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Skeletal Muscle Arteriole Dysfunction and Microvessel Density in Metabolic Syndrome and Depression: How Exercise Can Be Used as an Intervention

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

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ABSTRACT

Skeletal Muscle Arteriole Dysfunction and Microvessel Density in Metabolic Syndrome and Depression: How Exercise Can Be Used as an Intervention

Kayla W. Branyan

The present study examined the effect of unpredictable chronic mild stress (UCMS) on peripheral microvessel function in healthy and metabolic syndrome (MetS) rodents, and whether exercise training could prevent the vascular dysfunction associated with UCMS. Our initial hypothesis was that: 1) LZRs exposed to UCMS would have peripheral microvascular dysfunction similar to that evident in the OZR controls due to a reduction in NO bioavailability and an increase in ROS production; 2) the comorbidity between MetS exposed to UCMS will exacerbate the already existing peripheral microvascular dysfunction; 3) exercise could limit the peripheral microvascular dysfunction by decreasing oxidative stress and improving vasodilation associated with MetS exposed to chronic stress; 4) MVD will be reduced in rats exposed to UCMS but loss of MVD will be mitigated by exercise; and 5) the increase in MVD will be reflected by a change in angiogenesis and oxidative markers.

Lean and obese (model of MetS) Zucker rats (LZR; OZR) were exposed to 8 weeks of UCMS, exercise (Ex), UCMS+Ex, or control conditions. At the end of the intervention, gracilis arterioles (GAs) were isolated and hung in a pressurized myobath to assess endothelium-dependent (EDD) and -independent (EID) dilation. Levels of nitric oxide (NO) and reactive oxygen species (ROS) were measured through DAF-FM and DHE staining, respectively. Immunohistochemistry was used to determine the number of pericytes within the cortex and striatum of the brain.

The comorbidity between UCMS and MetS does not exacerbate the effects of one another on GA EDD responses, but does lead to the development of other vasculopathy adaptations, which can be partially explained by alterations in NO and ROS production. Importantly, exercise training alleviates most of the negative effects of UCMS on GA function. Compared to LZR controls, EDD and EID was lower in LZR-UCMS. The OZR-Ex group had a higher EDD and EID, compared to OZR-Controls; whereas only a difference in EDD was noted between LZR-Control and LZR-Ex groups. Importantly, EDD and EID were higher in the LZR and OZR UCMS+Ex groups compared to UCMS alone. Lower NO bioavailability and higher ROS were noted in the LZR-UCMS group, but not OZR-UCMS, compared to controls. Ex and UCMS-Ex groups had higher NO bioavailability compared to control and UCMS groups, but ROS levels remained high. UCMS significantly decreased MVD in LZR-UCMS but was not changed in OZR-UCMS. In LZR- and OZR-Ex, MVD was increased and when coupled with UCMS, Ex improved MVD in both LZR- and OZR-UCMS+Ex. RT-PCR showed no differences in mRNA expression between groups in any of the angiogenic and oxidative stress markers examined.

Corticosterone levels were elevated in the Ex and UCMS+Ex groups vs. the controls which may, in part, reflect the slight stress induced by use of the forced treadmill Ex protocol. It has been shown that glucocorticoids can increase ROS directly, including superoxide, hydrogen peroxide, and peroxynitrite (1). Given that DHE can interact with these oxidants, it could be speculated the higher ROS levels seen in LZR-Ex and UCMS+Ex are a byproduct of increased corticosterone. The fact that in Ex and UCMS+Ex EDD was improved despite elevated corticosterone levels, suggests EDD augmentation was likely mediated from non-corticosterone pathways. Previous

studies have also shown glucocorticoids can attenuate angiogenesis by inhibiting proliferation of cerebrovascular endothelial cells. In addition, pericyte apoptosis occurs with increased cortisol levels, where the glucocorticoid binds to receptors expressed on the pericytes to initiate cell death. L-NAME blunted maximum dilation back down to LZR- and OZR-Control+LNAME values, suggesting the Ex augmentation of EDD may be through a NO-dependent pathway. The expression and function of eNOS is upregulated after Ex training due to increased shear stress, thus could explain why EDD in LZR- and OZR-Ex was improved in the skeletal muscle arterioles. One possible factor that could have contributed to the increase in cerebral MVD could be the increase in NO production following Ex. Muscle contraction increases VEGF in the muscle interstitium, where VEGF acts on its receptors within the capillary endothelium to stimulate angiogenic processes, thus providing further support for exercise-induced angiogenesis.

Dedication

To Mom, Dad, and Adam for your continuous support and encouragement

To my “labmates” (or better yet partners in crime) Evan, Kent, Roy, Shinichi, and Sara

To the rest of my family and friends who helped keep everything fun

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I am grateful that Paul was willing and able to take me under his wing when I needed to leave my previous lab. I don't think I would have made it through my degree if that hadn't happened. Even though he's British, he's still a great mentor. I managed to avoid being called a "potted plant". Thankfully, getting my PhD didn't depend on how well I could play "football" because Paul would've been sorely disappointed in my foot skills. In all seriousness, Paul was always there for advice whether it was professional or personal, and for that I am grateful.

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Abbreviations

4-Hydroxy-TEMPO	(TEMPOL)
4-Amino-5-Methylamino-2',7'- Difluorofluorescein	(DAF-FM)
Acetylcholine	(Ach)
Angiotensin II	(Ang II)
Cardiovascular Disease	(CVD)
Cyclooxygenase	(COX)
Dihydroethidium	(DHE)
Endothelin-1	(ET-1)
Endothelial Nitric Oxide Synthase	(eNOS)
Endothelium-Dependent Dilation	(EDD)
Endothelium-Independent Dilation	(EID)
Exercise	(Ex)
Gracilis Arteriole	(GA)
Lean Zucker Rat	(LZR)
L-N ^G -nitroarginine methyl ester	(L-NAME)
Metabolic Syndrome	(MetS)
Microvessel Density	(MVD)
Middle Cerebral Artery	(MCA)
Nitric Oxide	(NO)
Obese Zucker Rat	(OZR)
Peripheral Vascular Disease	(PVD)
Phenylephrine	(PE)
Physiological Saline Solution	(PSS)
Reactive Oxygen Species	(ROS)
Sodium Nitroprusside	(SNP)
Unpredictable Chronic Mild Stress	(UCMS)

CHAPTER 1 : Review of the Literature

Peripheral Microvascular Structure and Function

Resistance arteries are essential to sufficient blood perfusion of tissue. The aorta has been the focus in previous studies involving pathologies of the vascular system. Even though this blood vessel is important, there is a need to explore the impact of disease on resistance arteries. Resistance arteries and arterioles are the primary vessels in regulating blood pressure and flow at/within an organ. They are located after small arteries but right before the capillaries (Figure 1-1). These resistance vessels further dampen the pulsatility of blood coming from the aorta. Once blood reaches the small arteries, there is a 50-70% decrease in pressure (2). This dissertation will focus specifically on resistance arterioles within the skeletal muscle, and partially on the cerebral microvasculature.

Figure 1-1 Cardiovascular Physiology Concepts. Klabunde 2016.

The structure of an arteriole is optimized for its function. The diameter of the vessel is small, and the tunica media consists of fewer smooth muscle cell layers than what is seen in muscular and elastic arteries. These vascular smooth muscle cells keep the arteriole in a partially constricted state, called basal vascular or vasomotor tone (3). Central control processes and local regulatory mechanisms alter the contractile activity of the vascular smooth muscle (2,4). On the smooth muscle, there are membrane receptors, ion channels, and mechanical sensors that respond to changes in blood flow, along with second messenger systems that are activated by gaseous signaling molecules. These factors illustrate the complexity of the regulation of vascular tone to control blood flow (4). Vasomotor control properties vary along the arteriolar branch but also among the different types of skeletal muscle fibers (5).

Skeletal muscle arteriole function

Resistance arteries and arterioles are highly innervated by the sympathetic nervous system (2). The sympathetic nervous system acts on peripheral arteries and arterioles during times of increased cardiac demand. Sympathetic input constricts these blood vessels to increase resistance and decrease distal blood flow. The downstream effect of resistance artery constriction is an increase in cardiac output, systemic vascular resistance, and arterial blood pressure. The main nerves innervating resistance arteries are sympathetic adrenergic nerves that release norepinephrine (NE) as a neurotransmitter. NE binds to α_1 -adrenoceptors to cause smooth muscle contraction, thus vasoconstriction. A common pharmaceutical drug that is used in studies because of its actions as a α_1 -adrenoceptor agonist is phenylephrine (PE). Vasodilation can be achieved by stimulation with epinephrine (EPI) which binds to β_2 -adrenoceptors, but this is highly

concentration dependent. Acetylcholine (Ach) is another neurotransmitter involved in vasodilation, where it binds to muscarinic receptors (specifically M_3) on the endothelium to trigger the production of nitric oxide (NO) to prevent smooth muscle contraction (2).

There are several vascular signal transduction mechanisms including G-protein coupled and cGMP-coupled signaling. G-protein coupled signaling involves three different G-proteins: Gs, Gi, and Gq. Gs-proteins stimulate adenylyl cyclase to catalyze the formation of cAMP. This causes relaxation of the smooth muscle because the increased cAMP inhibits myosin light chain kinase activity. The Gs-proteins are coupled to β_2 -adrenoceptors (EPI and isoproterenol), α_2 -purinergic receptors (adenosine), and IP receptors (prostacyclin). α_1 -adrenoceptors are coupled to Gi-proteins and when NE binds, there is a reduction in cAMP leading to smooth muscle contraction (2).

Gq-proteins are coupled to three different receptors: α_1 -adrenoceptors (NE), ET_A receptors (endothelin-1; ET-1), AT_1 receptors (angiotensin II), and V_1 receptors (vasopressin). Instead of stimulating adenylyl cyclase, Gq-proteins activate the phospholipase C pathway and the Rho-kinase pathway to cause smooth muscle contraction (2).

Eighty percent of ET-1 is released towards the vascular smooth muscle cells where it binds to ET_A receptors to cause vasoconstriction. Another isoform of the ET-1 receptor, ET_B , is expressed on both smooth muscle and endothelial cells (6). When ET-1 binds to this receptor on endothelial cells, it promotes the production of NO and prostacyclin which then diffuse into the smooth muscle to cause relaxation (4).

Under basal conditions, cyclooxygenase-1 (COX-1) is active in endothelial cells to convert arachidonic acid to prostaglandin H₂ (PGH₂). PGH₂ is then converted to either prostacyclin

(PGI₂) or thromboxane A₂ (TxA₂) by their respective synthases. PGI₂ causes vasodilation and TxA₂ induces vasoconstriction (7).

NO is produced from the combination of L-arginine and molecular oxygen by the enzymatic activity of endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS). cGMP-signaling within the vasculature involves the production of NO from eNOS, which diffuses into the smooth muscle to activate soluble guanylyl cyclase and increases cGMP formation to cause vasodilation (2) (Figure 1-2). Activation of eNOS is complicated and can be done through several different pathways. Adenosine, Ach, and NE can activate eNOS after binding to their respective receptors. During inflammatory conditions, bradykinin, calcitonin gene-related peptide, and substance P can also activate eNOS. Mechanical forces can activate eNOS, such as flow- or shear-dependent vasodilation. eNOS is also activated by the binding of calcium-calmodulin and post-translational control mechanisms including: phosphorylation of activation sites, fatty acid modification, and protein-protein interactions (8,9). Treatment with L-N^G-nitroarginine methyl ester (LNAME), a NOS inhibitor, abolishes

Figure 1-2. Normal endothelial function. Adapted from Bruyndonckx et al. 2013.

endothelium-dependent dilation and even causes significant vasoconstriction in a variety of vascular beds, providing evidence that NO is crucial to proper endothelial function (10–12).

Myogenic Response

Skeletal muscle arteriole smooth muscle responds to changes in transmural pressure, the difference between intravascular and extravascular pressure. When transmural pressure increases, smooth muscle contracts and luminal diameter is reduced to increase the resistance to blood flow. A decrease in transmural pressure causes an increase in luminal diameter. This reaction is called the myogenic response. The myogenic response keeps the arteriole in an intrinsic, steady-state of contraction, allowing it to change vessel diameter in either direction in response to stimuli (3,13,14). Shear stress-induced vasodilation operates by mechanisms inherent to the blood vessel wall such that alterations in smooth muscle tone independent of changes in intravascular or transmural pressure can be produced in vessels isolated from the surrounding tissue and perfused in an *ex vivo* system (15–18).

Autoregulation of blood flow

The maintenance of relatively constant perfusion of an organ is essential to proper function. Most tissues, like skeletal muscle, are capable of autoregulating their blood flow in relation to changes in mean arterial pressure (MAP). If MAP is reduced, skeletal muscle blood flow falls, at least initially. Decreased blood flow increases the number of vasodilator metabolites within the tissue to cause vasodilation; blood flow is restored to near normal levels. The opposite is true when

MAP increases. The increase in arterial pressure enhances vasodilator metabolite washout, leading to vasoconstriction and a restoration of blood flow toward normal levels (19,20).

Cerebral Microvascular Function

The brain is the most perfused organ in the body, and obviously for important reason. In order for essential nutrient exchange to occur, the microcirculation of the brain must be highly regulated. Capillary density varies significantly within the brain, depending on region and energy needs. This density is influenced by pathological, physiological, and environmental factors (21). For example, hypertension affects brain capillary density the same way it affects peripheral microcirculation, causing rarefaction and impaired microvessel formation that leads to increased vascular resistance. The structure of brain capillaries is unique compared to their peripheral counterparts (Figure 1-3). Pericytes and astrocytes are crucial to cerebral capillary function by regulating cerebral blood flow, upregulating tight junction proteins, contributing to ion and water homeostasis, and interfacing directly with neurons (22–25).

Pericytes are adjacent to capillaries and share a common basement membrane with endothelial cells. In fact, the ratio of pericyte-to-endothelia is high compared to the vasculature of other organs (1:3 in brain vs 1:100 in skeletal muscle) (26). These cells are thought to have several roles in the brain such as contributing to the stability of the vessel, release of growth factors and matrix important for microvascular permeability, remodeling, and angiogenesis (27). Another essential structural component to the brain vasculature is collateral circulation. The collateral vascular network allows for maintenance of cerebral blood flow, if and when primary inflow fails

due to occlusion or constriction. The circle of Willis provides low-resistance connections to the anterior and posterior circulations, allowing for reversal of blood flow if needed (28).

Figure 1-3. Schematic of the neurovascular unit. *Neuron* 2008;57:178–201

Autoregulation of the brain microvasculature is the most important mechanism to ensure constant and sufficient perfusion. The capillaries of the brain are perfused with blood at all times and each have their own neuron. When the resistance arteries and arterioles dilate, the microvascular pressure gradient increases, thus increasing capillary flow. Red blood cell velocity is high in the cerebral capillary microcirculation to provide efficient oxygen transport to the highly metabolic neuronal tissue (29). The microvessel innervation is intrinsic, meaning each vessel is innervated from within the brain tissue. The nerve terminals do not, however, contact the arteriole itself but rather targets astrocytes surrounding the vessel (30). Middle cerebral artery (MCA) smooth muscle cells are contracted by the binding of serotonin (5-HT) to α_1 -adrenoreceptors to cause activation of the phospholipase C-protein kinase C pathway (31). Interestingly, sympathetic stimulation has little effect on resting cerebral blood flow under normotensive conditions. In contrast, parasympathetic stimulation has potent effects on cerebral arteries, and increases cerebral blood flow (32). Neuronal NO is important for modulating cerebral blood flow autoregulation. Other vasoactive substances such as H^+ , K^+ , O_2 , and adenosine are released to cause vasodilation

when cerebral blood flow is reduced. When blood flow pressure is at the high end of the autoregulatory curve, regulation is probably due to the myogenic response of the cerebral smooth muscle to constrict in response to elevated pressure. Evidence for the crucial importance of autoregulation in normal brain function is provided by the fact that significant brain injury occurs when this autoregulatory mechanism is lost (33–35). Another protective mechanism for the cerebral circulation is segmental vascular resistance, meaning both large arteries and small arterioles contribute significantly to vascular resistance. This mechanism helps to provide constant blood flow in the brain to meet high metabolic demand, without pathologically increasing hydrostatic pressure (36,37).

