Molecular mechanisms of chromium(VI)-induced apoptosis and malignant transformation

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Molecular Mechanisms of Chromium (VI) - Induced Apoptosis and Malignant Transformation

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

Doctor of Philosophy
in
Pharmaceutical Sciences

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Keywords: Cr(VI), apoptosis, reactive oxygen species, nitric oxide, peroxynitrite, Bcl-2, Ubiquitin, Proteasome, S-nitrosylation, lung, malignant transformation.
ABSTRACT

Molecular Mechanisms of Chromium (VI) - Induced Apoptosis and Malignant Transformation

NEELAM AZAD

Hexavalent chromium [Cr(VI)] compounds are redox cycling environmental carcinogens that induce apoptosis as the primary mode of cell death. Apoptosis is abnormally regulated in various disorders including cancer. Therefore, to understand the etiologies of these diseases, it is important to delineate the biochemical and molecular pathways involved in the regulation of apoptosis. The main objective of this study was to characterize the molecular mechanisms involved in Cr(VI)-induced apoptosis and malignant transformation. We found that both death receptor and mitochondrial pathways of apoptosis are involved in Cr(VI)-induced apoptosis, with the latter being more dominant. Consequently, overexpression of the mitochondrial anti-apoptotic protein Bcl-2 blocked Cr(VI)-induced apoptosis in human lung epithelial cells. We further observed that reactive oxygen species (ROS) play a critical role in Cr(VI)-induced apoptosis by acting through the mitochondrial death pathway. Superoxide anion (·O$_2^-$) was found to be the major ROS involved in Cr(VI)-induced apoptosis that exerted its effect by degrading Bcl-2 protein through the ubiquitin-proteasomal pathway. Furthermore, nitric oxide (NO) scavenged ·O$_2^-$ to form peroxynitrite (ONOO$^-$) and negatively regulated Cr(VI)-induced apoptosis. The mechanism by which NO exerted its anti-apoptotic effect involved upregulation of Bcl-2 via S-nitrosylation that prevented its ubiquitination and subsequent proteasomal degradation. Additionally, we established an in vitro model for studying Cr(VI)-induced malignant transformation by subjecting non-tumorigenic human lung epithelial Beas-2B cell line to long-term Cr(VI) exposure. Cr(VI) transformed cells exhibited clear signs of malignancy such as loss of contact inhibition and increased colony formation as compared to the passage-matched original cell-line. Cr(VI) transformed cells showed decreased apoptosis and ROS production and increased NO as well as Bcl-2 expression. These observations confirmed that NO mediated stabilization of Bcl-2 is an important event in Cr(VI) induced carcinogenesis. Taken together, our study reveals a novel regulatory mechanism that could be important in apoptosis resistance in response to Cr(VI) exposure. Additionally, this study demonstrated Cr(VI)-induced malignant transformation of a human lung epithelial cell line, establishing an important in vitro model for studying the molecular mechanisms involved in Cr(VI)-induced carcinogenesis. This study provides new mechanistic insights about environmental carcinogen induced human lung cancer. Since Cr(VI) is a paradigm of carcinogenic transition metals, the inferences from this study may be broadly applied to general metal carcinogenesis.
Dedicated to

My Parents
Gianchand Azad and Kaushalya Azad

My Sisters
Sunita Azad, Neeta Azad and Reeta Azad

AND

My Beloved brother
Anurag Azad
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ABBREVIATIONS

Chromium (Cr); Trivalent chromium – Cr(III); Hexavalent chromium – Cr(VI); International Agency of Research on Cancer – IARC; United States – US; American Type Culture Collection – ATCC; Tumor necrosis factor receptor-1 – TNFR-1; Death-inducing signaling complex – DISC; Apoptotic protease activating factor 1 – Apaf 1; FLICE-inhibitory protein – FLIP; B-cell lymphoma-2 – Bcl-2; Bcl-2 Interacting Domain – Bid; Truncated Bid – tBid; Reactive oxygen species – ROS; Superoxide anion – \( \cdot O_2^- \); Hydrogen peroxide – \( H_2O_2 \); Hydroxyl radical – \( \cdot OH \); \( N \)-acetyl cysteine – NAC; 6-Anilinoquinoline-5,8-quinone – LY-83,583; Xanthine/Xanthine oxidase – X/XO; Diphenylene iodonium – DPI; Lactacystin – LAC; Mn(III)tetrakis (4-benzoic acid) porphyrin – MnTBAP; Superoxide Dismutase – SOD; Glutathione peroxidase – GPx; Phenylmethylsulfonyl fluoride – PMSF; Bicinchoninic acid – BCA; Nitric oxdie – NO; Peroxynitrite – ONOO\(^-\); Neuronal NOS – nNOS/NOS; Inducible NOS – iNOS/NOS-2; Endothelial NOS – eNOS/NOS-3; Sodium nitroprusside – SNP; Aminoguanidine – AG; 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide – PTIO; Buthionine sulfoximine – BSO; Dithiothreitol – DTT; Diaminonaphthalene – DAN; Mercury (II) chloride – HgCl\(_2\); Dipropylenetriamine – DPTA; Fas ligand – FasL; Tumor necrosis factor-\( \alpha \) – TNF- \( \alpha \); 3-morpholinosydonimine – SIN-1.
CHAPTER I

Background and Objective
1.1. Chromium Exposure

Chromium (Cr) is a naturally occurring element found in rocks, animals, plants, soil, volcanic dust, gases and living organisms (1, 2). Chromium commonly occurs in the trivalent [Cr(III)] and hexavalent [(Cr(VI)] forms. Cr(III) compounds are normal dietary constituents that affect sugar metabolism by potentiating insulin action via interaction with the insulin receptor on the cell surface, and act as a cofactor in the maintenance of normal lipid and carbohydrate metabolism (3-5). Cr(III) compounds are considered non-toxic, non-carcinogenic and non-mutagenic as they are poorly absorbed (6, 7). On the other hand, Cr(VI) compounds are strongly associated with adverse health effects such as lung cancer. Exposure to Cr(VI) has been associated with the induction of lung cancer in workers employed in chrome plating, electroplating, leather tanneries, pigment manufacturing and stainless steel welding (8-11). Non-occupational and environmental exposure to Cr occurs from areas near landfills, chromate industries, hazardous waste disposal sites, automobile emissions and cigarette smoke (1). In United States (US), increased incidence of lung cancer has been reported in smokers with Cr(VI) exposure, with average cigarettes containing 0.24/6.3 mg Cr/kg as estimated by the International Agency of Research on Cancer (IARC) (9, 12). Cr(VI) compounds are classified as Group I human carcinogens by IARC in 1990 (12). The carcinogenic potential of Cr(VI) depends upon various factors such as water solubility, chemical speciation and involvement of reactive oxygen species (ROS) (13). Although Cr(VI) does not interact with isolated DNA directly, it is carcinogenic as it can actively enter cells, in contrast to Cr(III), by non-specific anionic transport system (2). Inside the cell, it is metabolically reduced to its lower oxidation states. These intermediates, specifically Cr(III), together with oxidative stress and a cascade of cellular events are involved in Cr(VI)-induced toxicity and cell death (Fig. 1) (14). In the cells, Cr(III) is the ultimate DNA binding species of Cr but Cr(III) compounds are considered non-carcinogenic as they can not enter cells through the cell membrane transporters (15). Cell death induced by Cr(VI) occurs primarily through apoptosis (16), and its abnormal regulation has been associated with the initiation of Cr(VI)-induced cancer(17). It has been suggested that Cr(VI) exposure may result in cell cycle arrest, neoplastic transformation, and apoptosis induction (14, 18). However, the molecular mechanisms involved in Cr(VI)-induced apoptosis and carcinogenesis are largely unknown.

1.2. Apoptosis Signaling Cascades

Under normal conditions, cell cycle proceeds without interruptions. However, cell cycle surveillance mechanisms sets checkpoints for cell cycle progression and if the cells are damaged genetically or functionally, they undergo arrest in the G1 phase of the cell cycle where the damaged cell is repaired before they enter the replication phase. Nevertheless, if the cells are damaged beyond repair they undergo programmed cell death, i.e., apoptosis. Apoptosis is a regulated physiological process leading to cell death characterized by cell shrinkage, membrane blebbing and DNA fragmentation (19). Caspases, a family of cysteine proteases, are central regulators of apoptosis. Initiator caspases such as caspase-8 and -9 activate downstream effector caspases and apoptosis (20, 21). Caspase-8 and -9 are the key pro-apoptotic proteins of the two major pathways regulating apoptosis, viz., the intrinsic pathway (mitochondria) and the extrinsic pathway (death receptor) (Fig. 2).
1.2.1. **Extrinsic pathway** - This pathway of apoptosis is induced via signaling through a family of death receptors including Fas (CD95/APO-1) and tumor necrosis factor receptor - 1 (TNFR-1). Death receptor ligands characteristically initiate signaling by recruiting specialized adaptor proteins to their cytosolic death domains resulting in the recruitment of procaspase-8 to the death-inducing signaling complex (DISC) causing activation of caspase-8 (22-24). Activated caspase-8 stimulates apoptosis via two parallel cascades 1) by direct cleavage and activation of caspase-3 leading to apoptosis, and 2) by cleavage of Bid (a Bcl-2 family protein) leading to apoptosis through the intrinsic pathway.

1.2.2. **Intrinsic pathway** - This pathway can be induced by pro-apoptotic Bcl-2 family of proteins such as Bid, Bax and Bad that reside in the cytosol but translocate to the mitochondria following death signaling. In the mitochondria they promote the release of cytochrome c, which then binds to apoptotic protease activating factor 1 (Apaf 1) and forms an activation complex (apoptosome) with procaspase-9 leading to its cleavage and activation in the form of caspase-9 (25, 26). Caspase-9 activates effector caspases such as caspase-3 leading to apoptosis. The mitochondrial pathway is also activated when cytosolic Bid is cleaved by caspase-8 following signaling through the activation of death receptor (27-29). Its active fragment or truncated Bid (tBid) translocates to the mitochondria, inducing cytochrome c release, which sequentially activates caspases-9 and -3 leading to cell death by apoptosis (Fig. 2).

1.3. **Apoptosis Regulatory Proteins**
The apoptotic pathways are regulated at different levels by various proteins including FLICE-inhibitory protein (FLIP) and B-cell lymphoma-2 (Bcl-2). FLIP proteins are the regulators of the extrinsic death receptor pathway. These anti-apoptotic proteins are recruited to the DISC upon stimulation where they prevent procaspase-8 recruitment and processing, preventing apoptosis through the extrinsic pathway (30-32). FLIP is involved in rendering cells resistant to death receptor-mediated apoptosis in various cell types and elevated expression of FLIP is associated with tumor cells that can escape from immune surveillance *in vivo* (33-36). Bcl-2 is a key apoptosis-regulatory protein of the mitochondrial death pathway (25). Formation of heterodimers with pro-apoptotic proteins such as Bax, inhibition of cytochrome c release, and regulation of the mitochondrial transmembrane potential are some of the mechanisms by which Bcl-2 exerts its anti-apoptotic effect (37-39). The anti-apoptotic function of Bcl-2 is closely associated with its expression levels, which is regulated by various mechanisms, including proteasomal degradation. Selective modification of ε-NH₂ groups of lysine residues in the protein ready for degradation by ubiquitination is the initial step that targets the protein for degradation by the proteasome complex (40, 41). Various factors such as specific structural features, phosphorylation or a partially conserved sequence motif are implicated in making proteins susceptible to degradation (42). However, the physiological signals that lead to protein recognition by ubiquitin and degradation via the proteasomal pathway are unclear. The importance of Bcl-2 and FLIP in the development of apoptosis resistant phenotype and neoplastic development has been established. However, their role in Cr(VI)-induced apoptosis and carcinogeneis is largely unknown.

1.4. **Reactive oxygen species (ROS)**
ROS such as hydrogen peroxide (H$_2$O$_2$), superoxide anion (‘O$_2^{-}$), and hydroxyl radical (‘OH) are the byproducts of normal oxygen metabolism which participate in normal cell functions and act as intracellular signaling molecules in many biological processes (43, 44). However, excessive production of ROS may cause DNA damage and abnormal activation of certain cell growth regulators, thereby leading to carcinogenesis (43). Mitochondria are the primary but not the only source of ROS during apoptosis. The release of cytochrome c in response to various apoptotic stimuli disrupts the mitochondrial electron transport chain as cytochrome c is responsible for the transfer of electrons from complex III to IV (45). This leads to the generation of ‘O$_2^{-}$ as a result of the leakage of electrons due to the disruption of mitochondrial respiratory chain (45, 46). Cellular redox state plays a major role in its response to external stimuli, induction or inhibition of apoptosis. Cr(VI) compounds are redox cycling carcinogens that are reduced by certain flavoenzymes such as glutathione reductase to generate Cr(V), Cr(IV) and Cr(III) in the cellular system (Fig. 1) (17, 47-50). During this process, molecular oxygen is reduced to ‘O$_2^{-}$, which generates H$_2$O$_2$ via dismutation. The resultant lower oxidation state intermediates react with H$_2$O$_2$ generating ‘OH radical via a Haber-Weiss or Fenton like reaction (49) (Fig.1). Thus, during the cellular reduction of Cr(VI), along with the reduced intermediates, a whole spectrum of ROS are generated that cause diverse cytotoxic and genotoxic effects including DNA damage, mutagenesis, growth arrest and cell death (51). Although various studies have shown the involvement of ROS in Cr(VI)-induced apoptosis, its relationship with the well studied caspase activation are not established.

1.5. Nitric oxide (NO)

NO is an important signaling molecule produced endogenously in a NADPH-dependent reaction catalyzed by constitutively expressed or inducible NO synthases from the guanodino group of L-arginine (52). There are 3 different genes encoding NO synthases including 1) nNOS/NOS-1 (neuronal NOS) found in the neurons, 2) iNOS/NOS-2 (inducible NOS) found in macrophages, epithelial cells, T cells, endothelial cells and eosinophils, and eNOS/NOS-3 (endothelial NOS) found in the endothelial cells lining the lumen of blood vessels (53). The physiological levels of constitutively expressed NOS remain relatively steady, whereas in response to an appropriate stimuli, iNOS catalyzes the production of much higher levels of NO (53). NO is a multifunctional free radical that diffuses freely across cell membranes and interacts with many other molecules. For instance, it reacts rapidly with ‘O$_2^{-}$ in a diffusion controlled reaction to primarily form peroxynitrite (ONOO$^{-}$) in most cell systems (54). ONOO$^{-}$ is a potent and versatile oxidant that can attack a wide range of biological molecules. It is believed that the formation of ONOO$^{-}$ anions due to the overproduction of NO is the main culprit in most of the diseases related to NO. ONOO$^{-}$ plays a role in mediating cytotoxicity through alterations in protein, lipids and nucleic acid structure and function with resultant disruption of cellular homeostatic mechanisms leading to diseased conditions. The effects of ONOO$^{-}$ on protein function are mainly due to its reactivity and covalent modification of tyrosine and/or cysteine residues (55). One of the well-established mechanisms by which NO, through the formation of ONOO$^{-}$, regulates the function of various target proteins is via S-nitrosylation (56). This post-translational modification of proteins can positively or negatively regulate various signaling pathways, proteins, and metabolic processes (57). Thus, NO exhibits various physiological as well as pathophysiological effects. Subtle changes in its rate of
production may critically impact cellular homeostasis, consequently initiating a variety of cellular signaling processes, including apoptosis (58). Depending on a variety of factors, including the flux and dose of NO, type of cells involved and redox state of the cell, NO has been demonstrated to have both pro- and anti-apoptotic role (52). Even though NO has been widely implicated in apoptosis and its dysregulation, its role in carcinogenesis is not well characterized. In particular, the role of NO in Cr(VI)-induced apoptosis has never been elucidated.

1.6. Cr(VI)-induced malignant transformation
Lung cancer is the leading cause of cancer mortality worldwide and yet the etiology of lung cancer is poorly understood. Current research indicates that long-term exposure to inhaled carcinogens has the greatest impact on risk of lung cancer. Exposure to Cr(VI) is associated with the incidence of lung cancer in humans. Several epidemiological studies in the last few decades have associated exposure to Cr(VI) with the induction of lung cancer in workers in various occupational settings (8-11). In US, a recent air quality survey indicated that people in several residential areas are exposed to particulate airborne chromium at concentrations exceeding 100 times the chronic toxicity benchmark (59). Therefore, in addition to occupational exposure, environmental chromium is an emerging concern and is associated with long-term carcinogenic effect of the lungs. However, these observations are not supported by animal data. Several groups have established in various animal models that exposure to Cr(VI) compounds demonstrated no significant increase in lung tumors as compared to untreated control (60-62) and the reason for this discrepancy is not understood. It is widely believed that human lung cancer manifests mostly as epithelial cell derived carcinomas (14); however, it is still unknown whether human lung epithelial cells are directly susceptible to Cr(VI)-induced malignant transformation. This is due to the lack of a good model to study the molecular mechanisms of Cr(VI)-induced apoptosis and malignant transformation. Abnormal apoptosis regulatory mechanisms and ROS generation are considered as important factors in neoplastic development in response to Cr(VI). However, the molecular mechanisms underlying Cr(VI)-induced apoptosis and malignant transformation are poorly understood.
FIGURE 1. Schematic representation of cellular reduction and cytotoxic effects of Cr(VI).
FIGURE 2. Diagrammatic illustration of the death receptor pathway and mitochondrial pathway of apoptosis.
1.7. SUMMARY

- Cr(VI) compounds are ubiquitous environmental carcinogens that primarily induce cell death via apoptosis. Mitochondria-mediated intrinsic pathway and death receptor-mediated extrinsic pathway are the two major signaling cascades of apoptosis. However, the relative importance of these pathways in Cr(VI)-induced apoptosis are largely unknown.

- Bcl-2 and FLIP are key regulatory proteins of the intrinsic and extrinsic pathways, respectively. The relative importance of these apoptosis regulatory proteins in response to Cr(VI) exposure is largely unknown. Since defects in apoptosis regulatory mechanisms lead to neoplastic development, it is important to understand the role of these key proteins in Cr(VI)-induced apoptosis.

