Bcl-2, Bax, Apaf-1, AIF, cleaved caspase-3, cleaved/active caspase-9, HSP70, Cu/Zn-SOD, Mn-SOD, and actin were determined in the cytoplasmic protein fraction, while cleaved PARP was determined in the nuclear protein fraction.

Forty micrograms of soluble protein was boiled for 5 min at 100°C in Laemmli buffer, loaded on each lane of a 12% polyacrylamide gel, and separated by routine SDS-PAGE for 1.5 h at 20°C (48). The gels were blotted to nitrocellulose membranes (Bio-Rad, Hercules, CA) and stained with Ponceau S red (Sigma) to confirm equal loading and transfer of proteins to the membrane in each lane. Similar loading between the lanes was further verified by loading gels in duplicate with one gel stained with Coomassie blue. The membranes were blocked in 5% nonfat milk in Tris-buffered saline with 0.05% Tween 20 (TBS-T) at room temperature for 1 h and probed with anti-Bcl-2 mouse monoclonal antibody (1:200 dilution, sc-7382, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Bax rabbit polyclonal antibody (1:100 dilution, sc-493, Santa Cruz Biotechnology), anti-Apaf-1 rabbit polyclonal antibody (1:500 dilution, AB16503, Chemicon International, Temecula, CA), anti-AIF rabbit polyclonal antibody (1:1000 dilution, AB16501, Chemicon International), anti-cleaved PARP rabbit polyclonal antibody (1:500 dilution, 9545, Cell Signaling Technology, Beverly, MA), anti-cleaved caspase-3 rabbit polyclonal antibody (1:500 dilution, 9661, Cell Signaling Technology), anti-cleaved caspase-9 rabbit polyclonal antibody (1:500 dilution, 9507, Cell Signaling Technology), anti-active caspase-9 rabbit polyclonal antibody (1:200 dilution, ab2325, Abcam, Cambridge, UK), anti-HSP70 mouse monoclonal antibody (1:1000 dilution, SPA810, StressGen, Victoria, BC, Canada), anti-SOD-1 rabbit polyclonal antibody (1:200 dilution, sc-11407, Santa Cruz Biotechnology), anti-Mn-SOD goat antibody (1:1000 dilution, A300449A, Bethyl Lab, Montgomery, TX), or anti-actin rabbit antibody (1:1000 dilution, A2066, Sigma) diluted in TBS-T with 2% BSA. All primary antibody incubations were at 4°C overnight. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies at room temperature for 1 h (1:1000 dilution, AP124P for anti-mouse IgG antibody, AP132P for anti-rabbit IgG antibody, AP106P for anti-goat IgG antibody, Chemicon International), and the signals were developed by

chemiluminescence (34080, Pierce Biotechnology, Rockford, IL). The signals were visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak), and digital records of the films were captured with a Kodak 290 camera. The resulting bands were quantified as $OD \times \text{band area}$ by a one-dimensional (1-D) image analysis system (Eastman Kodak) and expressed in arbitrary units. The sizes of the immunodetected proteins were verified by using standard molecular-weight markers (Bio-Rad).

Caspase-3 activity assay

Enzymatic activity of caspase-3 was analyzed by a commercial caspase assay kit (APO-54A-019-KI01, Apotech, Switzerland) according to the manufacturer's procedure. Briefly, 50 μl of the cytoplasmic protein fraction without protease inhibitor of soleus and ventricle muscles were incubated in 50 μM of the 7-amino-4-trifluoromethyl coumarin (AFC)-conjugated substrate at 37°C for 3 h. Caspase specific inhibitor, Z-VAD-FMK (219007, Calbiochem, La Jolla, CA) was used as a control to confirm the specificity of caspase in the assay. Caspase activity was monitored by fluorometric detection. The change in fluorescence was measured on a spectrofluorometer with an excitation wavelength of 390 nm and an emission wavelength of 530 nm (CytoFluor, Applied Biosystems, Foster City, CA) before and after the 3-h incubation. Caspase activity was estimated as the change in arbitrary fluorescence units normalized to milligram protein. Measurements were performed in duplicate while CON and TR samples were run on the same microplate in the same setting.

DNA gel electrophoresis

Nuclear DNA fragmentation was determined by DNA laddering. DNA was isolated from ventricle muscles using DNeasy tissue DNA purification kit (Qiagen, Valencia, CA). Briefly, ventricle tissues were lysed using proteinase K in the lysis buffer overnight at 55°C. The lysed samples were then treated with DNase-free RNase (100 $\mu\text{g}\cdot\text{ml}^{-1}$) for 1 h at 50°C. DNA was then extracted and eluted using the silica-gel-membrane column provided with the kit. The concentration of isolated DNA was determined by measuring the absorbance at wavelength of 260 nm, and the purity of DNA was confirmed by A_{260}/A_{280} ratio of 1.8–2.0. Three micrograms of isolated DNA was electrophoresed on 1.5% agarose gel and visualized by staining with 1 $\mu\text{g}/\text{ml}$ ethidium bromide in Tris/acetate/EDTA (TAE) buffer (pH 8.5, 2 mM EDTA, and 40 mM Tris-acetate). Ten micrograms of 100-bp DNA ladder was included as a molecular size marker (15628019, Invitrogen Life Technologies). Gels were photographed under ultraviolet transillumination. DNA laddering was not performed on soleus muscles because the amount of soleus tissue was insufficient for qualitative analysis by DNA gel electrophoresis.

Cell death ELISA

Cell death detection ELISA kit (1544675, Roche Molecular Biochemicals, Mannheim, Germany) was used to quantitatively detect the cytosolic histone-associated DNA fragmentation, according to manufacturer's instructions. Briefly, the extracted cytoplasmic fractions of soleus and ventricle muscles were used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated to the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate for 10 min at 20°C. The change in color was measured at a wavelength of 405 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). All measurements were performed in duplicate, with CON and TR samples analyzed on the same microtiter plate in the same setting. The OD₄₀₅ reading was then normalized to the total amount of protein in the sample. The data were reported as an apoptotic index (OD₄₀₅·mg protein⁻¹) to indicate the level of cytosolic mono- and oligonucleosomes.

Statistics

Statistical analyses were performed using the SPSS 10.0 software package. Student's *t* test was used to examine differences between CON and TR groups. Relationships between given variables were examined by computing the Pearson product-moment correlation coefficient, *r*. Statistical significance was accepted at *P* < 0.05. All data are given as means ± SE.

RESULTS

DNA laddering and cell death ELISA

Representative ethidium bromide-stained DNA products after gel electrophoresis are shown in Figure 1A. We did not observe any apparent laddering pattern in the isolated DNA from the ventricles of our young adult rats. Nonetheless, there was no obvious qualitative difference between TR and CON groups. We further quantitatively estimated the DNA fragmentation by using a more sensitive technique of cell death ELISA in both ventricle and soleus muscles. The ELISA analyses indicated that the apoptotic index in ventricles of TR group was 15% lower than that in the ventricles of the CON group (*P* < 0.05, Figure 1B). We found that the apoptotic index in soleus of TR animals decreased by 33% when compared with that in soleus of CON animals, but the difference did not reach statistical significance (*P* = 0.05, Figure 1B).

Bcl-2 and Bax transcript and protein contents

Representative ethidium bromide-stained gels for the mRNA after RT-PCR are shown in Figures 2A

and 2B. Bcl-2 mRNA increased in soleus samples of TR animals by 48% higher as compared to soleus samples in CON animals ($P<0.01$), whereas a 35% higher content of Bcl-2 transcript was observed in ventricle muscles of TR animals when compared with that of CON animals ($P<0.01$, Figure 2A). Although Bax protein contents in TR animals were similar to that in CON animals, transcript contents of Bax in soleus muscle of TR group decreased by 35% when compared with that of CON group ($P<0.01$, Figure 2B). Bax transcript contents in ventricle muscles of TR and CON animals were not different ($P<0.05$, Figure 2B).

In our Western blot analyses, we found an immunoreactive band of ~25 kDa corresponding to the predicted molecular mass of Bcl-2 protein and a ~21 kDa immunoreactive band corresponding to Bax protein. Representative immunoreactive blots are shown in Figures 2C and 2D. Bcl-2 protein contents in soleus and ventricle muscles of TR animals estimated by Western analysis were 64% and 68% higher than that of CON animals, respectively ($P<0.05$, Figure 2C). However, no difference was found in Bax protein contents in both soleus and ventricle muscles between CON and TR groups ($P<0.05$, Figure 2D). Moreover, protein content of actin is not different between CON and TR groups in both the soleus and ventricle muscles ($P<0.05$, data not shown).

Apaf-1, AIF, cleaved PARP, and cleaved caspase-3 and -9 protein contents

Although Apaf-1 has been reported to be absent in human skeletal muscle (22), a detectable level of Apaf-1 was observed in Fischer 344 rat skeletal muscle by Western immunoblotting (21). In the present study, we also detected a ~130 kDa immunoreactive band corresponding to Apaf-1 protein by Western blotting. In the soleus of TR animals, Apaf-1 protein content was 49% lower than that in the soleus of CON animals ($P<0.05$, Figure 3A). No difference was found in the Apaf-1 protein content in the ventricle muscles between TR and CON groups ($P<0.05$, Figure 3A). AIF has been suggested to be a major factor mediating caspase-independent apoptosis (19). An immunoreactive band of ~67 kDa corresponding to the predicted molecular mass of AIF protein was detected in the cytoplasmic protein fraction of soleus and ventricle muscles. However, there was no difference in AIF protein contents in both soleus and ventricle muscles of TR animals when compared with that of CON animals ($P<0.05$, Figure 3B).

Caspase protease activity

Caspase-3 activity was measured biochemically in soleus and ventricle muscles of TR and CON animals using a fluorometric assay, which is a more sensitive technique to detect the activated caspase. However, caspase-3 activities in both the soleus and ventricle muscles were not different between TR and CON groups ($P<0.05$, Figure 4).

HSP70, Cu/Zn-SOD, and Mn-SOD protein levels

Western blot analyses demonstrated that the protein contents of HSP70 increased substantially in both soleus and ventricle muscles of TR animals when compared with that of CON animals. HSP70 protein content in the soleus of TR group increased by ~170% ($P<0.01$), whereas HSP70 protein content in the ventricle of TR group increased by ~2100% ($P<0.01$) when compared with that of CON groups (Figure 5A). The protein contents of Cu/Zn-SOD in both soleus and ventricle muscles of TR animals were similar to that of CON animals ($P<0.05$, Figure 5B). However, the protein contents of Mn-SOD increased in both soleus and ventricle muscles of TR animals when compared with that of CON animals. Mn-SOD protein contents increased by 64% and 39% in soleus and ventricle muscles of the TR group as compared with the CON group, respectively ($P<0.05$, Figure 5C).

Relationships of HSP70 and apoptotic factors

The relationships between HSP70 and Bcl-2 protein and transcript as well as Bax transcript were analyzed by examining the corresponding Pearson's correlation coefficient (r). When the soleus and ventricle muscles of both groups were collapsed and treated as a single group, HSP70 protein content was positively correlated with the Bcl-2 protein content ($r=0.483$, $P=0.005$, $n=32$, Figure 6A). We found that the HSP70 protein content was correlated with the Bcl-2 protein content in the ventricle muscles when the CON and TR animals were pooled as a group ($r=0.589$, $P=0.016$, $n=16$, data not shown). A positive relationship was also found between the HSP70 protein content and the Bcl-2 protein content when the soleus and ventricle muscles of CON animals were pooled together ($r=0.621$, $P=0.010$, $n=16$, data not shown). In addition, we found that the HSP70 protein content was correlated with the Bcl-2 protein content in the ventricle muscles of CON animals ($r=0.742$, $P=0.035$, $n=8$, data not shown).

Furthermore, HSP70 protein content was positively correlated with the Bcl-2 transcript content in the soleus muscles of CON animals ($r=0.739$, $P=0.036$, $n=8$, Figure 6B). When the CON and TR animals were pooled as a group, HSP70 protein content was negatively correlated with the Bax transcript content in the soleus muscles ($r=-0.525$, $P=0.037$, $n=16$, Figure 6C).

Relationships of Mn-SOD and apoptotic factors

Mn-SOD protein content was negatively correlated with the apoptotic index in the ventricle muscles of the CON animals ($r=-0.961$, $P=0.0001$, $n=8$, Figure 7A). When the ventricle muscles of the CON and the TR groups were collapsed and treated as a single group, Mn-SOD protein content was negatively correlated with the apoptotic index ($r=-0.539$, $P=0.031$, $n=16$, data not shown). A negative relationship was also found between the Mn-SOD protein content and the caspase-3 activity when the soleus and ventricle muscles of CON animals were pooled together ($r=-0.535$, $P=0.033$,

$n=16$, Figure 7B). Furthermore, Mn-SOD protein content was positively correlated with Bcl-2 transcript content when the soleus and ventricle muscles of the CON and TR animals were pooled ($r=0.381$, $P=0.032$, $n=32$, Figure 7C). When the soleus muscles of the CON and TR animals were pooled as a group, Mn-SOD protein content was positively correlated with HSP70 protein content ($r=0.729$, $P=0.001$, $n=16$, Figure 7D).

DISCUSSION

The present study provides evidence that exercise training is capable of influencing apoptosis in skeletal and cardiac muscles in young adult rats. We showed that exercise training attenuates the extent of apoptosis in cardiac and skeletal muscles when measurements are taken at 48 h after the last exercise bout. We demonstrated that positive relationships exist between the protein level of HSP70 and both Bcl-2 protein and mRNA levels, whereas an inverse relationship exists between the protein level of HSP70 and Bax mRNA level. We also found that Mn-SOD protein content is positively correlated to Bcl-2 transcript content and HSP70 protein content, whereas negative correlational relationships exist between Mn-SOD protein content and apoptotic index and caspase-3 activity. Our data are consistent with the hypothesis that HSP70 and Mn-SOD may play an anti-apoptotic role in modulating the homeostasis of apoptotic factors in skeletal and cardiac muscles after exercise.

Apoptosis and exercise

The influence of exercise training on apoptosis has not been fully defined, and only a few studies have attempted to study the relationship of acute strenuous exercise with apoptosis in skeletal muscle (24, 49–51). Sandri and colleagues investigated the influence of acute intense exercise on apoptosis in skeletal muscles of both young normal and dystrophin-deficient (*mdx*) mice (24). They found that the DNA double strand breaks (as an indicator of DNA fragmentation) measured by in situ terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) increased by ~20%, 48 h after a night of spontaneous wheel running in both normal and *mdx* mice skeletal muscles. Using DNA gel electrophoresis, they reported that the amount of fragmented chromosomal DNA was greater in the skeletal muscles from *mdx* mice after the 16 h spontaneous wheel running when compared with their nonexercise partners. The same investigators reported that TUNEL-positive myonuclei existed after 16 h of spontaneous running in skeletal muscles of both C57B and *mdx* mice (51). Furthermore, they found that Bcl-2 protein levels decreased after spontaneous exercise in muscle of *mdx* mice. Similar results of increased apoptotic nuclei and a decreased ratio of Bcl-2/Bax have also been documented in skeletal muscles after a 16 h spontaneous wheel running in normal healthy animals (49, 50).