Metabolic Syndrome (MetS)

Thirty-five percent of all American adults and 50% of those aged 60 years and older present with MetS (38). There is a 3-fold increased risk for CVD with MetS and the syndrome alone predicted 25% of all new-onset CVD (39). The conditions that underlie the metabolic syndrome, including most types of cardiovascular disease, have a cumulative cost of over \$300 billion in the US annually, which comprises the largest financial burden on the US healthcare system. For contrast, the annual cost of all types of cancer is just under \$90 billion (38,40). MetS is defined by the co-occurrence of several cardiovascular risk factors including insulin resistance, obesity, atherogenic dyslipidemia, and hypertension. Previous research has shown that these risk factors are not independent of one another and they share underlying causes, mechanisms, and features (41,42). The current definition of MetS is centered on insulin resistance and obesity. Insulin resistance is measured as impaired fasting glucose or glucose tolerance. An increased homeostatic

model assessment of insulin resistance (HOMA-IR) is also indicative of insulin resistance. The diagnosis of MetS is contingent on the patient presenting with three or more of the criteria.

Insulin stimulates the synthesis of glycogen from glucose and inhibits glycogenolysis in the liver and skeletal muscle. Lipolysis is inhibited, and glucose uptake is stimulated by insulin in adipose tissue. Insulin signaling activates two pathways: the phosphoinositide 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways. PI3K activates Akt kinase which phosphorylates and activates eNOS in vascular endothelial cells. Akt kinase also stimulates translocation of the insulin-responsive glucose transporter GLUT4 to the cell surface, leading to increased glucose uptake. Activation of the MAPK pathway mediates ET-1 production, leading to vasoconstriction; expression of the vascular cell adhesion molecules VCAM-1 and E-selectin, leading to more leukocytes-endothelial interactions; and growth and mitogenesis effects on vascular smooth muscle cells.

Low levels of ROS contribute to normal physiological cell signaling but when there is excessive production or impaired buffering of ROS, there can be deleterious effects. Many pathologies augment the production of ROS from nicotinamide adenine dinucleotide phosphate oxidase (NOX), xanthine oxidase (XO), cyclooxygenases (COX), and even eNOS. ROS, such as superoxide, degrades NO by binding to it to form peroxynitrite. Peroxynitrite is a highly unstable free radical that causes endothelial damage, and NO bioavailability is lowered. NOX is the predominant source of superoxide and is found in both endothelial and smooth muscle cells. Superoxide dismutase (SOD) usually breaks down superoxide anions into hydrogen peroxide but under pathological conditions, SOD function decreases and leads to an impaired buffering of ROS. Experimental inhibition of SOD results in impaired agonist-evoked endothelium-dependent, NO-mediated dilation (43–46).

Other endogenous antioxidant systems exist in the vasculature including glutathione and hydrogen peroxide-reducing enzymes, glutathione peroxidase and catalase. Supplemental treatment with antioxidants acutely improve endothelium-dependent relaxation *in vitro* and *in vivo* (47–50). 4-hydroxy-TEMPO (TEMPOL) is a common pharmaceutical agent used to scavenge free radicals. Previous studies have shown that both acute and chronic treatment with TEMPOL can increase NO bioavailability and improve endothelium-dependent dilation response (51,52). ROS can effect eNOS directly through S-glutathionylation to inactivate the enzyme (53).

Figure 1-4. Normal versus impaired endothelial function. Bruyndonckx et al. 2013.

MetS encompasses a variety of different factors that contribute to endothelial dysfunction (Figure 1-4). Other contributors to endothelial dysfunction associated with MetS include: increased ROS production by oxidase complexes leading to damages of the insulin receptors; increased insulin receptor substrate (IRS)-1 phosphorylation at Ser307; induction of ET-1 expression in the endothelium; exaggerated activation of endothelial mineralocorticoid receptors by aldosterone; and increased production of tumor necrosis factor- α (TNF- α) (54). MetS alters arachidonic acid metabolism and shifts the pathway from mainly vasodilatory to vasoconstrictor

metabolites (55,56). Results from previous studies suggest that insulin resistance and increase oxidative stress contribute to enhanced TP activation which is independent of increased TxA₂ or TP activity (57).

Blood flow must meet the metabolic demands of the tissue and where this is especially important is the skeletal muscle and brain. The microvascular network is optimized for meeting these demands but under pathological conditions, structural alterations to the network can elevate vascular resistance and impair functional hyperemic responses (58). This includes the loss of microvessels within the tissue, or microvascular rarefaction. Microvascular rarefaction can also decrease the ability of skeletal muscle to resist fatigue through impairments in the processes of mass transport and exchange (59,60). The extent of rarefaction is correlated with a loss in NO bioavailability, suggested by previous studies that used physiological and pharmacological interventions to maintain NO levels and the severity of microvascular rarefaction was blunted (61–65). Fluctuations in markers of inflammation and oxidative stress, such as TNF- α , nitrotyrosine, and thromboxane, have been shown to be predictive of microvessel density changes (66).

Previous studies have provided evidence for a link between the balance of systemic vascular oxidant stress and endothelial function and progression/severity of microvascular rarefaction (66–68). This suggests that NO bioavailability is also linked to microvascular rarefaction because rarefaction followed the reduction in NO levels, and interventions aimed at improving NO bioavailability were the most effective in blunting the severity of rarefaction (69,70).

Insulin Resistance

Insulin resistance affects the PI3K-Akt pathway but not the MAPK pathway. This means NO production is reduced and ET-1 and expression of adhesion molecules are unaffected (71). During insulin resistance conditions, adipose, skeletal muscle, and liver cells do not respond appropriately to insulin, and circulating glucose levels remain high. Insulin resistance causes a reduction in eNOS phosphorylation; thus, the endothelium-dependent, NO-mediated relaxation response is blunted. As a common pathway in CVD and in the development of atherosclerosis, endothelial dysfunction is affected by each of the constituent risk factors of MetS. The major feature of endothelial dysfunction is a reduction in NO bioavailability. eNOS activation by phosphorylation at Ser-1177 is diminished by insulin resistance. Activation of eNOS is essential to the hemodynamic actions of insulin, thus during insulin resistance blood flow to skeletal muscle is reduced. ET-1 expression and vascular smooth muscle mitogenic effects are not affected by insulin resistance, therefore exacerbating endothelial dysfunction. TNF- α blocks IRS-1 activation and directly activates NOX to increase superoxide generation. This cytokine also stimulates lipolysis, resulting in FFA release (72).

Visceral adiposity

Obesity causes a reduction in NO-mediated, endothelium-dependent relaxation because of increased ROS production and decreased phosphorylation of eNOS (73,74). The release of endothelium-derived vasoconstrictor prostanoids and ET-1 is also upregulated. Previous studies have found that caloric restriction, weight loss, and exercise training improve endothelium-dependent responses, which could partially be explained by deacetylation and activation of eNOS by SIRT1 (75). Free fatty acids diminish PI3K-Akt signaling, increase ROS, and increase ET-1

production (71,76–78). As another major risk factor of MetS, visceral obesity causes a decrease in insulin-mediated glucose uptake through adipokine signaling including TNF- α and IL-6. Adiponectin is a protective adipokine that couples insulin sensitivity with energy metabolism but adiponectin levels are decreased in obesity and MetS. Adiponectin attenuates ET-1-induced vasoconstriction in normal, healthy conditions but with obesity, adiponectin levels are decreased and the imbalance of ET-1 vs. adiponectin could contribute to insulin resistance. Further, ET-1 stimulates reactive oxygen species (ROS) production by NADPH oxidase, thus promoting oxidative stress-induced vascular dysfunction (4,79).

Atherogenic dyslipidemia

Dyslipidemia is characterized by high plasma triglyceride levels, low HDL cholesterol levels, and an increase in small dense LDL. Insulin resistance and dyslipidemia are causative factors of one another. Insulin normally suppresses lipolysis in adipocytes, thus when insulin signaling is impaired there is an increase in lipolysis and increase of free fatty acid levels. apoB, the major lipoprotein of very-low-density lipoprotein (VLDL) particles, is stabilized by free fatty acids resulting in more VLDL production. Insulin also regulates the activity of lipoprotein lipase, the major mediator of VLDL clearance. If HDL is triglyceride-rich, hepatic lipase can rapidly clear it from the circulation to leave fewer HDL particles to participate in reverse cholesterol transport from the vasculature. This perpetuates the dyslipidemia associated with MetS (39,71,80).

Hypertension

The generally accepted view of hypertension is it causes premature aging of the vasculature because it is exposed to chronically increased arterial blood pressure. It has been shown that NO bioavailability is reduced, thus causing a reduction in endothelium-dependent dilation response. A variety of different mechanisms have been proposed as to why NO is decreased. Perticone et al, (81) attributed lower NO bioavailability in hypertension to higher circulating levels of asymmetric dimethylarginine (ADMA). High blood pressure can cause epigenomic DNA methylation and RNA processing changes which alter gene expressions, increase oxidative stress, and precipitate senescence of endothelial cells (82). Increased intraluminal pressure can also increase local angiotensin signaling and further augment oxidative stress (83). All of these factors lead to endothelial dysfunction. A chronic increase in perfusion pressure, in the case of hypertension, also causes microvascular rarefaction (84).

Animal model of MetS

Lois Zucker discovered the *fa* mutation in a cross between Merck M-strain and Sherman rats, and rats that are homozygous for this allele present with a missense mutation in the leptin receptor (85). These rats are named obese Zucker rats (OZR). Leptin is released by adipose tissue in proportion to the amount of lipids stored and acts in the brain to determine a decrease in food intake and an increase in energy expenditure. By 3-5 weeks, the OZR become noticeably obese as a result of hyperphagia, and develop defective non-shivering thermogenesis and preferential deposition of energy into adipose tissue. These animals also have elevated circulating leptin levels as compared to their lean counterparts. At 14 weeks, the body composition of the obese Zucker is

approximately 40% lipid weight (85,86). The OZR_s also present with dyslipidemia, insulin resistance, and mild glucose intolerance.

Mesenteric arterial function is reduced in OZR_s. Wu et al. (87), found the endothelium-dependent dilation response to Ach was significantly reduced and incubation with LNAME inhibited dilation even further. This study and others suggest endothelial dysfunction in OZR_s, perhaps by a decrease in NO bioavailability (88–92). A study by Frisbee and Stepp (89) confirmed this finding, but in the cremaster muscle arteriole. Further, the study attributed the decrease in dilation response to increased levels of superoxide which in turn ameliorated NO levels.

MetS is associated with microvascular rarefaction along with endothelial dysfunction. Chantler et al. (70), showed a decrease in cerebral cortical MVD in OZR_s by 12-13 weeks. Cerebral MVD was reduced by 20% in 16-17-week-old OZR_s as compared to LZR controls. This study used two pharmaceuticals prescribed for glycemic control and found they were effective in reducing plasma levels of nitrotyrosine (marker of oxidative stress) and blunting microvascular rarefaction (70). It is important to note that MetS-associated loss of MVD is not solely present in the brain, but also in the skeletal muscle.

Lash et al. (93), compared the capillary density (CD) and capillary basement membrane (CBM) thickness in the plantar muscle of sedentary and trained OZR_s to those of lean Zucker rats (LZR). At 11 weeks of age, OZR_s had lower CD and thicker CBM than LZR_s. CD and CBM decreased proportionally greater in OZR_s from 11 weeks to 18 weeks. Exercise training for 6 or 12 weeks significantly increased CD in 18-week OZR_s. A study by Xiang et al. (94), showed exercise training could also improve skeletal muscle microvascular function, specifically by augmenting endothelium-dependent dilation.

The collective factors of MetS cause direct endothelial injury, resulting in vascular remodeling. Lumen remodeling occurs in three ways: hypotrophic (thinned), eutrophic (invariant), or hypertrophic (thickened). Thickening of the vessel wall usually occurs by vascular smooth muscle cell proliferation and migration, impaired integrity of elastin fibers, and deposition of extracellular matrix (95).

MetS causes an increase in circumferential wall stress and flow-mediate shear stress, leading to vascular remodeling. Specifically, carotid wall thickness is increased as is lumen diameter (96–98). In OZRs, the microvasculature has been shown to undergo atrophic remodeling, a reduced lumen size and thinner vascular walls (99). The coronary resistance microvessels in a swine model of MetS demonstrated inward hypertrophic remodeling (100). Vascular remodeling is variable, possibly site- and animal-dependent (101). MetS causes arterial stiffening, which may be due to decreased NO bioavailability, smooth muscle cell proliferation, and increased synthesis of structural proteins including collagen. The chronic inflammation associated with MetS can also induce release of matrix metalloproteinases (MMPs). Leukocytes can facilitate outward remodeling, while macrophages can expedite inward remodeling (102). Further, local hormones such as angiotensin II induces hypertrophy of vascular smooth muscle cells and increases collagen production by fibroblasts (103).

A disease that is often underdiagnosed and undertreated is peripheral artery disease (PAD). PAD is associated with an elevated risk of poor cardiovascular outcomes such as myocardial infarction, stroke and even death. More than 200 million people have PAD worldwide. Pain related to PAD causes patient quality of life to diminish as mobility and independence are progressively reduced, often exceeding the loss of function seen in patients with other forms of cardiovascular

disease (CVD) (104). The individuals most affected by PAD are those 50 years and older, with a sharp rise in prevalence around the age of 70 (105).

Figure 1-5. 10-year survival rate for patients with and without PAD. San Diego Artery Study, 1992.

The risk factors associated with PAD correlate with those of CVD. The first is diabetes mellitus which is estimated to attribute to PAD in 14% of patients (106). The duration of diabetes mellitus, level of glycemic control, and use of insulin are strongly correlated with the increased risk of PAD (107,108). Diabetic patients with PAD have worse outcomes such as an increased amputation risk and 3-times higher odds of mortality (109) (Figure 1-5).

Several other risk factors for PAD are hypertension, dyslipidemia, obesity, and increased C-reactive protein. These are not just risk factors for PAD, but also fall under the cluster of factors that constitute MetS. Hypertension and systolic blood pressure were found to be significantly and independently associated with PAD. The higher prevalence of PAD in elderly patients renders hypertension a major contributor to the total burden of PAD in the population (110). The ratio between total cholesterol and HDL-C is another measure for increased risk of PAD. The typical dyslipidemia seen in insulin-resistant individuals (low HDL-C and high triglycerides) is

significantly associated with PAD (111). Although obesity has not been found to be independently associated with PAD, the factors linked to obesity, like the ones previously stated, contribute to PAD. A higher waist/hip ratio has been found to be significantly correlated with a higher risk of PAD even though BMI or body fat percentage were not (108). An increase in inflammatory markers, such as C-reactive protein, have also been shown to contribute to PAD risk (112).