- Activation of apoptosis is tightly linked with generation of ROS. ROS have been implicated in the regulation of Cr(VI)-induced apoptosis and carcinogenicity. However, the identity of specific ROS, precise source of ROS and the underlying mechanism are largely unknown. The elucidation of the regulatory mechanisms of apoptosis by ROS is crucial in understanding Cr(VI)-induced carcinogenesis.

- Exposure to Cr(VI) is associated with the incidence of lung cancer in humans. Elevated levels of NO have been reported in human lung cancers (63-68). However, the role of NO in Cr(VI)-induced apoptosis and malignant transformation has not been elucidated.

- Lung cancers mostly manifests as epithelial cell derived carcinomas; however, it is still unknown whether human lung epithelial cells are directly susceptible to Cr(VI)-induced malignant transformation. This is due to the lack of a good model system to study the molecular mechanisms involved in Cr(VI)-induced malignant transformation.

1.8. SPECIFIC AIMS

The objective of this study was to characterize the molecular mechanisms of Cr(VI)-induced apoptosis and malignant transformation. To achieve this goal we accomplished the following specific aims:

**Aim I:** Delineate the signaling cascades and identify the key apoptosis-regulatory proteins involved in Cr(VI)-induced apoptosis.

**Aim II:** Identify specific ROS, their cellular sources, and the mechanism by which ROS regulates Cr(VI)-induced apoptosis.

**Aim III:** Study the role of NO in Cr(VI)-induced apoptosis and determine the underlying mechanisms.

**Aim IV:** Establish a model to study Cr(VI)-induced malignant transformation and study the mechanisms involved in the development of apoptosis resistant phenotype.
1.9. References

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CHAPTER II

Signaling Cascades Involved in Cr(VI)-Induced Apoptosis and its Regulation by Reactive Oxygen Species
2.1. Abstract

Cr(VI) compounds are redox cycling carcinogens that induce apoptotic cell death. Since defects in apoptosis regulatory mechanisms contributes to increased resistance to apoptosis induced by Cr(VI), it is critical to understand the molecular mechanisms involved in Cr(VI)-induced apoptosis. Activation of apoptosis is tightly linked with the generation of ROS. Likewise, ROS have been implicated in the regulation of Cr(VI)-induced apoptosis and carcinogenicity. However, the mechanism involved, the identity of specific ROS and the cellular source of ROS are largely unknown. The objective of this study was to characterize the signaling pathways of Cr(VI)-induced apoptosis and evaluate the role of ROS in this process. We observed that both extrinsic and intrinsic pathway are involved in Cr(VI)-induced apoptosis and there is a potential cross-talk between the two pathways. Further, it was found that the mitochondrial pathway is more dominant in response to Cr(VI) exposure. Consequently, overexpression of the mitochondrial anti-apoptotic protein Bcl-2 blocked Cr(VI)-induced apoptosis significantly as compared to Flip, which had minimal effect. Recent evidences suggest that Bcl-2 protein inhibits apoptosis by suppressing free radicals generation or by regulating cellular antioxidant defense mechanisms. In this study, we also found that ROS, specifically superoxide (·O$_2^\text{-}$), mediates Cr(VI)-induced apoptosis of human lung epithelial H460 cells. ·O$_2^\text{-}$ exerted its effect by degrading Bcl-2 protein through the ubiquitin-proteasomal pathway. H460 $\rho^0$ cells that lack mitochondrial DNA and a functional electron transport demonstrated a significant decrease in ROS production and apoptosis indicating that mitochondria is the major source of ROS in Cr(VI)-induced apoptosis of H460 cells. This study gives a mechanistic understanding of ROS-mediated Bcl-2 downregulation and apoptosis induction providing critical insight into the pro-oxidant role of Cr(VI) and the basis for differential susceptibility to apoptotic cell death in response to Cr(VI) exposure.
2.2. Introduction

Hexavalent chromium compounds are ubiquitous environmental carcinogens that have been listed as Group I human carcinogens by IARC in 1990 (1). Exposure to Cr(VI) has been associated with induction of lung cancer in various occupational settings (2-5). Non-occupational exposure to Cr(VI) compounds occurs from cigarette smoke, automobile emissions, areas of landfills and hazardous waste disposal sites (1, 3, 6). Cr(VI) has been shown to induce chromosomal aberrations, mutations, and transformations in cultured mammalian cells (7, 8), and a variety of DNA lesions such as strand breaks, DNA protein cross-links, DNA base modification leading to cell death (9-12). Cell death induced by Cr(VI) occurs primarily through apoptosis (13), and its abnormal regulation has been associated with the initiation of Cr(VI)-induced cancer (14). Apoptosis is a tightly regulated process characterized by shrinkage of the nucleus, blebbing of membranes, condensation or fragmentation of chromatin that eliminates damaged cells maintaining tissue homeostasis (15, 16). In general, two major pathways of apoptosis have been recognized; the mitochondria-mediated intrinsic pathway and death receptor-mediated extrinsic pathway. The extrinsic pathway of apoptosis is induced via signaling through a family of death receptors such as Fas and TNFR-1, connecting ligand binding at the cell surface to apoptosis induction (17-19). The intrinsic pathway is induced by pro-apoptotic proteins belonging to the Bcl-2 family of proteins that reside in the cytosol but translocate to the mitochondria following death stimuli and promote the release of cytochrome c leading to apoptosis (20, 21). The mitochondrial pathway is also activated following signaling through death receptor and caspase-8 that causes the cleavage, translocation and activation of Bid, which sequentially activates caspases-9 and caspase-3 leading to apoptosis (22-24). Therefore, there is a potential cross-talk between the two apoptotic pathways. Both apoptotic pathways are regulated at different levels by various proteins including Bcl-2 and FLIP. The anti-apoptotic protein FLIP is the key regulator of the extrinsic pathway. It is recruited to the DISC upon stimulation where it prevents procaspase-8 recruitment and processing (25-27). The anti-apoptotic protein Bcl-2 resides in the outer mitochondrial wall and regulates apoptosis by controlling mitochondrial permeability and the release of cytochrome c, thus, inhibiting apoptosis (20, 28, 29). Bcl-2 and Flip proteins are overexpressed in a variety of cancers and play a key role in these malignancies. However, the role of Bcl-2 and FLIP in Cr(VI)-induced caspase activation and apoptosis is largely unknown. Identifying the key mechanisms and apoptosis regulatory proteins involved in Cr(VI)-induced apoptosis are critical in understanding the development of apoptosis resistance phenotype in response to Cr(VI) exposure. However, the detailed mechanism of apoptosis in response to Cr(VI) has not been systemically investigated.

Several reports indicate that ROS can cause cell death via apoptosis (30, 31). ROS such as hydrogen peroxide (H$_2$O$_2$), superoxide anion (·O$_2^-$), and hydroxyl radical (·OH) are the byproducts of normal oxygen metabolism which act as intracellular signaling molecules in many biological processes (32, 33). However, excessive production of ROS may cause DNA damage and abnormal activation of certain cell growth regulators, thereby leading to carcinogenesis (32). Although recent evidences suggest that ROS have been implicated in the regulation of Cr(VI)-induced apoptosis and carcinogenicity, the mechanisms of
carcinogenesis are not yet clear (34-36). In the case of Cr(VI) compounds, cellular reduction leads to the generation of \( \cdot \text{O}_2^- \) along with the formation of lower oxidation state intermediates (14, 37, 38). \( \cdot \text{O}_2^- \) is further converted to \( \text{H}_2\text{O}_2 \) via dismutation, which reacts with the resultant intermediates to generate \( \cdot \text{OH} \) radicals. ROS generated during the one-electron reduction of Cr(VI) along with a cascade of cellular events causes diverse cytotoxic and genotoxic effects including DNA damage, mutagenesis, growth arrest and cell death (10). Programmed cell death or apoptosis can be modulated by the changes in the expression of distinct sets of genes (15). The proto-oncogene Bcl-2 is one of the major genes regulating apoptotic cell death. The anti-apoptotic function of Bcl-2 is regulated by its expression levels. Various mechanisms including dimerization, phosphorylation, degradation and post-translational modification regulate the stability and expression levels of Bcl-2 protein (39, 40). Degradation of Bcl-2 is mainly mediated through the ubiquitin-proteasomal pathway (39, 40). In vitro and in vivo studies suggest that Bcl-2 blocks apoptosis through regulation of cellular antioxidant defense mechanisms or by suppressing production of free radicals thus acting as an antioxidant (41-43). Even though ROS has been implicated in Cr(VI)-induced apoptosis, the cellular source of ROS generation and its relationship with the well-studied caspase activation has not been established.

The aim of this study was to delineate the signaling cascades involved in Cr(VI)-induced apoptosis and evaluate the role of ROS in this process. We found that both extrinsic and intrinsic pathway are involved in Cr(VI)-induced apoptosis and there is a potential cross-talk between the two pathways. Further, mitochondrial pathway of apoptosis was more dominant in response to Cr(VI) and consequently, overexpression of Bcl-2 blocked Cr(VI)-induced apoptosis significantly. Further, it was observed that generation of ROS induces apoptotic cell death in response to Cr(VI) exposure and \( \cdot \text{O}_2^- \) is the major ROS involved in Cr(VI)-induced apoptosis. Although, cytosolic ROS is involved, mitochondria is the major cellular source of ROS involved in Cr(VI)-induced apoptosis. Further, it was observed that \( \cdot \text{O}_2^- \) mediated its pro-apoptotic effect by causing down-regulation and degradation of Bcl-2 through the ubiquitin-proteasomal pathway thus inducing apoptosis through the mitochondrial pathway. The elucidation of the regulatory mechanisms of apoptosis by ROS is crucial in understanding carcinogenesis induced by genotoxic agents such as chromium. This study showing the role of \( \cdot \text{O}_2^- \) in Cr(VI)-induced cell death and Bcl-2 downregulation, documents a novel mechanism of apoptosis induction by Cr(VI) involving Bcl-2 stability linked to \( \cdot \text{O}_2^- \) generation. This study forms the basis of the mechanisms involved in the development of apoptosis resistant phenotype in response to Cr(VI) and can be exploited further to understand the molecular mechanisms involved in Cr(VI)-induced malignant transformation and carcinogenic development.
2.3. Materials and Methods

**Chemicals and Reagents**

*N*-acetyl cysteine (NAC), 6-Anilinoquinoline-5,8-quinone (LY-83,583), Hydrogen peroxide (H$_2$O$_2$), Xanthine, Xanthine oxidase, rotenone, diphenylene iodonium (DPI), lactacystin (LAC), ethidium bromide, sodium pyruvate, uridine and sodium dichromate (Na$_2$Cr$_2$O$_7$.2H$_2$O) [Cr(VI)] were obtained from Sigma Chemical Inc. (St. Louis, MO). Fluorogenic caspase substrates IETD amino-4-methylcoumarin (IETD-AMC), LEHD-AMC, caspase-8 inhibitor (z-IETD-FMK), caspase-9 inhibitor (z-LEHD-FMK), pan-caspase inhibitor (zVAD.FMK) and Flip antibody were from Alexis Biochemicals (San Diego, CA). Cell-permeable superoxide dismutase (SOD) mimetic, Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP), was purchased from Calbiochem (La Jolla, CA), and catalase was from Boehringer Mannheim (Indianapolis, IN). The oxidative probes, dichlorofluorescein diacetate (DCF-DA) and dihydroethidium bromide (DHE), and the apoptosis dye Hoechst 33342 were from Molecular Probes (Eugene, OR). Antibodies for caspase-8, caspase-9 and Bid were from Cell Signaling Technology, Inc. (Danvers, MA). Antibodies for Bcl-2, peroxidase-labeled secondary antibodies, anti-myc agarose beads, and protein A-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for ubiquitin, and β-actin were from Sigma (St. Louis, MO) and the transfecting agent Lipofectamine 2000 was from Invitrogen (Carlsbad, CA).

**Cell Culture**

The human lung epithelial cancer NCI-H460 cell line was obtained from the American Type Culture Collection (ATCC) (Rockville, MD). Cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) containing 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin in a 5% CO$_2$ environment at 37°C. Cells were passaged at preconfluent densities using a solution containing 0.05% trypsin and 0.5 mM EDTA (Invitrogen).

**Caspase Assay**

Caspase activity was determined by fluorometric assay using the enzyme substrate IETD-AMC for caspase-8 and LEHD-AMC for caspase-9, which are specifically cleaved by the respective enzymes at the Asp residue to release the fluorescent group, AMC. Cell extracts containing 20 µg of protein were incubated with 100 mM HEPES containing 10% sucrose, 10 mM dithiothreitol, 0.1% 3-((3-cholamidopropyl)-1) propane sulfonate, and 50 µM caspase substrate in a total reaction volume of 0.25 ml. The reaction mixture was incubated for 60 min at 37°C and quantified fluorometrically at the excitation and emission wavelengths of 380 nm and 460 nm, respectively using RF5301PC spectro-fluorometer (Shimadzu).

**Apoptosis Assay**

After specific treatments, apoptosis was determined by incubating cells with 10 µg/ml Hoechst 33342 nuclear stain for 30 min at 37°C and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by fluorescence microscopy (Axiovert 100; Carl Zeiss) using Pixera software.
**Transient Transfection**

H460 cells were seeded in 60-mm dishes until they reached 80% confluence. The cells were then transiently transfected in serum free medium with the expression plasmids encoding Flip or Bcl-2 and green fluorescent protein using Lipofectamine 2000 reagent. After 10 h, the medium was replaced with culture medium containing 5% fetal bovine serum. Approximately, 36 h after transfection, the cells were analyzed for apoptosis by Hoechst assay.

**Stable Transfection**

Stable transfectants of Flip, Bcl-2, glutathione peroxidase (GPx) and superoxide dismutase (SOD) were generated by culturing H460 cells in six-well plates until they reached 80% confluence. 1 µg of cytomegalovirus-neo vector and 15 µl of Lipofectamine 2000 reagent with 2 µg of Flip, Bcl-2, GPx, SOD, or control pcDNA3 plasmid were used to transfect the cells in the absence of serum. After 10 h, the medium was replaced with culture medium containing 5% fetal bovine serum. Approximately 36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin, and the cell suspensions were plated onto 75 ml culture flasks and cultured for 24 to 28 days with G418 selection (400 µg/ml). Stable transfectants were identified by Western blot analysis and were cultured in G418-free RPMI 1640 medium for at least two passages before each experiment.

**ROS Detection**

Intracellular peroxide and superoxide production was determined by flow cytometry and fluorescence microscopy using DCF-DA and DHE fluorescence probes, respectively. For flow cytometric analysis, cells (1x10^6/ml) were incubated with the fluorescent probes (10 µM) for 30 min at 37ºC, after which they were washed, resuspended in phosphate buffered saline (PBS), and analyzed for DCF (494/519 nm) and DHE fluorescence (535/617 nm) using FACS calibur, (Becton-Dickinson, Rutherford, NJ). The median fluorescence intensity was quantitated by CellQuest software (Becton-Dickinson) analysis of the recorded histograms. For fluorescence microscopy, cells were incubated with the probes as described above and then examined for DCF and DHE fluorescence using Carl Zeiss Axiovert microscope.

**Derivation of ρ^0 cells**

H460 ρ^0 were prepared as previously described (44). Briefly, H460 cells were cultured in the presence of ethidium bromide (100 ng/ml) to inhibit mitochondrial DNA replication for more than 20 generations until use (ρ^0 cells). ρ^0 cells were derived and maintained in complete medium supplemented with 100 µg/ml uridine and 100 µg/ml pyruvate.

**Western Blotting**

After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na3VO4, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor mixture for 20 min on ice. After insoluble debris was precipitated by centrifugation at 14,000 x g for 15 min at 4ºC, the supernatants were collected and assayed for protein content using bicinchoninic acid (BCA) method. Equal amount of proteins per sample (15 µg) were resolved on 10% SDS-PAGE and transferred onto a 0.45-µm nitrocellulose membrane. The transferred
membranes were blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween 20) and incubated with the appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immune complexes were detected by chemiluminescence (Supersignal, West Pico; Pierce) and quantified by imaging densitometry using UN-SCAN-IT automated digitizing software (Silk Scientific Corp.). Mean densitometry data from independent experiments were normalized to the control. The data were presented as mean ± S.D. and analyzed by Student’s t test.

**Immunoprecipitation**

Stable H460 cells overexpressing Bcl-2 were washed after treatments with ice-cold PBS and lysed in lysis buffer (20 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 0.2% NP40, 100 mM PMSF and a commercial protease inhibitor mixture) at 4°C for 20 min. After centrifugation at 14,000 x g for 15 min at 4°C, the supernatants were collected and the protein content was determined by BCA protein assay. Cleared lysates were normalized and 60 µg proteins were incubated with 8 µl of anti-myc agarose bead (Santa Cruz Biotechnology) diluted with 12 µl protein A-agarose for 4 h at 4°C. The immune complexes were washed 3 times with 500 µl lysis buffer, resuspended in 2x Laemmli sample buffer and boiled at 95°C for 5 min. The immune complexes were separated by 10% SDS-PAGE and analyzed by Western blot as described above.
2.4. Results

Cr(VI) Induced Cell Death – Cr(VI)-containing compounds are ubiquitous environmental carcinogens that induce apoptosis as the primary mode of cell death (13). To characterize the signaling pathways involved in Cr(VI)-induced apoptosis, we first characterized the apoptotic response to Cr(VI) treatment in human lung epithelial H460 cells. Cells were treated with different doses of Cr(VI) (0-100 µM), and apoptosis was determined after 12 h by Hoechst 33342 assay. Figs. 1, A and B, show that Cr(VI) treatment caused a dose-dependent increase in cell apoptosis over control level, as indicated by increased nuclear fluorescence and chromatin condensation of the treated cells. Cells were also treated with zVAD, a pan-caspase inhibitor, in addition to Cr(VI). Figs. 1, C and D, show that zVAD treatment blocked Cr(VI)-induced apoptosis, reducing it to control levels. This indicated that Cr(VI) induces apoptosis through the classical caspase dependent pathways.