Although activation of apoptosis in muscles following acute bout of exercise has not been fully elucidated, these studies provided evidence suggesting that apoptosis increases following acute

strenuous exercise in the skeletal locomotion muscles. However, it is unclear that the proposed increase in muscle apoptosis after intense exercise plays a protective role in hastening the muscle recovery process or accelerates the muscle damage. Nonetheless, increased production of ROS and glucocorticoids during intense exercise has been suggested to be the possible reason for increased apoptosis after acute intense exercise (52). In contrast to acute strenuous exercise, the effect of exercise training on apoptosis is unknown. Furthermore, although increased apoptosis after ischemia-reperfusion in cardiac myocytes has been generally documented (29), few data exist to describe the influence of exercise training in cardiac tissue.

In the present study, we have demonstrated that exercise training reduced DNA fragmentation, increased Bcl-2 protein and transcript levels, decreased Bax transcript levels, and decreased Apaf-1 protein levels in rat soleus and cardiac muscles when compared with sedentary control animals. This is the first study to show a relationship between regular moderate-intensity exercise and reduced levels of pro-apoptotic genes in postmitotic tissues (skeletal and cardiac muscles).

In the present study, we observed that apoptotic measurements differed in soleus and ventricle muscles after training. For example, we found that the apoptotic index in the ventricle was lower in the TR compared with the CON group. On the other hand, there was a trend but no significant differences in the apoptotic index in soleus muscle samples of TR and CON animals. Nevertheless, transcript levels of Bax and protein levels of Apaf-1 decreased in soleus muscle of TR animals, but these did not change in the ventricle muscles of TR animals as compared with their respective muscles in CON animals. The differences in the apoptotic markers between soleus and cardiac muscles may be explained by the differences in the total numbers of nuclei in skeletal and cardiac muscles. Structurally, skeletal muscle cells are multinucleated, whereas cardiomyocytes compose fewer nuclei per myocyte (typically ~1–2 nuclei in each cardiomyocyte), so that skeletal myofibers have a higher nuclei-to-cytoplasmic ratio than do cardiomyocytes (53–55). Furthermore, myocardial nuclei readily enlarge or decrease their DNA size depending on the loading conditions placed on the heart (56), and loading would be expected to increase with aerobic exercise. In contrast, endurance exercise would not be expected to result in increase DNA synthesis in skeletal muscles.

Because it has been proposed that apoptosis may be activated in particular “segmental” domains of a skeletal myofiber and cause elimination of individual myonuclei during skeletal muscle remodeling (e.g., muscle atrophy) (28, 57), it is reasonable to expect that some nuclei in the same myofiber may undergo apoptosis, whereas others do not. This would tend to “dilute” the overall apoptotic index, relative to the total skeletal muscle and nuclear samples. Nevertheless, the signals regulating apoptosis (e.g., Bax, Bcl2) may need to be expressed at higher levels to induce apoptotic control in skeletal muscles, simply because there is a greater number of nuclei per cytoplasm in

myofibers vs. cardiomyocytes (53–55). Thus, it is possible that Bax and Bcl2 proteins required greater relative changes in skeletal muscle to affect changes in the apoptotic environment as compared with cardiac muscle. Furthermore, according to our results, it appears that the regulators of the mitochondrial-mediated caspase-dependent apoptotic pathway (e.g., Bcl-2, Bax, and Apaf-1) are more responsive to moderate exercise training when compared with the components of caspase-independent apoptotic pathways (e.g., AIF). Additional studies will require additional non-caspase-dependent markers to determine whether this pathway is also sensitive to aerobic training.

A variety of proteins, including Bcl-2, Bax, cytochrome *c*, Apaf-1, caspase-9, and caspase-3, are critically involved in regulating mitochondrial-mediated apoptosis. Although we have demonstrated increases in Bcl-2 as well as decreases in Bax and Apaf-1 in our TR animals, we did not find differences in caspase-3 protease activity between TR and CON groups. The failure to obtain decreased caspase-3 protease activity in the TR animals may be due to the timing of tissue collection. Because our measurements were performed on the tissues that were collected 48 h after the last training session, it is possible that we missed changes in caspase-3 activity that may have occurred in the TR animals at other time points that were not examined in this study.

We were unable to detect immunoactive bands corresponding to the predicted molecular mass of cleaved PARP, cleaved caspase-3, and cleaved caspase-9 in our Western immunoblots. Although previous studies have shown immunopositive bands for these proteins in muscle tissues (22, 58), our data did not allow us to determine whether the failure to detect cleaved PARP, cleaved caspase-3, and cleaved caspase-9 in our immunoblots was the result of having levels of these proteins that were below the detectable limits of our assays or the result of low antibody sensitivity.

ROS has been widely believed to be one of the potent stimuli activating apoptosis in a variety of in vitro and in vivo experimental models of different cell types (15, 34). It has also been suggested that ROS influences apoptosis mainly through the modulation of the mitochondrial-mediated pathway (59). It has been hypothesized that a high oxidative stress level destabilizes the mitochondrial membrane homeostasis and therefore induces the formation of mitochondrial membrane permeability pores and release of pro-apoptotic factors (e.g., cytochrome *c*) (59). It has also been shown that high levels of oxidative stress induced by dietary selenium and vitamin E deficiencies increase caspase-like activity and DNA fragmentation (as measured by DNA gel electrophoresis) in chick skeletal myocytes (60). Bcl-2 family proteins are known to be responsible for the modulation of mitochondrial membrane pore formation and therefore regulating mitochondrial-mediated apoptosis.

We have shown that Bcl-2, an anti-apoptotic gene product, was up-regulated and that Bax, a pro-apoptotic gene product, was down-regulated in muscle samples taken 48 h after the last bout of an 8-wk exercise training program in rats. Exercise training of ~10 wk has been demonstrated to improve the antioxidant capacity in skeletal and cardiac muscles (41, 43). Several antioxidant

enzymes, including Cu/Zn-SOD, catalase, glutathione peroxidase, glutathione reductase, and mitochondrial Mn-SOD have been implicated as crucial endogenous antioxidant enzymes in biological systems. In the present study, we have demonstrated that the protein content of Mn-SOD increases (by 64% in soleus and 39% in ventricle muscle) in the TR animals relative to the CON animals after 8 wk of endurance training. These observations suggest that exercise training may attenuate muscle apoptosis. Our data are consistent with the idea that an increased antioxidant capacity and modulated oxidative stress from exercise training may be involved in reducing pro-apoptotic genes.

Apoptosis and HSP70

HSPs are a group of highly conserved proteins induced by a variety of stresses, including hyperthermia, pH disturbance, and oxidative stress. There is ample evidence showing that HSPs play an important role in maintaining the housekeeping cellular function and therefore cell survival. HSPs function by controlling the protein folding in cytosol, endoplasmic reticulum, and mitochondria; preventing protein denaturing and aggregation during stress; accelerating the breakdown of damaged and unwanted proteins; refolding the misfolded proteins; and performing as molecular chaperones (40, 42). Among the different classes of HSP, the HSP70 family constitutes the most conserved and extensively studied class of HSPs. It has been recently suggested that HSP70 is a potent anti-apoptotic protein (61). There is evidence supporting the hypothesis that HSP70 inhibits apoptosis by modulating the mitochondrial-mediated pathway (33, 62, 63). Li and colleagues (62) reported that HSP70 inhibits apoptosis by suppressing the formation of apoptosomes due to the effect on downstream of cytochrome *c* release and upstream of caspase-3 activation. Beere and colleagues have shown that HSP70 inhibits apoptosis by binding directly to Apaf-1, an adaptor protein involved in apoptosome formation, and thereby preventing the recruitment of procaspase-9 to the apoptosome (33). The suppressive effect of HSP70 on the formation of apoptosome leads to the reduced caspase-9 activation as well as caspase-3 cleavage and therefore decreases nuclear fragmentation and apoptosis. AIF, a mitochondrial-released protein with endonuclease activity, has also been suggested to be one of the molecular targets for the apoptosis-inhibiting effect of HSP70 (64, 65). The anti-apoptotic effect of HSP70 on AIF suggests that HSP70 inhibits apoptosis by suppressing both the caspase-dependent (apoptosome in mitochondrial-mediated apoptosis) and caspase-independent pathways (e.g., AIF). Although we have demonstrated significant negative relationships between HSP70 and apoptotic markers following exercise training, these findings do not indicate a causative function of HSP70 in regulating apoptosis. The underlying cellular and molecular mechanisms involved in training-induced adaptations in apoptosis and HSP70 are unknown and require further investigation.

In conclusion, these data support the hypothesis that exercise training is able to attenuate the extent of apoptosis in ventricle and skeletal muscles of young adult animals. We have demonstrated that significant correlational relationships exist between HSP70 and Bcl-2 protein and transcript levels as well as Bax transcript levels in skeletal and cardiac muscles. We have also shown significant negative correlations between Mn-SOD and apoptotic index and caspase-3 activity as well as significant positive correlations of Mn-SOD, Bcl-2 transcript levels, and HSP70. Although the molecular mechanism for the training-induced attenuation in apoptosis is still not completely understood, our findings are in agreement with the hypothesis that HSP70 and Mn-SOD play an anti-apoptotic role in training-induced decreases in apoptosis in postmitotic tissues, including skeletal and cardiac myocytes. Our data show that caspase-sensitive markers of apoptosis decrease with endurance training; however, additional research is required to determine whether caspase-independent pathways are also sensitive to exercise training in skeletal and cardiac muscles. Although there is evidence to suggest that apoptosis occurs in muscles with aging or disuse (21, 23, 28, 66–68), it is unclear to what extent apoptosis occurs in otherwise healthy postmitotic tissues of young individuals. Nevertheless, the findings of this study suggest that adopting exercise training as part of a lifelong regimen may provide a more favorable environment to reduce or delay apoptosis in skeletal and cardiac muscles when they are exposed to some apoptotic stimulus such as aging (sarcopenia) or disuse-induced atrophy.

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Figure 1A DNA Laddering

Legend for Figure 1A Qualitative analysis of DNA fragmentation by DNA gel electrophoresis in ventricle muscles of TR and CON animals. DNA was extracted from ventricle muscles and 3 μ g of DNA was loaded on 1.5% agarose gel. A 100-bp DNA ladder was used as a molecular size marker. DNA was visualized with ethidium bromide staining. CON, control; TR, trained.

Figure 1B Apoptotic index

Legend for Figure 1B Apoptotic index defined by the quantification of mono- and oligonucleosomes in soleus and ventricle muscles of CON and TR animals. The OD₄₀₅ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from CON animals. CON, control; TR, trained.

Figure 2 Bcl-2 and Bax mRNA and protein content**A. Bcl-2 transcript content** **C. Bcl-2 protein content** **B. Bax transcript content** **D. Bax protein content**

Legend for Figure 2A Bcl-2 mRNA content was estimated by RT-PCR. The insert shows a representative result of PCR in soleus and ventricle muscles isolated from CON and TR animals. PCR products were visualized with ethidium bromide staining. Quantification of PCR signals was obtained by densitometric analysis of the signal product optical density (OD) x resulting band area. Gene expression is normalized to the ribosomal 18S signal from the same RT product. The normalized data are presented as means $\pm$ SE. Data were run in duplicate on different gels for each gene. *P < 0.01, data are significantly different from CON animals. CON, control; TR, trained rats.

Legend for Figure 2B Bax mRNA content was estimated by RT-PCR. The insert shows a representative PCR results from soleus and ventricle muscles isolated from CON and TR animals. PCR products were visualized with ethidium bromide staining. Quantification of PCR signals was by densitometric analysis of the signal product optical density (OD) x resulting band area. Gene expression is normalized to the ribosomal 18S signal from the same RT product. The normalized data are presented as means $\pm$ SE. Data were run in duplicate on different gels. *P < 0.01, data are significantly different from CON animals. CON, control; TR, trained rats

Legend for Figure 2C Bcl-2 protein content was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10^7 . The insert shows a representative blot for Bcl-2 in soleus and ventricle muscles isolated from CON and TR animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from CON animals. CON, control; TR, trained rats.

Legend for Figure 2D Bax protein content was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10^8 . The insert shows a representative blot for Bax in soleus and ventricle muscles isolated from CON and TR animals. The data are presented as means $\pm$ SE. CON, control; TR, trained, rats.

Figure 3A Apaf-1 protein content

Legend for Figure 3A Apaf-1 protein content was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁷. The insert shows a representative blot for Apaf-1 in soleus and ventricle muscles isolated from CON and TR animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from CON animals. CON, control; TR, trained.

Figure 3B AIF protein content

Legend for Figure 3B AIF protein content was determined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁷. The insert shows a representative blot for AIF in soleus and ventricle muscles isolated from CON and TR animals. The data are presented as means $\pm$ SE. CON, control; TR, trained rats.

Figure 4 **Caspase-3 activity**

Legend for Figure 4 Caspases-3 activities for soleus and ventricle muscles in CON and TR animals. The change in fluorescence during the 3h incubation of caspase-3 activity assay is normalized to the total protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. CON, control; TR, trained rats.

Figure 5A HSP70 protein content

Legend for Figure 5A HSP70 protein content was examined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁷. The insert shows a representative blot for HSP70 in soleus and ventricle muscles isolated from CON and TR animals. The data are presented as means $\pm$ SE. **P < 0.01, data are significantly different from CON animals. CON, control; TR, trained rats.

Figure 5B Cu/Zn-SOD protein content

Legend for Figure 5B Cu/Zn-SOD protein content was examined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁷. The insert shows a representative blot for Cu/Zn-SOD in soleus and ventricle muscles isolated from CON and TR animals. The data are presented as means $\pm$ SE. CON, control; TR, trained rats.

Figure 5C Mn-SOD Protein Content

Legend for Figure 5C Mn-SOD protein content was examined by Western blot analysis. The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10⁷. The insert shows a representative blot for Mn-SOD in soleus and ventricle muscles isolated from CON and TR animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from CON animals. CON, control; TR, trained rats.

Figure 6 **Correlations between HSP70 and apoptotic factors**

Legend for Figure 6A The relationship between the protein contents of HSP70 and Bcl-2. The soleus and ventricles of CON and TR animals were collapsed and treated as a single pooled group. A positive correlation coefficient (r) is established between the protein contents of HSP70 and Bcl-2 ($N=32$). CON, control; TR, trained rats.

Legend for Figure 6B The relationship between the protein content HSP70 and the transcript content of Bcl-2. The Bcl-2 transcript content is normalized to 18S rRNA and is expressed as relative optical density (OD) x band area. A positive correlation coefficient (r) is established between the protein content of HSP70 and the transcript content of Bcl-2 in soleus of CON group ($N = 8$). CON, control rat.

Legend for Figure 6C The relationship between the protein content of HSP70 and transcript content of Bax. The soleus of CON and TR animals were collapsed and treated as a single pooled group. A negative correlation coefficient (r) is established between the protein contents of HSP70 and the transcript content of Bax ($N = 16$). CON, control; TR, trained rats.

Figure 7 Correlations between Mn-SOD and apoptotic factors

Legend for Figure 7A The relationship between the protein contents of Mn-SOD and apoptotic index. A negative correlation coefficient (r) is established between the protein contents of Mn-SOD and apoptotic index in the ventricle muscle of CON group ($N=8$). CON, control rats.

Legend for Figure 7B The relationship between the protein content of Mn-SOD and caspase-3 activity. The soleus and ventricle muscles of CON animals were collapsed and treated as a single pooled group. A negative correlation coefficient (r) is established between the protein content of Mn-SOD and caspase-3 activity ($N = 16$). CON, control rats.

Legend for Figure 7C The relationship between the protein content of Mn-SOD and transcript content of Bcl-2. The soleus and ventricles of CON and TR animals were collapsed and treated as a single pooled group. A positive correlation coefficient (r) is established between the protein contents of Mn-SOD and the transcript content of Bcl-2 ($N = 32$). CON, control; TR, trained rats.