Chronic Stress and Depression

Diagnosing depression is actually problematic because distinguishing mood changes between clinical and normally occurring is difficult. Depressive symptoms in and of themselves are complex, characterized by the absence of a positive effect (anhedonia), low mood, and a range of associated emotional, cognitive, physical and behavioral symptoms. Major depression is based on its severity, persistence, the presence of other symptoms, and the degree of functional and social impairment but there is no distinct ‘cut-off’ between ‘major’ and ‘normal’ degrees of depression (113,114).

In major depressive disorder, mood remains low throughout the day and does not change with circumstance. Within other forms of depression, mood can be reactive to positive experiences and events, but depressive feelings return/reemerge quickly after the event is over (115). Physical and behavioral symptoms include tearfulness, irritability, social withdrawal, and exacerbation of pre-existing pains, pains secondary to increased muscle tension, a lack of libido, and fatigue and diminished activity. In some patients sleep and appetite are lowered but in others, sleep and appetite are increased. Other behavioral changes associated with depression include: loss of interest and enjoyment in everyday life, feelings of guilt, worthlessness and that one deserves punishment, lowered self-esteem, loss of confidence, and suicidal ideation. Poor concentration and

reduced attention, pessimistic and recurrently negative thoughts about oneself, one's past and the future, and mental slowing are all cognitive changes that occur with depression (116,117).

It used to be thought that depression was time-limited and although it is episodic, incomplete recovery and relapse are very common. Fifty percent of people will have at least one more episode after their first, and after the second and third episodes, the risk of further relapse rises to 70 and 90%, respectively (118). As the most common mental disorder, depression is a major cause of disability across the world. There is a strong correlation between illness severity and the extent of disability (119). Depressive illness causes a greater decrement in health state than the major chronic physical illnesses such as angina, arthritis, asthma, and diabetes (120). The emotional and cognitive effects of depression substantially reduce a person's ability to work effectively, which increases dependence on welfare and benefits and reduces personal income. When coupled with pre-existing physical diseases, health is worsened as compared with physical disease alone and increases risk of death (117,120).

The etiology of depression spans many different physiological processes and factors. Diabetes, cardiac disease, diseases of the thyroid, Cushing's syndrome, and Addison's disease have all been shown to increase the risk of depression (117). Interestingly, some psychological disorders have been found to be significantly correlated with PAD such as anxiety and depressive symptoms (121). The prevalence of depression in PAD is similar to that in CVD, which is from 2-10%. Depression has been linked with certain risk factors for PAD including coronary artery disease, hypertension, and diabetes mellitus. Hippisley-Cox et al. (122), found that 20% of depressed patients also had coronary artery disease and Frasure-Smith et al. (123), showed a significant increased mortality in patients with comorbid depression and coronary artery disease. Patients with hypertension are three times as likely to have concurrent major depression (124). In

individuals with diabetes mellitus, the mean prevalence of depression is 32% (125). The fact that PAD risk factors are significantly related to depression suggests there is a bi-directional causality between the two. Brostow et al. (126) examined the link between the physical dysfunction caused by PAD and the negative affect of depression on walking ability. The study made the connection that depressed PAD patients may experience a multifactorial burden of disease because PAD directly impacts patients' ability to walk and depression is also an independent predictor of disability and loss of mobility (126). Another study by Smolderen et al. (127) found that depressed PAD patients performed worse on the treadmill exercise test.

Chronic Stress

The state of threatened homeostasis, or stress, is caused by intrinsic and/or extrinsic adverse forces (stressors). The body then tries to counteract these stressors by activating physiologic and behavioral responses to reestablish equilibrium. Chronic stressors have the most detrimental effects on physiological functions including growth, reproduction, metabolism, immunocompetence, and behavior and personality development (128). In addition to these effects, chronic stress acts as a pre-disposing and participating factor in the onset of depression in humans (129–131).

Part of the stress response system includes the hypothalamic-pituitary-adrenal (HPA) axis. Corticotropin-releasing hormone (CRH) acts to regulate the anterior pituitary adrenocorticotrophic hormone (ACTH). Under normal, non-stressful conditions, CRH and vasopressin are secreted in a circadian fashion but also pulsatile with a pulse of production every 1-2 hours. The production of CRH and vasopressin increase in the early morning hours (132,133). Acute stress augments CRH

and vasopressin production and induces the production of Ang II and various cytokines and lipid mediators of inflammation, leading to further activation of the HPA axis (134).

ACTH targets the adrenal cortex to regulate glucocorticoid and adrenal androgen secretion. Cortisol secretion is increased during times of stress, and can be regulated by other hormones and cytokines coming from the adrenal medulla or systemic circulation (135). Glucocorticoids increase hepatic gluconeogenesis and glucose plasma levels, induce lipolysis, and cause protein degradation in multiple tissues. Therefore, chronic HPA axis activation can have detrimental effects including increased visceral adiposity, suppressed osteoblastic activity, decreased lean body mass, and insulin resistance (136–138). Previous studies have shown that chronic stressors increased the incidence of visceral obesity and MetS (138,139). In addition to accumulation of visceral adipose tissue, chronic stress strongly correlates with behavioral changes that decrease physical activity and poor dietary habits, therefore leading to further weight gain and potentially to dysregulation of glucose and lipid metabolism (140,141).

Under chronic systemic inflammation situations, such as in MetS, prolonged increased circulating levels of pro-inflammatory cytokines can lead to hyperactivation and derangement of the HPA axis (142). Adipocytes specifically release pro-inflammatory cytokines such as TNF- α and IL-6 that can chronically stimulate the HPA axis to induce a vicious cycle whereby hypercortisolemia contributes to adipocyte accumulation and vice versa (80,143). After chronic exposure to high aldosterone levels NO-dependent relaxations are impaired, perhaps due to a reduction in BH4 production and an increase in oxidative stress, thus augmenting the release of vasoconstrictor prostaglandins (144). Endothelium-dependent reactivity is reduced under chronically high cortisol levels, probably due to a decrease in eNOS expression (145).

Little is known about how chronic stress and depression directly impact vascular structure and cause remodeling. Aldosterone has been shown to decrease vascular integrity by disrupting endothelial remodeling, specifically by modifying the actin cytoskeleton assembly. Cortisol was found to partially mimic this effect by aldosterone where it significantly increased paracellular permeability and influenced distribution of junction-associated proteins (146).

Unpredictable Chronic Mild Stress Model

Unpredictable Chronic Mild Stress (UCMS) is a well-known, validated animal model using chronic stress to induce depressive symptoms. Most studies that use this model have documented physiological changes in the brains of rodents, mainly reflecting an elevated physiological stress response (147). The effects of UCMS have been attributed to its impact on the HPA axis, shown by the prevention of a depressive phenotype when glucocorticoid receptors are inhibited (148–151) and development of depressive symptoms when exogenous corticosterone is given (149,150,152).

Changes in NO levels have been discovered in chronic stress-induced depression patients but it is unclear in humans whether bioavailability is increased or decreased (153,154). In animals, however, Gao et al. (155) have shown an increase in plasma NO levels and decrease in total NOS activity. Isingrini et al. (156), illustrated an overall decrease in endothelium-dependent dilation response of the aorta in UCMS. The maximum response was significantly lower in UCMS as compared to the control group (156). Oxidative stress associated with chronic stress and depression directly decrease NO bioavailability. Zhou et al. (157), found that nNOS mRNA and protein

content were elevated in the hippocampus. nNOS enzymatic activity was also enhanced, consistent with the increase in expression (157).

Exercise

Acute Effects

Initiation of exercise causes drastic changes in hemodynamics through different mechanisms. The sympathetic nervous system directs increased sympathetic outflow to the heart to increase cardiac output and evokes baroreflex-mediated vasoconstriction in peripheral organs to redirect blood flow to contracting skeletal muscles and maintain blood pressure (158,159). This process is called functional hyperemia. Exercise creates a ‘muscle-pump’ to increase the blood flow to the active muscles.

Dilation of the arterioles is important to provide sufficient blood flow to meet the increased demand for oxygen. Vasoactive metabolites such as potassium ions, adenosine, and NO from the muscle, all generate vasodilation (160,161). Through the production of adenosine and NO, sympathetic vasoconstrictor tone in exercising muscles is lessened, a process known as functional sympatholysis.

Exercise inherently increases shear stress within the lumen of blood vessels, which contributes to the regulation of vascular tone (162). The increase in shear stress acts to trigger vasodilation, mainly through NO release from endothelial cells, but also hydrogen peroxide, epoxyeicosatrienoic acids, and PGI₂. ATP released from endothelial cells stimulates purinergic receptors to induce NO production from endothelial cells. It has also been shown that in response to laminar shear stress, eNOS mRNA expression is upregulated (163). All of these factors caused by shear stress aid in vasodilation to provide proper perfusion of active skeletal muscle.

Sudden increases in shear stress induce bursts of PGI₂, a vasodilator, to be released. In the skeletal muscle arterioles, PGI₂ plays a larger role in flow-mediated vasodilation than it does in conduit arteries. After short-term daily exercise the gracilis muscle arteriole response to wall shear stress was upregulated and resulted in an augmented dilator response, perhaps due to increased NO and prostacyclin (164). Continuous release of NO and prostacyclin, enhanced by shear stress, can counteract neurogenic and myogenic vasoconstriction in vivo (165).

Previous studies have shown that short-term exercise increased endothelial NO synthesis in skeletal muscle arterioles and increased the vasodilator response to Ach. Endothelium-dependent dilation was improved in conduit arteries as well (166). These results suggest that short-term exercise training enhances NO production and activity to buffer increased shear stress.

Active muscles release myokines such as IL-6 and IL-8. IL-6 is known to influence AMP-activated protein kinase (AMPK). It is thought that through this pathway, vasodilation is activated by AMPK phosphorylating eNOS to produce NO. Skeletal muscle also highly expresses neuronal NOS (nNOS), which enhances the production of IL-6 (167).

Chronic Effects

Adaptive changes in skeletal muscle circulation are found after chronic exercise training to improve blood flow capacity and oxygen diffusing capacity. Mainly this occurs through angiogenic adaptations and remodeling of the vasculature (160,168–170), but it also exerts beneficial effects on vascular reactivity.

The periodic increases in blood flow improve endothelium-dependent vasodilation by modulating the expression of NO synthase. The magnitude and nature of shear stress has influence

on endothelial cell NO synthesis, where laminar shear stress dose dependently upregulates NO synthesis but turbulent shear stress has no effect on the NO synthase pathway (171). One mechanism by which flow augments NO synthase is through an increase in the involvement of shear stress responsive elements within the promoter region of the gene. The sequence for these shear stress responsive elements is also common in other endothelial genes that respond to shear stress including tissue plasminogen activator, intracellular adhesion molecule, TGF- β , PDGF- β , and ET-1 (172,173).

Flow-mediated vascular remodeling is endothelium-dependent. With chronic low flow, vessel diameter decreases but the opposite is true for a chronic increase in flow. It is proposed that the endothelium induces changes in vessel structure by producing vascular growth-mediators such as NO, TGF- β , PDGF- β , ET-1, PGI₂, and tissue plasminogen activator (15,164,174–178).

Exercise increases capillary density and arteriolar number. The stimulation of angiogenesis may be derived from perivascular or stromal cells responding to mediators and mechanical forces associated with exercise to release angiogenic factors such as vascular endothelial growth factor (VEGF) and NO, thus inducing vascular remodeling (179,180). IL-8 seems to be involved with the angiogenic response to exercise. This particular myokine can affect pericyte function, which is essential for endothelial lumen formation during angiogenesis (167,181,182).

Protective Effects

Dysfunction within the peripheral microcirculation contributes significantly to pathologic conditions but exercise can mitigate the pathologic consequences through its protective actions.

Major Depressive Disorder

Using a chronic mild stress model, Watanasriyakul et al., (183) illustrated the beneficial effects of voluntary wheel running in the prairie vole. This study found that exercise trained voles had significantly lower depressive- and anxiety-behaviors, along with lower corticosterone levels as compared to sedentary voles. Four weeks of swimming exercise reversed UCMS-induced depression-like behaviors (184). Vancampfort et al. (185) through meta-analysis found a significant negative association between physical activity and suicide ideation (SI) levels. Further, “active” people were less likely to have SI but also were protected against SI.

Ischemia

Ischemia in any tissue can be deleterious to its function, and therefore, blood flow is of utmost importance. Exercise can precondition the heart and skeletal muscles to induce ischemic tolerance. The periods of increased blood flow and shear stress stimulate the release of substances such as NO and prostacyclin, which not only decrease the permeability of arterial walls to plasma lipoproteins, but also attenuate inflammatory processes (186,187).

Inflammation

A general risk factor for poor cardiovascular outcomes is chronic inflammation. Diseases associated with this type of inflammatory state include MetS, PAD, depression, and CVD. The main target of the inflammatory response is the endothelium of both large and small blood vessels

alike. This creates endothelial barrier dysfunction, expansion of proinflammatory stimuli, enhanced adhesion/leukocyte molecule expression, platelet recruitment, impaired vasomotor function, and vascular remodeling (187,188). The proinflammatory response contributes to enhanced cell trafficking and to the translocation of large inflammatory molecules into the blood vessel due to increase permeability (189). Exercise can reduce the damaging effects of inflammation on the endothelium by downregulating transcription of proinflammatory and atherogenic genes.

Oxidative Stress

Endothelium-dependent dilation impairment is caused by increased ROS production by the vasculature and circulating immune cells, upregulated angiotensin II and ET-1 production, which in turn activate NADPH oxidase and can uncouple eNOS (190). There is a reduction in endothelial-derived bioavailable NO, which is partially responsible for the diminished responses to Ach or increased shear stress. NO and superoxide interact to form peroxynitrite. Peroxynitrite is detrimental to NO production because it interacts with the eNOS cofactor, tetrahydrobiopterin, to cause eNOS uncoupling (191). The protective effect of exercise acts to decrease ROS production and increase eNOS function.

Microvascular Rarefaction

Hypertension, diabetes, and obesity cause the skeletal muscle microcirculation to undergo significant degenerative remodeling, known as microvascular rarefaction (Figure 1-6). This results from an imbalance of pro- and anti-angiogenic factors, cleavage of vascular growth factor receptors by matrix metalloproteinases, oxidant-mediated inactivation of NO, impaired angiogenic responses to growth factors, and alterations in adipokine expression (69). However, exercise can attenuate microvascular rarefaction by increasing NO bioavailability and decreasing inflammatory cytokines such as MCP-1 and IL-1 β (69).

Figure 1-6. Protective effects of exercise. Korthius 2011.

Summary and Statement of Aims

Previous studies have focused on the causative mechanisms of MetS and chronic stress-induced depression separately, however, there is a high prevalence of comorbidity between these two. The effects of this comorbidity on the peripheral microvasculature are unknown. There is a clear association between the vasculopathy present in each disorder and the effect of increased oxidative stress on vascular reactivity and MVD. The endothelial functional deficits, as well as the loss of MVD, seen in MetS and chronic stress-induced depression are causal to the poor cardiovascular outcomes correlated with the disorders. It is important to determine if chronic stress-induced depression exacerbates the vascular dysfunction present in MetS. Exercise has been shown in many previous studies to augment NO bioavailability, decrease ROS, and improve endothelium-dependent dilation response. This raises the question whether exercise can directly improve the vascular dysfunction associated with MetS and chronic stress-induced depression, and perhaps the exacerbated effects of the comorbidity. Further, exercise may attenuate loss of MVD and, in fact, stimulate angiogenesis.