Signaling Pathways Induced by Cr(VI) – Various studies have demonstrated that caspase-8 and caspase-9 represent the apical caspases in the death receptor and mitochondrial pathways, respectively (45-48). Therefore, to investigate the potential involvement of the extrinsic and intrinsic pathway in Cr(VI)-induced apoptosis, cleavage and activation of caspase-8 and caspase-9 in response to Cr(VI) exposure was analyzed by Western blotting and caspase activation assays. Western blot analysis showed that both apoptotic pathways were activated in response to Cr(VI) (Figs. 2, A and B). Additionally, we also observed that Bid protein was cleaved and activated (tBid) in response to Cr(VI), suggesting a potential cross-talk between the two apoptotic pathways. Since caspase activation leads to the activation of Bid, this indicates that H460 cells are Type II cells (Fig. 2 C).

Caspase Activation Induced in Response to Cr(VI) Exposure – Caspase activity assays confirmed that Cr(VI) induced both caspase-8 and caspase-9 activation. Furthermore, it demonstrated that the mitochondrial pathway is more dominant in response to Cr(VI) exposure (Fig. 3 A). Since caspase-8 and caspase-9 are the key marker proteins of the death receptor pathway and mitochondrial pathway, the results of this study suggest that the mitochondrial pathway is the major apoptotic pathway induced in response to Cr(VI) exposure. Control studies using specific caspase-8 inhibitor (IETD-CHO) and caspase-9 inhibitor (LEHD-CHO) indicate the specificity of caspase activation under the test conditions (Fig. 3 B). It was also observed that both caspase inhibitors reduced apoptosis to the control levels further confirming the involvement of caspases in Cr(VI)-induced apoptosis (Fig. 3 C).

Effect of Cr(VI) on Apoptosis Regulatory Proteins – Bcl-2 is the key apoptosis-regulatory protein of the mitochondrial death pathway and FLIP is the key protein of the death receptor pathway (20, 25-29). To determine the effect of Cr(VI) exposure on these proteins, cells were treated with various doses of Cr(VI) and analyzed for Flip and Bcl-2 expression by immunoblotting. Both, Flip and Bcl-2 protein levels were downregulated in response to Cr(VI) exposure (Figs. 4, A and B). To determine whether these proteins are involved in the regulation of Cr(VI)-induced apoptosis, H460 cells were transiently transfected with Flip, Bcl-2 or control plasmid, and their effect on Cr(VI)-induced apoptosis was determined by Hoechst 33342 assay. Fig. 4 C shows that transfection with both Flip and Bcl-2 inhibited
Cr(VI)-induced apoptosis as compared with the vector-transfected control, with the effect of Bcl-2 being more pronounced as compared to the effect of Flip.

**Bcl-2 Overexpression Protects Cells from Cr(VI)-Induced Apoptosis** – To further verify the effect observed after transient transfection of Flip and Bcl-2 proteins, stable H460 cell lines overexpressing Flip and Bcl-2 proteins were established and overexpression was confirmed by Western blot analysis. Transfected cells showed an increase in Bcl-2 and Flip protein expression in the Bcl-2-transfected cells and Flip-transfected cells but not in the control transfectant (Figs. 5, C and D). Further, stably transfected H460 cells with Flip, Bcl-2 or control plasmid, were tested for their effect on Cr(VI)-induced apoptosis by Hoechst 33342 assay. Overexpression of Bcl-2 significantly inhibited Cr(VI)-induced apoptosis over a wide concentration range as compared to the vector-transfected control (Fig. 5A), whereas Flip overexpression had minimal effect (Fig. 5B). These results indicate the role of Bcl-2 as a negative regulator of Cr(VI)-induced apoptosis and further suggest that H460 cells are Type II cells, supporting the role of the mitochondrial pathway in Cr(VI)-induced cell death.

**Effect of Cr(VI) and ROS Modulators on Cellular ROS Levels** – In the cellular system, Cr(VI) is metabolically reduced to its lower oxidation states, generating ROS in this process (14, 37, 38). Therefore, we quantified the induction of cellular ROS production in response to Cr(VI) exposure by flow cytometry in treated cells using the fluorescent probes DCF and DHE, which detect hydroperoxide and ‘O₂‾, respectively. Cr(VI) induced hydroperoxide and ‘O₂‾ production in a dose-dependent manner, as indicated by the increase in DCF and DHE fluorescence intensity (Figs. 6, A-C). Various ROS modulators used in combination with Cr(VI) verified these results. DHE signal was increased by Cr(VI) and LY-83,583 (‘O₂‾ donor) and was inhibited by the addition of the SOD mimetic MnTBAP (‘O₂‾ scavenger) and the general antioxidant NAC (Fig. 7, A and F). Similarly, DCF signal was increased by Cr(VI) and H₂O₂ and was inhibited by NAC and catalase (H₂O₂ scavenger) (Fig. 7, B and C).

**ROS Mediated Cr(VI) Induced Apoptosis** – Apoptosis is strongly linked with ROS induction. To test whether the apoptosis-inducing effect of Cr(VI) is associated with ROS production, cells were treated with Cr(VI) in the presence or absence of various ROS modulators, including NAC, catalase, MnTBAP, and xanthine/xanthine oxidase (‘O₂‾ donor), and apoptosis was determined by Hoechst assay. The results show that all the tested antioxidants were able to inhibit apoptosis induced by Cr(VI) (Fig. 8, A and B), indicating that multiple ROS are involved in the apoptotic process. The potent inhibitory effects of catalase and MnTBAP further indicate that H₂O₂ and ‘O₂‾ play an important role in the process.

**GPx and SOD Overexpression Inhibits Cr(VI)-Induced Apoptosis and ROS Generation** - To confirm the role of ROS in Cr(VI)-induced apoptosis, cells were stably transfected with the antioxidant enzymes GPx, SOD, or control plasmid, and their effects on ROS generation and apoptosis were determined. Apoptosis assays showed a decrease in apoptotic response to Cr(VI) treatment in GPx- and SOD-transfected cells but not in mock-transfected cells (Fig. 9, A and B). Flow cytometric analysis of hydroperoxide generation and ‘O₂‾ also showed a substantial reduction in Cr(VI)-induced fluorescence in both GPx-
and SOD-transfected cells as compared with mock-transfected cells (Fig. 9, C and D). It was observed that SOD overexpressed cells showed more significant decrease in ROS and apoptosis induction. In SOD overexpressed cells, apoptosis did not change significantly even with 100 μM Cr(VI). This indicated significant involvement of ·O$_2^-$ anion in Cr(VI)-induced apoptosis.

*Mitochondrial ROS are Responsible for Cr(VI)-Induced Apoptosis* – To determine the cellular source of ROS involved in Cr(VI)-induced apoptosis, cells were treated with Cr(VI) in the presence or absence of DPI, a specific inhibitor of NADPH oxidase (49, 50), or rotenone, a mitochondrial electron transport chain interrupter (50, 51), and their effects on apoptosis and ROS generation were examined. The results show that both DPI and rotenone inhibited Cr(VI)-induced ROS generation and apoptosis, with the effect of rotenone being more pronounced (Fig. 10, A-D). These results indicate that even though cytosolic ROS may be involved, mitochondria is the major source of ROS involved in Cr(VI)-induced apoptosis. The differential inhibitory effect of rotenone on ·O$_2^-$ and peroxide generation could reflect the non-specific effect of this compound on ROS and the varying rates of reaction between the ROS, their probes, and the compound. The role of specific ROS in Cr(VI)-induced apoptosis could be more conclusively addressed using specific ROS modulators, as shown earlier.

*Cr(VI)-Induced ROS and Apoptosis is Reduced in H460 ρ$^0$ Cells* – Mitochondria is an important source of ROS that generates oxidative stress by incomplete reduction of oxygen from the electrons that escape normal oxidative phosphorylation (52). To confirm the role of mitochondrial ROS in Cr(VI)-induced apoptosis, we compared Cr(VI)-induced ROS formation in H460 and H460 ρ$^0$ cells as assessed by DHE and DCF fluorescence. It was observed that Cr(VI)-induced ·O$_2^-$ and ROS production in H460 cells was decreased significantly in H460 ρ$^0$ cells (Figs. 11, A and B). Further, it was observed that Cr(VI)-induced apoptosis was reduced by half in H460 ρ$^0$ cells as compared to H460 cells indicating that mitochondrial functional electron transport is important for mediating Cr(VI)-induced apoptosis (Fig. 11C).

*Effect of Cr(VI) on Bcl-2 Expression* – To provide mechanistic insight into the regulation of Cr(VI)-induced apoptosis by Bcl-2, the expression level of Bcl-2 in response to Cr(VI) treatment was determined by Western blot analysis. Having demonstrated the role of Bcl-2 in Cr(VI)-induced apoptosis, the effect of Cr(VI) treatment on Bcl-2 expression was characterized in these cells by immunoblotting. Fig. 12A shows that treatment of the cells with Cr(VI) caused a dose-dependent decrease in the expression level of Bcl-2. Fig. 12B shows that this effect of Cr(VI) was time-dependent with the effect clearly noticeable at 6 h post-treatment and thereafter.

*Effect of Antioxidants on Bcl-2 Expression* – Having shown the role of Bcl-2 in Cr(VI)-induced apoptosis, we next investigated the potential regulation of Bcl-2 by ROS. ROS have previously been shown to mediate tumor necrosis factor-induced proteasomal degradation of Bcl-2 in human umbilical vein endothelial cells (39). To test whether ROS might also mediate the effect of Cr(VI) on Bcl-2, cells were treated with Cr(VI) in the presence or absence of ROS modulators, and their effect on Bcl-2 expression was
determined. Figure 13A shows that treatment of the cells with MnTBAP completely inhibited Cr(VI)-induced Bcl-2 down-regulation, whereas catalase treatment showed no inhibitory effect suggesting an important role of $\cdot O_2^-$ in Bcl-2 degradation. These results were confirmed in GPx- and SOD-overexpressed cells. Figure 13B shows that SOD overexpression completely inhibited Cr(VI)-induced Bcl-2 down-regulation, whereas GPx overexpression was ineffective. These results indicate that $\cdot O_2^-$ is the primary ROS responsible for Cr(VI)-induced Bcl-2 down-regulation. Furthermore, the expression of Bcl-2 was upregulated in H460 $\rho^0$ cells suggesting that mitochondrial $\cdot O_2^-$ is important in Bcl-2 degradation (Fig. 13C). This is consistent with our earlier observation that mitochondrial $\cdot O_2^-$ is the major regulator of Cr(VI)-induced apoptosis.

**ROS Mediates Cr(VI)-Induced Ubiquitination of Bcl-2** - Previous studies have shown that Bcl-2 is down-regulated primarily through the proteasomal degradation pathway (39, 53), we tested whether such degradation is involved in the down-regulation of Bcl-2 by Cr(VI). Cells were treated with Cr(VI) in the presence or absence of lactacystin (LAC), a highly specific proteasome inhibitor, and its effect on Bcl-2 expression was determined. Figure 14A shows that LAC completely inhibited Bcl-2 down-regulation induced by Cr(VI). This finding indicates that proteasomal degradation is a key mechanism of Cr(VI)-induced Bcl-2 down-regulation. To further investigate the mechanism by which ROS mediates Cr(VI)-induced Bcl-2 degradation, we analyzed ubiquitination of Bcl-2 in response to Cr(VI) treatment by immunoprecipitation. Cells overexpressing myc-Bcl-2 were treated with Cr(VI) in the presence or absence of ROS donors and inhibitors. Cell lysates were prepared and immunoprecipitated using anti-myc antibody. The resulting immune complexes were analyzed for ubiquitination by Western blot using anti-ubiquitin antibody. The results show that Cr(VI) was able to induce ubiquitination of Bcl-2 and the $\cdot O_2^-$ donor LY-83,583 increased this effect (Fig. 14B). In contrast, $\cdot O_2^-$ scavenger MnTBAP inhibited Bcl-2 ubiquitination, suggesting that $\cdot O_2^-$ is the major ROS involved in Cr(VI) induced Bcl-2 degradation. $H_2O_2$ donor and inhibitor had minimal effect. This result supported the pro-apoptotic role of ROS in ubiquitin-mediated degradation of Bcl-2 by Cr(VI) and established a novel mechanism linking $\cdot O_2^-$ signaling with Bcl-2 stability.
2.5. Discussion

Cr(VI) is a known human carcinogen that has been associated with lung cancer (3). Since acquired resistance to genotoxic cell death is a hallmark of neoplastic progression, it is important to understand the molecular mechanisms by which cells acquire an apoptosis resistant phenotype in response to genotoxic insult such as exposure to Cr(VI). Therefore, we first characterized the apoptotic pathways and regulatory proteins involved in Cr(VI)-induced apoptosis. In this study, we demonstrated that Cr(VI) induces apoptosis in a dose dependent manner and zVAD completely blocked this effect (Fig.1). Since zVAD is a general caspase inhibitor, this result indicated that Cr(VI) induces apoptosis via classical caspase dependent manner. Apoptosis may be initiated through the stimulation of death receptors located on the cell surface or through the intrinsic pathway involving the release of apoptotic signals from mitochondria. To gain a mechanistic understanding of the susceptibility to cell death in response to Cr(VI) exposure, we further showed that both mitochondrial and death receptor pathways of apoptosis were activated by Cr(VI) (Fig. 2, A and B). There have been studies suggesting a potential cross-talk between the two pathways in many cell-types. In the so called ‘Type I’ cells, stimulation of death receptors has been reported to cause caspase-8 mediated cleavage and activation of caspase-3 (54, 55). This pathway does not require mitochondria-associated events, whereas in “Type II’ cells caspase-8 leads to the cleavage and activation of Bid protein (56-58). Truncated bid stimulates formation of Bax pores in the outer mitochondrial membrane, which mediates the release of cytochrome c and, consequently, formation of Apaf-1/caspase-9 apoptosome (59). We observed that there is a potential cross-talk between the two apoptotic pathways in response to Cr(VI) exposure in H460 cells (Fig. 2C). Bid cleavage also suggested that H460 cells are Type II cells. Spectrofluorometric analysis further confirmed that both apoptotic pathways are induced by Cr(VI) exposure, with the mitochondrial pathway playing a more dominant role as compared to the death receptor pathway (Fig. 3, A and B). This observation was in agreement with another report that suggested that apoptotic doses of Cr(VI) caused mitochondrial instability (60). Various pro- and anti-apoptotic proteins regulate the extrinsic and intrinsic pathways. Interestingly, induction of pro-apoptotic proteins does not necessarily lead to apoptosis induction (26, 27), indicating that inhibitors of both apoptotic pathways exist and are important. Flip is the negative regulator of the death receptor pathway (25, 61, 62) while Bcl-2 plays a key role in the mitochondrial pathway (20, 28, 29). We observed that Cr(VI) downregulated both FLIP and Bcl-2 in a dose dependent manner (Fig. 4, A and B). It was observed that Bcl-2 overexpression strongly blocked Cr(VI)-induced apoptosis, whereas Flip had minimal effect (Fig. 4C and 5, A and C). This further substantiated the role of the mitochondrial death pathway in Cr(VI)-induced apoptosis.

Cr(VI) is actively transported into cells by anionic transport system at the cellular level (64) and is metabolically changed to the ultimate reduced form, Cr(III), in a process that also produced ROS. Since generation of ROS is a definite consequence of Cr(VI) exposure we also demonstrated the role of ROS in Cr(VI)-induced apoptosis of human lung cancer epithelial H460 cells. Consistent with the observation that reduction of Cr(VI) inside the cell produces ROS, our results showed that Cr(VI) was able to induce \( \cdot \text{O}_2^- \) and peroxide generation in human lung epithelial H460 cells (Fig. 6 and 7). We also observed that ROS
was responsible for Cr(VI)-induced apoptosis (Fig. 8). This was verified in the presence of antioxidants such as NAC, catalase, and MnTBAP that effectively inhibited apoptosis induced by Cr(VI), indicating the role of ROS, specifically \( \cdot \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \), in this process. H460 cells overexpressing GPx and SOD showed reduced apoptotic responses to Cr(VI) treatment (Fig. 9, A and B). This effect was more pronounced in SOD-overexpressed cells suggesting that even though both \( \cdot \text{O}_2^- \) and \( \text{H}_2\text{O}_2 \) are important, \( \cdot \text{O}_2^- \) is the major ROS involved in Cr(VI) apoptosis. To determine the source of ROS involved in Cr(VI)-induced apoptosis, we utilized DPI and rotenone. Rotenone has previously been shown to inhibit mitochondrial ROS and DPI is known to inhibit cellular NADPH oxidase and are considered as indicators of the mitochondrial and cytosolic ROS, respectively (49-51). Cr(VI) induced ROS generation and apoptosis was significantly inhibited by DPI and rotenone, with the effect of the latter being more dominant (Fig. 10). This indicated that total ROS produced in the cell is important in Cr(VI) induced apoptosis, but mitochondrial ROS plays a major role. This was further confirmed in H460 \( \rho^0 \) cells which are prepared by long-term exposure to ethidium bromide that leads to the depletion of critical respiratory chain sub-units encoded by mitochondrial DNA (65, 66). Therefore these cells are incapable of generating mitochondrial ROS and normal oxidative phosphorylation (67). We observed decreased ROS production and apoptosis in H460 \( \rho^0 \) as compared to H460 cells (Fig. 11). Since \( \rho^0 \) cells have dysfunctional mitochondria, this observation indicated an important role of mitochondrial electron transport chain and ROS in Cr(VI) induced apoptosis (68, 69).