Legend for Figure 7D The relationship between the protein content of Mn-SOD and HSP70. The soleus of CON and TR animals were collapsed and treated as a single pooled group. A positive correlation coefficient (r) is established between the protein contents of Mn-SOD and HSP70 ($N = 16$). CON, control; TR, trained rats.

CHAPTER 12

Response of XIAP, ARC, and FLIP apoptotic suppressors to 8-weeks of treadmill running in rat heart and skeletal muscles

ABSTRACT

Although it has been demonstrated that exercise training has an anti-apoptotic effect on postmitotic myocytes, the mechanisms responsible for this effect are still largely unclear. Since the anti-apoptotic effect of exercise training in postmitotic myocytes could be possibly mediated by the upregulation of apoptotic suppressors, this study examined the effect of endurance training on endogenous apoptotic suppressors including XIAP, ARC, and FLIP in skeletal and cardiac muscles. Eight adult Sprague Dawley rats were trained 5 days weekly for 8 weeks on treadmill and eight sedentary rats served as controls. Soleus and ventricle muscles were dissected 2 days after the last training session. The mRNA content of XIAP, ARC, and FLIP was estimated by RT-PCR with ribosomal 18S RNA used as an internal control. The protein expression of XIAP, ARC, FLIP_S and FLIP_α was assessed by Western immunoblot. Following training, mRNA content of ARC and FLIP was not different between the control and trained animals whereas XIAP mRNA content was elevated by 22% and 14% in the trained soleus and cardiac muscles, respectively, relative to the control samples. No difference was found in the protein content of FLIP_S and FLIP_α between control and trained muscles while XIAP and ARC protein content was increased by 18% and 38%, correspondingly, in the soleus muscle of trained animals. Furthermore, negative relationships were found between XIAP and apoptotic DNA fragmentation as well as ARC and caspase-3 activity. These findings are consistent with the hypothesis that the modulation of apoptotic suppressors is involved in training-induced attenuation of apoptosis in skeletal and cardiac muscles.

Key words: Exercise; mitochondria; cell death receptor; apoptotic inhibitors

INTRODUCTION

Apoptosis is a highly organized and tightly coordinated biological process, which plays a vital role in monitoring a variety of cellular events (e.g., tissue turnover). Aberrant regulation of apoptosis has been implicated in the pathogenesis of certain severe muscle-related diseases including Duchenne and facioscapulo human muscular dystrophy (33-35). The cellular decision resulting in the activation and subsequent execution of the apoptotic program is principally controlled under the simultaneous influence of both pro- and anti-apoptotic signaling which are primarily orchestrated by a specific cluster of apoptotic regulatory proteins (e.g., BCL-2 family and caspases). Among a number of proteins that have been identified in regulating the apoptotic machinery, a group of endogenous proteins referring to X-linked inhibitor of apoptosis protein (XIAP), apoptosis repressor with caspases recruitment domain protein (ARC), and [Fas-associated death domain protein-like interleukin-1 β -converting enzyme]-like inhibitory protein (FLIP) constitute an assembly of elemental apoptotic suppressors in modulating the anti-apoptotic signaling.

XIAP has been suggested to be a potent member of a protein family named inhibitor of apoptosis (IAP) which is fundamentally conserved among various species (7, 39). All known IAP proteins have been exhibited to include at least one baculovirus IAP repeat (BIR) motif that is essential for their anti-apoptotic activity through the inhibition on the initiator and effector caspases (6, 32, 39). Furthermore, ARC and FLIP are two other endogenous proteins abundantly expressed in muscle tissues which have been identified in constituting the anti-apoptotic modulating mechanism (15, 19). Although it has been shown that the suppressive effect of ARC and FLIP on apoptosis is related to their negative effects on the selected caspases and is mostly associated with the death receptor-mediated apoptotic pathway (2, 15, 19), there have been recent data suggesting that the anti-apoptotic function of ARC may be also mediated by influencing the mitochondria-mediated apoptosis pathway (e.g., interferes with the activation of pro-apoptotic Bax) (12). In addition, the suppressive action of FLIP in apoptosis may be dependent on the individual splice variant or protein isoform of FLIP (10, 15, 18).

Previously, we have demonstrated that exercise training is capable of attenuating apoptosis in skeletal and cardiac muscles of adult rats and we reported that the regulatory mechanisms responsible for the anti-apoptotic effect of training comprise the alteration of Bcl-2, Bax, Apaf-1, HSP70, and MnSOD (41). Nonetheless, whether apoptotic suppressor proteins are involved in causing the attenuation of apoptosis following exercise training remains unknown. Thus, in this study, we examined the response of the apoptotic suppressors (XIAP, ARC, and FLIP) to 8-week endurance training in skeletal and cardiac muscles. We tested the hypothesis that the apoptotic suppressors are upregulated following exercise training, which is part of the cause for the anti-apoptotic adaptation in skeletal muscle and heart.

METHODS

Animals and treadmill training. Adult male Sprague Dawley rats aged 3 mo (Harlan, Indianapolis, IN) were studied. The rats were housed in pathogen-free conditions at $\sim 20^{\circ}\text{C}$. They were exposed to a reverse light condition of 12 h of light and 12 h of darkness each day and were fed rat chow and water ad libitum throughout the study period. Sixteen rats with similar body weights (range of 210-240 g) were randomly assigned to control ($N = 8$) or training ($N = 8$) groups. The animals were trained by running on a level motorized rodent treadmill (Columbus Instruments, Columbus, OH) 5 days weekly for 8 weeks. The training intensity and duration were progressively increased and we have shown that this training protocol stimulates moderate endurance training effect with a $\sim 25\%$ increase in citrate synthase activity in rats (42, 43). For the first 4 weeks, the speed of the treadmill and duration of the training sessions were gradually increased from a speed of $10 \text{ m}\cdot\text{min}^{-1}$ for 10 minutes, to a running speed of $28 \text{ m}\cdot\text{min}^{-1}$ for 55 minutes by the end of the 4th week. For the next 4 weeks, a 5-minute warm-up session at a speed of $20 \text{ m}\cdot\text{min}^{-1}$ was followed by the 55-minute training session at a speed of $28 \text{ m}\cdot\text{min}^{-1}$. Control animals were handled daily and were subjected to the noise of the running treadmill by placing their cages next to the treadmill when the training animals ran on the treadmill. This procedure aimed to minimize the possible confounding effect of external factors (e.g., handling, treadmill noise etc.). Training animals were sacrificed 48 h after the last training session while the control animals were euthanized at the same time as training animals. All animals were killed via CO_2 inhalation followed by decapitation at which time the soleus muscle and the apex cordis from the heart muscle were quickly removed and frozen immediately in liquid nitrogen and stored at -80°C until further analysis.

All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC) and following the policies and procedures detailed in the Guide for the Care and Use of Laboratory Animals as published by the U.S. Dept. of Health and Human Services.

RT-PCR. Total RNA was obtained from soleus and ventricle muscles of control and trained animals with TriReagent (Molecular Research Center, Cincinnati, OH), which is based on the guanidine thiocyanate method. Briefly, frozen muscles were minced then mechanically homogenized on ice in 1 ml of ice-cold TriReagent. Total RNA was solubilized in RNase-free H_2O and quantified in duplicate by measuring the optical density (OD) at 260 nm. Purity of RNA was determined by examining the $\text{OD}_{260}/\text{OD}_{280}$ ratio. Two micrograms of RNA was reverse transcribed with decamer primers and Superscript II reverse transcriptase (RT) in a total volume of 20 μl according to standard methods (Invitrogen Life Technologies, Bethesda, MD). A control RT reaction was done in which

the RT enzyme was omitted. The control RT reaction was PCR amplified to ensure that DNA did not contaminate the RNA. One μ l of complementary DNA (cDNA) was then amplified by PCR using 100 ng of forward and reverse primers, ribosomal 18S primer pairs (Ambion, TX), 250 μ M deoxyribonucleotide triphosphates (dNTPs), 1 x PCR buffer, and 2.5 units *Taq* DNA polymerase (USB Corp., Cleveland, OH) in a final volume of 50 μ l. PCR was performed using a programmed thermocycler (Biometra, Göttingen, Germany). The primer pairs were designed from sequences published in GenBank (**Table 1**) and PCR products were verified by restriction digestions. Preliminary experiments were conducted with each gene to assure that the number of cycles represented a linear portion for the PCR optical density curve for the muscle samples. The cDNA from all muscle samples were amplified simultaneously using aliquots from the same PCR mixture. After the PCR amplification, 30 μ l of each reaction was electrophoresed on 1.5% agarose gels, stained with ethidium bromide. Images were captured and the signals were quantified in arbitrary units as optical density (OD) x band area using Kodak image analysis system (Eastman Kodak, Rochester, NY). The size (number of base pairs) of each of the bands corresponded to the size of the processed mRNA. Ribosomal 18S primers were used as internal controls while all RT-PCR signals were normalized to the 18S signal of the corresponding RT product to eliminate the measurement error from uneven samples loading and provide a semi-quantitative measure of the relative changes in gene expression.

Protein preparation and Western immunoblot. The protein extraction method described by Rothermel et al. (30) was adopted with minor modification to obtain the total cytosolic protein fraction from the soleus and ventricle muscles. In brief, after removal of connective tissues, muscle was minced and homogenized on ice in ice-cold lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol). Following centrifuging at 1,000 rpm for 1 min at 4°C to pellet the nuclei and cell debris, the supernatants were further centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual nuclei and stored as total cytosolic protein extract at -80°C after a protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO) was added. The protein content of the extract was then measured in duplicate by DC Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay (23) As a further measure to assure the protein content, all the protein samples were quantified in duplicate on a different occasion by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation (44).

Forty micrograms of protein was boiled for 5 min at 95°C in Laemmli buffer (161-0737, BioRad, Hercules, CA) in the presence of 2-mercaptoethanol and was loaded on each lane of a 12%

polyacrylamide gel and separated by SDS-PAGE at room temperature. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and probed with anti-hILP/XIAP mouse monoclonal antibody (1:250 dilution, 610762, BD Biosciences, San Jose, CA), anti-ARC rabbit polyclonal antibody (1:200 dilution, sc-11435, Santa Cruz Biotechnology, Santa Cruz, CA), or an anti-FLIP_{S/L} mouse monoclonal antibody (1:100 dilution, sc-5276, Santa Cruz Biotechnology, Santa Cruz, CA) diluted in PBS-T with 2% BSA. It is noted that we did not detect the FLIP_L by using the anti-FLIP_{S/L} mouse monoclonal antibody (sc-5276), therefore an anti-FLIP _{α} (i.e., FLIP_L) rabbit polyclonal antibody (1:500 dilution, ab6144, Abcam, Cambridge, MA) was further used to detect the α -splice variant or long isoform of FLIP. Membrane was also probed with anti-beta tubulin rabbit polyclonal antibody (1:500 dilution, ab6046, Abcam, Cambridge, MA) to examine the equality of protein loading. All primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (1:3000 dilution, Chemicon International, Temecula, CA), and signals were developed by ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The predicted molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Statistical analyses. Statistics were performed using the SPSS 10.0 software package. Independent *t*-test was used to examine differences between control and trained groups. Relationships between given variables were examined by computing the Pearson product-moment correlation coefficient, *r*. Statistical significance was accepted at $P < 0.05$. All data are given as means $\pm$ standard error of mean (SE).

RESULTS

mRNA content of XIAP, ARC, and FLIP. Transcriptional expression of XIAP, ARC, and FLIP genes was analyzed by semi-quantitative RT-PCR in soleus and cardiac muscles of trained and control animals. Inverted images of the representative ethidium bromide-stained gels for the PCR amplified products are shown in the insets of **Figure 1**. Following exercise training, we found that the XIAP mRNA content was elevated by 22% ($P < 0.01$) and 14% ($P < 0.05$) in soleus and cardiac

muscles, respectively, when compared to the control muscles (**Figure 1**). The transcriptional expression of ARC and FLIP was similar in both soleus and cardiac samples obtained from trained and control animals ($P > 0.05$).

Insert Figure 1 —————→ mRNA content of XIAP, ARC, and FLIP

Protein content of XIAP, ARC, FLIP_S, and FLIP_α. An immunoreactive band of ~57 kDa corresponding to the predicted molecular mass of XIAP protein and ~25 kDa immunoreactive band corresponding to ARC protein was detected in our immunoblots. According to the Western analysis, XIAP protein content in soleus muscle of the trained animals was 18% higher ($P < 0.05$) than that of the control animals whereas the protein content of XIAP was similar in cardiac muscle obtained from the trained and control animals (**Figure 2**). Although the ARC protein content was similar in the control and trained cardiac muscles, in the trained soleus muscle, the protein content of ARC was elevated by 38% ($P < 0.05$) relative to the control soleus muscle (**Figure 2**). In contrast, no differences were found in FLIP_S or FLIP_α protein content between control and trained animals ($P > 0.05$).

Insert Figure 2 —————→ Protein content of XIAP, ARC, FLIP_S and FLIP_α

Relationships of XIAP, ARC, FLIP, and apoptotic markers. We have previously found relationships between XIAP, ARC and FLIP with apoptotic markers including caspase-3 activity and apoptotic DNA fragmentation (41). In this study we found a negative Pearson's correlation coefficient (r) between XIAP protein content and apoptotic DNA fragmentation (expressed as apoptotic index) in the control and trained soleus muscles ($r = -0.647$, $P = 0.007$, $N = 16$) when the soleus and ventricle muscles of control and trained animals were treated as a single group ($r = -0.512$, $P = 0.003$, $N = 32$). The ARC mRNA content was negatively correlated with caspase-3 activity when the soleus and ventricle samples of the control and trained animals were pooled as a single group ($r = -0.415$, $P = 0.018$, $N = 32$). Individually, the correlation between ARC mRNA and caspase-3 activity in the ventricle muscles of control and trained animals was $r = -0.624$ ($P = 0.01$, $N = 16$), and in the trained ventricle muscles this relationship was $r = -0.720$ ($P = 0.044$, $N = 8$).

DISCUSSION

We have previously demonstrated that 8-weeks of moderate endurance training attenuates apoptosis in skeletal and cardiac muscles of young adult rats (41). Here we extend our prior findings by showing that endogenous apoptotic suppressors (XIAP and ARC) appear to be involved in mediating the anti-apoptotic effect of exercise training in postmitotic myocytes. We demonstrated that the mRNA

and protein content of XIAP and the protein content of ARC were elevated in the soleus muscle while XIAP mRNA content was upregulated in the ventricle muscle of rats following 8-weeks of endurance training. Moreover, negative relationships existed between XIAP and apoptotic DNA fragmentation as well as ARC and caspase-3 activity. Overall, our data are in agreement with the hypothesis that modulation of apoptotic suppressors (e.g., XIAP and ARC) is involved in explaining the anti-apoptotic effect of exercise training in skeletal and cardiac muscles.

