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S-Nitrosylation of Bcl-2 Inhibits Its Ubiquitin-Proteasomal Degradation

*A NOVEL ANTIAPOPTOTIC MECHANISM THAT SUPPRESSES APOPTOSIS**

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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-tetramethylimidazoline-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 chromium (VI). In contrast, the NO donors dipropylenetriamine NONOate and sodium nitroprusside showed opposite effects. 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.

Bcl-2 (B-cell lymphoma-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)[2] 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 a pro- and antiapoptotic 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 glyceraldehyde-3-phosphate dehydrogenase-Siah1 cascades (14–17). The antiapoptotic 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-X_L with subsequent inhibition of cytochrome *c* release from the mitochondria (18–24). One of the well established mechanisms by which NO regulates the function of various target proteins is through *S*-nitrosylation (25). This post-translational modification of proteins can positively or negatively regulate various signaling pathways, proteins, and metabolic processes (26). For instance, NO inhibits the function of NF-κB, JNK and protein kinase C and activates Ras and ryanodine receptor via *S*-nitrosylation (27–31).

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[2] The abbreviations used are: NO, nitric oxide; AG, aminoguanidine; SNP, sodium nitroprusside; DPTA, dipropylenetriamine; PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; DTT, dithiothreitol; FasL, Fas ligand; TNF-α, tumor necrosis factor-α; BSO, buthionine sulfoximine; DAF-DA, diaminofluorescein diacetate; AMC, amino-4-methylcoumarin; PBS, phosphate-buffered saline; E3, ubiquitin-protein isopeptide ligase.

The objective of this study was to investigate the role of NO in the regulation of Bcl-2 function in chromium (VI)-induced apoptosis of human lung epithelial cancer cells. Cr(VI) is a naturally occurring heavy metal that has been classified as group I carcinogen by the International Agency for Research on Cancer (32). Exposure to Cr(VI) has been associated with lung cancers in various occupational settings (33–36). This compound is also present in cigarette smoke, and an increased incidence of lung cancer has been reported in smokers with Cr(VI) exposure (32, 34). Cell death induced by Cr(VI) occurs primarily through apoptosis (37), and its abnormal regulation has been associated with the initiation of Cr(VI)-induced cancer (38). 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 (39–41). 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, 42–44). 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. *S*-Nitrosylation of Bcl-2 prevents its down-regulation 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.

MATERIALS AND METHODS

Cells and Reagents—The human lung epithelial cancer cell line NCI-H460 was obtained from the American Type Culture Collection (Manassas, VA). 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% CO_2 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-oxy-3-oxide (PTIO), lactacystin, buthionine sulfoximine (BSO), dithiothreitol (DTT), diaminonaphthalene, mercury(II) chloride ($HgCl_2$), sodium hydroxide (NaOH), sulfonilamide, *N*-1-naphthyl ethylenediamine dehydrochloride, sodium nitrite ($NaNO_2$), and sodium dichromate ($Na_2Cr_2O_7 \cdot 2H_2O$) (Cr(VI)) were obtained from Sigma. The NO donor dipropylenetriamine (DPTA) NONOate, Fas ligand (FasL), tumor necrosis factor-α (TNF-α) and the fluorogenic caspase substrates IETD-amino-4-methylcoumarin (IETD-AMC) and LEHD-AMC were from Alexis Biochemicals (San Diego, CA). The NO fluorescent probe 4,5-diaminofluorescein diacetate (DAF-DA) and the apoptosis dye Hoechst 33342 were from Molecular Probes, Inc. (Eugene, OR). Antibodies for Bcl-2, phospho-Bcl-2 (Ser^{87}), 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.

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 was verified by Western blotting or immunoprecipitation.

Generation of Stable Bcl-2 Transfectant—Stable transfectant of Bcl-2 was generated by culturing H-460 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-cholamidiopropyl)-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 (1×10^6/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 (FACScalibur; BD Biosciences). For fluorescence microscopy, cells were incubated with the probe as described above and then examined for DAF fluorescence using Carl Zeiss Axiovert microscope.