Since we observed that the anti-apoptotic protein Bcl-2 overexpression plays a key role in Cr(VI)-induced apoptosis, we tested the effect of Cr(VI) on Bcl-2 expression. Exposure of the cells to Cr(VI) caused a dose- and time-dependent down-regulation of Bcl-2 (Fig. 12, A and B). We found that this down-regulation was ROS dependent as co-treatment of the cells with the \( \cdot \text{O}_2^- \) scavenger MnTBAP completely inhibited the down-regulation of Bcl-2 by Cr(VI) (Fig. 13A). \( \text{H}_2\text{O}_2 \) scavenger catalase failed to inhibit this effect, indicating that \( \text{H}_2\text{O}_2 \) has a minimal role in Cr(VI)-induced Bcl-2 down-regulation and apoptosis. Gene transfection studies using GPx- and SOD-overexpressed cells confirmed these results and indicated the critical role of \( \cdot \text{O}_2^- \) in this process (Fig. 13B). The observation that catalase and GPx failed to inhibit Bcl-2 down-regulation while exhibiting a protective effect on Cr(VI)-induced apoptosis suggests that other peroxide-mediated Bcl-2-independent mechanisms may be involved in the apoptotic effect of Cr(VI). It was further observed that H460 \( \rho^0 \) cells inhibited Bcl-2 down-regulation when treated with Cr(VI) as compared to H460 cells (Fig. 13C). This suggested that mitochondrial \( \cdot \text{O}_2^- \) plays a critical role in maintaining Bcl-2 stability. The anti-apoptotic function of Bcl-2 is closely associated with its expression levels, which is controlled by various mechanisms. Post-translational modifications, such as ubiquitination and phosphorylation, have emerged as important regulators of Bcl-2 function (39, 40, 70). The ability of the proteasome inhibitor lactacystin to inhibit Bcl-2 down-regulation strongly supports the role of the proteasomal pathway in Bcl-2 regulation (Fig. 14A). We observed that \( \cdot \text{O}_2^- \) scavenger MnTBAP inhibited Bcl-2 ubiquitination induced by Cr(VI), whereas \( \cdot \text{O}_2^- \) donor LY-83,583 promoted this effect (Fig. 14B). \( \text{H}_2\text{O}_2 \) modulators had minimal effect. These results indicate that \( \cdot \text{O}_2^- \) downregulates Bcl-2 by inducing its degradation via the ubiquitin-proteasomal pathway in response to Cr(VI) exposure.
In summary, our data provides evidence that apoptosis induced by Cr(VI) is mediated through the mitochondrion-dependent caspase-9 activation pathway, which is negatively regulated by the anti-apoptotic Bcl-2 protein. ROS plays an important role in Cr(VI)-induced apoptosis of human lung cancer epithelial H460 cells. The mechanism by which Cr(VI) induces apoptosis involves rapid generation of ROS and ·O$_2^-$ is the major ROS involved. Mitochondria is the main source of ROS that is involved in Cr(VI)-induced apoptosis. Cr(VI) induces down-regulation of Bcl-2 through a process that involves ·O$_2^-$-mediated proteasomal degradation of the protein. Exposure to Cr(VI) is associated with lung cancer and Bcl-2 has been shown to be overexpressed in various cancers including lung tumor cells and tissue specimen (71-73). This aim established the importance of mitochondrial pathway and Bcl-2 protein in Cr(VI)-induced apoptosis which will further help in understanding the carcinogenicity of Cr(VI). ·O$_2^-$ may represent a common regulator of Bcl-2 function that controls apoptotic cell death induced by various physiologic and pathologic stimuli. This study showing the role of ·O$_2^-$ in Cr(VI)-induced cell death and Bcl-2 downregulation, documents a novel mechanism of apoptosis induction by Cr(VI) involving Bcl-2 stability linked to ·O$_2^-$ generation. Signal transduction leading to apoptotic cell death has been of great interest in biomedical and pharmaceutical research mainly because successful apoptotic agents could be used to treat cancer.
FIGURE 1. Induction of apoptosis by Cr(VI) in human lung epithelial H460 cells. A, subconfluent (90%) monolayers of H460 cells were exposed to varying doses of Cr(VI) (0-100 µM) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. C, subconfluent (90%) monolayer of H460 cells were exposed to zVAD (25 µM) for 1 h and were then treated with Cr(VI) (20 µM) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. B and D, fluorescence micrographs of treated cells stained with Hoechst dye. Apoptotic cells exhibited shrunken and fragmented nuclei with bright nuclear fluorescence. Values are mean ± S.D. (n ≥ 3). *, p < 0.05 versus non-treated control.
FIGURE 2. **Signaling Cascades Induced by Cr(VI).** H460 cells were treated with varying doses of Cr(VI) (10, 20, 50 µM) for 12 h, and cell lysates were prepared and analyzed for

A, Caspase-8, B, Caspase-9 and C, Bid expression by Western blotting.
FIGURE 3. Caspase activation in response to Cr(VI) exposure. A, fluorometric assay of caspase activity in cells treated with varying doses of Cr(VI) (1-50 µM) for 12 h. Cell lysates (50 µg of protein) were prepared and analyzed for caspase activity using the fluorometric substrates IETD-AMC and LEHD-AMC for caspase-8 and -9, respectively. B, fluorometric assay of caspase activity in cells treated with Cr(VI) (20 µM) for 12 h in the presence or absence of the caspase-8 inhibitor IETD-CHO (10 µM) or caspase-9 inhibitor LEHD-CHO (10 µM). C, Apoptosis assay of caspase activity in cells treated with Cr(VI) (20 µM) for 12 h in the presence or absence of the caspase-8 inhibitor IETD-CHO (10 µM) or caspase-9 inhibitor LEHD-CHO (10 µM). Values are mean ± S.D. (n ≥ 3). *, p < 0.05 versus non-treated control.
FIGURE 4. **Effect of Cr(VI) on apoptosis regulatory proteins.** H460 cells were treated with varying doses of Cr(VI) (10, 20, 50 µM) for 12 h, and cell lysates were prepared and analyzed for A, Bcl-2 and B, Flip expression by Western blotting. Blots were reprobed with β-actin antibody to confirm equal loading of samples. C, H460 cells were transiently transfected with myc-tagged Bcl-2 plasmid, myc-tagged Flip plasmid or control pcDNA3 plasmid. Transfected cells were treated with Cr(VI) (10 and 20 µM) for 12 h and apoptosis was determined by Hoechst 33342 assay. Values are mean ± S.D. (n ≥ 3).
FIGURE 5. **Bcl-2 overexpression increases cell death resistance to Cr(VI).** *A* and *B*, H460 cells were stably transfected with myc-tagged Bcl-2 plasmid, myc-tagged Flip plasmid or control pcDNA3 plasmid. Transfected cells were treated with varying doses of Cr(VI) (0-100 µM) for 12 h and apoptosis was determined by Hoechst 33342 assay. Plots are mean ± S.D. (*n* = 4). *, *p* < 0.05 versus mock-transfected controls. *C*, Western blot analysis of Bcl-2 expression in mock- and Bcl-2-transfected cells. *D*, Western blot analysis of Flip expression in mock- and Flip-transfected cells. β-Actin was used as a loading control.
FIGURE 6. **Effect of Cr(VI) on cellular ROS levels.** *A, B and C*, subconfluent (90%) monolayers of H460 cells were treated with varying doses of Cr(VI) (0-100 µM) and analyzed for ·O₂⁻ and peroxide production by flow cytometry using DHE and DCF fluorescent probes.
FIGURE 7. Effect of ROS modulators on cellular ROS levels. A and B, subconfluent (90%) monolayers of H460 cells were pretreated with NAC (10 mM), MnTBAP (100 μM) or catalase (10000 U/ml) for 0.5 h, followed by Cr(VI) (20 μM) treatment. Plots show relative DHE or DCF fluorescence intensity over non-treated control determined at the peak response time of 1 h after Cr(VI) treatment. LY-83,583 (10 μM) and H2O2 (0.1 mM) were used as positive controls for DHE and DCF, respectively. C, Fluorescence micrographs of treated cells from above. Values are mean ± S.D. (n ≥ 3). *, p < 0.05 versus non-treated control.
FIGURE 8. Induction of apoptosis by ROS modulators in H460 cells. A, subconfluent (90%) monolayers of H460 cells were either left untreated or pretreated with NAC (10 mM), MnTBAP (100 µM), catalase (10000 U/ml) or xanthine/xanthine oxidase (1 mM/0.02 U) for 1 h. The cells were then treated with Cr(VI) (20 µM) for 12 h and analyzed for apoptosis by Hoechst assay. B, Fluorescence micrographs of treated cells stained with Hoechst 33342 dye. Values are mean ± S.D. (n ≥ 3). *, p < 0.05 versus non-treated control.
FIGURE 9. Effect of GPx and SOD overexpression on Cr(VI)-induced apoptosis and ROS generation. A and B, H460 cells were stably transfected with GPx, SOD, or control pcDNA3 plasmid as described under Materials and Methods. Transfected cells were treated with Cr(VI) (1-100 µM) for 12 h and analyzed for apoptosis by Hoechst 33342 assay. C and D, transfected cells were treated with Cr(VI) (1-100 µM) and analyzed for DCF and DHE fluorescence intensities at 1 h post-treatment. Plots show relative fluorescence intensity over non-treated control. Values are mean ± S.D. (n ≥ 3).
FIGURE 10. Cellular source of Cr(VI)-induced ROS. A and B, Cells were pretreated for 0.5 h with DPI (5 µM) or rotenone (5 µM), followed by Cr(VI) treatment (20 µM) and were analyzed for DHE and DCF fluorescence intensities at 1 h post-treatment. C, H460 cells were either left untreated or pretreated with the indicated concentrations of DPI or rotenone for 1 h. Cells were then treated with Cr(VI) (20 µM) and analyzed for apoptosis after 12 h. D, fluorescence micrographs of treated cells stained with Hoechst 33342 dye. Values are mean ± S.D. (n ≥ 3). *, p < 0.05 versus non-treated control.
FIGURE 11. Cr(VI)-induced ROS and apoptosis is reduced in H460 ρ0 cells. A and B, H460 and H460 ρ0 cells were treated with Cr(VI) (10, 20 and 50 µM) for 1 h and analyzed for DHE and DCF fluorescence intensities. Plots show relative fluorescence intensity over non-treated control. C, subconfluent (90%) monolayers of H460 cells and H460 ρ0 cells were treated with varying doses of Cr(VI) (0-100 µM) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. Values are mean ± S.D. (n ≥ 3). *, p < 0.05 versus non-treated control.
FIGURE 12. Effect of Cr(VI) on Bcl-2 expression. A, H460 cells were treated with varying doses of Cr(VI) (0 - 100 µM) for 12 h, and cell lysates were prepared and analyzed for Bcl-2 expression by Western blotting. B, cells were treated for various times (0–24 h) with Cr(VI) (20 µM) and Bcl-2 expression was determined. Blots were reprobed with β-actin antibody to confirm equal loading of samples. The immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the result obtained in cells in the absence of Cr(VI) (control). Plots are mean ± S.D. (n = 4). *, p < 0.05 versus non-treated control.
FIGURE 13. **Effect of ROS modulators on Bcl-2 expression.** A, H460 cells were either left untreated or pretreated with MnTBAP (100 µM), catalase (10000 U/ml), LY 83,583 (10 µM) or H$_2$O$_2$ (0.1 mM) for 1 h, followed by Cr(VI) treatment (20 µM) for 12 h. Cell lysates were then prepared and analyzed for Bcl-2 expression by Western blotting. B, GPx-, SOD-, or mock-transfected cells were treated with Cr(VI) (10, 20 µM) for 12 h, and Bcl-2 expression was determined by Western blotting. C, H460 and H460 $\rho^0$ cells were treated with Cr(VI) (10, 20 µM) for 12 h, and Bcl-2 expression was similarly determined. Blots were reprobed with β-actin antibody to confirm equal loading of samples. Densitometry was performed to determine the relative expression of Bcl-2 in treated cells compared with non-treated cells. Plots are mean ± S.D. ($n = 4$). *, $p < 0.05$ versus non-treated control.
FIGURE 14. **Effect of ROS on Bcl-2 ubiquitination.**

**A**, H460 cells were either left untreated or pretreated with LAC (10 μM) for 0.5 h, followed by Cr(VI) treatment (20 μM) for 12 h. Cell lysates were then prepared and analyzed for Bcl-2 expression by Western blotting. **B**, subconfluent monolayers of H460 cells overexpressing Bcl-2 were pretreated with MnTBAP (100 μM), catalase (10000 U/ml), LY-83,583 (10 μM) and H₂O₂ (0.5 mM) for 1 h. Subsequently, the cells were treated with Cr(VI) (20 μM) for 3 h in the presence of LAC (10 μM) to prevent proteasome-mediated Bcl-2 degradation. Cell lysates were immunoprecipitated with anti-myc antibody and the immune complexes were analyzed for ubiquitin by Western blotting. Analysis of ubiquitin was performed at 3 h post-Cr(VI) treatment where ubiquitination was found to be maximal. Plots are mean ± S.D. (n = 4). *, p < 0.05 versus non-treated control.
2.6. References

induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. Immunity, 9: 267-276, 1998.


CHAPTER III

Role of Nitric Oxide in Cr(VI)-Induced Apoptosis
3.1. Abstract

Bcl-2 is a key apoptosis regulatory protein of the mitochondrial death pathway whose function is dependent on its expression levels. Although Bcl-2 expression is controlled by various mechanisms, post-translational modifications, such as ubiquitination and proteasomal degradation, have emerged as important regulators of Bcl-2 function. However, the underlying mechanisms of this regulation are unclear. We report here that Bcl-2 undergoes S-nitrosylation by endogenous nitric oxide (NO) in response to multiple apoptotic mediators and that this modification inhibits ubiquitin-proteasomal degradation of Bcl-2. Inhibition of NO production by the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide and by NO synthase inhibitor aminoguanidine effectively inhibited S-nitrosylation of Bcl-2, increased its ubiquitination, and promoted apoptotic cell death induced by Cr(VI). In contrast, NO donors dipropylentriamine NONOate and sodium nitroprusside showed opposite effects. Furthermore, it was observed that NO scavenged ·O$_2$⁻ forming peroxynitrite, which was the major reactive nitrogen species involved in S-nitrosylation of Bcl-2. The effect of NO on Bcl-2 stability was shown to be independent of its dephosphorylation. Mutational analysis of Bcl-2 further showed that the two cysteine residues of Bcl-2 (Cys$^{158}$ and Cys$^{229}$) are important in the S-nitrosylation process and that mutations of these cysteines completely inhibited Bcl-2 S-nitrosylation. Treatment of the cells with other stress inducers, including Fas ligand and buthionine sulfoxide, also induced Bcl-2 S-nitrosylation, suggesting that this is a general phenomenon that regulates Bcl-2 stability and function under various stress conditions. These findings indicate a novel function of NO and its regulation of Bcl-2, which provides a key mechanism for the control of apoptotic cell death and cancer development.
3.2. Introduction

Bcl-2 is a key apoptosis-regulatory protein of the mitochondrial death pathway (1). Formation of heterodimers with proapoptotic proteins such as Bax, inhibition of cytochrome c release, and regulation of mitochondrial transmembrane potential are some of the mechanisms by which Bcl-2 exerts its antiapoptotic effect (2-4). The oncogenic potential of Bcl-2 protein is well characterized. It is overexpressed in 70% of breast cancer, 30–60% of prostate cancer, and 90% of colorectal cancer (5, 6). Additionally, its expression has been reported to be amplified in several apoptosis-resistant lung cell lines and tumor specimens (7-9). Bcl-2 expression is regulated by various mechanisms, including dimerization, post-translational modification, transcription, and degradation. Bcl-2 degradation is mainly mediated via the ubiquitin-proteasomal pathway, which is a major system for selective protein degradation in eukaryotic cells (10, 11). Nitric oxide (NO) is an important signaling molecule produced endogenously from L-arginine in a reaction catalyzed by NO synthases (12). Subtle changes in its rate of production may critically impact cellular homeostasis, consequently initiating a variety of cellular signaling processes, including apoptosis (13). NO has been demonstrated to have both pro- and anti-apoptotic role, depending on a variety of factors, including the type of cells involved, redox state of the cell, and the flux and dose of NO (12). Recent evidence indicates that NO activates a complex network of responses leading to apoptosis via mitochondrial, death receptor, p38/mitogen-activated protein kinase, and glyceralddehyde-3-phosphate dehydrogenase-Siah1 cascades (14-17). The anti-apoptotic effect of NO can be mediated through a number of mechanisms, such as caspase inactivation, induction of p53 gene expression, up-regulation of FLIP, and overexpression of Bcl-2 and Bcl-XL, with subsequent inhibition of cytochrome c release from the mitochondria (18-24). NO freely diffuses across cell membranes and interacts with many other molecules. For instance, it reacts rapidly with ·O$_2^-$ anions in a diffusion controlled reaction to primarily form peroxynitrite (ONOO¯) in most cell systems (25). The effects of ONOO¯ on protein function are mainly due to its reactivity and covalent modification of tyrosine and/or cysteine residues (26). One of the well-established mechanisms by which NO through the formation of ONOO¯ regulates the function of various target proteins is via S-nitrosylation (27). This post-translational modification of proteins can positively or negatively regulate various signaling pathways, proteins, and metabolic processes (28). For instance, NO inhibits the function of NF-κB, JNK and protein kinase C and activates Ras and ryanodine receptor via S-nitrosylation (29-33). Even though NO has been widely implicated in apoptosis and its dysregulation, its role in cancer is not well characterized. The involvement and the role of NO in Cr(VI)-induced apoptosis has never been studied.