Therefore, this dissertation aims to use a valid and reliable model of MetS to examine the hypothesis: 1) UCMS will cause peripheral microvascular dysfunction and microvascular dysfunction in LZRs similar to that seen in OZRs; 2) OZRs exposed to UCMS will have exacerbated peripheral microvascular dysfunction; 3) the peripheral microvascular dysfunction and loss of MVD are caused by reduced NO bioavailability and increase in oxidative stress; and 4) exercise can augment peripheral microvascular dysfunction and rarefaction by decreasing oxidative stress and improving endothelium-dependent dilation response.

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CHAPTER 2 : Role of Chronic Stress and Exercise on Microvascular Function in Metabolic Syndrome

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Abstract

Purpose: The present study examined the effect of unpredictable chronic mild stress (UCMS) on peripheral microvessel function in healthy and metabolic syndrome (MetS) rodents, and whether exercise training could prevent the vascular dysfunction associated with UCMS.

Methods: Lean and obese (model of MetS) Zucker rats (LZR; OZR) were exposed to 8 weeks of UCMS, exercise (Ex), UCMS+Ex, or control conditions. At the end of the intervention, gracilis arterioles (GAs) were isolated and hung in a pressurized myobath to assess endothelium-dependent (EDD) and -independent (EID) dilation. Levels of nitric oxide (NO) and reactive oxygen species (ROS) were measured through DAF-FM and DHE staining, respectively.

Results: Compared to LZR controls, EDD and EID was lower ($p=0.0001$) in LZR-UCMS. The OZR-Ex group had a higher EDD ($p=0.0001$) and EID ($p=0.003$), compared to OZR-Controls; whereas only a difference in EDD ($p=0.01$) was noted between LZR-Control and LZR-Ex groups. Importantly, EDD and EID were higher in the LZR ($p=0.0001$; $p=0.02$) and OZR ($p=0.0001$; $p=0.02$) UCMS+Ex groups compared to UCMS alone. Lower NO bioavailability and higher ROS were noted in the LZR-UCMS group ($p=0.0001$), but not OZR-UCMS, compared to controls. Ex and UCMS-Ex groups had higher NO bioavailability ($p=0.0001$) compared to control and UCMS groups, but ROS levels remained high.

Conclusions: The comorbidity between UCMS and MetS does not exacerbate the effects of one another on GA EDD responses, but does lead to the development of other vasculopathy adaptations, which can be partially explained by alterations in NO and ROS production. Importantly, exercise training alleviates most of the negative effects of UCMS on GA function.

Key words: obesity, UCMS, skeletal muscle arteriole, endothelial dysfunction

Introduction

Fifty-six million American adults are diagnosed with the metabolic syndrome (MetS) (1–4), which significantly increases an individual's risk of peripheral vascular disease (PVD). Indeed, Maksimovic et al. (5) showed that around 60% of patients with PVD also manifested with MetS. Our group, and others, have shown in a model of MetS (obese Zucker rat) there is a global reduction in vascular-derived nitric oxide (NO) bioavailability, leading to significant peripheral vascular dysfunction associated with PVD (6–9).

Depression is independent risk factor for PVD (10). Previous studies have shown that chronic stress is a major contributor to depressive illness and may be the link between depression and PVD (11–15). Stress-induced depression causes vascular dysfunction, in part, by impairing the bioavailability of dilator metabolites such as NO (16–18). Furthermore, it has been postulated that exposure to chronic psychosocial stress is a significant risk factor for the development of MetS. Indeed, approximately 43% of MetS patients present with depression (20, 23). Given that both MetS and depression result in significant vasculopathies, it remains unknown whether MetS exposed to chronic stress results in more severe vasculopathies thereby significantly increasing the risk of PVD events (myocardial infarction, heart failure, stroke, and/or limb ischemia).

Exercise, when used as an intervention, reduces the risk of PVD associated with MetS and depression, separately (19,20). Previous studies have shown that chronic exercise training can increase the expression of endothelial NO synthase (NOS) and its activity (21–24). Exercise also upregulates antioxidant activity which leads to an attenuation of reactive oxygen species (ROS) production, and therefore, decreases oxidative stress (25,26). Through these two mechanisms, aerobic exercise can increase NO bioavailability, improving vascular endothelial function (27) and perhaps reduce the risk of PVD associated with MetS and depression. However, it is unknown

whether chronic exercise can limit or restore the vasculopathies involving MetS exposed to chronic stress.

The current study used the obese (*fa/fa*) Zucker rat, a translational model of MetS that develops significant PVD risk (in the absence of significant atherosclerosis) that can impair skeletal muscle perfusion and performance, and its lean controls (LZR) to explore the role of unpredictable chronic mild stress (UCMS), exercise (Ex), and a combination of each on peripheral microvascular function (8,28). UCMS is a protocol used to induce depressive-like symptoms by exposing animal models to daily, mild stressors (29–31). The UCMS protocol is accepted as a relevant rodent model of depression that has been shown to reproduce clinical symptoms of depression, including anhedonia and increased anxiety-like behavior (32). Our initial hypothesis was that: 1) LZRs exposed to UCMS would have peripheral microvascular dysfunction similar to that evident in the OZR controls due to a reduction in NO bioavailability and an increase in ROS production; 2) the comorbidity between MetS exposed to UCMS would exacerbate the already existing peripheral microvascular dysfunction; and 3) exercise could limit the peripheral microvascular dysfunction by decreasing oxidative stress and improving vasodilation associated with MetS exposed to chronic stress.

Materials and Methods

Animals

Male LZR and OZR (Harlan) arrived at the West Virginia University Health Sciences Center (WVUHSC) animal facility at 7-8 weeks of age. After 1 week of acclimation to the local environment, LZRs ($n = 8$ per protocol) and OZR ($n = 8$ per protocol) were randomly assigned to

a specific protocol group for the subsequent 8-9 weeks including: 1) sedentary controls, 2) UCMS, 3) exercise (Ex), and 4) a combination of UCMS+Ex, resulting in 8 groups in total. All animals were fed standard chow and tap water ad libitum for all experiments. Protocols received prior approval from the WVUHSC Animal Care and Use Committee.

UCMS Protocol

Previous investigators developed the UCMS model for developing depression-like behaviors in rodents (212–215). The UCMS model is considered to be the most appropriate rodent model for clinical depression, based on its ability to reproduce the development of many clinical human depressive symptoms, including anhedonia and learned helplessness (147).

All rats were singly housed. In UCMS groups, rats were exposed to the following mild environmental stressors in randomly chosen sequences for 8 hours each day, 5 days/week, over the course of 8 weeks:

1. *Damp bedding* – 10 oz. of water was added to each standard cage
2. *Bath* – all bedding was removed and ~0.5 inches of water was added to empty cage. Water temperature was room temperature, ~24°C
3. *Cage Tilt* - cage was tilted to 45 degrees without bedding
4. *Social stress* – each rat was switched into a cage of a neighboring rat
5. *No bedding* – all bedding was removed from the cage
6. *Alteration of light/dark cycles* –turning lights off/on in random increments for scheduled period.

Exercise Training Protocol

LZR and OZR underwent 8 weeks of treadmill running, either concurrent with UCMS or as a standalone treatment. Animals ran 5 days/week on multi-lane motor driven treadmills set at a 5% grade. During the first week, animals were acclimatized to the treadmill by running for 20 min, then increasing by 10 min/day until sustainable duration of 60 minutes daily was achieved. A maximum speed test was performed on each animal and target-running speed was set for 60-70% of that maximum. After acclimatization, the first 15 minutes of the total 60 minutes consisted of a gradual increase until reaching target-running speed. Rats ran at this speed for the remaining 45 minutes. Mild electrical stimulus (≤ 0.3 mA) was used at the rear of the treadmill to discourage rats from stopping. There was a 48-hour wash out period between the last Ex bout and the terminal surgery at the end of 8-week treatment.

UCMS and Exercise Combination Protocol

OZRs and LZRs assigned to this group performed treadmill running first thing in the morning (8-9am) and then were immediately subjected to the UCMS protocol as described previously.

Coat Score

The rodents coats were evaluated throughout the duration of the 8-week protocol. Each week, the rats were weighed and inspected for grooming habits (216). The total cumulative coat score was computed by giving an individual score of 0 (clean) or 1 (dirty) to eight different body parts (i.e. head, neck, back, forelimbs, stomach, hindlimbs, tail, genitals).

Circulating Cortisol

Corticosterone is a glucocorticoid produced by the adrenal cortex in response to ACTH (corticotrophic hormone) and is the precursor to aldosterone. Corticosterone is the main glucocorticoid in rodents as cortisol is in humans. The production of glucocorticoids is increased by stress. Using a commercially available ELISA Kit (Cayman Chemical, Item #501320) serum samples, collected at time of terminal surgery, were examined for corticosterone levels in duplicate accordingly to the manufacturer's instructions

Plasma Clinical Markers

Fasting blood was drawn intravenously from anesthetized rats into lithium-heparin coated blood tubes and transported immediately to the laboratory for analysis. Levels of triglycerides (TG), total cholesterol (TC), high-density lipoprotein (HDL) were measured by the clinical laboratory service at Ruby Memorial Hospital (Morgantown, WV). Blood glucose was measured using a commercially available glucometer (FreeStyle, Abbott), and insulin was measured using a rat ELISA kit (Cayman Chemical, item #589501).

Isolation of the Gracilis Arteriole

After completion of the treatment period and at 17-18 weeks of age, each rat was anesthetized with pentobarbital sodium (50 mg/kg ip) and was intubated via the trachea to facilitate maintenance of a patent airway. In all rats, a carotid artery and an external jugular vein were cannulated for determination of arterial pressure and for infusion of heparin. Under anesthesia, an aliquot of blood was drawn from the inferior vena cava to be used for further analysis.

With specific attention to the skeletal muscle circulation and its role in the development of PVD we selected the gracilis arterioles (GA) to determine the effects of MetS and UCMS on peripheral vascular function. Under deep anesthesia, both right and left gracilis arterioles (GA) were isolated from their origin in the skeletal muscle of the thigh then placed in cold (4°C) physiological salt solution (PSS; in mM: 119 NaCl, 1.17 MgSO₄, 1.6 CaCl₂, 1.18 NaH₂PO₄, 24 NaHCO₃, 0.026 EDTA, and 5.5 glucose). Each GA was doubly cannulated in a heated (37°C) chamber that allowed perfusion and superfusion of the lumen and exterior of the vessel, respectively, with PSS from separate reservoirs. The PSS was equilibrated with a 21% O₂-5% CO₂-74% N₂ gas mixture. Vessel diameter was measured using microscopy and an on-screen video micrometer.

Measurements of vascular reactivity in isolated GA

After cannulation, GAs were extended to their in-situ length and equilibrated at 80% of the animal's mean arterial pressure (MAP) to approximate in vivo perfusion pressure. Active tone at the equilibration pressure was calculated as $(\Delta D/D_{max}) \times 100$, where ΔD is the diameter increase from rest in response to Ca²⁺-free PSS and D_{max} is the maximum diameter measured at the equilibration pressure in Ca²⁺-free PSS.

After equilibration, the dilator reactivity of GA was assessed in response to increasing concentrations (10⁻¹⁰-10⁻⁶M) of acetylcholine (ACh) to determine endothelium-dependent dilation (EDD) and sodium nitroprusside (SNP, 10⁻¹⁰-10⁻⁶M) to determine endothelium-independent dilation (EID). Constriction was assessed by exposing the GA to increasing doses of phenylephrine (PE, 10⁻¹⁰-10⁻⁷M). Vascular responses to ACh were also measured following acute (45-60 min)

incubation with nitro-L- arginine methyl ester (L-NAME, a NO synthase inhibitor, Sigma-Aldrich N5751) (10^{-4} M) and 4-Hydroxy-TEMPO (TEMPOL, Sigma-Aldrich 176141) (10^{-4} M) to assess the contributions of NO and oxidative stress, respectively, to modulation of vascular reactivity.

Following the experimental procedures for measuring ex-vivo reactivity, the perfusate and superfusate PSS were replaced with Ca^{2+} -free PSS containing the metal chelators EDTA (0.03 mM) and EGTA (2.0 mM). Vessels were stimulated with 10^{-7} M (PE) to facilitate Ca^{2+} release and eliminate active tone. Subsequently, intraluminal pressure within the isolated vessel was altered, in 20 mmHg increments, between 0 and 160 mmHg. To ensure that a negative intraluminal pressure was not exerted on the vessel, 5 mmHg was used as the “0 mmHg” intraluminal pressure point. After 7 mins at each intraluminal pressure, the inner and outer diameters of the passive GA were determined.

Measurement of Reactive Oxygen Species

Dihydroethidium (DHE, Invitrogen D1168) assays were performed on unfixed femoral arteries to evaluate superoxide and hydrogen peroxide levels in situ. Femoral artery was used rather than GAs because the entire section of isolated GA was used for reactivity. Femoral arteries were placed in individual wells of a 96 well plate containing 200µl of HEPES buffer. Femoral artery was incubated in control/drug treatment for 30 minutes at 37°C. Following incubation, 2µl of stock DHE solution was added to each well to a concentration of 10µM and incubated at 37°C for another 30 minutes. After completion of DHE incubation, arteries were washed in HEPES buffer, placed separately in Optimal Cutting Temperature compound (OCT, Fisher Healthcare™ Tissue-Plus™ O.C.T Compound), and flash frozen in liquid nitrogen to be stored at -80°C. DHE OCT blocks

were then cut into 8µm slices using a cryostat at -22°C and transferred to charged slides (Fisherbrand® Superfrost® plus microscope slides) and stained/mounted with DAPI mounting media (VECTORSHEILD antifade mounting media with DAPI, Vector laboratories). Four slices per animal were imaged with an EVOS fluorescent microscope (Invitrogen EVOS FL Auto Cell Imaging System) and then analyzed in ImageJ as fluorescent density/nucleus.

Measurement of NO Bioavailability

Aortic NO levels were measured by 4-Amino-5-Methylamino-2',7'- Difluorofluorescein Diacetate (DAF-FM-DA, Invitrogen) according to manufacturer's instructions. As stated previously, aortas were used because GA reactivity required the entire section of vessel. 3mm aortic rings were placed in individual wells of a 96 well plate containing 200µl of HEPES buffer supplemented with L-Arginine (100µM, MP Biomedical Inc. 100736). L-NAME was used as a negative control. After 30-minutes incubation with treatment, DAF-FM 10µM was added to each well and the vessel was stimulated with Acetyl-β-methylcholine chloride (methacholine (MCh), 1×10^{-6} , Sigma-Aldrich A2251). After 10 minutes, the aorta was removed and the conditioned solution was read in a plate reader excitation/emission at 495/515nm wavelength (BioTek Synergy HT). Fluorescence was normalized to aorta length and the L-NAME value to account for the reaction of the DAF assay with other molecules (i.e., hydrogen peroxide).

Data and Statistical Analyses

Data are presented as mean $\pm$ SD. Normality was evaluated by the Kolmogorov–Smirnov test. The maximal reactivity or the remodeling of the GA due to the experimental conditions were

analyzed by a multifactorial analysis of variance (ANOVA) [i.e., species (i.e., LZR vs. OZR), and experimental condition (i.e., Control, UCMS, Ex, and UCMS+Ex)] with an interaction term (species-by-group), and a Tukey post-hoc test was performed to determine differences between groups. The effects of TEMPOL and L-NAME on the maximal dilation of the GA was examined with a repeated measures ANOVA. Clinical characteristics between the animals were compared with a One-Way ANOVA with Tukey post-hoc test, as appropriate. DHE, was examined using Kruskal Wallis test, with within group comparisons examined using the Mann-Whitney test. In all cases, $p < 0.05$ was taken to reflect statistical significance.