The suppressive characteristic of XIAP in apoptosis has been well-examined in mitotic cell lineages (6). However, only a few studies have investigated XIAP in postmitotic myocytes. With a focus on mitochondrial encephalomyopathic human skeletal muscle, Ikezoe and colleagues reported that sarcoplasmic expression of XIAP was evident concomitant with the expressions of Bax and Apaf-1, cytochrome c release and caspase-3 activation but no significant increase in DNA fragmentation as estimated by light/electron microscopy-facilitated TUNEL (14). Accordingly, they concluded that XIAP might be involved in suspending the apoptotic process in mitochondrial encephalomyopathies. In addition, XIAP expression has been shown to increase in the skeletal muscle of the aged rats as compared to the young adult rats, even though acceleration of apoptosis in these aged muscles was still observed (i.e., increases in apoptotic DNA fragmentation and pro-/cleaved caspase-3 protein content) (9). While it appears that XIAP responds adaptively to mitochondrial encephalomyopathy and aging in skeletal muscle, in cardiomyocytes, it has been demonstrated that hypoxic or ischemic injury is related to the decline in XIAP (24, 40). Altogether, the exact role of XIAP in post-mitotic myocytes is still not clear, this is worth investigating in future studies. Nonetheless, it is generally agreed that XIAP has anti-apoptotic properties (6) and our present data showed that endurance training increases XIAP expression in skeletal and cardiac muscles, which is consistent with the training-induced anti-apoptotic response that we have reported (41).

The anti-apoptotic effect of XIAP and ARC has been initially shown to be related to their inhibitory action on selected caspases (2, 6, 19, 39). Although in the current study, we found that the expression of XIAP or ARC increased in soleus and cardiac muscles following training, we did not observe any influence in the enzyme activity of caspase-3, an essential effector caspase, from endurance training in these muscles (41). Since the present animals were killed and the muscle samples were obtained 48 hours after the last training session, one of the possible reasons may be the limitation of the time point being selected. The findings showing that ARC interacts with selected caspases (e.g., caspase-2 and -8) but it does not interact with other caspases including caspase-1, -3, and -9 (19). Thus, it was not surprising that caspase-3 activity did not change in our study despite increases in ARC levels. Furthermore, it is worth noting that the muscle samples examined in our study were obtained from healthy young adult rats, and we would expect a relatively low level of apoptotic activity (e.g., effector caspase-3 activation) in these post-mitotic tissues. This may also

preclude us from acquiring evidence that shows a direct role of increases in ARC or XIAP levels to suppress caspase-3 activity following training. Moreover, there have been data suggesting that both ARC and XIAP can demonstrate their anti-apoptotic properties by influencing multiple pathways involving the apoptotic factors other than caspases (12, 36, 37, 46). In addition to the findings showing that ARC can exhibit its anti-apoptotic action by interfering with the activation of Bax rather than via the caspases (12), XIAP may also inhibit apoptosis through an alternative caspase-independent mechanism which depends on the activation of JNK1 and/or neutralization of Smac/DIABLO (36, 37, 46). Although we cannot rule out the possibility that our observed changes in XIAP may have involved a caspase-dependent mechanism, it seems more likely that the exercise training-induced responses of XIAP and ARC may have contributed to the anti-apoptotic signaling through a caspase-independent mechanism. Nevertheless, this possibility warrants further investigation.

Considering that the aging-associated physiologic and functional impairments (e.g., sarcopenia) inevitably occur to all humans (31), active research has been undertaking with the goal of finding measures that can counteract the deteriorating effect of normal aging and thereby ameliorate the quality of life in the aged populations. Physical exercise training is an attractive strategy as it is a relatively economical and natural regimen, which does not introduce any drug- or chemical-induced side effects and can be readily applied to a large scale of populations. Resistance training has been suggested to be effective in alleviating the adverse outcomes with aging (e.g., falling due to frailty of musculoskeletal system) by preserving a considerable amount of muscle mass and muscular strength (13, 17). Although age-associated muscle and strength losses are still inevitable even in masters athletes who endurance train for many years (3), there is a decidedly lack of research that has investigated whether endurance type exercise training might reduce or delay the onset of sarcopenic muscle loss with aging. Clearly, appropriate endurance training causes a number of beneficial cellular adaptations (e.g., improved antioxidant defense capacity and stress protein-mediated protective mechanisms) and physiologic changes (e.g., improved cardiovascular functional capacity and decreased sympathetic neural tone) (1, 11, 16, 20, 22, 25, 29) that could be considered consistent with a favorable environment for the maintenance of muscle tissue. Since there has been evidence demonstrating that the loss of postmitotic tissues with aging may be attributed partly to the accelerated apoptosis (4, 5, 8, 9, 21, 26-28, 38, 45) and the present study and our previous findings showed that endurance training causes the anti-apoptotic cellular responses in adult postmitotic skeletal and cardiac muscles (41), it is appealing to hypothesize that endurance training may slow the apoptotic processes leading to sarcopenia. Providing that the process of aging-related postmitotic myocytes loss occurs gradually and progressively from the mid-life to later stages of one's lifespan, the anti-apoptotic adaptations from life-long endurance training may be capable of decreasing or

delaying the apoptosis-associated loss of postmitotic myocytes. Nonetheless, additional research is needed to examine whether exercise training also causes the similar apoptotic responses in postmitotic tissues from aged individuals.

In conclusion, the present data support the hypothesis that modulation of apoptotic suppressors is involved in mediating the anti-apoptotic effect of exercise training on postmitotic myocytes. We have demonstrated that the apoptotic suppressors (XIAP and ARC) are upregulated following endurance training while negative relationships exist between XIAP and apoptotic DNA fragmentation as well as ARC and caspase-3 activity in these animals. Although the cellular and molecular mechanisms responsible for the endurance training-induced attenuation in apoptosis are still unclear, our findings suggest that apoptotic suppressors contribute, at least in part, to the regulatory mechanisms for the anti-apoptotic adaptation from endurance training in postmitotic myocytes. Since exercise training is potentially a valuable regimen in counteracting the loss of postmitotic myocytes with aging, additional research in apoptosis and exercise training is warranted to fully understand the underlying mechanisms of the anti-apoptotic adaptation from exercise training.

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Figure 1 XIAP, ARC, and FLIP mRNA content

Legend for Figure 1 **The mRNA content of XIAP, ARC, and FLIP was estimated by RT-PCR.** PCR products were visualized with ethidium bromide staining. The insets show representative inverted images of PCR product in soleus and heart muscles isolated from control and trained animals. Quantification of PCR signals was obtained by densitometric analysis of the signal product optical density (OD) x resulting band area. Gene expression is normalized to the ribosomal 18S signal from the same RT product. The normalized data are presented as means $\pm$ SE. Data were run in duplicate on different gels for each gene. *P < 0.05, **P < 0.01, data are significantly different from control animals.

Figure 2 XIAP, ARC, FLIP_S, and FLIP_α protein content

Legend for Figure 2 **The protein content of XIAP, ARC, FLIP_S, and FLIP_α was determined by Western immunoblot.** The data are expressed as optical density (OD) x resulting band area, and expressed in arbitrary units x 10^7 (for XIAP and ARC) or in arbitrary units x 10^5 (for FLIP_S and FLIP_α). The insets show representative blots in soleus and heart muscles isolated from control and trained animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from control animals.

Table 1. Primers used for PCR amplification of cDNA

Product	Accession No.	Sequence	Position	T _A , °C	Cycles	Product length, bp	Restriction enzyme	Restriction products, bp
XIAP	AF183429	F: 5'-GCGGCAGTAGATAGATGGCAGTA-3' R: 5'-TGGGGCTTAAATGGGCATAGT-3'	202-224 524-544	54.9	36	343	<i>AhlI</i> <i>RsaI</i>	309,34 201,142
ARC	NM_053516	F: 5'-CCCCCCCACCAACAGTATTC-3' R: 5'-TGCGTGTGGCTTCTGTCTACTC-3'	1407-1426 1672-1693	56.2	36	287	<i>NcoI</i> <i>XbaI</i>	148, 139 239, 48
FLIP	NM_057138	F: 5'-GGCCCTGAGCACTGTGTCTG-3' R: 5'-TCGGCCTGTGTAATCCTTTGTAA-3'	56-75 412-434	56.4	36	379	<i>RsaI</i> <i>AhlI</i>	191, 188 195, 160, 19, 5

T_A, annealing temperature; Accession No., GenBank accession number; F, forward primer; R, reverse primer.

CHAPTER 13

Age-related apoptotic responses to stretch-induced hypertrophy in quail slow-tonic skeletal muscle

ABSTRACT

The present study examined the responses of apoptosis and apoptotic regulatory factors to muscle hypertrophy induced by stretch overload in quail slow-tonic muscles. The wing of young and aged Japanese quails from one side was loaded by attaching a tube weight corresponding to 12% of the bird's bodyweight for 7 or 21 days. Muscle from the contralateral side served as the intra-animal control. Relative to the intra-animal contralateral control side, the muscle wet weight increased by 96% in young birds whereas the muscle weight gain in aged birds was not significant following 7 days of loading. After 21 days of loading, muscle weight significantly increased by 179% and 102% in young and aged birds, respectively. HSP72 and HSP27 protein content in the loaded side was higher than the control side exclusively in young birds following 7 days of loading. Compared to the contralateral control muscle, the extent of apoptotic DNA fragmentation and the total cytosolic AIF protein content were reduced in all loaded muscles except for the 7 day loaded muscles from the aged birds. Bax protein content was diminished in the loaded muscle relative to the control side from all groups whereas Bcl-2 protein content was reduced in the young and aged muscles after 21 days of loading. The total cytosolic cytochrome c protein content was decreased and the XIAP protein content was elevated in 7 and 21 day-loaded muscles relative to the intra-animal control muscle from young birds. Furthermore, following 7 days of loading the muscles of aged birds, H₂O₂ content and the total cytosolic protein content of Smac/DIABLO were elevated when compared to the intra-animal control side. These data suggest that stretch overload-induced muscle hypertrophy is associated with changes of apoptosis in slow-tonic skeletal muscle. Moreover, discrepant apoptotic responses to muscle overload in young and aged muscles may in part account for the age-related diminishment of the capability for muscle hypertrophy.

Key words: aging; sarcopenia; Bcl-2; Bax; heat shock proteins; AIF

INTRODUCTION

Apoptosis is a cellular process that has been revealed to be essential in regulating a range of biological events (28,58). In addition to the etiologic role of apoptosis in the pathogenesis of several severe diseases (e.g., cancer) (58), it has been demonstrated that apoptosis may play a role in regulating muscle wasting under different physiologic and pathophysiologic conditions including muscle denervation, muscular dystrophy, neuromuscular disorders, hindlimb unweighting, muscle unloading, and strenuous physical exercise (1,6,8,31,47-51,53,54,57). In company with the hypothesized regulatory role of apoptosis in muscle atrophy, there has been evidence showing the relationship between apoptosis and aging muscle including accelerated apoptotic signaling in skeletal muscle with aging (6,7,19,20,30,31,43,44,56) and the influence of aging in the apoptotic response to muscle disuse (31,50,53,54).

Sarcopenia is an unavoidable age related loss of muscle mass that leads to significant muscle weakness (21). It has been suggested that sarcopenia is related to declined quality of life, increased morbidity as well as reduced life expectancy of the elderly population. Sarcopenia is attributed to both reductions in muscle fiber size and total fiber number (18,32). Although the mechanisms responsible for sarcopenia have not been fully elucidated, metabolic, hormonal, nutritional, neuropathic and immunological factors may contribute to the development of sarcopenia (46). In addition to the diminishment of muscle quality with aging (4,5,7,12,17,39), it has also been demonstrated that the hypertrophic response to either functional or stretch overload is decreased in aged muscle relative to the muscle from young adults (3,4,7,12,14-17,29). However, the exact mechanisms for the age-related attenuation in hypertrophic responses are largely unclear.

Recently, our laboratory has provided preliminary evidence suggesting that apoptosis may be related to muscle hypertrophy by showing the upregulation of apoptotic suppressor XIAP following stretch-induced overload (54). While apoptosis has been implicated in various muscle atrophic situations (1,6,8,31,47-51,53,54,57) as well as age-associated sarcopenic muscle loss (6,7,19,20,30,31,43,44,56), it is relatively unknown whether muscle hypertrophy is accompanied with the alteration of apoptotic components. Furthermore, it is unclear if apoptosis is related to the attenuated ability of aged skeletal muscle to achieve hypertrophy levels that are similar to that found in young animals in response to the same loading stimulus. Thus, the objective of this study was to examine the responses of apoptosis and apoptotic regulatory factors to muscle hypertrophy induced by stretch overload in slow-tonic skeletal muscles from young adult and aged quails. We hypothesized that apoptotic factors respond to muscle hypertrophy and aging influences the corresponding apoptotic responses to muscle overload.

METHODS

Animals. Japanese Coturnix quails were hatched and raised in pathogen-free conditions in the central animal care center at West Virginia University School of Medicine. The birds were housed at a room temperature of 22°C with a 12:12-h light:dark cycle and were provided with food and water *ad libitum*. Sixteen young adult birds (2 mo old) and sixteen senescent aged birds (48 mo old) were examined in the present study. Quail are physically and sexually mature by ~1.5 mo of age and do not grow thereafter (37,40). All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care.

Stretch-induced Overload. The anterior latissimus dorsi (ALD) muscle predominately contains slow-twitch β -fibers. It is flexed with the wing on the birds' back at rest, but it is stretched when the wing is extended. In our experimental stretch-overloading model, we placed a tube containing 12% of the bird's bodyweight over the left humeral-ulnar joint (9). Previous studies have consistently shown this stretch-overloading protocol results in various extent of muscle hypertrophy of the ALD muscles that is dependent in part, on the duration of the loading period (3,14-16). The unstretched right ALD muscle served as the intra-animal control muscle for each bird. Consistent with the fact that Japanese quails show no maturational changes in bodyweight and carcass composition beyond ~1.5-2 mo after hatching (35,36,61), it has been demonstrated that the bodyweights of Japanese quails do not change throughout stretch overloading and do not differ between adult and aged quails (2,9-11,16,33). Therefore, the responses to the same absolute and relative loads could be compared in muscles from young adult and aged quails.

The left wing of birds was loaded for 7 or 21 days (eight young and eight aged birds were included in each experimental setting) and then the birds were killed by an overdose of pentobarbital sodium. ALD muscles were dissected from the surrounding connective tissue, removed, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and then stored at -80°C until used for analyses.

Protein preparation. The fractionation method described by Rothermel et al. (45) was adopted with minor modification to extract the nuclei-free cytosolic protein fraction from the ALD muscle. We have previously obtained the fractionated cytosolic and nuclear proteins from skeletal or heart muscles using this modified protocol (50-53). Briefly, after removal of connective tissues, muscle was homogenized on ice in lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT) supplemented with a protease inhibitor cocktail containing 104 mM AEBSF, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO). Following centrifuging at

5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual nuclei. The final collected supernatants were stored as nuclei-free total cytosolic protein fraction. This cytosolic protein fraction was used for cell death ELISA, H₂O₂ assay, and Western immunoblots. The above described protein fractionation procedures have been routinely used in our laboratory to obtain high purity protein fractions as assessed by immunoblotting the fractions with an anti-histone H2B (a nuclear protein) and an anti-CuZnSOD (a cytosolic isoform of superoxide dismutase) antibody (51,53).

The protein contents of the extracts were quantified in duplicate by *DC* Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay (34). As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation (55).

Apoptotic cell death ELISA. A cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligo-nucleosomes. In brief, the nuclei-free cytosolic fraction of ALD muscle was used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated to the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate for 30 min at 20°C. The change in color was measured at a wavelength of 405 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). Measurements were performed with loaded and contralateral control samples analyzed on the same microtiter plate in the same setting. The OD₄₀₅ reading was then normalized to the mg of protein used in the assay and presented as an apoptotic index.