The objective of this study was to investigate the role of NO in the regulation of Bcl-2 function in Cr(VI)-induced apoptosis of human lung epithelial cancer cells. Cr(VI) is a naturally occurring heavy metal that has been associated with lung cancers in various occupational settings (35-38). This compound is also present in cigarette smoke, and an increased incidence of lung cancer has been reported in smokers with Cr(VI) exposure (34, 36). Cell death induced by Cr(VI) occurs primarily through apoptosis (39), and its abnormal regulation has been associated with the initiation of Cr(VI)-induced cancer (40). Several cellular factors and signaling pathways, such as reactive oxygen species, p53, and
NF-κB activation have been implicated in Cr(VI)-induced apoptosis and carcinogenicity (41-43). However, the mechanisms involved in the abnormal regulation of apoptosis in response to Cr(VI) exposure remain unclear. Elevated levels of Bcl-2 and NO production have been reported in human lung cancers (7-9, 44-46). In the present study, we found that NO up-regulates Bcl-2 expression, which provides a key mechanism against Cr(VI)-induced apoptosis. The mechanism by which NO regulates Bcl-2 involves S-nitrosylation of the protein, which was also observed under other stress conditions, including exposure to death ligand and glutathione depletion. ONOO$^-$ is the major reactive nitrogen species involved in S-Nitrosylation of Bcl-2 that prevents its downregulation via the ubiquitin-proteasome pathway. Mutational analysis indicated the involvement of Cys$^{158}$ and Cys$^{229}$ in S-nitrosylation and ubiquitination of Bcl-2. These findings reveal the existence of a novel mechanism of NO-mediated regulation of Bcl-2 that could be important in apoptosis resistance and the development of lung cancer induced by Cr(VI) and other carcinogens.
3.3. Materials and Methods

Cells and Reagents
The human lung epithelial cancer cell line NCI-H460 was obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 medium (Sigma) containing 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 µg/ml streptomycin in a 5% CO₂ environment at 37°C. The NO donor sodium nitroprusside (SNP), NO inhibitors aminoguanidine (AG) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxo-3-oxide (PTIO), lactacystin, buthionine sulfoximine (BSO), dithiothreitol (DTT), dianinonaphthalene (DAN), mercury (II) chloride (HgCl₂), sodium hydroxide (NaOH), sulfonilamide, N-1-naphthyl ethylenediamine dehydrochloride, sodium nitrite (NaNO₂), 6-Anilinoquinoline-5,8-quinone (LY-83,583) and sodium dichromate (Na₂Cr₂O₇·2H₂O) [(Cr(VI)] were obtained from Sigma (St. Louis, MO). The NO donor dipropylenetetramine (DPTA) NONOate, Fas ligand (FasL) and the fluorogenic caspase substrates IETD amino-4-methylcoumarin (IETD-AMC) and LEHD-AMC were from Alexis Biochemicals (San Diego, CA). Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) was purchased from Calbiochem (La Jolla, CA). The NO fluorescent probe 4,5-diaminofluorescein diacetate (DAF-DA), DHE and the apoptosis dye Hoechst 33342 were from Molecular Probes, Inc. (Eugene, OR). Antibodies for Bcl-2, phospho-Bcl-2 (Ser87), Myc, peroxidase-labeled secondary antibodies, anti-myc agarose beads, and protein A-agarose were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for S-nitrosocysteine, ubiquitin, and β-actin were from Sigma, and the transfecting agent Lipofectamine 2000 was from Invitrogen (Carlsbad, CA).

Plasmid and Transfection
The Bcl-2 plasmid was generously provided by Dr. Christian Stehlik (West Virginia University Cancer Center, Morgantown, WV). The open reading frame of Bcl-2 and ubiquitin were amplified by high fidelity PCR (Stratagene, La Jolla, CA) from corresponding expressed sequence tags and cloned into pcDNA3 expression vectors containing N-terminal myc epitope tag. The authenticity of all constructs was verified by DNA sequencing. Transient transfection was performed using Lipofectamine 2000 reagent according to the manufacturer’s instructions. The amount of DNA was normalized in all transfection experiments with pcDNA3. Expression of proteins were verified by Western blotting or immunoprecipitation.

Generation of Stable Bcl-2 Transfectant
Stable transfecntant of Bcl-2 was generated by culturing H460 cells in 60-mm dishes until they reached 80% confluence. 1 µg of cytomegalovirus-neo vector and 15 µl of Lipofectamine 2000 with 2 µg of myc-tagged Bcl-2 plasmid were used to transfect the cells in the absence of serum. After 10 h, the medium was replaced with culture medium containing 5% fetal bovine serum. Approximately 36 h after transfection, the cells were digested with 0.03% trypsin and plated onto 75-ml culture flasks and cultured for 24–28 days with G418 selection medium (400 µg/ml). The stable transfectant was identified by Western blotting of Bcl-2 and was cultured in G418-free RPMI medium for at least two passages before each experiment.
Caspase and Apoptosis Assays
Caspase activity was determined by fluorometric assay using the enzyme substrate IETD-AMC for caspase-8 and LEHD-AMC for caspase-9, which are specifically cleaved by the respective enzymes at the Asp residue to release the fluorescent group, AMC. Cell extracts containing 50 µg of protein were incubated with 100 mM HEPES containing 10% sucrose, 10 mM dithiothreitol, 0.1% 3-((3-cholamidopropyl)-1) propane sulfonate, and 50 µM caspase substrate in a total reaction volume of 0.25 ml. The reaction mixture was incubated for 60 min at 37°C and quantified fluorometrically at the excitation and emission wavelengths of 380 and 460 nm, respectively. Apoptosis was determined by incubating the cells with 10 µg/ml Hoechst 33342 nuclear stain for 30 min at 37°C and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by fluorescence microscopy (Axiovert 100; Carl Zeiss) using Pixera software.

NO Detection
Intracellular NO production was determined by flow cytometry and fluorescence microscopy using NO-specific probe DAF-DA. For flow cytometric analysis, cells (1 x 10⁶/ml) were incubated with the probe (10 µM) for 30 min at 37°C, after which they were washed, resuspended in phosphate-buffered saline (PBS), and analyzed for DAF fluorescence intensity using a 488-nm excitation beam and a 538-nm bandpass filter (FACS calibur; Becton-Dickinson). For fluorescence microscopy, cells were incubated with the probe as described above and then examined for DAF fluorescence using Carl Zeiss Axiovert microscope.

Griess Assay
NO production was confirmed by measuring its nitrite by-product using Griess assay. Cell supernatants (50 µl) were incubated with 50 µl of Griess reagent containing 0.1% N-1-naphthyl ethylenediamine dehydrochloride and 1% sulfanilamide for 10 min at room temperature. The optical density of the samples was measured using a microplate reader (model 550; Bio-Rad) at 550 nm. NO concentration was calculated from the standard curve produced during each assay by using NaNO₂ dissolved in 15 mM HEPES, pH 7.5.

Western Blotting
After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na₃VO₄, 50 mM NaF, 100 mM PMSF, and protease inhibitor mixture for 20 min on ice. After insoluble debris was precipitated by centrifugation at 14,000 x g for 15 min at 4°C, the supernatants were collected and assayed for protein content using BCA method. An equal amount of proteins per sample (15 µg) were resolved on 10% SDS-PAGE and transferred onto a 0.45-µm nitrocellulose membrane. The transferred membranes were blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl, pH 7.4, 125 mM NaCl, 0.05% Tween 20) and incubated with the appropriate primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The immune complexes were detected by chemiluminescence (Supersignal West Pico; Pierce) and quantified by imaging densitometry using UN-SCAN-IT automated digitizing software (Silk Scientific, Orem, UT). Mean densitometry data from independent experiments were normalized to the control. The data were presented as mean ± S.D. and analyzed by Student’s t test.
Immunoprecipitation

Cells were washed after treatments with ice-cold PBS and lysed in lysis buffer at 4°C for 20 min. After centrifugation at 14,000 x g for 15 min at 4°C, the supernatants were collected, and the protein content was determined as described. Cleared lysates were normalized, and 60 µg of proteins were incubated with 8 µl of anti-myc-agarose beads (Santa Cruz Biotechnology) diluted with 12 µl of protein A-agarose for 4 h at 4°C. The immune complexes were washed three times with 500 µl of lysis buffer, resuspended in 2 x Laemmli sample buffer, and boiled at 95°C for 5 min. The immune complexes were separated by 10% SDS-PAGE and analyzed by Western blot as described.

Fluorometric Measurement of S-Nitrosylation

S-Nitrosylation of Bcl-2 was measured as previously described (31, 47). In brief, cells were treated, harvested, lysed, and subjected to immunoprecipitation. The immunoprecipitates were rinsed four times with lysis buffer and twice with PBS. The pellets were resuspended in 500 µl of PBS and incubated with HgCl₂ (200 µM) and diaminonaphthalene (DAN) (200 µM) for 0.5 h in dark at room temperature followed by the addition of 1 M NaOH. A fluorescent triazole generated from the reaction between DAN and NO released from S-nitrosylated Bcl-2 was quantified using a fluorometer (FLUOstar OPTIMA, BMG Inc., Durham, NC) at the excitation and emission wavelengths of 375 and 450 nm, respectively.

Site-directed Mutagenesis of Bcl-2

Mutant Bcl-2 plasmids were constructed using the above-mentioned human Bcl-2 plasmid as template. Two sets of forward and reverse primers were constructed to introduce mutations at Cys158 and Cys229: C158A, CGGTGGGGTCATGGCTGTGGA GAGCGTCAACCG (forward) and CGGTTGACGCTCTCCACAGCCATGACCCCAG (reverse); C229A, GGCCCTGGTGGGAGCTGCCATCACCCTGGGTGCC (forward) and GGCACCCAGGGTGATGGCAGCTCCCACCAGGGCC (reverse). Three mutant plasmids (C158A, C229A, and a plasmid with both mutations) were constructed using the QuickChange XL site-directed mutagenesis kit (Stratagene). Mutagenesis was confirmed by automated nucleotide sequencing.

Proteasome Activity Assay

Proteasome activity was measured using an assay kit from CHEMICON (Temecula, CA), according to the manufacturer’s protocol. Briefly, cells were washed after treatments with ice-cold PBS and lysed in lysis buffer at 4°C for 20 min. After centrifugation at 14,000 x g for 15 min at 4°C, the supernatants were collected and protein content was determined by BCA method. Cleared lysates were normalized, and 20 µg of proteins were incubated with the proteasome substrate LLVY-AMC at 37°C for 1 h. The fluorophore AMC obtained after cleavage from the labeled substrate was quantified at the excitation and emission wavelengths of 380 and 460 nm, respectively.

ROS Detection

Intracellular ·O₂⁻ production was determined by spectrofluorometry using DHE as the fluorescence probe. Cells (1x10⁶/ml) were incubated with the fluorescent probe (10 µM) for 30 min at 37°C, after which they were washed, resuspended in phosphate buffered
saline (PBS), and analyzed for DHE fluorescence at the excitation and emission wavelengths of 535 nm and 617 nm) using FLUOstar OPTIMA (BMG, Inc.)
3.4. Results

NO Inhibits Cr(VI)-induced Apoptosis – As shown in the previous aim, Cr(VI) induces apoptosis in a dose-dependent manner mainly via the mitochondrial pathway. To investigate the potential role of NO in the regulation of Cr(VI)-induced apoptosis, cells were treated with Cr(VI) in the presence or absence of various NO inhibitors and donors, and apoptosis was determined by Hoechst assay. Figs. 1, A and B, show that NO inhibitors AG and PTIO effectively increased apoptotic cell death induced by Cr(VI), whereas NO donors SNP and DPTA NONOate inhibited this effect in a dose-dependent manner. Caspase activity assays similarly showed that the NO inhibitor AG and the NO donor SNP, respectively, increased and decreased the effect of Cr(VI) on caspase-9 activation (Fig. 1C). These results indicate that NO plays an anti-apoptotic role in Cr(VI)-induced apoptosis.

Effect of NO Modulators on Cellular NO Level - To provide a relationship between the apoptotic response and NO modulation by the test agents, we performed flow cytometric analysis assessing the effect of test agents on cellular NO production using the fluorescent probe DAF DA. Fig. 2, A and B, show that the NO inhibitors AG and PTIO significantly inhibited cellular NO production induced by Cr(VI), whereas the NO donors SNP and DPTA NONOate promoted this effect. Fig. 2C shows fluorescence micrographs of cells treated with Cr(VI) in the presence or absence of NO modulators. Since Cr(VI) exposure is known to produce ROS (39, 40) and due to the report challenging the specificity of DAF DA to NO in cells undergoing oxidative and nitrosative stress (50), we confirmed this effect by the Griess method. Griess assay measures nitrite, which is the stable breakdown product of NO. Fig. 2D shows that NO inhibitors inhibited cellular nitrite production induced by Cr(VI), whereas the NO donors SNP and DPTA NONOate increased its levels. These results indicate the modulation of cellular NO levels by the test agents and support the role of NO in the regulation of Cr(VI)-induced apoptosis.

Effect of NO Modulators on Bcl-2 Expression – Having demonstrated the role of Bcl-2 in Cr(VI)-induced apoptosis in the previous aims, we next examined the potential regulation of Bcl-2 by NO in H460 cells. We treated the cells with Cr(VI) in the presence or absence of various NO modulators, including AG, PTIO, SNP, and DPTA NONOate, and the expression level of Bcl-2 was similarly determined. Fig. 3A shows that the NO inhibitors AG and PTIO significantly increased the down-regulation of Bcl-2 by Cr(VI), whereas the NO donors SNP and NONOate inhibited this down-regulation and further increased the Bcl-2 expression level over control level. Fig. 3B shows the effect of AG and PTIO alone as controls on Bcl-2 expression. Since previous studies have shown that Bcl-2 is rapidly down-regulated by proteasomal degradation (10), we tested whether Bcl-2 down-regulation by Cr(VI) is also mediated by this pathway. Cells were treated with lactacystin, a highly specific proteasome inhibitor, and its effect on Cr(VI)-induced Bcl-2 down-regulation was determined by immunoblotting. The result in Fig. 3A shows that lactacystin completely inhibited Bcl-2 down-regulation induced by Cr(VI), indicating a dominant role of proteasome-mediated degradation of Bcl-2 by Cr(VI).

Effect of NO on Bcl-2 Phosphorylation - Accumulating evidence indicates that Bcl-2 phosphorylation induces a conformational change in Bcl-2 that controls its stability and
apoptotic function (51-53). Phosphorylation of Bcl-2 at Thr 74 and Ser 87 has been shown to regulate its stability and dephosphorylation at Ser 87 is the initial step of Bcl-2 degradation (10, 54). Since our results demonstrated that NO can prevent Bcl-2 degradation, we further investigated the effect of NO on Bcl-2 phosphorylation. Cells were treated with Cr(VI) in the presence or absence of various NO modulators, and their effect on Bcl-2 phosphorylation was determined by Western blot using phosphospecific Bcl-2 (Ser 87) antibody. The results show that Cr(VI) had minimal effect on Bcl-2 phosphorylation, and likewise NO donors and inhibitors had no significant effect on the phosphorylation level (Fig. 3C). These results suggest that NO regulates Bcl-2 stability via a mechanism that is independent of its phosphorylation.

**NO Prevents Cr(VI)-induced Ubiquitination of Bcl-2** - To further investigate the mechanism by which NO inhibits Cr(VI)-induced Bcl-2 degradation, we analyzed ubiquitination of Bcl-2 in response to Cr(VI) treatment by immunoprecipitation. Cells overexpressing myc-Bcl-2 were treated with Cr(VI) in the presence or absence of NO donors and inhibitors. Cell lysates were prepared and immunoprecipitated using anti-myc antibody. The resulting immune complexes were analyzed for ubiquitination by Western blot using anti-ubiquitin antibody. The results show that Cr(VI) was able to induce ubiquitination of Bcl-2 and that NO inhibitors AG and PTIO increased this effect (Fig. 4). In contrast, NO donors SNP and DPTA NONOate inhibited Bcl-2 ubiquitination, thus supporting the inhibitory role of NO in ubiquitin-mediated degradation of Bcl-2 by Cr(VI).

**NO-mediated S-Nitrosylation of Bcl-2 Inhibits its Ubiquitination** - NO can control the function of several proteins by S-nitrosylation process (55-57). To determine whether NO could nitrosylate Bcl-2 and whether this process could affect Bcl-2 stability, we performed immunoprecipitation experiments evaluating the effect of NO on Bcl-2 S-nitrosylation. Cells expressing ectopic Bcl-2-myc were treated with Cr(VI) and NO modulators, and cell lysates were immunoprecipitated and analyzed by Western blot analysis using anti-S-nitrosocysteine antibody and by spectrofluorometry. Figs. 5, A and B, show that treatment of the cells with Cr(VI) induced S-nitrosylation of Bcl-2, and this effect was enhanced by NO donors SNP and DPTA NONOate. In contrast, NO inhibitors AG and PTIO inhibited this nitrosylation process. These results suggest that NO, through its ability to nitrosylate Bcl-2, may interfere with the ubiquitination process and inhibit Bcl-2 degradation by the proteasome. To test this possibility, we treated cells with a known inhibitor of S-nitrosylation, DTT (58, 59), and its effects on Bcl-2 S-nitrosylation and ubiquitination were determined. The results show that DTT was able to prevent S-nitrosylation of Bcl-2 (Fig. 5A). Furthermore, it negated the effect of NO donors SNP and DPTA NONOate on Bcl-2 ubiquitination in Cr(VI)-treated cells (Fig. 5C). These results indicate that S-nitrosylation might be a key mechanism utilized by NO to regulate ubiquitination and degradation of Bcl-2 by the proteasome. Recent reports suggest that NO could directly regulate proteasome activity by S-nitrosylation of E3 ubiquitin ligases (parkin, Mdm2) (60, 61). To confirm that the effect observed on Bcl-2 ubiquitination is specifically due to its S-nitrosylation, we treated cells with DTT in the presence and absence of Cr(VI) and determined its effects on proteasome activity. Cells expressing ectopic Bcl-2-myc were treated with Cr(VI) and DTT, and cell lysates were immunoprecipitated and analyzed for proteasome activity by spectrofluorometry. The results show that DTT had no significant
effect on proteasomal activity as compared with Cr(VI) treatment alone (Fig. 5D), confirming that this effect is specifically due to modification and S-nitrosylation of Bcl-2.

**S-Nitrosylation of Bcl-2 under Other Stress Conditions** - To determine whether S-nitrosylation of Bcl-2 was a general phenomenon, we performed immunoprecipitation experiments evaluating the effect of other stress inducers including FasL and BSO on Bcl-2 S-nitrosylation. The results show that these test agents also induced S-nitrosylation of Bcl-2 (Figs. 6, A and B), suggesting that this process is a general phenomenon that regulates the anti-apoptotic function of Bcl-2.