Results

Animal Characteristics

The baseline characteristics of the animals used in the present study are summarized in Table 2-1. In comparison to LZR and OZR controls, the intervention groups had a lower body mass at the end of the intervention. Mean arterial pressure did not differ between UCMS or Ex groups. Higher fasting glucose concentrations were noted in the LZR and OZR UCMS group compared to their respective controls. Further, glucose concentrations were lower in the OZR-Ex group, but higher in the OZR-UCMS+Ex compared to OZR-controls. Markers and symptoms of stress were elevated in both LZRs and OZRs using the UCMS protocol as seen by an increase in corticosterone and coat scores in both LZR and OZR-UCMS, and UCMS+Ex groups compared to controls. Also, there was an increase in adrenal weights with UCMS in LZRs, from 16.7g to 25.1g ($p=0.0001$) and in OZRs, from 27.2g to 32.7g ($p=0.0001$) (data not shown). Adrenal weight did not significantly change in LZR- or OZR-UCMS+Ex as compared to their respective controls.

Effects of Experimental Conditions on GA Reactivity

Figure 2-1 illustrates the EDD, EID, and constriction responses of the GA for each treatment group. After 8 weeks of UCMS in LZR, there was a significant impairment in EDD (73%, $p=0.0001$) compared to LZR-Controls. EDD was higher in LZR-Ex (41%, $p=0.009$) compared to LZR-Controls, and EDD was also higher in the LZR-UCMS+Ex (209%, $p=0.0001$) vs. LZR-UCMS. Importantly, now EDD in the LZR-UCMS+Ex group did not statistically differ compared to LZR-Control. In the OZR groups, UCMS did not attenuate EDD further (Fig 2-1A); however, in the OZR-Ex group, EDD was higher (141%, $p=0.0001$) compared to OZR-Controls. When Ex was combined with UCMS, the EDD was higher (174%, $p=0.0001$) vs. OZR-UCMS, and EDD in the OZR-UCMS+Ex group was also higher (83%, $p=0.02$) than OZR-Controls (Fig. 2-1A). Of note, there was a significant ($p=0.0006$) species (LZR vs. OZR) by group (Con, UCMS, Ex, UCMS+Ex) interaction for the GA EDD response.

Next, we examined EID of the GA, and found that EID was lower (53%, $p=0.001$) in LZR-UCMS compared to LZR-Controls (Fig. 2-1B), but no differences were noted in EID between LZR-Ex and LZR-Control groups. Importantly, the concurrent exposure of LZR to UCMS and Ex resulted in a higher EID (56%, $p=0.02$) compared to LZR-UCMS, and made EID similar between LZR-UCMS+Ex and LZR-Control groups. As for the OZR groups, no significant differences in EID were noted between OZR-UCMS and OZR-Controls; however, EID was higher in the OZR-Ex (88%, $p=0.003$) vs. OZR-Controls (Fig. 2-1B). Further, EID in OZR-UCMS+Ex was higher (17%, $p=0.02$) compared to OZR-UCMS (Fig. 2-1B). We also noted a close to significant ($p=0.054$) species-by-group interaction for the GA EID response.

The GA constriction response was evaluated by stimulating the GA with increasing doses of PE (Fig. 2-1C). In LZR, UCMS resulted in a smaller constriction response (57%, $p=0.002$)

compared to LZR-Controls. The GA constriction response was similar between LZR-Ex, LZR-UCMS+Ex, and LZR-Control groups. In the OZR, the GA constriction response was similar between all groups. As such, a significant ($p=0.001$) species-by-group interaction was noted for the GA constriction response.

Effect of Acute TEMPOL or L-NAME on GA

To examine whether the impairment in the GA EDD was a reflection of increased oxidative stress and reduced NO bioavailability, the GA was acutely incubated with TEMPOL and L-NAME (Fig. 2-2). Of note, all comparisons here are compared to within the experimental group without the TEMPOL, or L-NAME incubation (i.e., LZR control-TEMPOL vs. LZR control). Acute TEMPOL incubation did not significantly impact EDD in LZR-Control, LZR+Ex, and LZR-UCMS+Ex (Fig. 2-2A); however, in the LZR-UCMS group, TEMPOL significantly increased EDD (81%, $p=0.009$), suggesting a role of oxidative stress on the impaired EDD with UCMS in LZRs. Acute TEMPOL incubation significantly increased EDD in OZR-Control (76%, $p=0.01$) and OZR-UCMS (149%, $p=0.03$) (Fig. 2-2B). Neither OZR-Ex nor OZR-UCMS+Ex had any improvements in EDD with acute TEMPOL incubation. A significant ($p=0.0012$) species-by-group interaction was noted for the EDD-TEMPOL response.

As with TEMPOL, we also incubated the GA with L-NAME to examine the role of NO. Acute L-NAME incubation severely blunted the EDD in LZR-Control (75%, $p=0.0001$), LZR-Ex (76%, $p=0.0001$), and LZR-UCMS+Ex (64%, $p=0.0001$) groups but had minimal effect on EDD in the LZR-UCMS group (9%, $p=0.99$) (Fig. 2-2C). In the OZR groups, acute L-NAME incubation only significantly blunted EDD in OZR-Ex (71%, $p=0.0001$) and OZR-UCMS+Ex

(65%, $p=0.0001$), with no significant effects in the OZR-Controls, and OZR-UCMS groups (Fig. 2-2D). We also noted a significant ($p=0.0001$) species by group interaction for the EDD-L-NAME response.

Effects of Experimental Conditions on GA Remodeling

Following assessment of vessel reactivity in our experimental groups, we then examined, under passive conditions, the GA remodeling. There were minimal changes in ID, OD, and WT in the LZR-UCMS vs. LZR-Control groups (Table 2-2); however, an increase in the β -slope of the GA stress-strain relationship ($p=0.05$) was noted in LZR-UCMS vs. LZR-Control. In contrast, no differences were noted between LZR-Ex, LZR-UCMS+Ex, or LZR-Control groups for ID, OD, WT, or β -slope, suggesting that Ex limited the increased GA stiffness noted with UCMS alone in LZRs. As for the effects of the experimental conditions in OZR, we noted a significantly lower ID and OD in OZR-UCMS vs. OZR-Controls ($p=0.01$), and as such WT did not differ between OZR-UCMS vs. OZR-Controls (Table 2-2). Further, an increase in the β -slope of the GA stress-strain relationship ($p=0.01$) was noted in the OZR-UCMS vs. OZR-Controls (Table 2-2). No significant differences were noted in ID, OD, WT, and the β -slope of the stress-strain relationship between OZR-Ex and OZR-Control groups. However, when Ex was combined with UCMS, the ID and OD were reduced (16%-18%, $p<0.05$) and the β -slope was increased (80%, $p=0.001$) in OZR-UCMS+Ex vs. OZR-Controls, as such the effects of UCMS+Ex on GA remodeling was similar to that noted with UCMS alone in OZRs (Table 2-2). No significant species-by-group interactions were identified for GA remodeling.

NO and ROS Levels

The improvement in EDD with acute TEMPOL or Ex suggested that the endothelial dysfunction in MetS with and without UCMS was in part, mediated by changes in ROS and NO levels. We therefore, examined NO levels in the aorta as determined by DAF-FM diacetate assay. NO bioavailability was lower in LZR-UCMS (68%, $p=0.0001$) compared to LZR-Controls, but higher in LZR-Ex (15%, $p=0.001$) vs. LZR-Control, and NO bioavailability was higher (25%, $p=0.0001$) in the LZR-UCMS+Ex vs. LZR-UCMS group. As for the OZR groups, NO bioavailability was similar between OZR-UCMS and OZR-Controls; however, NO bioavailability was higher (146%, $p=0.0001$) in OZR-Ex vs. OZR-Controls, and slightly higher (14%, $p=0.057$) in OZR-UCMS+Ex vs. OZR-UCMS group (Fig. 2-3A).

Figure 2-3B illustrates ROS levels in the femoral artery. Levels of ROS were higher in LZR-UCMS (99%, $p=0.0001$), LZR-Ex (99%, $p=0.003$), and LZR-UCMS+Ex (100%, $p=0.0001$) vs. LZR-Controls. Further, the ROS levels were higher in LZR-UCMS+Ex vs. LZR-Ex (130%, $p=0.0001$), and LZR-UCMS (101%, $p=0.04$). In the OZR groups, as with NO bioavailability, ROS levels did not differ between OZR-UCMS and OZR-Controls. No differences in ROS levels were noted between the OZR-UCMS+Ex vs. OZR-Controls, or OZR-UCMS groups.

Discussion

The present study is the first to look at the comorbidity between MetS undergoing chronic stress and the effects of Ex on limiting the peripheral vascular effects associated MetS and UCMS. Our results suggest that: 1) exposure to UCMS in LZR resulted in vascular pathologies similar to that evidence in OZR-Controls without the major changes in body mass, glucose or lipid profiles;

2) exposure to UCMS in OZR did not further affect EDD; and 3) Ex combined with UCMS improved peripheral microvascular dysfunction associated with UCMS.

The Impact of UCMS on the GA

Chronic psychosocial stress has been proposed as a risk factor for the development of the MetS (35,36). Thus, we were interested in understanding to what extent exposing a healthy rat (LZR) to the UCMS protocol would result in a MetS phenotype. The UCMS protocol resulted in some mild changes in fasting glucose, with a slight, but non-significant increase in triglycerides and MAP, and an actual reduction in BM in the LZR. Thus, one cannot say that the LZR now resembles a MetS phenotype based on the classification of MetS (i.e., BM, blood pressure, glucose, cholesterol); however, the LZR established substantial peripheral microvascular dysfunction that would represent the vasculopathies noted in an OZR. Indeed, the UCMS protocol severely blunted EDD and EID in LZR-Control. We have shown that UCMS significantly decreased NO and increased ROS production, which is the most likely cause of the impairment of EDD in LZRs. These results were also supported by the fact that acute incubation with the ROS scavenger, TEMPOL, improved EDD in LZR-UCMS, likely allowing NO levels to rise. Further, we also noted an increased production of ROS and a corresponding decrease in NO-bioavailability in the LZR-UCMS group. The UCMS-induced impairment of EID found in the present study is contrary to a similar study using UCMS in mice (37), but is in concurrence with a study using chronic social isolation in prairie voles (38). Factors that may have contributed to the smooth muscle damage include dysfunction of certain secondary messengers and their receptors, and increased cyclooxygenase signaling. One such secondary messenger is soluble guanylyl cyclase, which can be inhibited by increased levels of ROS. Inhibition of cyclic adenosine monophosphate expression

by overproduction of cortisol could also influence smooth muscle function (39–41). Furthermore, an increase in thromboxane production can lead to smooth muscle cell dysfunction, thus creating chronic vasoconstriction (42,43). However, further research is needed to address the potential reasons for the impaired EID with UCMS.

Previous studies by our group and others have examined the effects of MetS and UCMS on microvascular function separately (5,6,11,37,44,45) but to what extent the development of vascular pathologies occurs with the progression of MetS undergoing UCMS remained unclear. The condition of MetS already has significant microvascular dysfunction, therefore, it was important to examine the vascular pathologies during comorbidity. The UCMS protocol did not have a significant impact on EDD or EID in the OZR. The lack of UCMS effect on EDD in OZR may be due to the fact OZR-Controls demonstrate elevated ROS levels, and thus diminished bioavailable NO, unlike LZR-Control. Our group has previously shown in the GA that NO levels in OZR-Control are substantially lower than LZR-Control, as well as in other models of disease states such as hypertension, high-fructose diet, and high-salt diet (9). Therefore, the severity of impairment in OZRs may make them less susceptible to the effects of UCMS than in LZRs. TEMPOL increased EDD in OZR-Control and OZR-UCMS, suggesting that ROS scavenging can recover the reduction in NO caused by UCMS and MetS. These data were confirmed by the effect of acute incubation with L-NAME, which blunted dilation response in the Ex and UCMS+Ex groups.

There are various other mechanisms that could be affecting the microvasculature during the comorbidity between UCMS and MetS. For example, with chronic stress and depression there is an increase in cortisol/corticosterone levels as we have seen here in our LZR- and OZR-UCMS groups. Chronically elevated cortisol results in overproduction of angiotensin II (Ang II), which

causes vasoconstriction. Ang II has also been shown to cause an increase in endothelin-1, thromboxane, and ROS through activation of AT₁ type receptors (46). Thus, the increase in circulating Ang II *in vivo* causes changes in the smooth muscle and endothelium, possibly contributing to the impairment in EDD seen *in vitro* in LZR- and OZR-UCMS groups.

Peripheral microvascular wall dimensions were not affected by UCMS in LZRs but the stress-strain relationship was shifted to the left, suggesting an increase in stiffness. This response could reflect changes in the collagen deposition and elastin fractionation in the LZR GA (47,48). In contrast, UCMS significantly decreased both inner and outer diameters in OZRs as compared to their controls, indicating eutrophic inward remodeling. Eutrophic inward remodeling could be caused by repositioning of vascular smooth muscle cells to adapt to the chronic circumferential stress (49). OZR-UCMS also had a significant leftward shift in the stress-strain relationship, implying a stiffening of the vessels. Physical inactivity, a by-product of depression and a cause of MetS, produces oxidative stress and inflammation (50). Therefore, Ex is the logical intervention to augment the deleterious effects of MetS and depression.

Exercise Can Improve Aspects of the Comorbidity of MetS and UCMS

Exercise increased LZR EDD but Ex had a much greater effect on OZR EDD response overall. Exercise significantly enhanced NO bioavailability in LZRs and OZRs, which could explain why there was a greater improvement in dilation in LZR- and OZR-Ex. An earlier study found NO production and arginine conversion (indicating eNOS activity) were both increased with Ex (20). Following Ex, EDD was unaffected by the ROS levels as supported by the lack of TEMPOL effect in both LZR- and OZR-Ex groups. The specific cause of the elevation of ROS

levels is unclear given Ex has been shown to have antioxidant effects itself (25,26). However, corticosterone levels were elevated in the Ex and UCMS+Ex groups vs. the controls which may, in part, reflect the slight stress induced by use of the forced treadmill Ex protocol. It has been shown that glucocorticoids can increase ROS directly, including superoxide, hydrogen peroxide, and peroxynitrite (12). Given that DHE can interact with these oxidants, it could be speculated the higher ROS levels seen in LZR-Ex and UCMS+Ex are a byproduct of increased corticosterone. The fact that in Ex and UCMS+Ex EDD was improved despite elevated corticosterone levels, suggests EDD augmentation was likely mediated from non-corticosterone pathways. L-NAME blunted maximum dilation back down to LZR- and OZR-Control+LNAME values, suggesting the Ex augmentation of EDD may be solely through a NO-dependent pathway. The expression and function of eNOS is upregulated after Ex training due to increased shear stress (23,51), thus could explain why EDD in LZR- and OZR-Ex was improved in the skeletal muscle arterioles. Ex did not change EID response in LZRs or OZRs as compared to their respective controls, which was supported by a previous study from our group also showing no change in EID in LZRs and OZRs (20).

