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Issue: *Oxidative/Nitrosative Stress and Disease***Role of oxidative/nitrosative stress-mediated Bcl-2 regulation in apoptosis and malignant transformation**Neelam Azad,¹ Anand Iyer,¹ Val Vallyathan,² Liying Wang,² Vincent Castranova,² Christian Stehlik,³ and Yon Rojanasakul⁴¹Department of Pharmaceutical Sciences, School of Pharmacy, Hampton University, Hampton, Virginia. ²Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health, Morgantown, West Virginia. ³Department of Medicine, Northwestern University, Chicago, Illinois. ⁴Department of Pharmaceutical and Pharmacological Sciences, School of Pharmacy, West Virginia University, Morgantown, West Virginia

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Bcl-2 is a key apoptosis regulatory protein of the mitochondrial death pathway. The oncogenic potential of Bcl-2 is well established, with its overexpression reported in various cancers. The antiapoptotic function of Bcl-2 is closely associated with its expression levels. Reactive oxygen and nitrogen species (ROS/RNS) are important intracellular signaling molecules that play a key role in various physiological processes including apoptosis. We have recently reported that ROS and RNS can regulate Bcl-2 expression levels, thereby impacting its function. Superoxide anion ($\cdot\text{O}_2^-$) plays a proapoptotic role by causing downregulation and degradation of Bcl-2 protein through the ubiquitin-proteasomal pathway. In contrast, nitric oxide (NO)-mediated S-nitrosylation of Bcl-2 prevents its ubiquitination and subsequent proteasomal degradation, leading to inhibition of apoptosis. Interestingly, NO-mediated S-nitrosylation and stabilization of Bcl-2 protein was the primary mechanism involved in the malignant transformation of nontumorigenic lung epithelial cells in response to long-term carcinogen exposure. We describe a novel mechanism of Bcl-2 regulation by $\cdot\text{O}_2^-$ and NO, providing a new dimension to reactive species-mediated Bcl-2 stability, apoptotic cell death, and cancer development.

Keywords: Bcl-2; nitric oxide; superoxide; apoptosis; malignant transformation

Introduction

Lung cancer is one of the major causes of mortality worldwide, with over one million deaths reported annually.¹ Dysregulation of apoptosis or programmed cell death is one of the major mechanisms implicated in the neoplastic evolution of abnormal cells. Apoptosis is a tightly regulated process characterized by shrinkage of the nucleus, blebbing of membranes, condensation and fragmentation of chromatin. The caspase family of proteins is the central regulator of the two major apoptosis signaling pathways, viz., the extrinsic or death receptor pathway and the intrinsic or the mitochondrial pathway. Caspase-8 and caspase-9 are the key initiator caspases of the extrinsic and the intrinsic pathway, respectively, which cleave and activate downstream effector caspases such as caspase-3, leading to apop-

totic cell death.² Both intrinsic and extrinsic pathways of apoptosis are regulated at different levels by various proteins. The proto-oncogene Bcl-2 is one of the major regulators of the mitochondrial apoptotic pathway.³

Apoptosis is also regulated by various reactive oxygen and nitrogen species (ROS/RNS). ROS such as hydrogen peroxide (H_2O_2), superoxide anion ($\cdot\text{O}_2^-$), and hydroxyl radical ($\cdot\text{OH}$) are byproducts of normal oxygen metabolism, which participate in normal cellular functions and act as intracellular signaling molecules in a number of biological processes.⁴ However, excessive ROS production may lead to DNA damage and abnormal activation of certain cell growth regulators, thereby leading to carcinogenesis. Furthermore, subtle changes in the rate of production of RNS such as nitric oxide (NO) may critically impact cellular homeostasis,

consequently initiating a variety of cellular signaling processes including apoptosis.⁵ Nitric oxide (NO) is an important signaling molecule produced endogenously from L-arginine in a reaction catalyzed by NO synthases. NO has been demonstrated to have both pro- and antiapoptotic roles, depending on a variety of factors including the type of cells involved, the redox state of the cell, and the flux and dose of NO.⁶