Western immunoblot analyses. Protein expression of B-cell leukemia/lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), apoptosis inducing factor (AIF), second mitochondria-derived activator of caspase (Smac/DIABLO), X-linked inhibitor of apoptosis (XIAP), X-link inhibitor of apoptosis protein (XIAP), apoptosis repressor with caspases recruitment domain (ARC), superoxide dismutases (MnSOD and CuZnSOD), heat shock proteins (HSP72, HSP27, and HSP60) were determined in the total cytosolic protein fraction.

Thirty micrograms of protein from the total cytosolic fraction was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with

Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and probed with the following primary antibodies diluted in PBS-T with 2% BSA: anti-Bcl-2 mouse monoclonal antibody (1:200 dilution, sc-7382), anti-Bax rabbit polyclonal antibody (1:200 dilution, sc-6236), anti-hILP/XIAP mouse monoclonal antibody (1:250 dilution, 610762, BD Biosciences, San Jose, CA), anti-ARC rabbit polyclonal antibody (1:200 dilution, sc-11435), anti-AIF mouse monoclonal antibody (1:500 dilution, sc-13116HRP), anti-Smac/DIABLO mouse monoclonal antibody (1:500 dilution, 612244, BD Biosciences, San Jose, CA), anti-MnSOD goat antibody (1:2000 dilution, A300449A, Bethyl Lab, Montgomery, TX), anti-CuZnSOD rabbit polyclonal antibody (1:500 dilution, sc-11407), anti-HSP72 rabbit polyclonal antibody (1:2000 dilution, SPA812), anti-HSP27 rabbit polyclonal antibody (1:2000 dilution, SPA801) or anti-HSP60 rabbit polyclonal antibody (1:2000 dilution, SPA804). Bcl-2, Bax, ARC, AIF, and CuZnSOD antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while HSP72, 27, and 60 antibodies were purchased from StressGen (Victoria, BC, Canada). All primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed by ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Cytochrome c ELISA. A cytochrome c ELISA kit (MBL International, Woburn, MA) was used to assess the total cytosolic cytochrome c protein content. According to the manufacturer's protocol, sixty micro-liters of the total cytosolic fraction protein was used as an antigen source in a sandwich ELISA with a horseradish peroxidase-conjugated anti-cytochrome c polyclonal antibody in microwell strips coated with an anti-cytochrome c antibody. After washing, the peroxidase retained in the immunocomplex was detected by incubating with a chromogenic substrate, tetramethylbenzidine/hydrogen peroxide (TMB/ H₂O₂) followed by adding an acid solution to terminate the enzyme reaction and to stabilize the developed color. The change in color was monitored at a wavelength of 450 nm using a Dynex MRX plate reader. Measurements were performed with the loaded and contralateral control samples analyzed on the same microplate and the cytochrome c content was expressed as OD₄₅₀ per mg protein.

H₂O₂ fluorometric assay. The content of hydrogen peroxide (H₂O₂) in the ALD muscle homogenate was measured in the total cytosolic fraction using a fluorometric H₂O₂ detection kit (FLOH 100-3, Cell Technology Inc., Mountain View, CA). By following the manufacturer's instruction, 50 µl of the total cytosolic fraction protein of the muscles was incubated in 50 µl of reaction cocktail containing horseradish peroxidase and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP, a non-fluorescent substrate turns fluorescent after oxidized by H₂O₂) in sodium phosphate buffer at room temperature in dark for 10 min. The fluorescence was measured on a spectrofluorometer with an excitation wavelength of 530/25 nm and an emission wavelength of 590/35 nm (CytoFluor, Applied Biosystems, Foster City, CA) after the incubation. H₂O₂ content was estimated as the arbitrary fluorescence units normalized to milligram protein used in the assay. Measurements were performed with the loaded and contralateral control samples run on the same microplate in the same setting.

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package. ANOVA followed by Tukey HSD post hoc analysis was used to examine differences between groups. Statistical significance was accepted at $P < 0.05$. All data are given as means $\pm$ standard error of mean (SE).

RESULTS

Change in muscle mass following stretch overload. The percent difference in the whole ALD muscle wet weight between the loaded and the intra-animal contralateral control wings was examined in order to estimate the degree of muscle hypertrophy. After 7 days of loading, there was a 96% of hypertrophy in muscles of young adult birds, whereas no significant extent of hypertrophy was found in muscles of aged birds (**Figure 1A**). Following 21 days of loading, there was a 179% and 102% hypertrophy in muscles of young and aged birds, respectively (**Figure 1A**). The percents of hypertrophy in young muscles following 7 and 21 days of loading were both significantly greater than the aged muscles (**Figure 1B**). Our results confirmed that the extent of hypertrophy, as estimated by increase in whole ALD muscle wet weight, in response to stretch overload was attenuated in aged muscle when compared to the muscle from young adult birds.

Insert Figure 1A —→ ALD muscle mass

Insert Figure 1B —→ Percent change of muscle hypertrophy

HSP72, HSP27, and HSP60 protein content. In all groups of birds, we did not find any differences in the protein contents of HSP72 and 27 between the loaded and the intra-animal control sides except for the young birds following 7 days of loading (**Figure 2**). In the young adult muscle following 7 days of loading, the protein content of HSP72 and 27 was 33% and 34%, respectively,

higher than the control muscle (**Figure 2A and 2B**). No significant difference was found in the protein content of HSP60 between the loaded and control muscles in all birds examined in the present study (data not shown).

Insert Figure 2A ———→ HSP72 protein content

Insert Figure 2B ———→ HSP27 protein content

H₂O₂ content in muscle homogenate and protein content of MnSOD and CuZnSOD. As estimated by the fluorometric assay, no difference was found for the H₂O₂ content between the loaded and control muscles in all groups except the aged birds after 7 days of loading (**Figure 3**). Following 7 days of loading, the H₂O₂ content in the aged loaded muscle was 40% higher than the contralateral control muscle (**Figure 3**). There was no difference in the protein content of MnSOD and CuZnSOD between the loaded and control muscles in all groups of birds (data not shown).

Insert Figure 3 ———→ H₂O₂ content in muscle homogenate

Apoptotic DNA fragmentation and total cytosolic cytochrome c content. The cell death ELISA analysis showed that the level of the apoptotic DNA fragmentation was decreased with loading in all animals except the aged birds following 7 days of loading (**Figure 4A**). The level of DNA fragmentation was 33% lower than the control side in muscles from the young birds after 7 days of loading (**Figure 4A**). Following 21 days of loading, the level of DNA fragmentation in the loaded muscle was ~34% lower than the control muscle in young and aged birds (**Figure 4A**). The cytochrome c ELISA analysis indicated that the total cytosolic cytochrome c protein content was reduced with loading in the young but not the aged birds (**Figure 4B**). For the young adult birds, we found that the cytochrome c protein content in the loaded side was 34% and 31% lower than the contralateral control muscle following 7 and 21 days of loading, respectively (**Figure 4B**).

Insert Figure 4A ———→ Apoptotic DNA fragmentation

Insert Figure 4B ———→ Total cytosolic cytochrome c protein content

Bax and Bcl-2 protein content. According to our immunoblot data, the Bax protein content was decreased in the loaded side relative to the intra-animal control side in all groups of animals (**Figure 5A**). The protein content of Bax was 53% and 45% lower in muscles loaded for 7 days as compared to the control muscle in young and aged birds, respectively (**Figure 5A**). Following 21 days of loading, Bax protein content in the loaded muscle was 44% and 42% lower than the control muscle

in young and aged birds, respectively (**Figure 5A**). The Bcl-2 protein content was did not change with 7 days of loading in both young and aged muscle but it was 41% and 53% lower in the loaded muscle when compared to the control muscle in young and aged birds, respectively, following 21 days of loading (**Figure 5B**).

Insert Figure 5A ———→ Bax protein content

Insert Figure 5B ———→ Bcl-2 protein content

Total cytosolic AIF, Smac/DIABLO, XIAP, and ARC protein content. Relative to the intra-animal contralateral control side, the AIF protein content in the total cytosolic fraction was reduced in the loaded muscle from all groups of animals except for the aged birds after 7 days of loading (**Figure 6A**). Following 7 days of loading, the total cytosolic AIF protein content in the loaded muscle was decreased by 21% when compared to the control muscle in young birds while the protein content of AIF in the loaded side was 36% and 28% lower than the control side following 21 days of loading in young and aged birds, respectively (**Figure 6A**). We did not find any difference in the total cytosolic protein content of Smac/DIABLO between the loaded and control muscles in all birds except for the aged birds after 7 days of loading. In the 7 days-loaded aged muscle, the Smac/DIABLO protein content was 29% lower than the contralateral control muscle (**Figure 6B**).

Our immunoblot analysis indicated that the XIAP protein content was higher in the loaded side when compared to the contralateral control side following loading in the young but not aged birds (**Figure 6C**). The XIAP protein content in the loaded muscle was ~40% greater than the control muscle in the young birds following 7 or 21 days of loading (**Figure 6C**). No difference was found in the protein content of ARC between the loaded and control sides in all groups of animals (data not shown).

Insert Figure 6A ———→ AIF protein content in total cytosolic fraction

Insert Figure 6B ———→ Smac/DIABLO in total cytosolic fraction

Insert Figure 6C ———→ Total cytosolic XIAP

DISCUSSION

An expanding body of published data from several laboratories has shown that apoptosis may play a role in regulating the process of muscle loss under various conditions including aging (1,6,8,19,20,30,31,44,47,49,50,52,53,56,57). However, it is relatively unknown whether apoptosis is involved in remodeling of skeletal muscle during hypertrophy. By using an avian model of stretch-induced muscle hypertrophy, our laboratory has preliminarily shown that the elevation of apoptotic

suppressor XIAP is evident in loaded patagialis muscle which is a muscle predominately composed of fast α -fibers(54). In the present study, we further extend our previous findings by demonstrating that muscle hypertrophy as induced by stretch overload is accompanied with changes of apoptosis and apoptotic regulatory factors in slow β -fiber-predominated ALD muscle. Our findings indicated that, in the young adult ALD muscle after stretch-induced overload, apoptotic DNA fragmentation, Bax, Bcl-2, total cytosolic cytochrome c and AIF content are reduced, whereas XIAP, HSP72 and HSP27 are elevated with hypertrophy. Furthermore, we observed age-related differences in the apoptotic responses to muscle overload as shown by the increases in total cytosolic Smac/DIABLO and H₂O₂ content and unchanged total cytosolic cytochrome c, XIAP, HSP72 and HSP27 in the loaded muscle exclusively from aged animals.

Apoptotic responses to stretch-induced muscle overload. In accordance with the destructive or removal nature of apoptosis, it is generally accepted that apoptotic program is initiated by the internal and/or external stimuli linking to cellular stress or damage. The activation of apoptosis has been consistently demonstrated in atrophying skeletal muscle, (1,6,8,19,20,30,31,43,44,47-51,53,54,56,57). This apoptotic activation may be accompanied by other cellular signaling involving NF κ B, ubiquitin ligases MuRF1/MAFbx, and/or myostatin, which, are believed to be triggered by the largely unidentified atrophy-related stimuli during muscle disuse or with aging (23,25). All these atrophy-associated cellular changes presumably lead to decreased protein synthesis/accumulation and/or increased protein breakdown/degradation so the muscle approaches a catabolic state (23,25,27). In contrast to muscle atrophy, overload causes muscle hypertrophy by increasing protein synthesis and accumulation of proteins as a result of adjusting the rate of anabolism over catabolism in muscle cells. Noticeably, these anabolic responses necessitate increases in transcription and/or translation of muscle-related genes (13,33), and various signaling pathways are apparently required to connect the hypertrophic stimuli to the upregulation of transcriptional and/or translational processes (23,25). Indeed, several signaling components (e.g., insulin-like growth factor-1 or IGF-1, phosphatidylinositol 3-kinase or PI3K, PKB/Akt, mammalian target of rapamycin or mTOR, p70 ribosomal protein S6 kinase or p70(S6K), RhoA, serum response factor or SRF, focal adhesion kinase or FAK, and/or mechano growth factor or MGF) have been demonstrated to be potentially involved in mediating the anabolic process of muscle hypertrophy (22-26,38,42). In conjunction with the anabolic or survival nature of the demonstrated signaling proteins, it appears that a cellular milieu favoring survival/growth is anticipated for the overloading muscle to achieve a hypertrophic state. In line with this postulated idea, our results showed that muscle hypertrophy as induced by stretch overload is accompanied with the anti-apoptotic changes including modulation of XIAP, AIF, Bax, cytochrome c, and DNA fragmentation in quail slow twitch skeletal muscle. Although our data did not allow us to identify the exact physiologic role of the observed anti-apoptotic responses to muscle overload, the

present findings are the first to demonstrate that apoptotic components are responsive to stretch-induced muscle hypertrophy.

Aging influences the apoptotic responses to muscle overload. Consistent with the previous reports showing that the ability of aged skeletal muscle to achieve muscle hypertrophy is diminished (3,4,7,12,14-17,29,38,42,60), the data from muscles overloaded for 7 or 21 days in the current study demonstrate the impairment of the ability for the aged muscle to gain muscle mass with overload. Although the exact underlying mechanisms contributing to the attenuation of muscle enlargement in the aged muscle are not completely understood, potential molecular and cellular factors (e.g., MRF/Id myogenic repressor, mTOR/p70(S6K)/ERK1/2 signaling, AMP-kinase, and IGF-1/MGF/IGF-1 receptor) have been preliminarily established in accounting for the age-related limitation for muscle hypertrophy (7,26,33,41,42,59).

By examining hypertrophy of plantaris muscle induced by denervating its agonist plantarflexor muscles (i.e., gastrocnemius and soleus), Alway and colleagues (7) have demonstrated that myogenic regulatory factor (MRF) myogenin and MyoD and myogenic repressor Id2 are elevated in overloaded muscle from young adult rats but these changes are not present in the aged overloaded muscle. Similar observations for the attenuated increase in myogenic regulatory factors in the aged muscle have also been reported at the mRNA level in a quail stretch-induced muscle overload model (33). By examining 6 and 30 mo old Fischer 344 x Brown Norway rats, Parkington and coworkers (42) showed that the increases in the phosphorylation of mTOR, p70(S6K), and extracellular signal-regulated kinase1/2 (ERK1/2) after a single bout of *in situ* muscle contractile activity as elicited by high-frequency electrical stimulation of the sciatic nerve are attenuated in the aged muscle when compared to the muscle from adult animals. Additionally, impairment of the elevation of MGF or IGF-1 receptor and hyperphosphorylation of AMP-kinase during muscle overload have also been found in the aged skeletal muscle with muscle overload (26,41,59). In the present study, we observed that there are age-related differences in the apoptotic changes in the muscles following stretch-induced overload. We found that total cytosolic Smac/DIABLO protein content and H₂O₂ content are increased exclusively in the loaded muscle from aged birds. Moreover, the changes of total cytosolic cytochrome c, XIAP, HSP72 and HSP27 that we have observed in the loaded young muscle are not found in identically loaded muscles from aged birds. These observations designate the reduced pro-apoptotic or increased anti-apoptotic tendencies that were observed in the young muscle with overloading were not present in the aged loaded muscle. We speculate that the aged muscle might have defects in handling the anticipated apoptotic changes during overload and unsuccessfully assemble a survival-/growth-favoring cellular environment, and therefore fails to achieve a considerable muscle enlargement when compared to young adult muscle. This speculation is supported by findings showing that muscles from old animals have increased pro-apoptotic tendencies

as compared to muscles from young adult animals (6,7,19,20,30,31,43,44,56) and aging influences the apoptotic responses to muscle disuse (31,50,53,54). Nonetheless, more research is needed to fully exploit the possible contribution of apoptosis to the impairment of muscle-hypertrophied capacity in aged muscle.