Ex did not result in GA structure remodeling in either the LZRs or OZRs, but Ex was able to prevent the level of impairment in EDD seen in LZR- and OZR-UCMS. Perhaps the increase in NO bioavailability in the UCMS+Ex groups, relative to the UCMS groups, limited the GA structure remodeling. LZR- and OZR-UCMS+Ex did not show an increase in EDD of the GA when incubated with TEMPOL as compared to their respective Control+TEMPOL values, which would suggest that TEMPOL did not have an additive effect when paired with exercise. EID was also increased with exercise in LZR- and OZR-UCMS+Ex. Furthermore, in the LZR, the combination of Ex with UCMS prevented the leftward shift (and increase GA stiffness) in the

stress strain relationship as evident in the LZR-UCMS group, suggesting a global improvement in microvascular function (i.e., EDD, EID with reduced arterial stiffness). However, the combination of Ex with UCMS in OZR was unable to prevent the increase in GA arterial stiffness. Perhaps here the comorbidity of MetS and UCMS was too much of stressful stimulus for 8 weeks of Ex to prevent. Further, research is needed to identify whether longer periods of Ex or a combination of Ex with ‘destiffening’ agents (i.e., ALT-711) could be used to prevent the increase in arterial stiffness with UCMS in the OZR.

Limitations

The present study is not without limitations. We realize the limitation associated with measuring tissue superoxide levels by fluorescence-based assessment of DHE oxidation. While our excitation/emission gating does detect signals produced by oxidation products that may originate from reactions independent of superoxides, the spectral overlap does not exclude considerable contributions from 2-hydroxyethidium (2-OH-E⁺), the superoxide-specific oxidation product of DHE (232). The possible explanation for the higher ROS levels in LZR-Ex and LZR-UCMS+Ex is the generation of hydrogen peroxide by Ex. Hydrogen peroxide has been shown to be a vasodilator and produced during Ex (233). Further, it was interesting to note that corticosterone levels were elevated in the Ex and UCMS+Ex groups vs. the controls, which could also have contributed to more hydrogen peroxide production, thus increasing fluorescence intensity in the Ex and UCMS+Ex groups. Future research should use HPLC to look for superoxide and 2-OH-E⁺ levels to obtain a more quantitative indicator of these products.

Another limitation that may, in part, affect the translational relevance of our study was that we deployed a forced treadmill running protocol as opposed to voluntary wheel running, which in itself can cause stress. However, given the sedentary nature of the OZR, we wanted to ensure that all rats were exposed to a similar Ex stimulus. In addition, given the size of the OZR (by 12-15 weeks), it would be difficult for them to fit into a running wheel. Thus, although voluntary Ex would be more translationally relevant, unfortunately, in the OZR rats forced treadmill exercise was the most feasible option, we believe the benefits of forced Ex outweighed the stress the rats might undergo.

Conclusions

The data presented here shows that the chronic exposure to stressful conditions in healthy rats leads to substantial vasculopathies similar to that in an OZR. Further, the comorbidity between chronic stress and MetS, does not exacerbate the effects of one another on skeletal muscle arteriole EDD response. It does, however, lead to the development of other vasculopathy adaptations in relation to constriction response. We have found that Ex can improve these pathological maladaptations and that the NO pathway has the potential to be a therapeutic target in clinical settings.

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Conflict of Interest

The authors declare no conflicts of interest. The results of the present study do not constitute endorsement by the American College of Sports Medicine and are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation.

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Table 2-1. Baseline characteristics of animal groups.

	LZR				OZR			
	<i>Con</i>	<i>UCMS</i>	<i>Ex</i>	<i>UCMS+Ex</i>	<i>Con</i>	<i>UCMS</i>	<i>Ex</i>	<i>UCMS+Ex</i>
BM, g	400 ± 36	361 ± 25*	358 ± 28*	343 ± 21*	641 ± 54	585 ± 39*	599 ± 53*	567 ± 46*
MAP, mmHg	112 ± 8	122 ± 13	120 ± 15	120 ± 11	139 ± 14	142 ± 16	140 ± 14	132 ± 13
Glucose, mg/dl	98 ± 15	124 ± 17*	101 ± 20	115 ± 15	184 ± 29	230 ± 34*^	154 ± 29	219 ± 36*^
Insulin, mg/dl	1.2 ± 0.9	2.1 ± 0.9	1.4 ± 0.7	1.8 ± 0.9	6.6 ± 2.6	7.8 ± 3.7	4.6 ± 2.4	6.2 ± 2.4
TG, mg/dl	25 ± 7	54 ± 40	31 ± 8	36 ± 8	124 ± 19	114 ± 35	82 ± 38	103 ± 40
TC, mg/dl	86 ± 11	87 ± 12	95 ± 15	88 ± 4	256 ± 63	213 ± 42	181 ± 20*	187 ± 27*
Corticosterone, ng/ml	7.0 ± 0.4	8.8 ± 1.7*	9.4 ± 2.9	10.7 ± 4.0	13.8 ± 1.4	17.3 ± 5.2*	10.6 ± 2.9*	15.8 ± 4.0*
Coat Scores, AU	0.6 ± 0.4	1.5 ± 0.4*	0.6 ± 0.5	1.8 ± 0.8*	2.4 ± 0.4	4.8 ± 0.8*	3.2 ± 0.6	4.5 ± 0.4*^

Con, control; UCMS, Unpredictable Mild Chronic Stress; Ex, exercise; BM, body mass; MAP, mean arterial pressure; TG, triglycerides; TC, total cholesterol. *p<0.05 vs. control, ^p<0.05 vs. Ex. Mean ± SD. n=6-8 per group.

Table 2-2. Gracilis arteriole remodeling due to MetS and UCMS in the GA.

	LZR				OZR			
	<i>Con</i>	<i>UCMS</i>	<i>Ex</i>	<i>UCMS+Ex</i>	<i>Con</i>	<i>UCMS</i>	<i>Ex</i>	<i>UCMS+Ex</i>
ID (μm)	210 ± 8	204 ± 50	194 ± 11	192 ± 15	184 ± 31	151 ± 5*	189 ± 31	168 ± 24*
OD (μm)	306 ± 4	294 ± 38	294 ± 24	292 ± 28	273 ± 28	230 ± 5*	281 ± 28	258 ± 31*#
WT (μm)	48 ± 2	45 ± 11	50 ± 6	50 ± 6	45 ± 3	40 ± 2	46 ± 2	45 ± 6
Wall:Lumen Ratio	0.5 ± 0.04	0.5 ± 0.19	0.5 ± 0.04	0.5 ± 0.03	0.5 ± 0.11	0.5 ± 0.03	0.5 ± 0.11	0.5 ± 0.09
β-Slope Coefficient	4.5 ± 0.9	6.0 ± 0.9*	3.5 ± 0.7	4.3 ± 1.0	4.5 ± 1.3	8.1 ± 3.4*	4.6 ± 1.2	8.7 ± 5.0*

Con, control; UCMS, Unpredictable Mild Chronic Stress; Ex, exercise; ID, inner GA diameter; OD, outer GA diameter; WT, wall thickness. *p<0.05 vs. control, #p<0.05 vs. UCMS. Mean ± SD. n=6-8 per group.

Figure 2-1. The effects of UCMS and Ex on GA reactivity. A) Assessment of endothelium-dependent dilation (EDD) to a maximal dose of Ach (10^{-6}M); B) endothelium-independent dilation (EID) to a maximal dose of SNP (10^{-6}M); and C) GA constriction to a maximal dose of PE (10^{-7}M). $n = 6-8/\text{group}$. Mean $\pm$ SD. * $p < 0.05$ vs. control, # $p < 0.05$ vs. UCMS, ^ $p < 0.05$ vs. Ex, ° $p < 0.05$ vs. matched treatment group in opposite species (i.e., LZR vs. OZR), ‡ $p < 0.05$ species (LZR vs. OZR) by group (Con, UCMS, Ex, UCMS+Ex) interaction. Con=control, UCMS=Unpredictable Mild Chronic Stress, Ex=exercise.

Figure 2-2. The effects of acute TEMPOL or L-NAME incubation on GA reactivity. The effects of acute TEMPOL incubation on EDD reactivity in LZR (A) and OZR (B). The effects of acute L-NAME incubation on EDD reactivity in LZR (C) and OZR (D). $n = 6-8/\text{group}$. Mean $\pm$ SD. * $p < 0.01$ vs. control, # $p < 0.01$ vs. UCMS, ^ $p < 0.05$ vs. exercise, + $p < 0.05$ within group comparison for change in TEMPOL or L-NAME. ‡ $p < 0.05$ species (LZR vs. OZR) by group (Con, UCMS, Ex, UCMS+Ex) interaction. Con=control, UCMS=Unpredictable Mild Chronic Stress, Ex=exercise.

Figure 2-3. The effects of UCMS and Ex on NO and ROS levels. A) NO levels in the aorta as determined by DAF-FM diacetate assay; and B) DHE staining indicating ROS levels in the aortas of each group. Mean $\pm$ SD. * $p < 0.05$ vs. control, # $p < 0.05$ vs. UCMS, ^ $p < 0.05$ vs. exercise, ° $p < 0.05$ vs. matched treatment group in opposite species. ‡ $p < 0.05$ species (LZR vs. OZR) by group (Con, UCMS, Ex, UCMS+Ex) interaction. Con=control, UCMS=Unpredictable Mild Chronic Stress, Ex=exercise.

Supplemental Data for Chapter 2

Figure S-1. The Effect of UCMS and Exercise on EDD in LZRs and OZRs. A) Assessment of endothelium-dependent dilation by exposure to increasing doses of ACh (10^{-10} - 10^{-6} M) in control vs. UCMS, B) in control vs. exercise, C) in control vs. UCMS+Exercise. $n = 6-8/\text{group}$. Mean $\pm$ SEM. * $p < 0.01$ vs. control, # $p < 0.01$ vs. UCMS, ^ $p < 0.01$ vs. exercise.

Figure S-2. The Effect of UCMS and Exercise on EID in LZRs and OZRs. A) Assessment of endothelium-independent dilation by exposure to increasing doses of SNP (10^{-10} - 10^{-6} M) in control vs. UCMS, B) in control vs. exercise, C) in control vs. UCMS+Exercise. $n = 6-8/\text{group}$. Mean $\pm$ SEM. * $p < 0.01$ vs. control, # $p < 0.01$ vs. UCMS, ^ $p < 0.01$ vs. exercise.

Figure S-2. The Effect of UCMS and Exercise on Constriction in LZRs and OZRs. A) Assessment of endothelium-independent dilation by exposure to increasing doses of PE (10^{-10} - 10^{-7} M) in control vs. UCMS, B) in control vs. exercise, C) in control vs. UCMS+Exercise. $n = 6-8/\text{group}$. Mean $\pm$ SEM. * $p < 0.01$ vs. control, # $p < 0.01$ vs. UCMS, ^ $p < 0.01$ vs. exercise.

CHAPTER 3 : Microvessel Density in the Brain is Attenuated with MetS and Depression but Improved with Exercise: A Preliminary Study

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Introduction

Endothelial cells play a primary role in maintaining microvascular function in the brain. These cells produce metabolic factors in response to stimuli and induce vasodilation or constriction. Proper endothelial function is essential to blood perfusion in any tissue, but especially in the most perfused organ in the body, the brain (1). In the cerebral vasculature, pericytes are adjacent to capillaries and share a common basement membrane with endothelial cells. Pericytes are important in cerebral capillary function by regulating cerebral blood flow, upregulating tight junction proteins, contributing to ion and water homeostasis, and interfacing directly with neurons (2–5). Further, pericytes also have several roles in regards to the cerebrovasculature such as contributing to the stability of the vessel, release of growth factors and matrix important for microvascular permeability, remodeling, and angiogenesis (6).

Metabolic syndrome (MetS), which is a collection of risk factors including hypertension, dyslipidemia, abdominal obesity, and high blood sugar (7), affects 56 million American adults (8). Through the collaborative deleterious effects of the MetS pathophysiologies, the vascular system is severely impacted including impairments in endothelial function and microvessel density. MetS has been found to reduce capillary-to-fiber ratio and microvessel density (MVD) in both the skeletal muscle and heart (9). In addition, MetS has also been shown to decrease functional brain capillary density and microvascular blood perfusion (10). A reduction of MVD in any vascular bed can cause disruption in patterns of blood flow and increased peripheral vascular resistance leading to an elevated risk of poor cardiovascular outcomes (11–13).

It has been postulated that exposure to chronic psychosocial stress is a significant risk factor for the development of MetS (14–16). Indeed, approximately 43% of MetS patients present with depression (17). Unpredictable Chronic Mild Stress (UCMS) is a validated animal model

using chronic stress to induce depressive symptoms (18,19). Most studies that use this model have documented physiological changes in the brains of rodents, mainly reflecting an elevated physiological stress response (19). An elevated stress response can lead to vascular impairments due to increased cortisol levels and oxidative stress (20–22). Isingrini et al. (23), illustrated an overall decrease in endothelium-dependent dilation response of the aorta in UCMS-exposed mice as a result of decreased nitric oxide (NO) bioavailability. Although not much is known about the direct effect of chronic stress on cerebral microvascular rarefaction, an increase in cortisol has been shown to inhibit endothelial cell proliferation and induce apoptosis in pericytes in the brain (21,24). As with MetS, the chronic stress-induced loss in MVD can significantly increase an individual's risk of stroke or myocardial infarction (11,25).

Exercise is often used to reduce the risk factors of MetS through lifestyle changes and has also proven to have beneficial effects on depression (26–30). One of these health benefits is an augmentation of microvascular rarefaction in pathological states. In a study by Machado et al. (31), two and a half hours of exercise per week resulted in an increase in capillary density in skeletal muscle, but only ninety minutes per week was required to reverse the microvascular rarefaction caused by a high fat diet in the left ventricle. Four weeks of aerobic exercise has been shown to increase both capillary density and surface area within the white matter of the brain (32). Further, previous studies have found that exercise by downregulating TNF- α and IL-1 β could perhaps protect pericytes from apoptosis and enhance angiogenesis through upregulation of VEGF (33). These results suggest exercise could be a candidate therapeutic in individuals with comorbid MetS and depression to improve MVD.

The present study used the obese Zucker rat (OZR), an accepted, translational model of MetS, and induced depressive symptoms in these rats using the UCMS protocol. We hypothesized

that: 1) cerebral MVD, along with pericyte number, would be reduced in rats exposed to UCMS; 2) cerebral MVD and pericytes would be increased with exercise; 3) exercise could mitigate the loss of cerebral MVD caused by the comorbidity between MetS and depression; and 4) the increase in MVD and number of pericytes would be reflected by an increase in VEGF and decrease in oxidative stress markers.

Materials and Methods

Animals

Male LZR and OZR (Harlan) arrived at the West Virginia University Health Sciences Center (WVUHSC) animal facility at 7-8 weeks of age. After 1 week of acclimation to the local environment, LZRs ($n = 8$ per protocol) and OZR ($n = 8$ per protocol) were randomly assigned to sedentary control or UCMS for the subsequent 8-9 weeks. All animals were fed standard chow and tap water ad libitum for all experiments. Protocols received prior approval from the WVUHSC Animal Care and Use Committee.

UCMS Protocol

Previous investigators developed the UCMS model for developing depression-like behaviors in rodents (34–37). The UCMS model is considered to be the most appropriate rodent model for clinical depression, based on its ability to reproduce the development of many clinical human depressive symptoms, including anhedonia and learned helplessness (19).

All rats were singly housed. In UCMS groups, rats were exposed to the following mild environmental stressors in randomly chosen sequences for 8 hours each day, 5 days/week, over the course of 8 weeks:

7. *Damp bedding* – 10 oz. of water was added to each standard cage
8. *Bath* – all bedding was removed and ~0.5 inches of water was added to empty cage. Water temperature was room temperature, ~24°C
9. *Cage Tilt* - cage was tilted to 45 degrees without bedding
10. *Social stress* – each rat was switched into a cage of a neighboring rat
11. *No bedding* – all bedding was removed from the cage
12. *Alteration of light/dark cycles* –turning lights off/on in random increments for scheduled period.