Although cells are constantly subjected to ROS/RNS generated from endogenous sources, such production is routinely triggered by several exogenous sources as well, including exposure to heavy metals that are fairly ubiquitous in the environment. For instance, exposure to hexavalent chromium [Cr(VI)] compounds leads to increased production of various reactive species. Cr(VI) compounds have been classified as group I human carcinogens by the International Agency of Research in Cancer in 1990.⁷ For more than a century, exposure to Cr(VI), be it occupational (e.g., chrome plating) or nonoccupational (e.g., cigarette smoke), has been associated with the induction of lung cancer.⁸ Upon inhalation, chromate particles dissolve to form soluble Cr(VI) anions that enter cells through nonspecific anionic transporters, where they are metabolically reduced to their lower oxidation states.⁹ During the one-electron reduction of Cr(VI), in addition to the reduced intermediates of Cr(VI), a whole spectrum of ROS is generated that cause diverse cytotoxic and genotoxic effects.¹⁰

ROS/RNS produced during different cellular reactions may either be beneficial or harmful to the cells, thereby acting as “double-edged swords” in cellular reactions. ROS and RNS induce damage to macromolecules such as DNA, lipids, and proteins through their ability to induce biochemical alterations.¹¹ However, these species are also important in many physiological functions. It is well recognized that a balance between oxygen use, reactive species formation, and antioxidant activity is essential for normal physiological functions. In this paper, we will focus on the physiological role of ROS/RNS in regulating the pro-survival protein Bcl-2 and the consequent impact on apoptosis as well as the pathological manifestations of dysregulation of ROS/RNS-mediated Bcl-2 expression that leads to malignant transformation of cells.

B-cell lymphoma-2

The antiapoptotic protein Bcl-2 resides in the outer mitochondrial wall and regulates apoptosis by controlling mitochondrial permeability. The oncogenic potential of Bcl-2 protein is well characterized, with its overexpression reported in nearly 70% of breast cancer cases, 30–60% of prostate cancer cases, and 90% of colorectal cancers.¹² In addition, several studies demonstrate that expression levels of Bcl-2 are amplified in many apoptosis-resistant lung cell lines and tumor specimens.^{13–15}

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.^{16,17} The antiapoptotic function of Bcl-2 is dictated by its expression levels, which may be regulated by various mechanisms including dimerization, phosphorylation, posttranslational modification, transcription, and degradation. Bcl-2 degradation is mediated primarily via the ubiquitin-proteasomal pathway, which is a major system for selective protein degradation in eukaryotic cells.¹⁸ Modification of ϵ -NH₂ groups of lysine residues in the substrate protein is the initial step that targets it for degradation by the proteasome complex.¹⁸ Various factors including structural stereospecificity, phosphorylation status, or conservation of specific structural motifs are implicated in the proteasomal degradation of susceptible proteins.¹⁹ However, the physiological signals that lead to protein recognition by ubiquitin and subsequent degradation via the proteasomal pathway are unclear. This is true particularly for Bcl-2, where the underlying mechanism involved in its degradation is not well understood.

Regulation of Bcl-2 by ROS

As mentioned previously, the redox state of a cell plays a major role in its response to any external stimuli, leading to either induction or inhibition of apoptosis. ROS can mediate apoptosis by regulating the expression of various apoptosis regulatory proteins.^{20,21} The stability and expression levels of Bcl-2 protein can be regulated by different ROS through various mechanisms. Posttranslational modifications such as ubiquitination and phosphorylation have emerged as some of the

most important regulatory mechanisms of Bcl-2 function.