In summary, we reported anti-apoptotic changes including decreases in apoptotic DNA fragmentation, Bax, total cytosolic cytochrome c and AIF content and increases in protein content of XIAP, HSP72 and HSP27 in young adult slow-tonic skeletal muscles after 7 or 21 days of stretch-induced overloading. These findings first indicate that muscle overload is associated with alteration of apoptosis and apoptotic regulatory factors. While the exact physiologic function of these anti-apoptotic changes during muscle overload remains to be identified, we speculate that these anti-apoptotic changes may have provided a cellular milieu favoring survival/growth of activated satellite cells, which facilitates the cellular processes (e.g., anabolism) resulting in muscle enlargement with overload. Nevertheless, more investigation is needed to fully comprehend the role of these apoptotic changes in muscle hypertrophy. Furthermore, we observed differences in the apoptotic responses to overload between young adult and aged muscles. There are increases in total cytosolic Smac/DIABLO and H₂O₂ content whereas the total cytosolic cytochrome c, XIAP, HSP72 and HSP27 are unchanged exclusively in the aged loaded muscle. These findings are consistent with the hypothesis that the age-related apoptotic responses explain, at least in part, the attenuated ability of aged muscle to achieve considerable extent of hypertrophy during overload. However, further research is required to fully explore the contribution of apoptosis to the age-related impairment in muscle plasticity.

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Figure 1 **ALD Muscle Data**

Legend for Figure 1A ALD muscle data. The extent of muscle hypertrophy following stretch-induced overload was estimated by examining the muscle mass gain between the loaded and the contralateral control whole ALD muscle wet weight. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from control muscles. 7d loading, 7 days of loading; 21d loading, 21 days of loading; Young, young adult quails; Aged, aged quails.

Legend for Figure 1B Percent of muscle hypertrophy relative to the contralateral side. The extent of muscle hypertrophy was presented as the percent difference in the muscle weight between the loaded and the contralateral control whole ALD muscle wet weight. The data are presented as means $\pm$ SE. *P < 0.05, percent change is significantly different from the animals of the same age following 7 days of loading. #P < 0.05, percent change is significantly different from young animals following the same days of loading.

Figure 2A HSP72

Legend for Figure 2A HSP72. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for HSP72 in control and loaded muscles from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 2B HSP27

Legend for Figure 2B HSP27. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for HSP27 in control and loaded muscles from young adult and aged animals. The data are presented as means $\pm$ SE. * $P < 0.05$, data are significantly different from the corresponding intra-animal control muscles.

Figure 3 H₂O₂ content in muscle homogenate

Legend for Figure 3 H₂O₂ content in muscle homogenate. The H₂O₂ content was determined by a fluorometric assay. The fluorescence unit is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 4A Apoptotic DNA fragmentation

Legend for Figure 4A Apoptotic DNA fragmentation. The extent of apoptotic DNA fragmentation was estimated by measuring the cytosolic mono- and oligo-nucleosomes. The OD₄₀₅ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 4B Total cytosolic cytochrome c

Legend for Figure 4B Total cytosolic cytochrome c. The cytochrome c protein content was estimated by ELISA analysis on the total cytosolic fraction. The OD₄₅₀ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 5A Bax protein content

Legend for Figure 5A Bax protein content. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Bax in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 5B Bcl-2 protein content

Legend for Figure 5B Bcl-2 protein content. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Bcl-2 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 6A Total cytosolic AIF protein content

Legend for Figure 6A Total cytosolic AIF. The AIF protein content was estimated by immunoblotting analysis on the total cytosolic fraction. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for AIF in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 6B Total Smac/DIABLO protein content

Legend for Figure 6B Total cytosolic Smac/DIABLO. The Smac/DIABLO protein content was estimated by immunoblotting analysis on the total cytosolic fraction. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for Smac/DIABLO in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. * $P < 0.05$, data are significantly different from the corresponding intra-animal control muscles.

Figure 6C Total XIAP protein content

Legend for Figure 6C XIAP. The XIAP protein content was estimated by immunoblotting analysis on the total cytosolic fraction. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for XIAP in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. * $P < 0.05$, data are significantly different from the corresponding intra-animal control muscles.

CHAPTER 14

Stretch overload-induced hypertrophy is associated with apoptotic changes in young and aged quail fast muscles

ABSTRACT

Although apoptosis occurs during aging and muscle wasting, it is not known if apoptosis has a role in muscle remodeling during muscle hypertrophy. Here we examined the responses of apoptosis and apoptotic regulatory factors to muscle hypertrophy induced by stretch overload in quail fast-twitch skeletal muscles. One wing of young and aged Japanese quails was loaded by attaching a tube weight corresponding to ~12% of the animal's bodyweight for 7 or 21 days to induce muscle hypertrophy. The contralateral wing served as the intra-animal control. Following 7 days of loading, muscle weight increased by 28% in young bird but no significant hypertrophy was found in aged birds. The muscle weight significantly increased by 49% and 29% after 21 days of loading in young and aged birds, respectively. There were decreases in the level of apoptotic DNA fragmentation and the release of mitochondrial cytochrome c in all loaded muscles except for the muscles loaded for 7 days from the aged bird. ARC protein content was reduced and H₂O₂ content was elevated in muscles from aged birds following 7 days of loading, when compared to the intra-animal control muscles. The protein content of XIAP, an apoptotic suppressor was also increased in the loaded muscle of all groups exclusive of the aged birds after 7 days of loading. Moreover, AIF protein content in the total cytosolic and the mitochondria-free cytosolic fractions was reduced after 7 days of loading in muscles from young and aged birds. The elevation of HSP72 and HSP27 content was evident in all loaded muscles. Our results suggest that muscle remodeling during hypertrophy induced by stretch overload is accompanied with changes of apoptosis and apoptotic regulatory proteins in fast-twitch skeletal muscle. Furthermore, age-related attenuation of the ability of muscle to achieve hypertrophy may be partly explained by the differential apoptotic response to muscle overload between young and aged skeletal muscles.

Key words: aging; sarcopenia; AIF; XIAP

INTRODUCTION

It is well known that various biochemical processes and physiologic functions are significantly impaired with normal aging and thereby result in a number of detrimental health consequences in the elderly populations (25). This includes the loss of muscle fiber size and number leading to age-associated losses of muscle mass and strength with aging which is also called sarcopenia (30). The functional capability of skeletal muscle is diminished with aging (4,5,7,12,17). However, the hypertrophic response to overload is partly retained, as indicated by the findings showing that the extent of muscle hypertrophy induced by either functional or stretch overload is attenuated in aged when compared to young adult individuals (3,4,7,12,14-17,27). Nevertheless, the exact mechanisms underlying these attenuated age-related hypertrophic responses remain unclear.

Apoptosis is a well-conserved biological process that has been extensively investigated and exhibited to be crucial in modulating a variety of physiologic events (e.g., tissue turnover) (26). In addition to the etiologic role of apoptosis in the pathogenesis of several diseases (e.g., cancer and neurodegenerative diseases) (57), there has been a growing body of evidence suggesting that apoptosis is implicated in regulating the process of muscle wasting under different physiologic and pathophysiologic situations (1,6,8,29,44-48,50,56). Along with the proposed atrophic role of apoptosis, it has also been demonstrated that apoptotic machinery is accelerated in postmitotic muscle with age-related sarcopenia (18,19,28,29,40,41,50,54). Furthermore, it has been shown that aging has an impact on the apoptotic response during disuse-induced muscle atrophy (29,47,50,51).

While apoptosis has been consistently demonstrated under various muscle atrophic conditions (1,6,8,29,44-48,50,56), our laboratory has recently provided preliminary evidence showing that muscle hypertrophy is related to the upregulation of apoptotic suppressor XIAP (51). However, investigations are lacking on whether apoptosis has a role in muscle remodeling in response to muscle overload. Moreover, providing that an association of apoptosis and aging has been evidently established (18,19,28,29,40,41,50,54), it is worthwhile to examine whether apoptosis explains, at least in part, the attenuated ability of aged muscle to achieve similar hypertrophy to that found in young adult models of loading. Therefore, the purpose of this study was to examine the responses of apoptosis and apoptotic regulatory factors to muscle hypertrophy induced by stretch overload in young adult and aged quails. We hypothesized that apoptotic responses to muscle overload would occur in an age-dependent manner.

METHODS

Animals. Japanese Coturnix quails were hatched and raised in pathogen-free conditions in the central animal care center at West Virginia University School of Medicine. The birds were housed at a room temperature of 22°C with a 12:12-h light:dark cycle and were provided with food and water

ad libitum. Sixteen young adult birds (~2 mo old) and sixteen senescent aged birds (~48 mo old) were examined in the present study. The quail are physically and sexually mature by 1.5 mo of age and do not grow thereafter (35,37). The old group exceeded the median age and lifespan of Japanese quails in our colony.(35,37)All experimental procedures carried approval from the Institutional Animal Use and Care Committee from West Virginia University School of Medicine. The animal care standards were followed by adhering to the recommendations for the care of laboratory animals as advocated by the American Association for Accreditation of Laboratory Animal Care (AAALAC).

Stretch-induced Overload. The patagialis (PAT) is a muscle that contains predominately fast-twitch α -fibers. It is flexed with the wing on the birds' back at rest, but it is stretched when the wing is extended. In our experimental stretch-overloading model, we placed a tube containing ~12% of the bird's bodyweight over the left humeral-ulnar joint (9). This maintains the joint in extension throughout the period of stretch and induces stretch at the origin of the PAT muscle. Previous studies have consistently shown this stretch-overloading protocol results in PAT hypertrophy which is dependent on the duration of the overload (4,8). The unstretched right PAT muscle served as the intra-animal control muscle for each bird. Consistent with the fact that Japanese quails show no maturational changes in bodyweight and carcass composition beyond ~1.5-2 mo after hatching (33,34,59), it has been demonstrated that the bodyweights of Japanese quails do not change throughout stretch overloading and do not differ between adult and aged quails (2,9-11,16,31). Therefore, the responses to the same absolute and relative loads could be compared in muscles from young adult and aged quails.

The left wing of eight young and eight aged birds was loaded for 7 days and then the birds were killed by an overdose of pentobarbital sodium. The remaining young and aged quails were loaded for 21 days and then sacrificed. Whole PAT muscles were dissected from the surrounding connective tissue, removed, weighed, and frozen in isopentane cooled to the temperature of liquid nitrogen and then stored at -80°C until used for analyses.

Subcellular protein fractionation. The fractionation method described by Rothermel et al. (43) was adopted with minor modification to extract the nuclei-free cytosolic protein fraction from the PAT muscle. We have previously obtained the fractionated cytosolic proteins from skeletal or heart muscles using this modified protocol (47-50). Briefly, after removal of connective tissues, muscles were homogenized on ice in lysis buffer (10 mM NaCl, 1.5 mM MgCl₂, 20 mM HEPES, pH 7.4, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol or DTT) supplemented with a protease inhibitor cocktail containing 104 mM AEBSE, 0.08 mM aprotinin, 2 mM leupeptin, 4 mM bestatin, 1.5 mM pepstatin A, and 1.4 mM E-64 (Sigma-Aldrich, St Louis, MO). Following centrifuging at 5,000 rpm for 5 min at 4°C to pellet the nuclei and cell debris, the supernatants were collected and these supernatants were further centrifuged three times at 6,000 rpm for 5 min at 4°C to remove residual

nuclei. The supernatants were stored as nuclei-free total cytosolic protein fraction. This cytosolic protein fraction was used for cell death ELISA, H₂O₂ assay, and Western immunoblots.

Furthermore, in order to estimate the release of mitochondria-resided apoptotic factors including cytochrome c, AIF, and Smac/DIABLO to the cytosol, a nuclei-free, mitochondria-free cytosolic protein fraction was prepared as described by Rokhlin et al (42) and the protein contents of these mitochondrial apoptotic factors were measured in this mitochondria-free cytosolic fraction as described in the later sections. Muscle was dissected from the connective tissues and minced in ice-cold extraction buffer (250 mM sucrose, 20 mM HEPES, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and 0.1 mM PMSF, pH 7.4) in the presence of protease inhibitor cocktail. Following a gentle homogenization with a Teflon pestle motorized with an electronic stirrer, homogenates were centrifuged at 800 × g for 10 min at 4°C to pellet the nuclei and cell debris. The supernatants were then spun twice at 16,000 × g for 20 min at 4°C to pellet the mitochondria and the final supernatants were collected as nuclei-free, mitochondria-free cytosolic protein fractions. The above described subcellular protein fractionation procedures have been routinely used in our laboratory to obtain high purity protein fractions as assessed by immunoblotting the fractions with an anti-histone H2B (a nuclear protein), an anti-CuZnSOD (a cytosolic isoform of superoxide dismutase), and an anti-MnSOD (a mitochondrial isoform of superoxide dismutase) antibody (48,50).

The protein contents of the extracts were quantified in duplicate by *DC* Protein Assay (BioRad, Hercules, CA) based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent, which was similar to Lowry assay (32). As a further means to confirm the protein contents, all the protein samples were measured in duplicate on a different occasion by BCA Protein Assay (Pierce, Rockford, IL) based on the biuret reaction and the bicinchoninic acid detection of cuprous cation (52).

Apoptotic cell death ELISA. Cell death detection ELISA kit (Roche Applied Science, Indianapolis, IN) was used to quantitatively determine the apoptotic DNA fragmentation by measuring the cytosolic histone-associated mono- and oligo-nucleosomes. In brief, the extracted nuclei-free cytosolic fraction of PAT muscle was used as an antigen source in a sandwich ELISA with a primary anti-histone mouse monoclonal antibody coated to the microtiter plate and a second anti-DNA mouse monoclonal antibody coupled to peroxidase. The amount of peroxidase retained in the immunocomplex was determined photometrically by incubating with 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) as a substrate for 30 min at 20°C. The change in color was measured at a wavelength of 405 nm by using a Dynex MRX plate reader controlled through PC software (Revelation, Dynatech Laboratories, CA). Measurements were performed with loaded and contralateral control samples analyzed on the same microtiter plate in the same setting. The OD₄₀₅

reading was then normalized to the mg of protein used in the assay and presented as an apoptotic index.

Western immunoblot analyses. Protein expression of B-cell leukemia/lymphoma-2 (Bcl-2), Bcl-2 associated X protein (Bax), apoptosis inducing factor (AIF), X-linked inhibitor of apoptosis (XIAP), X-link inhibitor of apoptosis protein (XIAP), apoptosis repressor with caspases recruitment domain (ARC), superoxide dismutases (MnSOD and CuZnSOD), heat shock proteins (HSP72, HSP27, and HSP60) was determined in the total cytosolic protein fraction.