Coat Score

Coat score evaluation was done for every group throughout the duration of the 8-week protocol. Each week, the rats were weighed and inspected for grooming habits (38). The total cumulative score was computed by giving an individual score of 0 (clean) or 1 (dirty) to eight different body parts (i.e. head, neck, back, forelimbs, stomach, hindlimbs, tail, genitals).

Surgical Procedures

After completion of the treatment period and at 17-18 weeks of age, each rat was anesthetized with pentobarbital sodium (50 mg/kg ip) and was intubated via the trachea to facilitate maintenance of a patent airway. In all rats, a carotid artery and an external jugular vein were cannulated for determination of arterial pressure and for infusion of heparin. Under anesthesia, an aliquot of blood was drawn from the inferior vena cava. Tissues of interest were isolated from each animal for further analysis.

Immunohistochemistry

After rats were euthanized, surface cerebral blood vessels were removed for further analysis and the brain was placed in a matrix to cut 2 mm coronal slices. These slices were then flash-frozen in liquid nitrogen-cooled isopentane and store at -80°C for later use. Brain slices were sectioned into 8 µm slices using a cryostat at -24°C and transferred to positively charged microscope slides (Fisherbrand® Superfrost® plus microscope slides).

Cerebral microvessel and capillary density was evaluated by fluorescence immunohistochemistry using purified rat anti-mouse CD31 antibody (BD Biosciences, San Diego, CA) and rat anti-rabbit desmin antibody (Abcam, Cambridge, MA). Briefly, brain sections were fixed with 10% formalin, permeabilized with 0.2% Triton X-100, and blocked with blocking solution (5% BSA in PBS) and then incubated with anti-CD31 (1:100) and anti-desmin (1:200) overnight in a humidified chamber at 4°C. Following three washes with PBS, cells were incubated with AlexaFluor 555-conjugated anti-mouse IgG (1:1000; Invitrogen) and AlexaFluor 488-conjugated anti-rabbit IgG (1:1000; Invitrogen) for 2 h at room temperature in a humidified chamber. After a final wash with PBS, cover glass was mounted with hard-set DAPI medium (Vector Laboratories, Burlingame, CA). Negative control was performed with the same procedure without primary antibody. Images were obtained with an epifluorescence microscope (EVOS, ThermoFisher Scientific). The microvessel density of co-labeled cells were analyzed in 6 regions of interest (3 in cortex and 3 in striatum) in the MCA territory using ImageJ, as described previously (39).

Real-Time Quantitative PCR

RNA was extracted with the Direct-zol™ RNA MiniPrep Kit (Zymo Research) and reverse transcription was done using the QuantiTect Reverse Transcription Kit (Qiagen), according to manufacturer's protocols. QuantiTect primers (Qiagen) were used including: catalase, SOD, GSR, CD68, Nox2, VEGF, and TSP-1. Quantitative RT-PCR analysis was performed in duplicates for each sample using QuantiTect SYBR Green master mix (Qiagen) according to manufacturer's instructions and on the Roto-Gene thermocycler (Qiagen). Rat *18S* rRNA was used as a housekeeping gene to normalize expression of gene of interest depending on the experiment. Relative expression was calculated by the comparative CT ($2^{-\Delta\Delta C_t}$ values indicate fold change of the gene of interest in the samples relative to a selected control, $2^{-\Delta C_t}$ values indicate relative expression of gene interest between samples normalized to *18S*).

Determination of VEGF concentration

Brain tissue was homogenized using a bead mill and protein concentration was assessed by Bradford assay (DC Protein Assay, BioRad). Brain VEGF concentration was then determined with a commercially available rat VEGF-A sandwich-based ELISA kit (RayBiotech).

Determination of TSP-1 concentration

As with the VEGF ELISA, brain tissue was homogenized using a bead mill and protein concentration was assessed by Bradford assay (DC Protein Assay, BioRad). TSP-1 concentrations were determined as part of a rat inflammation V-plex panel ELISA (MesoScale Discovery).

Statistical Analysis

Data are presented as mean $\pm$ SEM. CD31+Desmin IHC was analyzed by a two-way analysis of variance (ANOVA), with a Tukey's post-hoc. RT-PCR was analyzed by a one-way repeated-measured ANOVA, with a Greenhouse-Geisser correction was performed to determine differences between groups. Protein levels were analyzed by a two-way analysis of variance (ANOVA), with a Tukey's post-hoc. In all cases, $p < 0.05$ was taken to reflect statistical significance.

Results

Figure 3-1 represents the immunohistochemistry (IHC) of the endothelial cell marker, CD31, and its co-labeling with desmin, a pericyte marker, in the cortex and striatum of the brain. The co-labeled cells are considered to be angiogenic microvessels. In LZRs, UCMS decreased the amount of CD31+Desmin⁺ cells within the cortex as compared to controls (37%, $p < 0.01$). However, UCMS did not affect MVD in OZR-UCMS. Exercise had higher MVD as compared to LZR Controls (18%, $p < 0.05$), and in OZR-Ex vs. their controls (79%, $p < 0.05$). In fact, when exercise was paired with UCMS, exercise improved MVD to control levels in LZRs (38%, $p < 0.01$) and higher than control levels in OZRs (74%, $p < 0.01$). The same trend occurred in the striatum. UCMS decreased CD31+Desmin⁺ cells by 27% ($p < 0.01$) as compared to LZR- and OZR-Controls, while in exercise, these cells were higher by 32% ($p < 0.01$) compared to controls. Exercise also improved MVD by 25% ($p < 0.01$) in LZR-UCMS+Ex, and also improved OZR-UCMS+Ex. MVD. The CD31⁺ cells mirrored the CD31+Desmin⁺ results in both the cortex and striatum for all treatment groups.

Figure 3-2 illustrates mRNA expression and protein levels of angiogenic markers, VEGF and TSP-1. There were no significant changes in mRNA expression of VEGF between treatment

groups (Fig. 3-2A), along with no change in protein levels (Fig. 3-2B). In TSP-1, no significant changes in mRNA expression were found between treatment groups (Fig. 3-2C), but OZR-UCMS had a significant decrease in TSP-1 protein levels as compared to OZR-Control and OZR-Ex ($p < 0.01$; Fig. 3-2D). Figure 3-3 represents mRNA expression in several oxidative stress markers. There were also no significant changes in mRNA expression between groups with catalase, SOD, GSR, or Nox2 (Fig. 3-3A-D). No significant changes in mRNA expression occurred in CD68, a macrophage marker (Fig. 3-3).

Discussion

The results outlined here are part of preliminary data on the change in cerebral MVD during the comorbidity of MetS and depression, and what effect exercise had on the microvascular rarefaction associated with these conditions. Our data suggests that globally in LZR, UCMS decreased cerebro-angiogenesis but this can be attenuated with the addition of exercise. In OZR, UCMS did not further decrease angiogenesis but exercise did improve MetS-derived microvascular rarefaction. Also, when exercise was in combination with UCMS in OZR, MVD was augmented as compared to OZR-Controls. Interestingly, there were no significant changes seen in mRNA expression of angiogenesis or oxidative markers. The lack of variation in mRNA expression between treatment groups could suggest that angiogenesis had already occurred in the brain, and transcription of these markers was back to basal levels.

The effect of depression on MVD has not been thoroughly studied. The present study showed that CD31+Desmin⁺ cells were decreased with UCMS. There are several possible mechanisms that could have induced microvascular rarefaction. Patients with recurrent depressive disorder had lower circulating VEGF levels, which would attenuate angiogenesis (40). Endothelial

progenitor cell numbers have also been found to be decreased in depressed patients, slowing angiogenesis in the brain (41). The elevated stress response associated with depression causes an increase in cortisol levels (16). Previous studies have also shown glucocorticoids can attenuate angiogenesis by inhibiting proliferation of cerebrovascular endothelial cells (21). In addition, pericyte apoptosis occurs with increased cortisol levels, where the glucocorticoid binds to receptors expressed on the pericytes, but when pericytes were treated with a glucocorticoid receptor antagonist, glucocorticoid-induced apoptosis was inhibited (24).

Exercise has been shown to reduce the physical symptoms of MetS and depression but also improve the pathophysiologies associated with each of the conditions (9,29,42–44). Therefore, it was hypothesized that microvessel density would be higher with exercise in pre-existing MetS and in the comorbidity between MetS and depression. We have shown that MVD was higher with exercise in both LZRs and OZR. Further, we also observed an improved MVD in LZR- and OZR-UCMS+Ex. NO is important in capillary recruitment. One possible factor that could have contributed to the increase in cerebral MVD could be the increase in NO production following exercise. Exercise increases NO bioavailability in several ways including: increased eNOS expression (45–47), increased SOD activity (48), and reduced ROS production from NOX2 (49,50) in skeletal muscle vasculature. Thus, NO bioavailability would be higher in the exercise groups, possibly leading to higher MVD. In addition, during acute bouts of exercise, mechanical forces such as shear stress and passive stretch lead to enhanced expression of angiogenic factors, including VEGF (51). Muscle contraction increases VEGF in the muscle interstitium, where VEGF acts on its receptors within the capillary endothelium to stimulate angiogenic processes (9).

Limitations and Future Directions

One reason there were no significant changes in mRNA expression in any of the genes examined could be attributed to the variability in time it took to isolate and section the brain at time of terminal surgery. The period of time the brain was out of the rat to when it was flash frozen was not standardized, therefore, RNA degradation could have occurred. Stress has been known to cause different physical effects in individuals (52,53). Exercise could also have been a stressor to certain rats, especially in the OZR. The individual response of the animals to stress and exercise may have contributed to the variability in mRNA expression, influencing the statistical power of the data and preventing small changes to be detected.

Even though RT-PCR did not reveal any significant changes in mRNA expression, it was still important to determine if protein levels changed because mRNA to protein synthesis is not guaranteed (54,55). The process of making protein from the mRNA transcript could be disrupted by many different mechanisms including: post-transcriptional adaptations, regulation by microRNAs, change in secondary structure of the transcript, disruption in ribosomal subunit recruitment, and proteasome activity (56–58). A change in any of these mechanisms could cause a decrease in protein synthesis or even an increase; therefore, western blots would need to be performed to determine if protein levels were affected by the experimental conditions.

Western blots for catalase, SOD, GSR, Nox2, and CD68 should have been done to determine the protein expression in the brain for each of these genes. Tissue is limited due to the many experiments already performed on the brain. ELISAs for VEGF and TSP-1 on brain homogenate are shown in this chapter, however, this is whole brain homogenate and more specific regional data is needed for correct evaluation of MVD changes in the cortex and striatum. It would be beneficial to separate these two regions and look at protein and mRNA expression in each. This

would allow for direct comparison between the IHC results and factors that may be contributing to the higher MVD in exercise and lower MVD in UCMS.

RT-PCR and western blots for members of the MMP family should also be performed on the brain as these proteins are involved in angiogenesis. MMPs help with the degradation of the vascular basement membrane and remodeling of the ECM to allow endothelial cells to migrate and invade the surrounding tissue. They also enhance angiogenesis by several methods including: helping to detach pericytes from vessels, releasing ECM-bound angiogenic growth factors, exposing cryptic pro-angiogenic integrin binding sites in the ECM, generating pro-migratory ECM component fragments, and cleaving endothelial cell-cell adhesion. In contrast, MMPs can also inhibit angiogenesis by proteolytic cleavage of certain collagen chains, plasminogen, and certain receptor ligand-binding domains (59). A recent study found that pericyte-release of MMP-9 may contribute to early blood-brain barrier degradation after injury (60). Another target for PCR and westerns could be thromboxane A₂. This metabolite, along with ATP, is released by platelets into the brain where they both act as potent constrictors of pericytes. Following constriction of the pericytes, contraction of the adjoining blood vessel wall would occur and could affect cerebral blood flow (61).

Another experiment that could have been done involves IHC probing for CD31 and Ki67. Unfortunately, we do not have enough brain slices to complete the investigation, nor are the slices reliably fresh to provide proper results. Ki67 is used as a cell proliferation marker because during mitosis, the Ki67 antigen is expressed on the surface of chromosomes. The colocalization of CD31 and Ki67 would indicate endothelial cells that are proliferating, giving a better idea to the extent of angiogenesis occurring within the brain. This process would involve the same procedure as the CD31 and desmin IHC previously shown.

An increase in peroxynitrite levels can cause a reduction in NO bioavailability, but also pericyte contraction (62). In healthy conditions, pericytes help to regulate blood flow by contracting or relaxing (63). If peroxynitrite levels are chronically high, such as in MetS, pericyte contraction could become chronic and affect blood flow in the brain. Thus, staining for nitrotyrosine in the brain could have been beneficial to assess the amount of peroxynitrite present. Staining for nitrotyrosine would serve two purposes: 1) to support that the microvascular dysfunction shown in UCMS was at least in part mediated by ROS production and; 2) estimate the amount of pericyte dysfunction that would lead to chronic constriction of cerebral blood vessels.

Collagen staining could have provided evidence for tissue remodeling due to the fibrotic response induced by pericyte death (61). Perivascular fibroblasts are recruited to sites of necrotic capillary pericytes and deposit extracellular matrix molecules that form a fibrotic scar within the brain. This could cause a number of problems including a disruption in neuronal signaling and cerebral perfusion (61). To evaluate the number of pericytes that are damaged in the case of decreased MVD, TUNEL and desmin staining could have been used to show DNA-fragmentation within pericytes. Paired with collagen staining, we would have been able to determine the sites of tissue remodeling and compare that to pericyte damage/apoptosis.

Monocyte-derived macrophages secrete VEGF-A, which seems to be crucial to induction of angiogenesis in tissue repair. Both M1 and M2 macrophage phenotypes are suggested to be involved with angiogenesis, in which M1 macrophages express genes involved with angiogenesis initiation and M2 macrophages secrete MMP-9 and recruit pericytes (64). Macrophages interact directly with pericytes, promoting pericyte-endothelial interactions. Macrophages also stimulate collateral artery diameter enlargement through smooth muscle cell hypertrophy and proliferation (65). Therefore, macrophage recruitment could have been evaluated in order to explain the higher

MVD with exercise. Staining brain slices with CXCL9 or CD86 would detect M1 macrophages and CXCR2 or CD23 would mark M2 macrophages (66). Flow cytometry could have been done on fresh brain slices to separate the macrophage phenotypes and compare population sizes.

Clinical Implications and Conclusions

Many pathologies that result in poor cardiovascular outcomes stem from vascular dysfunction, whether that dysfunction is in the endothelium or is due to a reduction in perfusion. The data presented here show that UCMS has an effect on cerebral angiogenesis and causes a loss of MVD in healthy animals. More importantly, exercise augments MVD in pre-existing MetS and in the case of the comorbidity between UCMS and MetS. Clinically, these data are relevant because they suggest a possible mechanism contributing to loss of MVD. These results also suggest that exercise can be used as a therapeutic for patients.

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Cortex

Striatum

Figure 3-1. Quantification of CD31/Desmin⁺ cells in the cortex and striatum. A, B) CD31⁺ and CD31+Desmin⁺ cells in the cortex were decreased with UCMS in LZR but not in OZR. Increased in both Ex and UCMS+Ex; C, D) CD31⁺ and CD31+Desmin⁺ cells in the cortex were decreased with UCMS in LZR but not in OZR. Increased in both Ex and UCMS+E. *n* = 6-8/group. Mean ± SEM. **p*<0.01 vs. control, +*p*<0.05 vs. UCMS, ^*p*<0.05 vs. Ex. Please see text for additional details.