Accumulating evidence indicates that Bcl-2 phosphorylation induces a conformational change that directly impacts Bcl-2 stability and apoptotic function.²² Phosphorylation of Bcl-2 at Thr⁷⁴ and Ser⁸⁷ in response to proapoptotic stimuli such as tumor necrosis factor- α (TNF- α) has been shown to regulate its stability, with dephosphorylation at Ser⁸⁷ serving as the initial step in Bcl-2 degradation. H₂O₂ was considered to be the primary ROS involved in Bcl-2 phosphorylation and proteasomal degradation.²³ However, our study indicated that $\cdot\text{O}_2^-$ was the major ROS involved in the downregulation and degradation of Bcl-2 by inducing its ubiquitination (Fig. 1), whereas H₂O₂ had a minimal role.²⁴ A possible explanation for this discrepancy is that we used Cr(VI) as the inducing agent. Cr(VI)-induced apoptosis is mainly mediated through the mitochondrion-dependent caspase-9 activation whereas TNF- α is a death ligand that induces apoptosis via the death receptor pathway. It is plausible that the role of a particular type of reactive species involved in mediating Bcl-2 stability in various biological systems is dictated by the mode of apoptosis that is triggered.

Furthermore, $\cdot\text{O}_2^-$ donor, 6-anilinoquinoline (LY83583) also caused degradation and downregulation of Bcl-2 by inducing its ubiquitination.²⁴ Therefore, $\cdot\text{O}_2^-$ may represent a common regulator of Bcl-2 function that controls apoptotic cell death induced by various physiologic and pathologic stimuli. In addition, *in vitro* and *in vivo* studies suggest that Bcl-2 can also block apoptosis through regulation of cellular antioxidant defense mechanisms or by suppressing production of free radicals, thus acting as an antioxidant.²⁵ Stable cell-lines that overexpressed Bcl-2 demonstrated significantly lower $\cdot\text{O}_2^-$ levels, and consequently lower apoptosis levels. Because, Bcl-2 overexpression significantly blocked ROS-mediated Cr(VI)-induced apoptosis, it is plausible that Bcl-2 may be acting as an antioxidant in response to Cr(VI) exposure. This provides new mechanistic insights into the interaction of Bcl-2 with $\cdot\text{O}_2^-$, which may be exploited in the treatment of cancer and related apoptosis disorders.

Regulation of Bcl-2 by RNS

Recent evidences suggest a dichotomous role for NO in determining cellular fate. NO can trigger apopto-

sis via mitochondrial, death receptor, p38/mitogen-activated protein kinase, and glyceraldehyde-3-phosphate dehydrogenase-Siah1 cascades.^{26–28} On the other hand, the antiapoptotic effect of NO can be mediated through several mechanisms including caspase inactivation, induction of p53 gene expression, upregulation of c-FLIP, or overexpression Bcl-X_L leading to inhibition of cytochrome *c* release from the mitochondria.^{29–31} One of the well-established mechanisms by which NO directly regulates the function of various target proteins is through S-nitrosylation.^{32,33} Such posttranslational modification of proteins has been shown to have either positive or negative effects on various signaling pathways, proteins, and metabolic processes.³⁴

Similar to Bcl-2 overexpression, NO has been shown to be elevated in many cancer cells;^{35,36} however, its potential role in the regulation of Bcl-2 and the underlying mechanisms has not been demonstrated. Our results show that the increased NO generated due to Cr(VI) exposure leads to S-nitrosylation of Bcl-2 protein leading to its stability, thereby decreasing cellular apoptosis⁶ (Fig. 1). This was verified using NO donors such as sodium nitroprusside (SNP) and dipropylentriamine (DPTA) NONOate, which stabilized Bcl-2 levels, and NO inhibitors such as 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazole-1-oxyl-3-oxide (PTIO) and aminoguanidine (AG) that downregulated Bcl-2 and increased apoptosis. Elevated NO levels led to a concomitant decrease in Bcl-2 ubiquitination, suggesting that NO may regulate Bcl-2 stability by preventing its degradation via the ubiquitin-proteasomal pathway. Furthermore, no effect of NO was observed on the phosphorylation of Bcl-2, positing that NO-mediated S-nitrosylation of Bcl-2 was sufficient for its stability and precluded its degradation. These results were confirmed by using site-directed mutagenesis, which demonstrated that Cys¹⁵⁸ and Cys²²⁹ were critical for Bcl-2 S-nitrosylation, and prevented its degradation through the ubiquitin-proteasomal pathway.⁶