Also, 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 $NaNO_2$ 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 Na_3VO_4, 50 mM NaF, 100 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture for 20 min on ice. After insoluble debris was precipitated by centrifugation at 14,000 × *g* for 15 min at 4 °C, the supernatants were collected and assayed for protein content using bicinchoninic acid 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% nonfat 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 × *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× 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 (28, 45). 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 $HgCl_2$ (200 μm) and diaminonaphthalene (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 diaminonaphthalene 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 Cys^{158} and Cys^{229}: C158A, CGGTGGGGTCATGGCTGTGGAGAGCGTCAACCG (forward) and CGGTTGACGCTCTCCACAGCCATGACCCCACCG (reverse); C229A, GGCCCTGGTGGGAGCTGCCATCACCCTGGGTGCC (forward) and GGCACCCAGGGTGATGGCAGCTCCCACCAGGGCC (reverse). Three mutant plasmids (C158A, C229A, and a plasmid with both mutations) were constructed using the QuikChange® 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 × *g* for 15 min at 4 °C, the supernatants were collected and determined for protein content. 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.

RESULTS

Apoptosis and Caspase Activation Induced by Cr(VI)—Chromium (VI)-containing compounds are ubiquitous environmental carcinogens that induce apoptosis as the primary mode of cell death (37). To study the role of NO and Bcl-2 in Cr(VI)-induced apoptosis, we first characterized the apoptosis response to Cr(VI) treatment in human lung epithelial H-460 cells. Cells were treated with different doses of Cr(VI) (0–100 μM), and apoptosis was determined after 12 h by a 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. Significant apoptosis was observed as early as 6 h and peaked at about 16 h post-treatment (data not shown). Caspase activity assays show that Cr(VI) was able to induce both caspase-8 and caspase-9 activation (Fig. 1*C*); however, the latter effect was much more pronounced. Since caspase-9 serves as the apical caspase of the intrinsic (mitochondrial) death pathway, whereas caspase-8 represents the apical caspase of the extrinsic (death receptor) pathway (46, 47), the results of this study suggest that the mitochondrial pathway is the major apoptosis pathway induced by Cr(VI). 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. 1*C*).

NO Inhibits Cr(VI)-induced Apoptosis—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. Fig. 2, *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 capase-9 activation (Fig. 2*C*).