Figure 3-2. mRNA and protein expression of angiogenic factors in the brain. A) There were no significant changes in VEGF mRNA expression between treatment groups; B) There were no changes seen in VEGF protein expression between groups; C) There were no significant changes in TSP-1 mRNA expression between treatment groups; D) There was a significant decrease in TSP-1 protein levels in OZR-UCMS as compared to OZR-Control and -Ex. $n = 6-8/\text{group}$. Mean $\pm$ SEM. * $p < 0.01$.

Figure 3-3. mRNA expression of oxidative stress markers in the brain. A) There were no significant changes in catalase mRNA expression between treatment groups; B) There were no significant changes in SOD mRNA expression between treatment groups; C) There were no significant changes in glutathione reductase (GSR) mRNA expression between treatment groups; D) There were no significant changes in Nox2 mRNA expression between treatment groups; E) There were no significant changes in CD68 mRNA expression between treatment groups. $n = 6-8/\text{group}$. Mean $\pm$ SEM.

CHAPTER 4 : Dissertation Discussion

The effect of the comorbidity between MetS and depression on the peripheral microvasculature was unknown. Previous studies had examined the causative mechanisms of MetS and depression separately, but this dissertation focused on determining the impact of the comorbidity on the microvasculature of the skeletal muscle and brain, along with MVD. The studies performed previously have suggested a direct association between the vasculopathy caused by each disorder and the increase in oxidative stress on vascular reactivity and MVD. Poor cardiovascular outcomes are brought on by the endothelial dysfunction caused by MetS and depression. Therefore, it was important to determine if depression exacerbated the vascular dysfunction already present with MetS. This study used exercise as a therapeutic, which has been proven previously to augment NO bioavailability and decrease ROS, to improve endothelium-dependent dilation response in OZR subjects subjected to UCMS. Further, exercise attenuated loss of MVD.

The work detailed in this dissertation used the obese Zucker rat, a model of MetS, to determine that: 1) UCMS causes peripheral microvascular dysfunction in LZR subjects similar to that seen in OZR subjects; 2) Peripheral microvascular dysfunction seen in OZR subjects was not exacerbated with UCMS; 3) the peripheral microvascular dysfunction and loss of MVD are caused by reduced NO bioavailability and increase in oxidative stress; and 4) exercise improves peripheral microvascular dysfunction and rarefaction by increasing NO.

The second chapter of this dissertation focused on characterizing the effect of MetS and depression, together, on the skeletal muscle arterioles using an *in vitro* pressurized myobath system to evaluate endothelium-dependent dilation and constriction response. Previous studies from our

collaborators have established the independent effects of MetS on the skeletal muscle arterioles and the effects of depression on the aorta (1,2). In both cases, endothelium-dependent dilation was impaired but did not find a significant change in constriction response. The study presented in Chapter 2 subjected OZR and LZR to UCMS for 8 weeks, and at the end of the 8 weeks of treatment GAs were isolated to determine dose response to Ach and PE. A significant reduction in endothelium-dependent dilation in LZR-UCMS but the same impairment was not seen in OZR-UCMS. Both LZR- and OZR-UCMS endothelium-independent dilation were attenuated, and constriction response was augmented only in OZR-UCMS. Treatment with the ROS scavenger, TEMPOL, increased dilation response in LZR-UCMS, OZR-Control, and OZR-UCMS. As to be expected, treatment with a NOS inhibitor, LNAME severely blunted LZR- and OZR-Control endothelium-dependent dilation. NO levels were lower in LZR-UCMS compared to their controls but did not significantly change between OZR-Control and OZR-UCMS. The same trends were seen with ROS levels in the respective groups.

The findings of this chapter provide evidence that a severe decrease in NO levels induced by UCMS and the added increase in ROS levels was the main cause of endothelium-dependent and -independent dilation in LZRs. The results of TEMPOL and LNAME treatment support this theory. In OZR, the effect of UCMS on endothelium-dependent dilation is less drastic, which could be due to the fact that OZR already have elevated ROS levels and reduced NO levels. This suggests the pre-existing impairment in OZR may make them less susceptible to UCMS effects. The interesting finding involves the attenuation of OZR endothelium-independent dilation, which seems to be due to another NO-independent mechanism. Elevated thromboxane or Ang II could be the cause of the impairment, but further experiments are needed to confirm this hypothesis. Both of these metabolites have been shown to directly affect endothelium dilator function (3–6).

The third chapter aimed to explore the possibility of using exercise as a therapeutic during the comorbidity of MetS and UCMS. This chapter built off the second chapter directly, meaning the same animals previously used in LZR- and OZR-Control and UCMS were used again, only LZR- and OZR-Ex and UCMS+Ex groups were added. Exercise by itself improved endothelium-dependent dilation in both LZRs and OZRs. It did not influence endothelium-independent dilation in LZRs; however, OZR-UCMS EID was significantly improved. Acute TEMPOL treatment did not further augment endothelium-dependent dilation in LZR- and OZR-Ex, but LNAME once again abolished dilation response in these groups. With exercise, NO production was significantly increased in both LZRs and OZRs as compared to controls. Of note, LZR-Ex had higher levels of ROS as compared to LZR-Control but ROS levels did not change in OZR-Ex. The most novel finding of this chapter was that exercise eliminated the deleterious effects of UCMS in LZR- and OZR-UCMS+Ex. In fact, OZR-UCMS+Ex EDD was improved beyond control values. Endothelium-independent dilation in both LZR- and OZR-UCMS+Ex was augmented as compared to UCMS groups. No further improvement was seen with TEMPOL and these effects were abolished with LNAME treatment. NO levels were subsequently increased in LZR- and OZR-UCM+Ex, while ROS levels remained high in these groups.

The outcomes of this chapter suggest that exposure to UCMS in LZRs resulted in vascular pathologies similar to those presented in OZR-Controls but without the other major changes in body mass, glucose, or lipid profile. Interestingly, the initial hypothesis for this paper was incorrect because exposure to UCMS in OZR did not further affect EDD when we had speculated that it would. The principle finding was that when Ex training was combined with UCMS, GA function was significantly improved in both LZRs and OZRs.

LZR-UCMS sustained EID dysfunction, which means the vascular smooth muscle was affected. There are several mechanisms that could damage the smooth muscle and most involve cell signaling dysfunction on the level of second messengers and their receptors. Previous studies have shown that an increase in cortisol levels, as seen in MetS and UCMS, can inhibit cyclic adenosine monophosphate expression; therefore, myosin light chain kinase activity is not blocked leading to vasoconstriction (7). Sustained high cortisol levels also result in overproduction of Ang II, a vasoconstrictor. Ang II directly effects the blood vessel but also causes an increase in ET-1, TxA₂, and ROS through the activation of AT₁ receptors. Thus, increased circulating Ang II causes vasoconstriction and smooth muscle damage (8). Another secondary messenger, soluble guanylyl cyclase, is inhibited by increased levels of ROS. This disrupts the action of NO on the smooth muscle and reduces vasodilatory response (9,10). Furthermore, increased cyclooxygenase signaling can lead to an imbalance of PGI₂ and TxA₂, causing an increase in TxA₂. An increase in TxA₂ induces vasoconstriction and smooth muscle cell dysfunction (5,11).

There is a possibility that the elevated cortisol seen in the Ex and UCMS+Ex groups were caused by the forced treadmill Ex protocol. The rats were made to run to keep total work similar between the species. It is not surprising that this could cause some stress in the animals. The increase in cortisol could have caused an increase in hydrogen peroxide, superoxide, and peroxynitrite (12), which perhaps explains why higher ROS levels were seen in LZR-Ex and -UCMS+Ex. It is known that DHE can interact with hydrogen peroxide and peroxynitrite as well as superoxide, meaning fluorescence intensity can only give a global measure of oxidative stress. Thus, the fact that ROS levels were higher in LZR-Ex and -UCMS+Ex could mean that hydrogen peroxide was significantly increased and not just superoxide. Exercise can increase hydrogen peroxide, where it acts as a vasodilator (13,14). This is a limitation of this chapter because some

of the improvement to EDD could be from hydrogen peroxide production in the UCMS+Ex groups.

The fourth chapter of this dissertation aimed to determine whether MVD in the brain was influenced by the comorbidity between MetS and depression. Ex training was also employed to see if MVD loss due to MetS and depression could be prevented. The first experiment performed was double IHC for the endothelial cell marker, CD31, and a pericyte marker, desmin. Pericytes are important for both microvessel function and angiogenesis. The CD31/Desmin⁺ cells within the cortex and striatum were considered to be pro-angiogenic. UCMS significantly decreased MVD in LZR-UCMS but was not changed in OZR-UCMS. In LZR- and OZR-Ex, MVD was increased and when coupled with UCMS, exercise improved MVD in both LZR- and OZR-UCMS+Ex. RT-PCR showed no differences in mRNA expression between groups in any of the angiogenic and oxidative stress markers examined.

All of the chapters presented here are connected, meaning the future directions of this research are interrelated. MetS and depression effect many different systems/mechanisms of the body and within those mechanisms are feed-forward and feed-back loops. Exercise also influences some of the same systems and more. In this regard, there are many avenues in which the research can branch, but for the sake of brevity this dissertation will only detail a few target areas.

MetS, depression, and exercise are the main focuses of this dissertation. There are several aspects of the studies presented previously that could be changed. For instance, the behavioral and physical characteristics of the rats undergoing UCMS and Ex could be further pursued. This could include better coat score analysis, implementation of the sucrose splash test, and perhaps use of the elevated plus maze to examine changes in behavior. Assessment of fat deposition could have been tracked using a rodent DEXA. Metabolic cages would have provided a more accurate way of

measuring food intake, and a way to separate urine and feces samples so that circulating levels of metabolites of interest could be measured.

The first logical future experiment would be to determine the impact of UCMS on the arachidonic acid pathway in pre-existing MetS. Previous studies have shown that circulating PGI₂ levels are decreased and TxA₂ are increased in OZR_s as compared to LZR_s (4). This study also showed that endothelium-dependent dilation was impaired in skeletal muscle arterioles. Therefore, the impairment in EDD in LZR_s and OZR_s along with the attenuation of EID in LZR_s, may be partially due to an imbalance of PGI₂ and TxA₂. Future experiments would involve microvessels, MCAs or GAs, hung in a pressurized myobath system and vessel reactivity would be assessed. The vessels would be exposed to increasing doses of iloprost, a synthetic analogue of PGI₂, to evaluate dilation response in control, UCMS, Ex, and UCMS+Ex LZR_s and OZR_s. Vasoconstriction would be assessed by increasing doses of U46618, a TxA₂ mimetic. Incubation with indomethacin, a COX antagonist, would help to determine how much of the dilation response is due to arachidonic acid pathway involvement versus the NO pathway. Whether the mechanism is caused by dysfunction in COX2 or in synthase activity needs to be clarified, and incubation with carboxyheptyl imidazole (CHI), a TxA₂ synthase inhibitor, in addition to the indomethacin incubation would help verify this. ELISAs or westerns should be done to examine the protein expression of TxA₂ and PGI₂ derivatives, and then compared to RT-PCR results for their respective synthases. These experiments would give a comprehensive view into how the arachidonic acid pathway is affected by the comorbidity between MetS and UCMS, especially when paired with the results previously outlined in this dissertation.

Exercise has been shown to increase production of hydrogen peroxide, where this molecule causes vasodilation. Our results showed an increase in ROS levels in LZR-Ex and -UCMS+Ex,

when we would have expected a decrease as compared to LZR-UCMS. TEMPOL incubation did not further improve endothelium-dependent dilation in LZR-Control, -Ex, or -UCMS+Ex. It also did not increase endothelium-dependent dilation in OZR-Ex and -UCMS+Ex. Hydrogen peroxide could have partially caused the augmentation of EDD seen in the Ex and UCMS+Ex groups, and also explain the increase in ROS in LZR-Ex and -UCMS+Ex due to the previously outlined limitations in DHE staining. Therefore, to determine how much of the improvement was due to NO versus hydrogen peroxide, AmplexRed could be used to detect hydrogen peroxide levels as EPR does not detect nonradical oxidants (15). TEMPOL catalyzes the process of turning superoxide into oxygen and hydrogen peroxide (16). It would, therefore, be interesting to test if adding TEMPOL to the Ex and UCMS+Ex vessels when probing for DHE would increase fluorescence intensity even further. To determine if hydrogen peroxide was causing the increase in fluorescence intensity, the vessels exposed to DHE could also have been incubated with catalase, a hydrogen peroxide scavenger. Further, endothelium-dependent dilation could be assessed in the presence of catalase and compared to the maximum dilation found with TEMPOL, to differentiate how much of the augmentation was because of increased NO bioavailability or hydrogen peroxide production. Mitochondrial production of hydrogen peroxide is increased in nonphosphorylating respiration during endurance exercise (17,18). It may be, therefore, beneficial to measure mitochondrial function through amperometric O₂ sensors or ATP production via bioluminescence (19,20).

VEGF expression has been thoroughly studied as a regulator of angiogenesis (21). Tang et al. (22) found that after one hour of exercise, VEGF transcriptional activity increased 38% in the brain, mRNA levels increased by 88%, and protein levels increased by 66%. Further, Tang and colleagues showed the major increase in VEGF expression occurred in the hippocampal region of

the brain. The hippocampus has inhibitory control over HPA-axis activity and has a role in stress regulation (23). Depression has been shown to alter the hippocampus, including causing a decrease in VEGF expression in the region, which could contribute to the downregulation of neurogenesis by stress (24). A study by Morland et al. (25), illustrated the effect of exercise-induced lactate release on brain angiogenesis. They found that pericyte-like cells express lactate receptor, HCAR1, and when this receptor is activated, VEGFA expression and cerebral angiogenesis is induced. This discovery could be interesting to expand on in the realm of using exercise to improve MetS and depression. We have shown in this dissertation that MVD is decreased with MetS and depression, but that exercise can improve MVD in these groups. Lactate could be involved in this process as evidenced by the study by Morland et al. A future study expanding on the experiments presented in this dissertation could employ lactate injections in place of exercise and include the following groups in LZRs and OZR: control, UCMS, daily lactate injections, and UCMS+lactate injections.

After 8 weeks of UCMS and lactate injections, evaluations of microvessel reactivity and MVD could be performed. Briefly, this would include baseline endothelium-dependent dilation response in the microvessels along with endothelium-dependent dilation response after incubations with TEMPOL and L-NAME, and IHC with CD31 and desmin to determine MVD in the brain. This experiment would isolate one aspect of Ex, lactate production, and demonstrate the ability of lactate to enhance cerebral angiogenesis. In addition, probing for CD31 and Ki67 would indicate endothelial cells that are proliferating and give a better idea to the extent of angiogenesis occurring within the brain. To evaluate involvement of pericytes expressing HCAR1 in cerebral angiogenesis, co-staining with desmin and HCAR1 antibodies could be used to quantify the number of pericytes in each treatment group that is expressing the receptor. Thus, if the number of HCAR1-expressing pericytes is higher in the lactate injection groups, it may suggest that lactate

is mediating the increase in MVD. Determining VEGFA expression would also be important and would be done via western blots and RT-PCR. VEGFA expression would be expected to rise in the lactate injection groups, in addition to the increase in HCAR1-expressing pericytes. Such a study could offer an alternative therapy to Ex for patients that may be unwilling or unable to daily.

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