**Mutations at Cys^{158} and Cys^{229} Prevents Bcl-2 S-Nitrosylation** - To confirm Bcl-2 S-nitrosylation and to determine the cysteine residue(s) that may be involved in the process, we constructed Bcl-2 mutant plasmids replacing the two cysteines in Bcl-2 with alanines. The mutant plasmids and the original Bcl-2 plasmid were individually introduced into H460 cells by transient transfection. Transfected cells were treated with Cr(VI), and cell lysates were prepared and analyzed for S-nitrosylated Bcl-2 by Western blotting using anti-S-nitrosocysteine antibody. The results show that treatment of the cells with Cr(VI) induced S-nitrosylation of Bcl-2, which was completely inhibited by one or both cysteine mutations (Fig. 7, A and B). The results also show that although both cysteines undergo S-nitrosylation in response to Cr(VI) treatment, Cys^{229} is the major site of Bcl-2 nitrosylation. To test whether S-nitrosylation of Bcl-2 could stabilize the protein and prevent its degradation, we tested the effect of mutant plasmids on Bcl-2 ubiquitination. Cells were transfected with the plasmids and treated with Cr(VI) as described. Cell lysates were immunoprecipitated and analyzed by Western blot using anti-ubiquitin antibody. The results show that cysteine mutations led to increased ubiquitination of Bcl-2 by Cr(VI) (Fig. 8A), indicating that S-nitrosylation of the protein prevents its ubiquitination and subsequent degradation. We also observed that transient transfection with the mutant plasmids increased apoptotic response to Cr(VI) exposure as compared to the wild-type plasmid (Fig. 8B). Increased apoptosis was observed in cells transfected with Cys^{229} mutant plasmid as compared to cells transfected with Cys^{158} mutant plasmid confirming that Cys^{229} is the major site of Bcl-2 nitrosylation. This observation indicates that S-nitrosylation of Bcl-2 stabilizes the protein and maintains its anti-apoptotic function.

**NO Reacts with \( \cdot O_2^- \) to Form ONOO\(^-\) that Causes S-nitrosylation of Bcl-2** - We demonstrate that NO plays an anti-apoptotic role in Cr(VI)-induced apoptosis by stabilizing Bcl-2 protein via S-nitrosylation. However, recent evidences suggest that ONOO\(^-\) is the major nitrogen species involved in S-nitrosylation, specifically in systems with increased \( \cdot O_2^- \) production. As shown in the previous aim, \( \cdot O_2^- \) mediates Cr(VI)-induced apoptosis by the down-regulation of Bcl-2 protein, we therefore tested the possibility of the involvement of ONOO\(^-\) in Bcl-2 nitrosylation in response to Cr(VI) exposure. H460 cells were treated with \( \cdot O_2^- \) modulators in addition to Cr(VI) and were assessed for NO levels by spectrofluorometry. Similarly, we treated cells with NO modulators and tested the effect on \( \cdot O_2^- \) level. We observed increased NO levels in cells treated with \( \cdot O_2^- \) inhibitor (MnTBAP) and decreased levels in cells treated with \( \cdot O_2^- \) donor (LY-83,583) (Fig. 9A). Similarly, treatment with NO donors decreased \( \cdot O_2^- \) levels whereas NO inhibitors increased \( \cdot O_2^- \) levels (Fig. 9B). This suggested a direct correlation between NO and \( \cdot O_2^- \) levels and...
indicated that NO may be scavenging ·O$_2^-$ forming ONOO$^-$ in response to Cr(VI) exposure. We also found that ONOO$^-$ donor (SIN1) had a protective effect in Cr(VI) induced apoptosis (Fig. 10, A and B). Furthermore, ·O$_2^-$ modulators affected S-nitrosylation with donors showing increased effect and inhibitors completely blocking the effect (Fig. 10 C). These results suggest that ONOO$^-$ formation in response to Cr(VI) treatment leads to S-nitrosylation of Bcl-2 that prevents apoptosis.
3.5. Discussion

Many cell-based studies have demonstrated that overexpression of Bcl-2 increases resistance to apoptotic cell death induced by various DNA-damaging agents, which is an important feature of malignant cells (7-9). Similarly, NO has been shown to be elevated in many cancer cells (44-46); however, its potential role in the regulation of Bcl-2 and the underlying mechanisms has not been demonstrated. In the present study, we show that NO, through the formation of ONOO⁻ regulates Bcl-2 protein via S-nitrosylation and that this mechanism stabilizes the protein, maintaining its anti-apoptotic function. In the previous aims, we have established that ROS, specifically ·O₂⁻, mediates Cr(VI)-induced apoptosis via the mitochondrial death pathway. Further, we showed that Bcl-2 overexpression inhibited Cr(VI)-induced apoptosis and ROS mediated its effect by down-regulation of Bcl-2. In this study, we observed that the addition of NO donors SNP and DPTA NONOate effectively inhibited this down-regulation and decreased cell death (Figs. 1 and 3). In contrast, NO inhibitors AG and PTIO showed opposite effects, indicating the anti-apoptotic role of NO and its regulation of Bcl-2 in the test system. It has been reported that apoptotic doses of Cr(VI) caused mitochondrial instability (62). The anti-apoptotic function of Bcl-2 is closely associated with its expression levels. Although Bcl-2 expression is controlled by various mechanisms, post-translational modifications, such as ubiquitination and phosphorylation, have emerged as important regulators of Bcl-2 function (7, 8). Our results show that down-regulation of Bcl-2 by Cr(VI) was associated with an increase in Bcl-2 ubiquitination and proteasomal degradation (Figs. 4 and 5). The ability of the proteasome inhibitor lactacystin to inhibit Bcl-2 down-regulation strongly supports the role of the proteasomal pathway in Bcl-2 regulation. The NO donors SNP and DPTA NONOate inhibited Bcl-2 ubiquitination by Cr(VI), whereas the NO inhibitors AG and PTIO promoted this effect (Fig. 4). These results indicate that NO regulates Bcl-2 stability by preventing its degradation via the ubiquitin-proteasomal pathway. It has been reported that Bcl-2 stability is regulated by phosphorylation at Thr 74 and Ser 87 and dephosphorylation at Ser 87 in response to proapoptotic stimuli, such as TNF-α, is the initial step of Bcl-2 degradation (10). Since NO prevented down-regulation of Bcl-2 in our system, we studied the effect of NO on Bcl-2 phosphorylation. However, we found that NO had no significant effect on Bcl-2 phosphorylation (Fig. 3), indicating that NO may regulate Bcl-2 stability through a mechanism that does not require Bcl-2 dephosphorylation. TNF-α is a death ligand that induces apoptosis via the death receptor pathway, whereas Cr(VI) induces cell death primarily via the mitochondrial pathway. Therefore, its mechanism of Bcl-2 regulation may be different from Cr(VI). Our results suggest that dephosphorylation of Bcl-2 might not be a necessary event for triggering Bcl-2 ubiquitination and that different mechanisms of Bcl-2 regulation exist and are utilized by different apoptosis-inducing agents.

The mechanism by which NO regulates Bcl-2 ubiquitination is not known but may involve S-nitrosylation of the protein, which may prevent its recognition and subsequent attachment of ubiquitin by the enzyme ubiquitin ligases. S-Nitrosylation of proteins, such as FLIP and caspases, has been reported to modulate their apoptosis activities (19, 55-57). To determine the effect of NO on Bcl-2 ubiquitination and degradation, we analyzed NO-mediated S-nitrosylation of Bcl-2 in Cr(VI)-treated cells. We found that Cr(VI) induced S-nitrosylation
of Bcl-2 and that this effect was inhibited by the NO inhibitors AG and PTIO and enhanced by the NO donors SNP and PTIO (Fig. 5). Inhibition of S-nitrosylation by the known inhibitor DTT completely inhibited the effect of NO on Bcl-2 ubiquitination (Fig. 5D), thus confirming the protective role of S-nitrosylation on Bcl-2 ubiquitination. Recent evidence suggests that NO can nitrosylate E3 ubiquitin ligase (parkin, Mdm2) (60, 61). S-Nitrosylation of this enzyme inhibits its activity and protective function. To test whether the effect of NO was due to S-nitrosylation of Bcl-2 specifically or due to nitrosylation of ubiquitin ligase, we performed a proteasome activity assay and tested the effect of DTT on the enzyme activity in the presence or absence of Cr(VI). We found that DTT had no significant effect on proteasomal activity as compared with Cr(VI) treatment alone (Fig. 5D). If the effect on Bcl-2 degradation was due to S-nitrosylation of ubiquitin ligase, treatment with DTT should have shown an increase in proteasomal activity. This result suggests that the effect of NO on Bcl-2 stability was specifically due to S-nitrosylation of Bcl-2. This result was confirmed by mutation assay in which the cysteine residues of Bcl-2 (Cys158 and Cys229) were mutated to alanines. Such mutations resulted in a complete inhibition of Bcl-2 S-nitrosylation by Cr(VI) (Fig. 7 and 8). The stronger inhibitory effect of the Cys229 mutation indicates that this cysteine is the major site of Bcl-2 S-nitrosylation. Further, cysteine mutations led to increased ubiquitination and increased apoptosis, confirming that S-nitrosylation of Bcl-2 prevents its ubiquitination and stabilizes the activity of the protein. Recent evidences suggest that under physiological conditions NO does not react with thiol (-SH) groups directly as it requires the presence of a strong electron acceptor such as Fe3+ (63). The kinetically favored reaction for NO is interaction with ·O₂⁻ which leads to the formation of ONOO⁻ (64). NO derived reactive species such as ONOO⁻ readily reacts with thiols to form nitrosothiols (RS-NO). As shown in the previous aim, we observed that ·O₂⁻ is the major ROS involved in Cr(VI)-induced apoptosis, we therefore tested the possibility of ONOO⁻ involvement in S-nitrosylation of Bcl-2. We observed a direct correlation between NO and ·O₂⁻ levels, with the increase in one leading to the decrease in the levels of the other and vice versa (Fig. 9). We observed that ·O₂⁻ donor further increased Cr(VI) induced Bcl-2 S-nitrosylation and ·O₂⁻ inhibitors reduced it to the control levels. Further, SIN1 treatment decreased Cr(VI) induced apoptosis significantly. All these results suggest that ONOO⁻ causes S-nitrosylation of Bcl-2 that leads to its stabilization (Fig. 10).

Since this study shows a novel mechanism of Bcl-2 regulation by S-nitrosylation, we further tested the generality of this process by treating the cells with other well established stress inducers, including FasL and BSO. BSO is an amino acid that depletes cells of glutathione, resulting in free radical-induced apoptosis (65). FasL is a death ligand that is known to induce apoptosis via the death receptor pathway (48, 49). We observed that Bcl-2 was S-nitrosylated in response to these stress inducers, suggesting that S-nitrosylation is a general process that can regulate Bcl-2 stability and function under various stress conditions (Fig. 6). In summary, our data provide evidence that NO plays an important role as a negative regulator of Cr(VI)-induced cell death through the mitochondrial pathway by preserving Bcl-2 stability and function. NO exerts this effect through the formation of ONOO⁻, which nitrosylates the protein and inhibit its ubiquitination and proteasomal degradation. S-Nitrosylation of Bcl-2 was observed to be a general mechanism that can regulate its stability under various stress conditions. In demonstrating S-nitrosylation of
Bcl-2, we document a novel layer of regulation that links NO signaling with Bcl-2 stability and function, which may represent an important mechanism that controls cancer development and progression. Since increased NO production and Bcl-2 expression have been associated with several human tumors, NO may be one of the key regulators of cell death resistance and cancer development through its ability to S-nitrosylate Bcl-2. This finding on the novel function of NO on Bcl-2 regulation may have important implications in carcinogenesis and its prevention.
FIGURE 1. Effect of NO modulators on Cr(VI)-induced apoptosis and caspase activation. A, subconfluent (90%) monolayers of H460 cells were pretreated with the NO donor SNP (500 µg/ml) or DPTA NONOate (400 µM) or with the NO inhibitor AG (300 µM) or PTIO (300 µM) for 1 h. The cells were then either left untreated or treated with Cr(VI) (20 µM) for 12 h and analyzed for apoptosis by Hoechst assay. B, dose effect of NO modulators on Cr(VI)-induced apoptosis. Cells were treated with Cr(VI) (20 µM) for 12 h after pretreatment with SNP (250, 500, and 750 µg/ml), DPTA NONOate (200, 400, and 600 µM), AG (100, 300, and 500 µM), or PTIO (100, 300, and 500 µM) for 1 h. C, cells were similarly treated with Cr(VI) with or without NO modulators, and caspase-9 activity was determined by fluorometric analysis. Plots are mean ± S.D. (n≥ 3). *, p < 0.05 versus Cr(VI)-treated control.
FIGURE 2. Effect of NO modulators on cellular NO levels. A and B, flow cytometric analysis of NO production in H460 cells. Cells were treated with the NO inhibitor AG (300 μM) or PTIO (300 μM) or with the NO donor SNP (500 μg/ml) or DPTA NONOate (400 μM) for 1 h, after which they were treated with Cr(VI) (20 μM) and analyzed for NO levels by flow cytometry using DAF DA as the fluorescent probe. Plots show relative DAF fluorescence intensity determined at the peak response time of 1 h after Cr(VI) treatment. C, fluorescence micrographs of treated cells from above. D, measurement of nitrite production (direct breakdown product of NO) by the Griess method. Cells were treated as mentioned above, and 50 μl of the culture medium in each plate was collected and analyzed for NO production by Griess method. Values are mean ± S.D. (n ≥ 3). *, p < 0.05 versus non-treated control; **, p < 0.05 versus Cr(VI)-treated control.
FIGURE 3. Effect of NO modulators on Bcl-2 expression and phosphorylation.  

A, H460 cells were pretreated for 1 h with one of the following agents: SNP (500 µg/ml), DPTA NONOate (400 µM), AG (300 µM), PTIO (300 µM), and lactacystin (LAC) (10 µM). The cells were then treated with Cr(VI) (20 µM) for 12 h, and cell lysates were prepared and analyzed for Bcl-2 expression by Western blots using anti-Bcl-2 antibody. B, H460 cells were treated with AG (300 µM) and PTIO (300 µM) for 12 h, and cell lysates were prepared and analyzed for Bcl-2 expression by Western blots using anti-Bcl-2 antibody. C, H460 cells were pretreated for 1 h with SNP (500 µg/ml), DPTA NONOate (400 µM), AG (300 µM) and PTIO (300 µM) and were then treated with Cr(VI) (20 µM) for 12 h, and cell lysates were prepared and analyzed for Bcl-2 phosphorylation by Western blots using phospho-specific Bcl-2 (Ser 87) antibody. Densitometry was performed to determine the relative levels of Bcl-2 and phospho-Bcl-2 after reprobing the blots with β-actin antibody. Plots are mean ± S.D. (n = 4). *, p < 0.05 versus non-treated control.
FIGURE 4. Effect of NO on Bcl-2 ubiquitination. Subconfluent monolayers of H460 cells overexpressing Bcl-2 were pretreated with the NO inhibitor AG (300 µM) or PTIO (300 µM) or with the NO donor SNP (500 µg/ml) or DPTA NONOate (400 µM) for 1 h. Subsequently, the cells were treated with Cr(VI) (20 µM) for 3 h in the presence of lactacystin (10 µM) to prevent proteasome-mediated Bcl-2 degradation. Cell lysates were immunoprecipitated with anti-myc antibody, and the immune complexes were analyzed for ubiquitin by Western blotting. Analysis of ubiquitin was performed at 3 h post-Cr(VI) treatment, where ubiquitination was found to be maximal. Data are mean ± S.D. (n = 4). *, p < 0.05 versus non-treated control. **, p < 0.05 versus Cr(VI)-treated control.
FIGURE 5. Effect of NO on S-nitrosylation and ubiquitination of Bcl-2. A, subconfluent monolayers of H460 cells overexpressing Bcl-2 were pretreated with SNP (500 µg/ml), DPTA NONOate (400 µM), AG (300 µM), PTIO (300 µM), or DTT (10 mM) for 1 h. The cells were then treated with Cr(VI) (20 µM) for 3 h, and cell lysates were prepared for immunoprecipitation using anti-myc antibody. The resulting immune complexes were analyzed for S-nitrosocysteine by Western blotting. Densitometry was performed to determine the relative S-nitrosocysteine levels after reprobing the membranes with anti-Bcl-2 antibody. B, S-nitrosylation of Bcl-2 determined by spectrofluorometry. Immunopellets from above were incubated with 200 µM HgCl₂ and 200 µM DAN. NO released from S-nitrosylated Bcl-2 was quantified at 375/450 nm. C, effect of NO modulators and DTT on Cr(VI)-induced Bcl-2 ubiquitination. Cells overexpressing Bcl-2 were treated with the indicated test agents in the presence of lactacystin (10 µM). Cell lysates were then immunoprecipitated with anti-myc antibody, and the immune complexes were analyzed for ubiquitin. D, cells overexpressing Bcl-2 were pretreated with DTT (10 mM) for 0.5 h and then treated with Cr(VI) (20 µM) for 3 h. Proteasome activity was determined spectrofluorometrically at 380/460 nm. Data are mean ± S.D. (n = 4). *, p < 0.05 versus non-treated control. **, p < 0.05 versus Cr(VI)-treated controls. #, p < 0.05 versus NO-modulated controls.
FIGURE 6. Effect of stress inducers on Bcl-2 S-nitrosylation. A, Subconfluent monolayers of H460 cells overexpressing Bcl-2 were treated with FasL (200 ng/ml), BSO (100 µM), or Cr(VI) (20 µM) for 3 h, and cell lysates were prepared for immunoprecipitation using anti-Myc antibody. The resulting immune complexes were analyzed for S-nitrosocysteine by Western blotting. Densitometry was performed to determine the relative S-nitrosocysteine levels after reprobing of the membranes with anti-Bcl-2 antibody. B, S-nitrosylation of Bcl-2 determined by spectrofluorometry. Immunopellets from above were incubated with 200 µM HgCl₂ and 200 µM DAN. NO released from S-nitrosylated Bcl-2 was quantified at 375/450 nm. Data are mean ± S.D. (n = 4). *, p < 0.05 versus non-treated control.
FIGURE 7. Effect of cysteine mutations on S-nitrosylation of Bcl-2. A, H460 cells were transiently transfected with myc-tagged original Bcl-2 plasmid (WT) or with myc-tagged C158A, C229A, or C158A/C229A mutant plasmid. 36 h later, the cells were treated with or without Cr(VI) (20 µM) for 3 h, and cell lysates were prepared for immunoprecipitation using anti-myc antibody. The immune complexes were analyzed for S-nitrosocysteine by Western blotting. Densitometry was performed to determine the relative S-nitrosocysteine levels after reprobing of the membranes with anti-Bcl-2 antibody. B, S-nitrosylation of Bcl-2 determined by spectrofluorometry. Immunopellets from above were incubated with 200 µM HgCl₂ and 200 µM DAN. NO released from S-nitrosylated Bcl-2 was quantified at 375/450 nm. Data are mean ± S.D. (n = 4). *, p < 0.05 versus non-treated control; **, p < 0.05 versus Cr(VI)-treated control.
FIGURE 8. **Effect of cysteine mutations on ubiquitination of Bcl-2.**  

**A**, H460 cells were transiently transfected with myc-tagged original Bcl-2 plasmid (WT) or with myc-tagged C158A, C229A, or C158A/C229A mutant plasmid. 36 h later, the cells were treated with or without Cr(VI) (20 µM) for 3 h, and cell lysates were prepared for immunoprecipitation using anti-myc antibody. Immunoprecipitates were prepared as described above and analyzed for ubiquitin by Western blotting. Data are mean ± S.D. (n = 4).  