Eighty micrograms of total cytosolic protein was boiled for 5 min at 95°C in Laemmli buffer and was loaded on each lane of a 12% polyacrylamide gel and separated by SDS-PAGE. The gels were blotted to nitrocellulose membranes (VWR, West Chester, PA) and stained with Ponceau S red (Sigma Chemical Co, St Louis, MO) to verify equal loading and transferring of proteins to the membrane in each lane. As another approach to validate similar loading between the lanes, gels were loaded in duplicate with one gel stained with Coomassie blue. The membranes were then blocked in 5% non-fat milk in phosphate buffered saline with 0.05% Tween 20 (PBS-T) at room temperature for 1 h and probed with the following primary antibodies diluted in PBS-T with 2% BSA: anti-Bcl-2 mouse monoclonal antibody (1:200 dilution, sc-7382), anti-Bax rabbit polyclonal antibody (1:200 dilution, sc-6236), anti-hILP/XIAP mouse monoclonal antibody (1:250 dilution, 610762, BD Biosciences, San Jose, CA), anti-ARC rabbit polyclonal antibody (1:200 dilution, sc-11435), anti-AIF mouse monoclonal antibody (1:500 dilution, sc-13116HRP), anti-MnSOD goat antibody (1:2000 dilution, A300449A, Bethyl Lab, Montgomery, TX), anti-CuZnSOD rabbit polyclonal antibody (1:500 dilution, sc-11407), anti-HSP72 rabbit polyclonal antibody (1:2000 dilution, SPA812), anti-HSP27 rabbit polyclonal antibody (1:2000 dilution, SPA801) or anti-HSP60 rabbit polyclonal antibody (1:2000 dilution, SPA804). Bcl-2, Bax, ARC, AIF, and CuZnSOD antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) while HSP72, 27, and 60 antibodies were purchased from StressGen (Victoria, BC, Canada). All primary antibody incubations were performed overnight at 4°C. Secondary antibodies were conjugated to horseradish peroxidase (Chemicon International, Temecula, CA), and signals were developed by ECL detection kit (Amersham Biosciences, Piscataway, NJ). The signals were then visualized by exposing the membranes to X-ray films (BioMax MS-1, Eastman Kodak, Rochester, NY), and digital records of the films were captured with a Kodak 290 camera. Resulting bands were quantified as optical density (OD) $\times$ band area by a one-dimensional image analysis system (Eastman Kodak, Rochester, NY) and recorded in arbitrary units. The molecular sizes of the immunodetected proteins were verified by using pre-stained standard (LC5925, Invitrogen Life Technologies, Bethesda, MD).

Estimation of mitochondrial cytochrome c, Smac/DIABLO, and AIF release. Cytochrome c, AIF, and Smac/DIABLO (second mitochondria-derived activator of caspase) are

apoptotic factors normally confined to mitochondria and their release into the cytosol has been demonstrated during the activation of apoptosis (55). In the present study, the release of Smac/DIABLO and AIF into the cytosol was estimated by measuring their protein contents in the mitochondria-free cytosolic protein fraction by immunoblotting with an anti-Smac/DIABLO mouse monoclonal antibody (1:500 dilution, 612244, BD Biosciences, San Jose, CA) and an anti-AIF monoclonal mouse antibody. Moreover, a cytochrome c ELISA kit (MBL International, Woburn, MA) was used to assess the protein content of cytochrome c in the mitochondria-free cytosol fraction to evaluate the release of the mitochondrial cytochrome c into the cytosol. According to the manufacturer's protocol, 60 μ l the mitochondria-free cytosolic fraction was used as an antigen source in a sandwich ELISA with a horseradish peroxidase-conjugated anti-cytochrome c polyclonal antibody in microwell strips coated with an anti-cytochrome c antibody. After washing, the peroxidase retained in the immunocomplex was detected by incubating with a chromogenic substrate, tetramethylbenzidine/hydrogen peroxide (TMB/ H_2O_2) followed by adding an acid solution to terminate the enzyme reaction and to stabilize the developed color. The change in color was monitored at a wavelength of 450 nm using a Dynex MRX plate reader. Measurements were performed with the loaded and contralateral control samples analyzed on the same microplate and the cytochrome c content was expressed as OD₄₅₀ per mg protein.

H_2O_2 fluorometric assay. The content of hydrogen peroxide (H_2O_2) in the muscle homogenate was measured in the total cytosolic fraction using a fluorometric H_2O_2 detection kit (FLOH 100-3, Cell Technology Inc., Mountain View, CA). By following the manufacturer's instruction, 50 μ l of the total cytosolic fraction protein of the PAT muscles was incubated in 50 μ l of reaction cocktail containing horseradish peroxidase and 10-acetyl-3,7-dihydroxyphenoxazine (ADHP, a non-fluorescent substrate turns fluorescent after oxidized by H_2O_2) in sodium phosphate buffer at room temperature in dark for 10 min. The fluorescence was measured on a spectrofluorometer with an excitation wavelength of 530/25 nm and an emission wavelength of 590/35 nm (CytoFluor, Applied Biosystems, Foster City, CA) after the incubation. H_2O_2 content was estimated as the arbitrary fluorescence units normalized to milligram protein used in the assay. Measurements were performed with the loaded and contralateral control samples run on the same microplate in the same setting.

Statistical analyses. Statistical analyses were performed using the SPSS 10.0 software package. ANOVA followed by Tukey HSD post hoc analysis was used to examine differences between groups. Statistical significance was accepted at $P < 0.05$. All data are given as means $\pm$ standard error of mean (SE).

RESULTS

Change in muscle mass following stretch overload. The extent of muscle hypertrophy was estimated by examining the percent difference in the whole PAT muscle wet weight between the loaded and the intra-animal contralateral control wings. Following 7 days of loading, there was a 28% of hypertrophy in young adult birds whereas no significant extent of hypertrophy was found in aged birds (**Figure 1A**). After 21 days of loading, there was a 49% and 29% hypertrophy in young and aged birds, respectively (**Figure 1A**). The percents of hypertrophy in young muscles following 7 and 21 days of loading were both significantly greater than the aged muscles (**Figure 1B**). Our results confirmed that the extent of hypertrophy, as estimated by increase in whole patagialis muscle wet weight, in response to stretch overload was attenuated in aged muscle when compared to the muscle from young adult birds.

Insert Figure 1A ———→ PAT muscle mass

Insert Figure 1B ———→ Percent change of muscle hypertrophy

HSP72, HSP27, and HSP60 protein content. In all groups of birds, both the HSP72 and HSP27 protein contents of the loaded side were significantly higher than the intra-animal control side (**Figure 2**). In muscles loaded for 7 days the protein content of HSP72 was 125% and 111% higher than the control side in young and aged birds, respectively (**Figure 2A**) while HSP72 protein content was elevated by 79% and 84% in the loaded muscle relative to the intra-animal control muscle in young and aged birds, respectively (**Figure 2A**). For the protein content of HSP27 following 7 days of loading, it was increased by 107% and 95% in the loaded side when compared to the control side in young and aged birds, correspondingly (**Figure 2B**). HSP27 protein content was 104% and 65% higher in muscles loaded for 21 days than the control muscle in young and aged birds, respectively (**Figure 2B**). No significant difference was found in the protein content of HSP60 between the loaded and control muscles in all birds examined in the present study (data not shown).

Insert Figure 2A ———→ HSP72 protein content

Insert Figure 2B ———→ HSP27 protein content

H₂O₂ content in muscle homogenate and protein content of MnSOD and CuZnSOD. As estimated by the fluorometric assay, no difference was found for the H₂O₂ content between the loaded and control muscles in all groups except the aged birds following 7 days of loading (**Figure 3**). Following 7 days of loading, the H₂O₂ content in the aged loaded muscle was 38% higher than the contralateral control muscle ($P < 0.05$, **Figure 3**). In contrast, we did not find any difference in the

protein content of MnSOD and CuZnSOD between the loaded and control muscles in all groups of birds (data not shown).

Insert Figure 3 ———→ H₂O₂ content in muscle homogenate

Apoptotic DNA fragmentation, Bax and Bcl-2 protein content, and release of mitochondrial cytochrome c. The cell death ELISA analysis indicated that the extent of the apoptotic DNA fragmentation was reduced with loading in all animals except the aged birds following 7 days of loading (**Figure 4A**). In the young birds after 7 days of loading, the level of DNA fragmentation in the loaded side was 52% lower than the control side (**Figure 4A**). Following 21 days of loading, the level of DNA fragmentation in the loaded muscle was 53% and 61% lower when compared to the control side in young and aged birds, respectively (**Figure 4A**). The protein content of Bax, a pro-apoptotic member and Bcl-2, an anti-apoptotic member of BCL-2 family was assessed by immunoblot analysis. However, no difference was found in either Bax or Bcl-2 protein content between the loaded and control muscles in all groups of birds (data not shown). Our cytochrome c ELISA analysis on the mitochondria-free cytosolic fraction demonstrated that the release of mitochondrial cytochrome c decreased with loading in all birds except for the aged birds after 7 days of loading (**Figure 4B**). The cytochrome c protein content in the mitochondria-free cytosolic fraction of muscles loaded for 7 days was 44% lower than the contralateral control muscles (**Figure 4B**). Following 21 days of loading, the cytochrome c protein content in the mitochondria-free cytosolic fraction in the loaded side was 32% and 36% lower than the control side in young and aged birds, respectively (**Figure 4B**).

Insert Figure 4A ———→ Apoptotic DNA fragmentation

Insert Figure 4B ———→ Cytochrome c content in mitochondria-free cytosolic fraction

Total cytosolic AIF protein content and release of mitochondrial AIF and Smac/DIABLO. Relative to the intra-animal contralateral control side, the AIF protein content in both the total cytosolic and the mitochondria-free cytosolic fractions was reduced in young and aged muscles following 7 but not 21 days of loading (**Figure 5**). Following 7 days of loading, the total cytosolic AIF protein content was 48% and 35% lower than the control muscle in young and aged birds, respectively (**Figure 5A**). The mitochondria-free cytosolic AIF protein content, was 41% and 29% lower in muscles loaded for 7 days relative to the contralateral control muscles in young and aged birds, respectively (**Figure 5B**). In contrast, no difference was found in the protein content of Smac/DIABLO in the mitochondria-free cytosolic fraction between the loaded and control muscles in all groups of animals (data not shown).

Insert Figure 5A ———→ AIF protein content in total cytosolic fraction

Insert Figure 5B ———→ AIF protein content in mitochondria-free cytosolic fraction

Apoptotic suppressor XIAP and ARC. According to our immunoblot analysis, the XIAP protein content was higher in the loaded side when compared to the contralateral control side in all animals except for the aged birds following 7 days of loading (**Figure 6A**). The XIAP protein content in the loaded muscle was 61% greater than the control muscle in the young birds after 7 days of loading (**Figure 6A**). In the muscle following 21 days of loading, XIAP protein content was increased by 107% and 67% relative to the control muscle in young and aged birds, respectively (**Figure 6A**). The ARC protein content was not different between the loaded and control muscles in all animals except for the aged birds after 7 days of loading (**Figure 6B**). We found that the protein content of ARC in the loaded side was 41% lower than the control side in the aged birds following 7 days of loading (**Figure 6B**).

Insert Figure 6A ———→ XIAP protein content

Insert Figure 6B ———→ ARC protein content

DISCUSSION

Apoptosis has been implicated in regulating the process of muscle wasting. On the contrary, it is relatively unknown whether apoptotic proteins respond to muscle hypertrophy. Recently, by using an experimental model of stretch-induced muscle hypertrophy in quails, we have preliminarily demonstrated that upregulation of apoptotic suppressor XIAP occurs following overload in skeletal muscles from both young adult and aged birds (51). In the present study, we further extend our previous findings by showing that muscle hypertrophy induced by stretch overload is accompanied with changes of apoptosis and apoptotic regulatory factors. Our data indicated that apoptotic DNA fragmentation, mitochondrial cytochrome c release, and AIF content are reduced whereas XIAP, HSP72 and HSP27 are elevated with stretch-induced muscle hypertrophy. Furthermore, changes including a decrease in ARC and an increase in H₂O₂ content were found in the loaded muscle exclusively from aged animals suggesting that there are clear age-related differences in the apoptotic responses to muscle overload.

Alteration of apoptotic components during muscle hypertrophy. Apoptosis has been identified as a cellular machinery that functions to eliminate or remove the non-functional, misplaced, abnormal, damaged or harmful cells, thereby having an indispensable role in embryonic development, tissue turnover, and immunological defense (26,57). It is commonly recognized that the apoptotic program is activated in response to a variety of internal and/or external stimuli, which are linked to

cellular stress or damage. Although the physiologic significance and functional role of apoptosis in skeletal muscle loss have yet to be fully elucidated, there have been consistent data demonstrating that apoptotic signaling is activated in muscle undergoing atrophy (1,6,8,18,19,29,40,41,44-46,48,50,54,56). This apoptotic activation together with other cellular signaling mediated by NFkB, myostatin, and/or ubiquitin ligases MuRF1/MAFbx are most likely triggered by the atrophic stimuli that are provoked during muscle disuse and with aging (21,23).

In opposition to muscle atrophy, muscle hypertrophy resulting from overload involves increased protein synthesis and accumulation of proteins by modulating the rate of anabolism over catabolism in skeletal muscle cells. The hypertrophy-associated anabolic response necessitates increases in transcription and/or translation of muscle genes, and these upregulations of transcriptional and/or translational machineries need to be mediated by a number of signals triggered by the hypertrophic stimuli (13,31). Recently, there have been several reports that establish that various potential signaling components (e.g., IGF-1, mechano growth factor, PI3 kinase, PKB/Akt, mTOR, p70 ribosomal protein S6 kinase, RhoA, serum response factor, focal adhesion kinase) are likely involved in mediating the process of muscle hypertrophy (20-24,36,39). Along with the anabolic or survival nature of these established signals, it is likely that a cellular environment that favors cell survival or growth is required for the overloading muscle to approach a hypertrophic state. Consistent with this idea, our data indicate that muscle hypertrophy induced by stretch overload is accompanied by several anti-apoptotic changes including modulation of XIAP, AIF, cytochrome c, and DNA fragmentation in quail fast twitch skeletal muscle. Although the exact physiologic and functional roles of our observed anti-apoptotic responses to muscle overload remain to be resolved, we have provided evidence illustrating that apoptotic changes accompany stretch-induced muscle hypertrophy.

Age-related apoptotic responses to stretch-induced muscle overload. It has been documented that the degree of muscle hypertrophy following functional or stretch-induced overload is attenuated in the aged muscle when compared to the muscle from young individual (3,4,7,12,14-17,27). The data from muscles loaded for 7 and 21 days in our current study further confirm the diminishment of the ability for the aged skeletal muscle to achieve enlargement. With the aim of understanding the underlying mechanisms that contribute to the limitation of hypertrophied capability in the aged muscle, active research has been undertaking to determine the molecular and cellular factors, which may have been responsible for the age-related decrease in muscle plasticity. Indeed, there are several potential pathways that have been identified to be possibly related to the age-associated impairment of hypertrophied ability. These include phosphorylation of AMP-kinase (58), mTOR/p70(S6K)/ERK1/2 signaling (39), IGF-1/mechano growth factor/IGF-binding protein (24,38,53), and myogenic regulatory factors/Id myogenic repressor (7,31). In this study, we found differences in the apoptotic changes in the loaded muscles between young adult and aged birds. We

observed that the protein content of apoptotic suppressor ARC was reduced while the H_2O_2 content was increased in the aged muscle after 7 days of loading but these changes were not found in the young loaded muscles. Correspondingly, in all the loaded muscles except for the muscles from aged birds that were loaded for 7 days, there were decreases in the extent of DNA fragmentation and mitochondrial cytochrome release and increase in XIAP protein content. These findings indicate the reduced pro-apoptotic or increased anti-apoptotic tendencies that were observed in the muscles from young birds with overloading were not present in the loaded muscles from aged birds. In support of the findings demonstrating that aged muscles have increased pro-apoptotic tendencies (6,7,18,19,28,29,40,41,54) and aging influences the apoptotic responses to muscle disuse (29,47,50,51), it is reasonable to speculate that muscles from the aged animals may have defects in regulating the apoptotic changes during overload with a failure to assemble a survival- or growth-favoring cellular milieu. Such a pro-apoptotic environment would reduce overload-induced muscle enlargement relative to young adult muscles. Nevertheless, more work in the area of aging, muscle hypertrophy and apoptosis are needed. Specifically, further research is required to verify that this age-related apoptotic changes also occur with muscle overload using other muscle hypertrophy models (e.g., compensatory hypertrophy, electrical stimulation-induced hypertrophy, and resistance training).