As with Cr(VI), we observed a similar pattern of Bcl-2 regulation via S-nitrosylation in response to other stress inducers such as Fas ligand (FasL) and buthioninesulfoximine (BSO), suggesting that S-nitrosylation is a general process that can regulate Bcl-2 stability and function under various stress conditions. These findings indicate a novel function

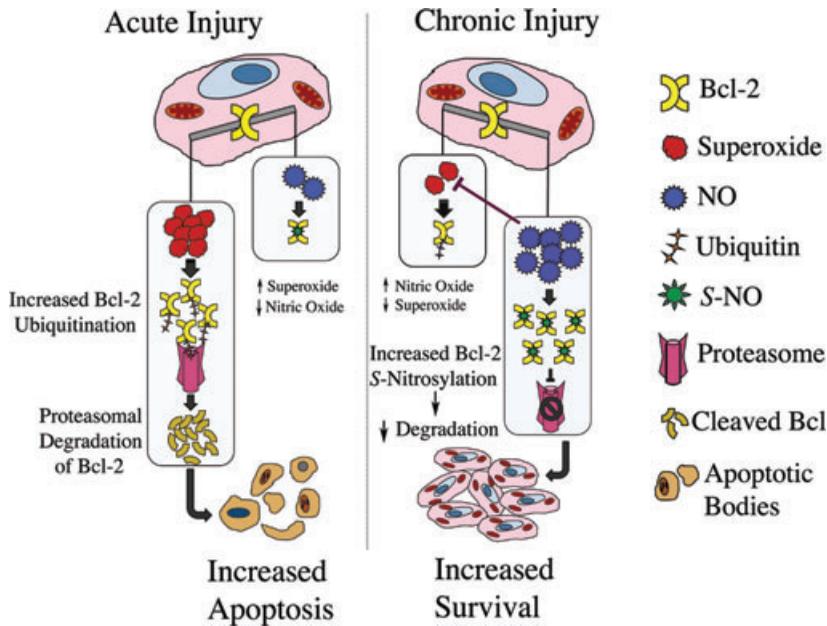


Figure 1. The scales that determine the balance between life and death. A schematic representation of various mechanisms of Bcl-2 regulation by ROS and RNS. Although both $\cdot\text{O}_2^-$ and NO are produced in response to acute injury, $\cdot\text{O}_2^-$ is the predominant reactive species that dictates cellular response. Increased $\cdot\text{O}_2^-$ levels lead to ubiquitination of Bcl-2 protein, leading to its degradation through the ubiquitin-proteasomal pathway. Therefore, acute exposure to heavy metals typically results in cellular apoptosis. On the other hand, chronic injury leads to sustained production of NO, which in turn inhibits the production of $\cdot\text{O}_2^-$. This increased NO leads to significantly higher levels of S-nitrosylated Bcl-2 protein that preclude its ubiquitination and subsequent degradation, thereby leading to its stability. This is sufficient to shift the balance toward cell survival, ultimately leading to the malignant transformation of cells.

of NO in regulating Bcl-2, which provides a key mechanism for the control of apoptotic cell death and cancer development.

Bcl-2 in malignant transformation

The importance of Bcl-2 in the development of apoptosis resistant phenotype and neoplastic development has been well established. Several cell-based studies have demonstrated that overexpression of Bcl-2 increases resistance to apoptotic cell death induced by various DNA-damaging agents, and evasion of apoptosis is an important precipitator of malignant transformation of normal cells.^{13–15} In our study, we investigated the role of Bcl-2 in malignant transformation of cells chronically exposed to subtoxic doses of Cr(VI).