**B**, H460 cells transfected with original (WT) and mutant plasmids as mentioned above were exposed to Cr(VI) (10, 20 µM) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay.
FIGURE 9. NO reacts with \( \cdot \text{O}_2^- \) to form ONOO\(^-\). A, H460 cells were treated with \( \cdot \text{O}_2^- \) donor LY-83,583 and \( \cdot \text{O}_2^- \) scavenger MnTBAP for 0.5 h, after which they were treated with Cr(VI) (20 µM) and analyzed for NO levels by spectrofluorometry using DAF-DA as the fluorescent probe. B, Cells were treated with the NO inhibitor AG (300 µM) or PTIO (300 µM) or with the NO donor SNP (500 µg/ml) or DPTA NONOate (400 µM) for 0.5 h, after which they were treated with Cr(VI) (20 µM) and analyzed for \( \cdot \text{O}_2^- \) levels by spectrofluorometry using DHE as the fluorescent probe. Plots show relative DAF and DHE fluorescence intensity determined at the peak response time of 1 h after Cr(VI) treatment. *, \( p < 0.05 \) versus non-treated control; **, \( p < 0.05 \) versus Cr(VI)-treated control.
FIGURE 10. ONOO⁻ inhibits apoptosis and causes S-nitrosylation of Bcl-2. A and B, subconfluent (90%) monolayers of H460 cells were exposed to Cr(VI) (20 μM) in the presence or absence of SIN-1 (100 μg/ml), and the cells were analyzed for apoptosis by Hoechst 33342 assay after 12 h exposure. E, Subconfluent monolayers of H460 cells overexpressing Bcl-2 were treated with LY 83,583 and MnTBAP followed by Cr(VI) (20 μM) treatment for 3h, and cell lysates were prepared for immunoprecipitation using anti-myc antibody. Immunopellets from above were incubated with 200 μM HgCl₂ and 200 μM DAN. NO released from S-nitrosylated Bcl-2 was quantified at 375/450 nm by spectrofluorometry. *, p < 0.05 versus non-treated control.
3.6. References


CHAPTER IV

Malignant Transformation of Human Lung Epithelial Cells in Response to Chronic Cr(VI) Exposure
4.1. Abstract

Exposure to Cr(VI) is associated with the incidence of lung cancer in humans. Human lung cancer manifests mostly as epithelial cell derived carcinomas; however, it is still unknown whether human lung epithelial cells are directly susceptible to Cr(VI)-induced malignant transformation. This study was designed to determine whether the non-tumorigenic human lung epithelial Beas-2B cell line could be malignantly transformed in vitro by subjecting them to long-term Cr(VI) exposure. Additionally, human lung cancer epithelial H460 cells were used and the effects in Beas-2B cells were compared to this cell-type. Beas-2B and H460 cells were continuously exposed to 5 μM Cr(VI). After 24 weeks of exposure, Cr(VI) exposed cells exhibited loss of contact inhibition and increased colony formation in soft agar as compared to the passage-matched original cell-lines. Further, an increase in proliferation, invasion and migration rates was observed in the transformed cells. Cr(VI) transformed cells showed decreased ROS production and apoptosis as well as an increase in NO levels and inhibition of Bcl-2 down-regulation. This confirmed our earlier observations that dysregulation of ROS mediated Cr(VI)-induced apoptosis by NO through the stabilization of Bcl-2 is an important molecular event in the development of apoptosis resistant phenotype in response to Cr(VI) exposure. This is the first report demonstrating Cr(VI)-induced malignant transformation of a human lung epithelial cell line and provides an important in vitro model for studying the mechanisms involved Cr(VI)-induced carcinogenesis in humans. This study strongly fortifies the potential role of Cr(VI) in lung cancer.
4.2. Introduction

Lung cancer is the leading cause of cancer mortality worldwide, with over 1,000,000 deaths annually (1). In US, 160,440 deaths were attributed to cancer of the lung and bronchus in 2003 (1, 2). Even though it is one of the major fatalities, the etiology of lung cancer remains poorly understood. Current research indicates that long-term exposure to inhaled carcinogens has the greatest impact on the risk of lung cancer. Inhalation of Cr(VI) compounds has been associated with the induction of human lung cancer for more than a century (3). In US, Machle and Gregorius reported the first epidemiologic evidence to demonstrate increased lung mortality among chromium-exposed workers (4). Subsequently, several epidemiological studies in the last few decades have confirmed that exposure to Cr(VI) in chrome plating, electroplating, leather tanneries, pigment manufacturing and stainless steel welding is associated with the induction of lung cancer in workers employed in these industries (3, 5-7). In US, an air quality survey indicated that people in several residential areas are exposed to particulate airborne chromium at concentrations exceeding 100 times the chronic toxicity benchmark (8). Therefore, in addition to occupational exposure, environmental chromium is an emerging concern and is associated with long-term carcinogenic effects of the lung. Even though several epidemiological studies have demonstrated the carcinogenic potential of Cr(VI) compounds, animal studies do not support this observation. Several groups have established in various animal models including rat, mice, guinea pigs and rabbits that exposure to Cr(VI) compounds demonstrated no significant increase in lung tumors with respect to the untreated control (9-11). The problems associated with the development of animal models to study Cr(VI)-induced carcinogenesis is poorly understood. Therefore, lack of a good model becomes a major hindrance in studying the molecular mechanisms involved in Cr(VI)-induced carcinogenesis. In this study, we developed an in vitro model for studying the molecular mechanisms underlying Cr(VI)-induced malignant transformation.

The carcinogenic potential of chromium is attributed to various factors including chemical speciation, water solubility and intracellular reductive metabolism. Chromates with low solubility are less carcinogenic as compared to soluble chromates (12). Cr(VI) does not interact with the DNA directly. It has to be converted to Cr(III) inside the cell which then intercalates with the DNA causing DNA strand breaks, DNA-protein cross-links and Cr-DNA adduct formation (13). In this process, ROS is generated that are believed to play an important role in the cytotoxicity induced by Cr(VI) (14) by causing cell cycle arrest, neoplastic transformation, and apoptosis induction (13, 15). It is well-established that dysregulated apoptosis contributes to tumor promotion, autoimmune and immunodeficiency diseases, and neurodegenerative disorders (16). Abnormal ROS generation and apoptosis regulatory mechanisms have been considered as the most important factors in neoplastic development in response to Cr(VI). Since human lung cancer manifests mostly as epithelial cell derived carcinomas (13), we used normal human lung epithelial Beas-2B cell-line and human lung cancer H460 cell-line for this study. Since it is not known whether Cr(VI) can directly transform human lung epithelial cells, we developed an in vitro model of Cr(VI)-induced lung carcinogenesis by subjecting the non-
tumorigenic human lung epithelial Beas-2B cell line to long-term Cr(VI) exposure in vitro. We also exposed human lung cancer epithelial H460 cells to Cr(VI) and compared the effects in Beas-2B cells to this cell-type. We observed that long-term exposure of Cr(VI) led to malignant transformation of the cells as exhibited by loss of contact inhibition, increase in colony formation, cell proliferation, invasion and migration. In the previous aims, we showed that ROS mediated Cr(VI)-induced apoptosis by the down-regulation of the anti-apoptotic protein Bcl-2. NO inhibited Cr(VI)-induced apoptosis by nitrosylating Bcl-2 protein that led to the stabilization and increased expression of Bcl-2 causing inhibition of apoptosis. Cr(VI) transformed cells showed decreased ROS production and apoptosis. They further demonstrated an increase in NO and inhibition of Bcl-2 down-regulation verifying our earlier observations. The development of such an in vitro model allows detailed examination of the molecular changes underlying Cr(VI)-induced carcinogenesis in a system that is of clear relevance to human exposure and validates human lung epithelial cells as a direct target for Cr(VI)-induced carcinogenesis.
4.3. Materials and Methods

**Chemicals and Reagents**
Sodium dichromate (Na$_2$Cr$_2$O$_7$·2H$_2$O) [Cr(VI)], 6-Anilinoquinoline-5,8-quinone (LY-83,583), Hydrogen peroxide (H$_2$O$_2$), NO donor sodium nitroprusside (SNP), NO inhibitors aminoguanidine (AG) and 2-(4-carboxyphenyl)-4,4,5,5-tetramethyl-imidazoline-1-oxy-3-oxide (PTIO) were obtained from Sigma Chemical Inc. (St. Louis, MO). Mn(III)tetrakis (4-benzoic acid) porphyrin (MnTBAP) was purchased from Calbiochem (La Jolla, CA), and catalase was from Boehringer Mannheim (Indianapolis, IN). NO donor dipropyltriamine (DPTA) NONOate was from Alexis Biochemicals (San Diego, CA). The oxidative probes, dichlorofluorescein diacetate (DCF-DA), dihydroethidium bromide (DHE), diaminofluorescien diacetate (DAF-DA) and the apoptosis dye Hoechst 33342 were from Molecular Probes (Eugene, OR). Antibodies for Bcl-2 and peroxidase-labeled secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). β-actin antibody was from Sigma (St. Louis, MO).

**Cell Culture**
The normal bronchial epithelial Beas-2B cell line and human lung cancer epithelial H460 cell line were obtained from ATCC (Rockville, MD). Beas-2B cells were cultured in DMEM media (Sigma, St. Louis, MO) containing 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin and H460 cells were cultured in RPMI 1640 medium (Sigma, St. Louis, MO) containing 5% fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin in a 5% CO$_2$ environment at 37ºC. Cells were passaged at preconfluent densities using a solution containing 0.05% trypsin and 0.5 mM EDTA (Invitrogen).

**Derivation of transformed cells**
Beas-2B and H460 cells were exposed continuously to 5 µM Cr(VI) and incubated in a 5% CO$_2$ environment at 37ºC. Cells were passaged weekly at preconfluent densities using a solution containing 0.05% trypsin and 0.5 mM EDTA (Invitrogen). Henceforth, Cr(VI) exposed cells are designated as B-Cr and H-Cr cells to distinguish them from the parental Beas-2B and H460 cells, respectively. Parallel cultures grown in Cr(VI) free medium provided passage-matched controls. After 24 weeks of exposure, phenotypic changes were observed in both B-Cr and H-Cr cells. The transformed cells were then grown in normal media and their tumorigenic potential was assessed by various experiments, described below.

**Cyquant cell proliferation assay**
Beas-2B, B-Cr, H460 and H-Cr cells were plated in a 96-well plate at a density of 1 × 10$^4$ in growth media and were incubated for 24 h. The growth media was changed with 100 µl of 1X Cyquant dye binding solution (Invitrogen) and were incubated for 30-60 min at 37ºC. The fluorescence intensity of each sample was measured at 485/535 nm.

**Cell invasion and migration**
*In vitro* invasion and migration was determined by a modified Boyden chamber assay using cell culture inserts with a polycarbonate-filter coated with Matrigel (BD Biosciences, NJ)
for invasion or control inserts for migration in a 24-well format. Briefly, $3 \times 10^4$ cells (invasion) or $1.5 \times 10^5$ cells (migration) in 500 μl of serum-free media were added to the inserts. The lower chambers were filled with 5% FBS in media. Chambers were incubated at 37°C in a 5% CO$_2$ atmosphere for 24 h for migration and 48 h for invasion. The non-invading or non-migrating cells were removed from the insides of the inserts by cotton swabs. The cells that invaded the matrigel or migrated to the underside of the coated membrane were fixed and stained with Diff-Quik fixative and stain. The cell nuclei stain purple and the cytoplasm stains pink. The inserts were directly examined under a light microscope and the number of cells migrated or invaded was counted.

**Soft agar colony assay**

Soft agar assays were performed as described previously with minor modifications (17). Beas-2B, B-Cr, H460 and H-Cr cells ($3 \times 10^4$ cells for each cell-type) were mixed with tissue culture media containing 0.5 % agar to result in a final agar concentration of 0.33 %. Cell suspension (1.5 ml) was immediately plated in 60 mm dishes coated with 7 ml/plate of 0.5 % agar in tissue culture media. After 2 weeks, the number of colonies per $3 \times 10^4$ cells were counted under a light microscope.

**Apoptosis Assay**

After specific treatments, apoptosis was determined by incubating the cells with 10 μg/ml Hoechst 33342 nuclear stain for 30 min at 37°C and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by fluorescence microscopy (Axiovert 100; Carl Zeiss) using Pixera software.

**ROS and NO Detection**

Intracellular peroxide, $\text{O}_2^-$ and NO production were determined by spectrophotometry using DCF-DA, DHE or DAF-DA fluorescence probes, respectively. Cells (1x10$^6$/ml) were incubated with the fluorescent probes (10 μM) for 30 min at 37°C, after which they were washed with PBS, and analyzed for DCF (494/519 nm), DHE (535/617 nm) or DAF (488/ 538nm) fluorescence using FLUOstar OPTIMA (BMG Inc., Durham, NC).

**Western Blotting**

After specific treatments, cells were incubated in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, 10% glycerol, 1 mM Na$_3$VO$_4$, 50 mM NaF, 100 mM PMSF, and a commercial protease inhibitor mixture (Roche) for 20 min on ice. After insoluble debris was precipitated by centrifugation at 14,000 x g for 15 min at 4°C, the supernatants were collected and assayed for protein content using BCA method (Pierce Biotechnology, Rockford, IL). Equal amount of proteins per sample (15 μg) were resolved on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto 0.45-μm nitrocellulose membrane (Pierce). The transferred membranes were blocked for 1 h in 5% non-fat dry milk in TBST (25 mM Tris-HCl pH 7.4, 125 mM NaCl, 0.05% Tween-20) and incubated with the appropriate primary antibodies and horseradish peroxidase-conjugated isotype-specific secondary antibodies. The immune complexes were detected by chemiluminescence (Supersignal™ West Pico, Pierce).
4.4. Results

Cr(VI)-Induced Malignant Transformation of Normal Lung Epithelial Cells – Normal human bronchial epithelial cells Beas-2B were continuously exposed to 5 µM Cr(VI) and were passaged weekly. After 24 weeks of exposure, phenotypic differences were observed between the Cr(VI)-treated and passage-matched control cells. The treated cells, designated as B-Cr, were subsequently cultured in Cr(VI)-free medium and soon began to form cell mounds, even when subconfluent, whereas mounding was not observed in passage-matched control cells (Fig. 1). This mounding indicated loss of contact inhibition and was the first indication of the transformation of Beas-2B cells. This was also observed in the cancer cell line used. As expected, cell mounding was observed in passage-matched control lung cancer epithelial H460 cells; however, treatment with Cr(VI) led to increased malignant transformation of the H460 cells. Cr(VI) transformed cells were stained with Hoechst stain to elucidate the colony formation clearly (Fig. 1). As seen in the figure normal Beas-2B cells undergo malignant transformation leading to colony formation and loss of contact inhibition. Loss of contact inhibition is a characteristic in vitro feature of tumor progression (18).

Confirmation of Cr(VI)-Induced Malignant Transformation – Soft agar colony formation assay was performed to confirm Cr(VI)-induced malignant transformation (Fig. 2). Beas-2B, B-Cr, H460 and H-Cr cells were subjected to colony formation by growing them on agar plates as described in Materials and Methods to assess anchorage independent growth. After 2 weeks, B-Cr cells showed a 4 fold increase in colony formation as compared to the Beas-2B cells and H-Cr cells showed a 2 fold increase in colony formation as compared to the H460 cells (Fig. 2, A and B). These experiments demonstrated significant colony formation in Cr(VI) transformed cells, although very small, slowly growing colonies were also formed by normal human lung epithelial Beas-2B cells. It was also observed that the number of colonies formed by B-Cr cells were approximately equal to the number of colonies formed by the passage controlled H460 cells, indicating the carcinogenic potential of normal human lung epithelial cells, as H460 is a cancer cell-line.

Effect of Cr(VI)-Induced Malignant Transformation on Cellular Proliferation – We further examined the proliferation of control and Cr(VI)-transformed lung epithelial cells in a complete medium. The transformed B-Cr cells proliferated approximately twice as fast as the control cells (Fig. 3), in keeping with their malignant behavior. Although H460 cells are lung cancer epithelial cells, exposure to Cr(VI) transformed this cell-line, further making it more malignant. H-Cr cells proliferated slightly faster than H460 cells. Overall, the transformed cells showed rapid growth as compared to the original cell-lines.

Effect of Cr(VI)-Induced Malignant Transformation on Cellular Invasion and Migration – Cr(VI) exposed cells were assessed in vitro for the potential to migrate through and invade matrigel. The invasive and migratory properties of the transformed cells were compared to the original cell-lines. B-Cr cells demonstrated approximately 4.5 fold increase in invasion and nearly 4 fold increase in the migration rate as compared to passage-matched Beas-2B cells (Fig. 4A). H-Cr cells demonstrated more than 5 fold increase in invasion and 4.5 fold increase in the migration rate as compared to passage-matched H460 cells (Fig. 4A).
result indicated the aggressive and malignant nature of the Cr(VI) transformed cells.