In conclusion, we have demonstrated anti-apoptotic changes including decreases in apoptotic DNA fragmentation, mitochondrial cytochrome c release, and AIF protein content and increases in protein content of XIAP, HSP72 and HSP27 in quail fast twitch skeletal muscles after 7 or 21 days of stretch-induced overloading. Moreover, we found decrease in apoptotic suppressor ARC protein content and increase in H_2O_2 content exclusively in the 7 days-loaded muscle from aged animals. These observations suggest that the apoptotic responses to muscle overload may be different in young adult and aged muscles and support the hypothesis that the age-related apoptotic responses to overload may partly account for the attenuated ability of aged muscle to achieve considerable extent of hypertrophy. Although the precise physiologic function of these anti-apoptotic changes during muscle overload is unclear, these data are the first to indicate that muscle overload is accompanied with alteration of apoptosis and apoptotic regulatory factors in fast twitch muscles. We speculate that these anti-apoptotic changes may have facilitated the anabolic process and/or muscle growth with overload by providing a cellular milieu favoring cell survival or growth and therefore muscle enlargement. Nonetheless, future research is needed to explore the role of these apoptotic changes in muscle hypertrophy and to establish the contribution of apoptosis to the diminishment of muscle function and plasticity in aging skeletal muscle.

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Figure 1 PAT Muscle Data

A. Muscle weight

B. Percent of muscle hypertrophy relative to the contralateral side

Legend for Figure 1A PAT muscle data. The extent of muscle hypertrophy following stretch-induced overload was estimated by examining the muscle mass gain between the loaded and the contralateral control whole PAT muscle wet weight. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from control muscles. 7d loading, 7 days of loading; 21d loading, 21 days of loading; Young, young adult quails; Aged, aged quails.

Legend for Figure 1B Percent of muscle hypertrophy relative to the contralateral side. The extent of muscle hypertrophy was presented as the percent difference in the muscle weight between the loaded and the contralateral control whole PAT muscle wet weight. The data are presented as means $\pm$ SE. *P < 0.05, percent change is significantly different from the animals of same age following 7 days of loading. **P < 0.05, percent change is significantly different from young animals following the same loading.

Figure 2A HSP72

Legend for Figure 2A HSP72. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for HSP72 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 2B **HSP27**

Legend for Figure 2B **HSP27.** The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for HSP27 in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 3 H_2O_2 content in muscle homogenate

Legend for Figure 3 H_2O_2 content in muscle homogenate. The H_2O_2 content was determined by a fluorometric assay. The fluorescence unit is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. * $P < 0.05$, data are significantly different from the corresponding intra-animal control muscles.

Figure 4A Apoptotic DNA fragmentation

Legend for Figure 4A Apoptotic DNA fragmentation. The extent of apoptotic DNA fragmentation was estimated by measuring the cytosolic mono- and oligo-nucleosomes. The OD₄₀₅ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 4B Release of mitochondrial cytochrome c

Legend for Figure 4B Release of mitochondrial cytochrome c. The release of mitochondrial cytochrome c was estimated by measuring the protein content of cytochrome c in the mitochondria-free cytosolic fraction using an ELISA. The OD₄₅₀ is normalized to the total milligrams protein content of the sample used in the assay. The normalized data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 5A Total cytosolic AIF protein content

Legend for Figure 5A Total cytosolic AIF. The AIF protein content was estimated by immunoblotting analysis on the total cytosolic fraction. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for AIF in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 5B Release of mitochondrial AIF

Legend for Figure 5B Release of mitochondrial AIF. The release of mitochondrial AIF protein content was estimated by immunoblotting analysis on the mitochondria-free cytosolic fraction. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for AIF in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 6A XIAP protein content

Legend for Figure 6A XIAP. The XIAP protein content was estimated by immunoblotting analysis on the total cytosolic fraction. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for XIAP in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

Figure 6B **ARC protein content**

Legend for Figure 6B **ARC.** The ARC protein content was estimated by immunoblotting analysis on the total cytosolic fraction. The data are expressed as OD x resulting band area, and expressed in arbitrary units. The insets show representative blots for ARC in control and loaded muscles isolated from young adult and aged animals. The data are presented as means $\pm$ SE. *P < 0.05, data are significantly different from the corresponding intra-animal control muscles.

GENERAL DISCUSSION

Apoptosis has been implicated to be involved in muscle wasting during muscle disuse and with aging. However, the biological significance of apoptosis in muscle remodeling remains to be identified. In this dissertation, I examined the apoptotic signaling components and cellular stress markers including Bax, Bcl-2, Apaf-1, cytochrome c, caspases, Smac/DIABLO, XIAP, ARC, FLIP, AIF, p53, Id2, p21, c-Myc, PARP, HSP70, HSP27, HSP60, MnSOD, CuZnSOD, catalase, H₂O₂, MDA/4-HAE, nitrotyrosine or 8-OHdG. The data from my dissertation provides convincing results demonstrating the incidence of apoptosis, activation of the pro-apoptotic signaling, and elevation of oxidative stress in the skeletal muscles following denervation, hindlimb suspension, and unloading following overload in mice, rats, or quails. Moreover, data from this dissertation show that Id2 and p53 respond to muscle unloading or overload in a subcellular compartmentalized manner. Furthermore, anti-apoptotic alterations are exhibited in the muscles following endurance treadmill training or muscle overloading. These findings are consistent with the hypotheses that apoptosis has an important role in regulating muscle loss and exercise training is able to alter the apoptotic signaling in skeletal muscle.

Apoptosis is associated with skeletal muscle wasting

Apoptosis requires regulation of specific genes to tightly coordinate the corresponding apoptotic events and has been widely accepted to be crucial in monitoring the balance between cell survival and death in mitotic cell lineages (5, 35). Recently, apoptosis has been reported in postmitotic skeletal muscle under certain physiological and pathophysiological conditions (e.g., muscle denervation, muscle dystrophy, muscle unloading, neuromuscular disorders, hindlimb unweighting, strenuous physical exercise, and aging-associated sarcopenia) and these consistent observations of activated apoptotic machinery under muscle atrophic conditions call attention to the existence of a physiologic role of apoptosis in regulating the atrophic process of muscle remodeling during disuse or inactivity (1-4, 6, 7, 15, 16, 18, 20-23, 27-31, 37, 38). In the present dissertation, the relationship of apoptosis muscle wasting is further confirmed by examining three different animal muscle atrophic models including muscle denervation model in mice and rats, hindlimb suspension-induced muscle disuse model in rats, and muscle unloading following muscle overload model in quails. It is noted that these muscle wasting models have different characteristics regarding the nature of muscle loss. In the muscle denervation model, muscle disuse/inactivity was induced by removing the nerve innervation to the experimental muscles. The resulting muscle loss is related to both the disuse/inactivity of muscle and the absence of neural stimulation, and therefore it is a partial neural-dependent muscle atrophic model. For the hindlimb suspension model, muscle atrophy is induced by removing the gravitational

loading on the hindlimb muscles or unweighting the hindlimb muscles. Throughout the hindlimb suspension period, the neural components remain intact. Nonetheless, the muscle wasting in both models of muscle denervation and hindlimb suspension occur where muscle mass is reduced below the basal muscle mass level. In the quail muscle unloading following overload model, the skeletal muscle is first hypertrophied by stretch-induced overload and then atrophied by subsequent unloading. Thus, this quail unloading model investigates the process of muscle regression from the “overloaded state” to the “normally loaded state” of the muscle. The term “unloading” used in this dissertation refers to the removal of the load from the hypertrophied muscle and this induces the atrophy of the previously hypertrophied muscle. In addition, stretch-induced muscle hypertrophy in this quail model is attributed to the mechanical component of stretch (i.e., passive stretch). This has been shown by observations that the electromyographic (EMG) activity in muscle does not change with stretch (12) and stretch-induced hypertrophy occurs independent of the neuromuscular activity or innervation in skeletal and hemidiaphragm muscles of chickens and rats (8, 10, 11, 33, 34, 36). With these differences in the characteristics among models, the results of this dissertation demonstrate that apoptotic machinery is consistently activated in the atrophied skeletal muscle during muscle denervation in mice and rats, hindlimb suspension-mediated muscle disuse in rats, and muscle unloading following overload in quails. Furthermore, the findings that the extent of denervation-induced muscle loss is attenuated in transgenic mice that are deficient of the pro-apoptotic *Bax* gene strongly suggest that apoptotic machinery is important in promoting muscle loss. These data support the general hypothesis that apoptosis has a physiologic role in regulating the process of muscle wasting in response to muscle disuse.

Another novel finding of this dissertation is that the apoptotic response to muscle unloading is influenced with aging. The data of the present dissertation show that the changes of the apoptotic components or cellular stress markers are different in the atrophied muscles between young adult and aged/senescent animals in both the rat hindlimb suspension and quail unloading models. Previously, by using the technique of immunohistochemistry, it has been demonstrated that apoptosis in soleus muscle of old rats is partly mediated by the subsarcolemmal mitochondria through endonuclease G (a mitochondrial caspase-independent apoptogenic factor) translocation to the myonucleus in response to hindlimb suspension. These results suggest that the underlying pathways involved in apoptosis may be distinct in young and old muscles (16). The findings of this dissertation extend our understanding of the relationship of apoptosis and hindlimb suspension in old muscle by showing that the release of mitochondrial AIF (another caspase-independent apoptogenic factor) into the cytosol is evident exclusively in the aged muscle after suspension but this change is not present in the young muscle. These AIF findings are in accordance to the endonuclease G data that have been recently reported in the old suspended soleus muscle (16). Together the data support that different apoptotic mechanisms

are responsible for the activation of apoptosis in young adult and old skeletal muscles. In line with these age-related findings, the present dissertation also demonstrates that, in the quail muscle unloading following overload model, the apoptotic signaling components (e.g., Bax, Bcl-2, AIF, Id2, and p53) respond to unloading differently in the hypertrophied muscles from young adult and old birds. Together, the present dissertation supports the proposition that aging influences the apoptotic response to muscle disuse.

Oxidative stress is related to apoptosis-associated muscle loss

Free radicals together with reactive oxygen or nitrogen species are produced in large quantities in contracting skeletal muscle but the production of reactive oxygen or nitrogen species is still present in muscle under an inactive state (19, 25, 26). Reasonably, it has been proposed that oxidative stress may have an important role in mediating the process of muscle atrophy during disuse (24). Since hindlimb unloading has been demonstrated to increase oxidative stress and disrupts antioxidant capacity in young adult rat muscle (14), the findings of hindlimb suspension in this dissertation further extend the knowledge by showing that oxidative stress is increased not only in the atrophied young but also the atrophied old rat skeletal muscle following hindlimb unloading. The present dissertation also demonstrates that the elevation of oxidative stress is evident in the atrophied mice skeletal muscle after denervation. It is noted that the increased oxidative stress is accompanied with the activation of apoptotic signaling in the atrophied muscles in both the hindlimb suspension and denervation models. Since oxidative stress may have a role in the activation of apoptosis (17, 32), this raises the possibility that the elevated oxidative stress maybe associated with the activation of apoptosis in muscle undergoing atrophy. Indeed, the findings in the skeletal muscle cell culture of this dissertation show that H₂O₂-mediated oxidative stress induces the activation of apoptosis in a dose- and time-dependent manner. These observations provide strong evidence supporting the concept that oxidative stress may have a role in mediating muscle wasting through the interaction with the activation of the apoptotic signaling during muscle disuse.

Id2 and p53 is responsive to muscle unloading and overload

Although apoptosis has been implicated in having a physiologic role in mediating unloading-induced muscle atrophy, the underlying cellular and molecular regulatory mechanism contributing to the activation of the subsequent apoptotic signaling pathway that results in unloading-induced apoptosis is not currently well understood. Therefore, one of the intentions of this dissertation is to identify the potential candidates that maybe involved in mediating the activation of apoptosis during muscle disuse. According to the results of the present works, two apoptosis-associated cellular factors including inhibitor of DNA-binding/differentiation Id2 and tumor suppressor p53 maybe involved in

muscle wasting. By examining the atrophied skeletal muscle in the models of rat hindlimb suspension and quail unloading, Id2 and p53 both respond together with the activation of apoptosis as well as muscle wasting. Moreover, the responses of Id2 and p53 appear to be restricted to a subcellular compartmentalized fashion. For the change of Id2 during muscle loss, the elevation of Id2 is exclusively occurs in the cytosolic fraction while the nuclear Id2 does not change in both the rat hindlimb suspension and quail unloading models. These findings of Id2 subcellular response are in support of the fact that the apoptotic property of Id2 is related to the pro-apoptotic protein Bax which primarily resides in the cytosolic compartment (9, 13). On the other hand, it appears that both the nuclear and cytosolic p53 maybe be related to the apoptosis-related muscle loss but it is dependent on particular atrophic situations. The cytosolic p53 increases but nuclear p53 remains unaltered in the rat muscle following hindlimb unloading whereas both the cytosolic and nuclear p53 elevate in the quail muscle during unloading following overload. These discrepancies in the change of nuclear p53 may be reasonably attributed to the different nature of muscle loss in these models (i.e., muscle loss that stops above the basal control muscle level in the quail model vs. muscle loss below the basal level in the rodent hindlimb suspension model) and between species (i.e., birds vs. rodents). Overall, the findings in both models of rat hindlimb suspension and quail unloading generally agree with the hypotheses that p53 is associated with muscle atrophy induced by muscle disuse and the atrophic role of p53 maybe related to the subcellular location of the protein accumulation.

Endurance exercise and muscle overload are able to alter the apoptotic signaling

A few studies have attempted to study the relationship of acute strenuous exercise with apoptosis in skeletal muscle. However, the effect of exercise training (endurance training and muscle hypertrophic overload) has not been investigated. This dissertation provides novel data showing that both endurance treadmill training and hypertrophic overload can influence the apoptotic signaling in mature skeletal or heart muscles. This dissertation demonstrates that endurance exercise training is capable of attenuating apoptosis in skeletal and heart muscles of adult rats and the regulatory mechanisms responsible for the anti-apoptotic effect of training comprise the alteration of Bcl-2, Bax, Apaf-1, HSP70, MnSOD, XIAP, and/or ARC. In response to muscle hypertrophic overloading, it appears that a cellular milieu favoring survival/growth is anticipated for the overloading muscle to achieve a hypertrophic state. In agreement with this idea, the data of this dissertation demonstrate that muscle hypertrophy as induced by stretch overload is accompanied with the anti-apoptotic changes including modulation of XIAP, AIF, Bax, cytochrome c, or DNA fragmentation in quail slow and fast twitch skeletal muscles. These findings first suggest that apoptotic components are responsive to stretch-induced muscle hypertrophy. Overall, the present dissertation demonstrates that endurance training or muscle overload causes the anti-apoptotic cellular responses in adult postmitotic skeletal or

cardiac muscles and it is appealing to hypothesize that exercise training maybe capable of decreasing or delaying the apoptosis-associated loss of postmitotic myocytes during muscle disuse/inactivity (e.g., bed rest and space flight) and with aging (e.g., sarcopenia). It opens a new prospectus that exercise training may results in beneficial effects on health by altering the apoptotic events in mature muscles.

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