Long-term Cr(VI) exposure led to the malignant transformation of nontumorigenic Beas-2B lung epithelial cells.⁷ In addition to visible phenotypic changes, Cr(VI) transformed cells (designated as B-Cr cells henceforth) exhibited loss of contact inhibition with cell mounding, and increased rates

of invasion and colony formation, all of which are hallmarks of tumorigenesis.^{37,38}

Interestingly, elevated levels of NO were observed in the apoptosis-resistant B-Cr cells as compared with passage-matched control cells. NO inhibitor (AG) inhibited cell invasion, migration, proliferation, and colony formation of Cr(VI)-transformed cells, whereas NO donor (DPTA NONOate) promoted these effects as compared to controls, suggesting a key role of NO in apoptosis resistance and malignant transformation induced by chronic exposure to Cr(VI).

In addition, Bcl-2 levels were sustained in B-Cr cells in response to Cr(VI) as opposed to passage-matched controls, primarily through increased S-nitrosylation and decreased ubiquitination of Bcl-2. B-Cr cells transfected with a non-nitrosylable Bcl-2 mutant demonstrated decreased migration, proliferation, and invasion as compared with B-Cr cells transfected with empty vector. This confirmed that NO-mediated S-nitrosylation of Bcl-2 was a critical event in the malignant transformation of

nontumorigenic lung epithelial cells in response to Cr(VI) exposure. Furthermore, basal levels of $\cdot\text{O}_2^-$ anions in B-Cr cells were also decreased as compared with passage-matched control cells, and were susceptible to NO modulators. It is plausible that the high basal levels of NO in B-Cr cells contribute to the decreased levels of $\cdot\text{O}_2^-$ in our system, leading to increased Bcl-2 levels regulated by NO-mediated nitrosylation and stabilization. Overall, the data suggest that NO-mediated S-nitrosylation of Bcl-2 and downregulation of $\cdot\text{O}_2^-$ are critical events in the malignant transformation of nontumorigenic lung epithelial cells in response to Cr(VI) exposure (Fig. 1).

Summary

Sensitivity to apoptosis depends on the expression levels of various apoptosis-regulatory proteins. A functional loss of proapoptotic proteins and/or increased expression of antiapoptotic protein can confer resistance to apoptotic stimuli. Bcl-2 is one of the most important antiapoptotic proteins that play a critical role in cancer development. In this study, we describe various mechanisms by which Bcl-2 expression levels and thus its function may be regulated. By linking Bcl-2 and $\cdot\text{O}_2^-$, we document a novel mechanism that forms the basis for differential susceptibility of cells to apoptotic cell death. This is also the first study demonstrating S-nitrosylation of Bcl-2, and sheds light on the hitherto unknown link between NO signaling and Bcl-2 stability.

With respect to metal-induced carcinogenesis, although several epidemiological studies demonstrated the tumorigenic potential of Cr(VI) compounds, *in vivo* studies in various animal models, including rat, mouse, guinea pig, and rabbit, have demonstrated no significant increase in lung tumors in Cr(VI)-treated animals versus untreated controls.^{39,40} Therefore, the establishment of this *in vitro* model is an important step toward delineating the molecular and genetic events associated with Cr(VI)-induced carcinogenesis. The *in vitro* model recapitulates the importance of Bcl-2 in tumor development, and highlights the role of S-nitrosylation in contributing toward cellular transformation. Because 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 study reveals an important mechanism that impli-

cates $\cdot\text{O}_2^-$, NO, and Bcl-2 regulation in the development of an apoptosis-resistant, malignant phenotype. This novel mechanism may also be critical in malignant transformation of normal cells under various conditions in response to other human carcinogens, and warrants investigation in the future with similar *in vitro* approaches.

Conflicts of interest

The authors declare no conflicts of interest.

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