**Apoptosis Levels in Cr(VI) Transformed Cells** – Dysregulation of apoptosis regulatory mechanisms in response to Cr(VI) exposure has been implicated in Cr(VI)-induced malignant transformation (19). Therefore, to study the mechanism involved in Cr(VI) induced malignant transformation, we characterized the apoptotic response in transformed cells as compared to normal cells. Cells were treated with different doses of Cr(VI) (0-100 µM), and apoptosis was determined after 12 h by Hoechst 33342 assay. Figs. 5, A and B, show that Cr(VI) treatment caused a dose-dependent increase in cell apoptosis in Beas-2B and H460 cells and, comparatively, B-Cr and H-Cr cells demonstrated reduced apoptosis. This indicated that continuous exposure of Cr(VI) modifies cellular system such that the damaged cells escape apoptosis and undergo malignant transformation.

**Cellular ROS Levels in Cr(VI) Transformed Cells** – As demonstrated in aim II, Cr(VI) induced ROS production is involved in apoptosis induction. Since we observed a decrease in apoptosis in Cr(VI) transformed cells, we quantified the ROS levels in these transformed cells. When we treated B-Cr and H-Cr cells with Cr(VI), we observed that hydroperoxide and ·O₂‾ production was significantly reduced as compared to Beas-2B and H460 cells in a dose-dependent manner, as indicated by the decrease in DCF and DHE fluorescence intensity (Figs. 6, A-D). This observation was verified by treating cells with various ROS modulators along with Cr(VI). ROS modulators showed significantly decreased ROS levels in Cr(VI) transformed cells as compared to the passage-matched control cell-lines (Fig. 7, A-D). This suggests that cells acquire apoptosis resistant phenotype by decreasing ROS production that leads to decreased apoptosis in response to Cr(VI).

**Cellular NO Levels in Cr(VI) Transformed Cells** – We demonstrated in aim III that NO inhibits Cr(VI)-induced apoptosis by nitrosylating and stabilizing Bcl-2 protein. Therefore, NO regulates cellular events such that cells become resistant to apoptosis. NO mediated regulation of Cr(VI)-induced apoptosis may be a key mechanism by which cells acquire apoptosis resistant phenotype. To test this possibility we quantified the NO levels in Cr(VI) transformed cells as compared to the passage-matched control cells. It was observed that NO production induced in response to Cr(VI) in Beas-2B and H460 cells was further increased significantly in B-Cr and H-Cr cells in a dose-dependent manner, as indicated by the increase in DAF fluorescence intensity (Figs. 8, A and B). Further, NO modulation studies confirmed this result (Fig. 8, C and D). This indicated that malignant transformation involves increased NO production and thus confirmed our earlier study that NO exerts an anti-apoptotic role in response to Cr(VI).

**Bcl-2 Expression Levels in Cr(VI) Transformed Cells** – The oncogenic potential of Bcl-2 is well-established with its overexpression reported in various cancers including lung cancer (20-24). In our earlier aims, we found that Bcl-2 is the key anti-apoptotic protein involved in inhibiting Cr(VI)-induced apoptosis. Having demonstrated the important role of Bcl-2 in Cr(VI)-induced apoptosis, we examined the potential modification of Bcl-2 expression in Cr(VI) transformed cells. To provide a mechanistic insight into the dysregulation of Cr(VI)-induced apoptosis, the expression level of Bcl-2 in response to Cr(VI) treatment was determined in B-Cr and H-Cr cells as compared to Beas-2B and H460 cells by
Western blot analysis. Figs. 9, A and B show that Bcl-2 is down-regulated in Beas-2B and H460 cells, however this effect was reversed and Bcl-2 was stabilized in B-Cr and H-Cr cells. This suggested that persistent and long-term exposure to Cr(VI) leads to the stabilization and overexpression of the anti-apoptotic protein Bcl-2 that causes inhibition of apoptosis and malignant transformation of the cells.
4.5. Discussion

Cr(VI) is a known human carcinogen that induces lung cancer (3, 5-7). Several epidemiological studies have demonstrated the carcinogenic potential of Cr(VI) compounds. However, the mechanisms involved in Cr(VI) induced carcinogenesis are poorly understood. One of the major hindrances is the lack of an appropriate model for studying Cr(VI) induced malignant transformation because exposure to Cr(VI) does not induce lung tumor formation in animals (9-11). Since there is no direct evidence demonstrating the carcinogenic potential of Cr(VI), we developed an in vitro model of Cr(VI)-induced lung carcinogenesis by subjecting the non-tumorigenic human lung epithelial Beas-2B cell line to long-term Cr(VI) exposure in vitro. In addition, we also exposed human lung cancer epithelial H460 cells to Cr(VI) further transforming these cells and compared the effects in Beas-2B cells to the control and transformed H460 cells to validate our observations. We observed that long-term exposure of these cells to Cr(VI) led to malignant transformation of the cells as observed by the loss of contact inhibition in vitro of the non-tumorigenic human lung epithelial Beas-2B cell-line (Fig. 1). This is an exclusive property of cancer cells and demonstrates the malignant transformation of normal cells in response to Cr(VI) exposure. B-Cr and H-Cr cells demonstrated anchorage-independent growth and increased colony formation in soft agar as compared to the passage-matched original cell-line confirming the malignant potential of Cr(VI) transformed cell-lines (Fig. 2). The number of colonies formed by B-Cr was comparable to the number of colonies formed by H460 cells confirming the carcinogenic potential of Cr(VI) transformed Beas-2B cells. Although Beas-2B cells are normal human bronchial epithelium obtained from autopsy of non-cancerous individuals, they also developed significantly less number of slow growing colonies. Beas-2B cells are widely reported to possess mutated and non-functional p53 gene (tumor suppressor gene) (25, 26) and ATCC product datasheet indicates that Beas-2B cell-line forms colonies in semisolid medium but were non-tumorigenic in immunosuppressed mice. Therefore, the colony formation observed in Beas-2B cells is due to inherent cell properties and not due to its tumorigenic potential. Cr(VI) transformed cells demonstrated an increase in the proliferation, invasion and migration rates as compared to the passage-matched control cells demonstrating their aggressive and malignant behavior (Fig. 3 and 4).

We further characterized the mechanism involved in the malignant transformation of normal lung epithelial cells in response to Cr(VI) exposure. We have earlier shown that Cr(VI) induces apoptosis through the mitochondrial pathway and Bcl-2 plays an important role in this system. ROS mediates Cr(VI)-induced apoptosis by the down-regulation of the anti-apoptotic Bcl-2 protein and NO inhibited this effect by S-nitrosylating the protein that led to the stabilization and increased expression of Bcl-2 causing inhibition of apoptosis. Therefore, we tested the possibility of the involvement of this mechanism in Cr(VI) induced malignant transformation. We observed that the transformed cells showed decreased ROS production and increased NO production (Fig. 6, 7 and 8). Moreover, it was observed that apoptosis was inhibited and Bcl-2 was up-regulated in the Cr(VI) transformed cells (Fig. 5 and 9). These observations in the Cr(VI) transformed cells confirmed our earlier studies and revealed one of the important mechanisms responsible for Cr(VI)-induced malignant transformation. This study is important because it is the first, to
our knowledge, description of Cr(VI)-induced malignant transformation of a human lung epithelial cell-line analogous to a potential in vivo target cell population relevant to Cr(VI)-induced carcinogenesis in humans. This study is also biologically significant, as there is established epidemiologic evidence that human lung is a potential target of Cr(VI) induced-carcinogenicity. More importantly, it provides a model in which the molecular and genetic events associated with Cr(VI)-induced carcinogenesis in humans can be studied. Since Cr(VI) is a paradigm of carcinogenic transition metals, the inferences from this study may be broadly applied for studying molecular mechanisms associated with general metal carcinogenesis.
FIGURE 1. Cr(VI)-induced malignant transformation of normal human lung epithelial cells. Normal human bronchial epithelial Beas-2B cells were exposed to 5 µM Cr(VI) for 24 weeks. A, Passage-matched control Beas-2B and B-Cr cells stained with Hoechst 33342 dye (top). Passage-matched control Beas-2B and B-Cr cells under light microscope (bottom). B, Fluorescence micrographs of B-Cr cells after 24 weeks of Cr(VI) exposure.
FIGURE 2. Cr(VI)-induced malignant transformation assessed by Soft Agar colony formation. A, Beas-2B, B-Cr, H460 and H-Cr cells (3 x 10^4 cells) were seeded on 0.5 % agar plates and were incubated at 37°C in a 5% CO₂ incubator. After 2 weeks, colonies were scored under a light microscope. B, Representative micrographs of colonies formed by Beas-2B, B-Cr, H460 and H-Cr cells in soft agar. Values are mean ± S.D. (n ≥ 3). *, p < 0.05 versus the respective passage-matched control cell-line.
FIGURE 3. Effect of Cr(VI)-induced malignant transformation on cellular proliferation. Beas-2B, B-Cr, H460 and H-Cr cells were plated in a 96-well plate at a density of $1 \times 10^4$ in growth media and were incubated for 24 h. The growth media was changed with 100 µl of 1X Cyquant dye binding solution and were incubated for 30-60 min at 37°C. The fluorescence intensity of each sample was measured at 485/535 nm. Values are mean ± S.D. (n > 3). *, $p < 0.05$ versus the respective passage-matched control cell-line.
FIGURE 4. Effect of Cr(VI)-induced malignant transformation on cellular invasion and migration. A and B, Beas-2B, B-Cr, H460 and H-Cr cells at a density of $3 \times 10^4$ or $1.5 \times 10^4$ cells/ml were added to inserts coated with matrigel (invasion) or control inserts (migration), respectively. The inserts were incubated for 48 h for invasion assay and 24 h for migration assay. Cells were counted under fluorescence microscope after staining and the average number of cells was scored in each case. Values are mean ± S.D. ($n \geq 3$). *, $p < 0.05$ versus the respective passage-matched control cells. C, Representative micrographs of cells stained for invasion.
FIGURE 5. Apoptosis in Cr(VI) transformed cells. A and B, subconfluent (90%) monolayers of Beas-2B, B-Cr, H460 and H-Cr cells were exposed to varying doses of Cr(VI) (0-100 µM) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. Plots are mean ± S.D. (n = 4). *, p < 0.05 versus non-treated control.
FIGURE 6. Cellular ROS levels in Cr(VI) transformed cells. A, B, C and D - spectrophotometric measurements of DHE and DCF fluorescence intensities in Beas-2B, B-Cr, H460 and H-Cr cells. Cells were treated with Cr(VI) (0-100 µM) for 1 h. Plots show relative fluorescence intensity over non-treated control. Values are mean ± S.D. (n ≥ 3). E, F, G and H, Beas-2B, B-Cr, H460 and H-Cr cells were either left untreated or pretreated with MnTBAP (100 µM), catalase (10000 U/ml), LY-83,583 (10 µM) or H$_2$O$_2$ (0.1 mM) for 0.5 h, followed by Cr(VI) treatment (20 µM) for 1 h. Plots show relative fluorescence intensity over non-treated control. Values are mean ± S.D. (n ≥ 3).
FIGURE 7. Effect of ROS modulators on Cr(VI) transformed cells. A, B, C and D - spectrophotometric measurements of DHE and DCF fluorescence intensities in Beas-2B, B-Cr, H460 and H-Cr cells. Cells were treated with Cr(VI) (0-100 µM) for 1 h. Plots show relative fluorescence intensity over non-treated control. Values are mean ± S.D. (n ≥ 3). E, F, G and H, Beas-2B, B-Cr, H460 and H-Cr cells were either left untreated or pretreated with MnTBAP (100 µM), catalase (10000 U/ml), LY-83,583 (10 µM) or H2O2 (0.1 mM) for 0.5 h, followed by Cr(VI) treatment (20 µM) for 1 h. Plots show relative fluorescence intensity over non-treated control. Values are mean ± S.D. (n ≥ 3).
FIGURE 8. **Cellular NO levels in Cr(VI) transformed cells.** A and B - spectrophotometric measurement of DAF fluorescence in Beas-2B, B-Cr, H460 and H-Cr cells. Cells were treated with Cr(VI) (0-100 µM) for 1 h. Plots show relative fluorescence intensity over non-treated control. C and D, Beas-2B, B-Cr, H460 and H-Cr cells were pretreated for 0.5 h with SNP (500 µg/ml), DPTA NONOate (400 µM), AG (300 µM), and PTIO (300 µM). The cells were then treated with Cr(VI) (20 µM) for 1 h and were analyzed for DAF fluorescence. Plots show relative fluorescence intensity over non-treated control. Values are mean ± S.D. (n ≥ 3).
FIGURE 9. **Bcl-2 expression in Cr(VI) transformed cells.** A and B, Beas-2B, B-Cr, H460 and H-Cr cells were treated with Cr(VI) for 12 h, and cell lysates were prepared and analyzed for Bcl-2 by Western blotting.
4.6. References


OVERALL CONCLUSIONS

- Both extrinsic and intrinsic pathways of apoptosis are induced in human lung epithelial cells in response to Cr(VI) exposure, with the mitochondrial pathway being more dominant.

- Overexpression of the mitochondrial anti-apoptotic protein Bcl-2 significantly inhibits Cr(VI)-induced apoptosis.

- ROS, specifically ·O₂⁻, is important in mediating apoptosis in response to Cr(VI) exposure.

- Mitochondrion is the main source of ROS production during Cr(VI)-induced apoptosis.

- ·O₂⁻ induces apoptotic cell death, at least in part, by inducing proteasomal degradation of Bcl-2 protein in response to Cr(VI) exposure.

- NO plays an anti-apoptotic role in Cr(VI)-induced apoptosis.

- NO mediates its effect, at least in part, by the inhibition of proteasome-mediated degradation of Bcl-2.

- The mechanism by which NO regulates Bcl-2 involves S-nitrosylation of the protein, which was also observed under other stress conditions, including exposure to death ligand and glutathione depletion.

- NO mediated ONOO⁻ is the major reactive nitrogen species involved in S-nitrosylation of Bcl-2 that prevents its downregulation via the ubiquitin-proteasome pathway.

- Both Cys¹⁵⁸ and Cys²²⁹ are involved in S-nitrosylation and ubiquitination of Bcl-2, with Cys²²⁹ being the major site for nitrosylation.

- Chronic Cr(VI) exposure can induce malignant transformation of normal human lung epithelial cells.

- The in vitro model of Cr(VI)-induced malignant transformation verified that the molecular mechanisms recognized in our study are important in Cr(VI)-induced carcinogenesis.

- Cr(VI)-induced malignant transformation of human lung epithelial cells is of clear relevance to human exposure and validates human lung epithelial cells as a direct target for Cr(VI)-induced carcinogenesis.
OBJECTIVE

Continue research for the discovery and development of novel therapeutic targets utilizing my expertise in pharmaceutics and molecular biology.

EDUCATION

• PhD, Basic Pharmaceutical Sciences, May 2007
  Department of Basic Pharmaceutical Sciences, West Virginia University, WV, USA.

• BS, Pharmacy, Sep 2001
  K. M. Kundnani College of Pharmacy, University of Mumbai – Mumbai, India.

MERITS & HONORS

Research Awards:
- 1st place - Sigma Xi Research Award in the basic science category (2006).
- 1st place – WVU Health Science Center ‘Van Liere’ Research Award in the basic science category (2006).
- 1st place - Department of Basic Pharmaceutical Sciences annual Research Award (WVU) (2006).
- 2nd Place - WVU Health Science Center ‘Van Liere’ Research Award (2005).

Teaching Award:
- Best Teaching Assistant Award - Department of Pharmaceutical and Pharmacological Sciences (WVU) (2004).

SKILL SET

- Cell culture of various human cell lines.
- Assays for cell proliferation, migration, invasion, viability and death.
- Western blotting, Immunoprecipitation, Fluorescence microscopy.
- DNA and siRNA transfection, preparation of stable cell-lines.
- DNA cloning using PCR and Site-directed mutagenesis.
- Flow cytometry, Spectrophotometry, Spectrofluorometry.
- ELISA, Soft agar assay, collagen deposition assay.
- DNA fragmentation assays, Southern Blotting and RT-PCR.
- Protein expression and characterization.
PUBLICATIONS

SCIENTIFIC PAPERS:


• Neelam Azad, Rojanasakul Y., ROS Mediated Proteasomal Degradation of Bcl-2 Determines Lung Cell Susceptibility to Cr(VI)-Induced Apoptosis (under preparation).

• Neelam Azad, Rojanasakul Y., Molecular Mechanisms Involved in Malignant Transformation of Normal Human Lung Epithelial Cells in Response to Cr(VI) Exposure (under preparation).

SCIENTIFIC REVIEWS:


BOOK CHAPTERS:


WORK EXPERIENCE

• Jan 2004 - present – Doctoral Dissertation Research, Department of Basic Pharmaceutical Sciences, West Virginia University.
**Mentor – Dr. Yon Rojanasakul**

- Identification of key molecular targets involved in lung cancer induced by environmental and occupational carcinogens such as hexavalent chromium [Cr(VI)] compounds.
- Findings will recognize novel therapeutic targets and important biological mediators such as nitric oxide and reactive oxygen species (ROS) in lung carcinogenesis induced by Cr(VI) and other related compounds.

*Mentored* five graduate students and two summer interns during the period of my dissertation at WVU. Currently, one graduate student is an assistant professor and the other three are pursuing their respective graduate careers.

- **Aug 2002 - Aug 2004 – Teaching Assistant,** Department of Basic Pharmaceutical Sciences, West Virginia University.
  - Taught and managed pharmacy and microbiology labs, graded homework, proctored exams, conducted and supervised experiments for PCL-1 (Pharmacy) and PCL-2 (Pharmacy/microbiology) labs.

- **Aug 2002 - Dec 2003 – Graduate Study Laboratory Rotations,** Department of Basic Pharmaceutical Sciences, West Virginia University.
  - Synthesized and characterized CYP2D6.N, a novel isoform of CYP2D6 sub-family of cytochrome P450s (major drug metabolizing enzymes).

  - Worked in production, quality control, marketing, research and development departments.
  - Worked on tablet coating and quality control of a syrup formulation.

**ORAL & POSTER PRESENTATIONS**

- **Neelam Azad.** S-Nitrosylation of Bcl-2 - A Novel Anti-Apoptotic Mechanism. AAPS, 2006 (*Podium*).
PROFESSIONAL AFFILIATIONS

- Student member of the American Association of Pharmaceutical Scientists (AAPS) since 2004.
- Honorary member of Sigma Xi Research Society since 2006.