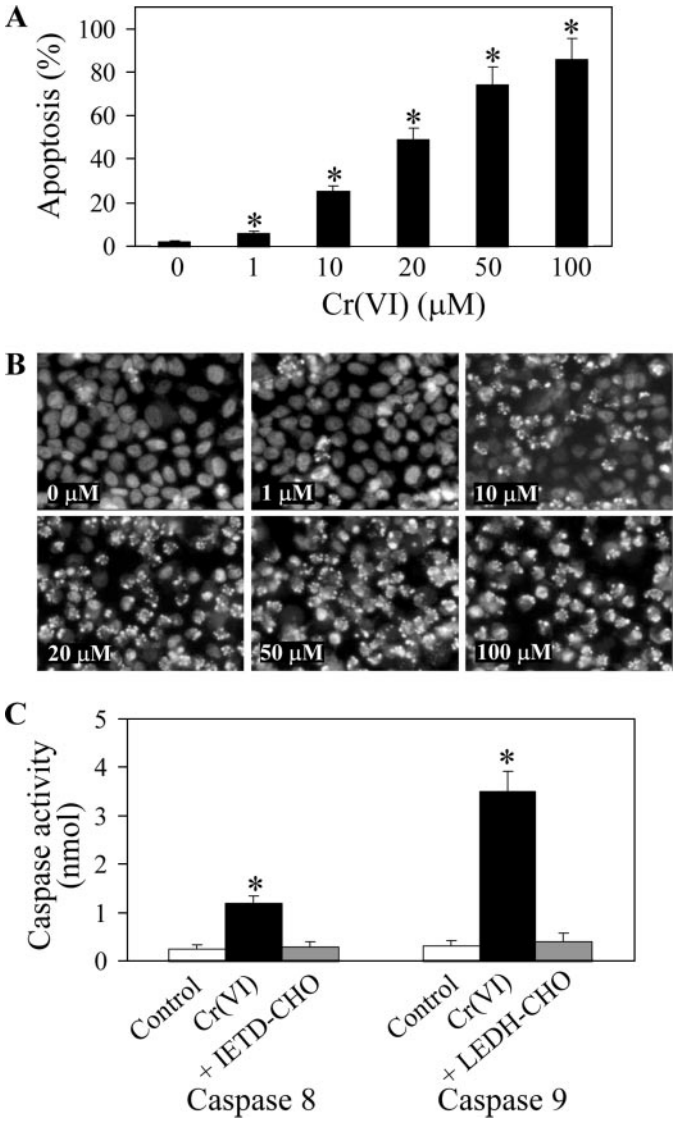

FIGURE 1. **Induction of apoptosis and caspase activation by Cr(VI) in human lung epithelial H-460 cells.** *A*, subconfluent (90%) monolayers of H-460 cells were exposed to varying doses of Cr(VI) (0–100 μM) for 12 h, and the cells were analyzed for apoptosis by Hoechst 33342 assay. *B*, fluorescence micrographs of treated cells stained with Hoechst dye. Apoptotic cells exhibited shrunken and fragmented nuclei with bright nuclear fluorescence. *C*, 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). 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. Data are mean ± S.D. ($n = 4$). *, $p < 0.05$ *versus* nontreated control (original magnification, ×200).

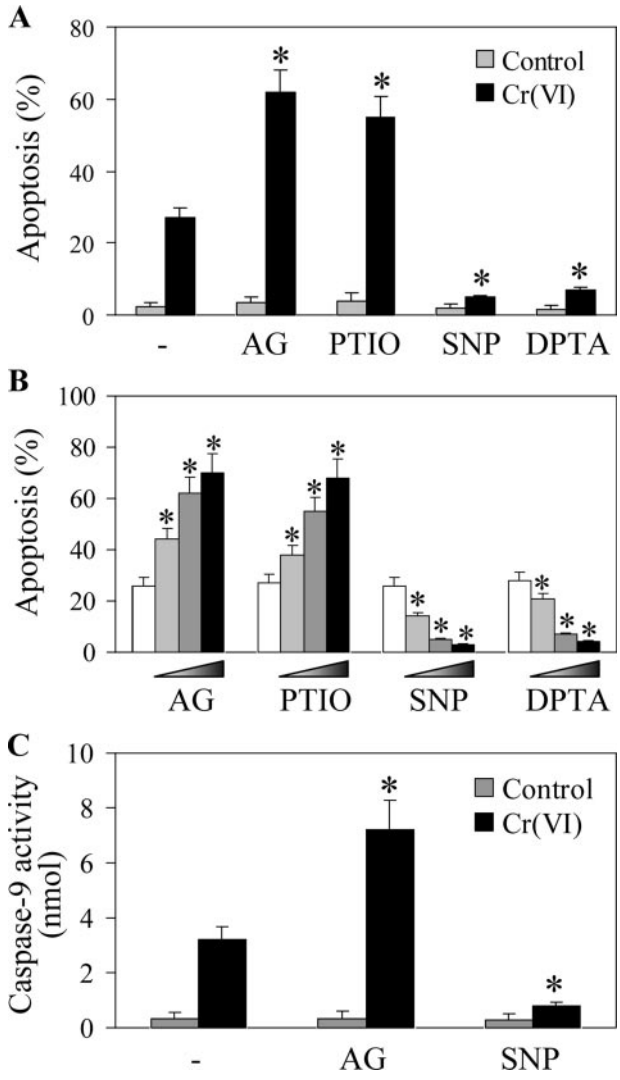

FIGURE 2. **Effect of NO modulators on Cr(VI)-induced apoptosis and caspase activation.** *A*, subconfluent (90%) monolayers of H-460 cells were pretreated with the NO donor SNP (500 μg/ml) or DPTA NONOate (400 μM) or with the NO inhibitor AG (300 μM) or PTIO (300 μM) for 1 h. The cells were then either 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. ($n \geq 3$). *, $p < 0.05$ *versus* Cr(VI)-treated control.

These results indicate that NO plays an antiapoptotic 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. 3, *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. 3*C* 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 reactive oxygen species (39, 40) and due to the report challenging the specificity of DAF DA to NO in cells undergoing oxidative and nitrosative stress (48), we confirmed this effect by the Griess method. Griess assay measures nitrite, which is the stable breakdown product of NO. Fig. 3*D* 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.

Bcl-2 Overexpression Protects Cells from Cr(VI)-induced Apoptosis—Bcl-2 is a key apoptosis-regulatory protein of the mitochondrial death pathway (1). To determine whether this

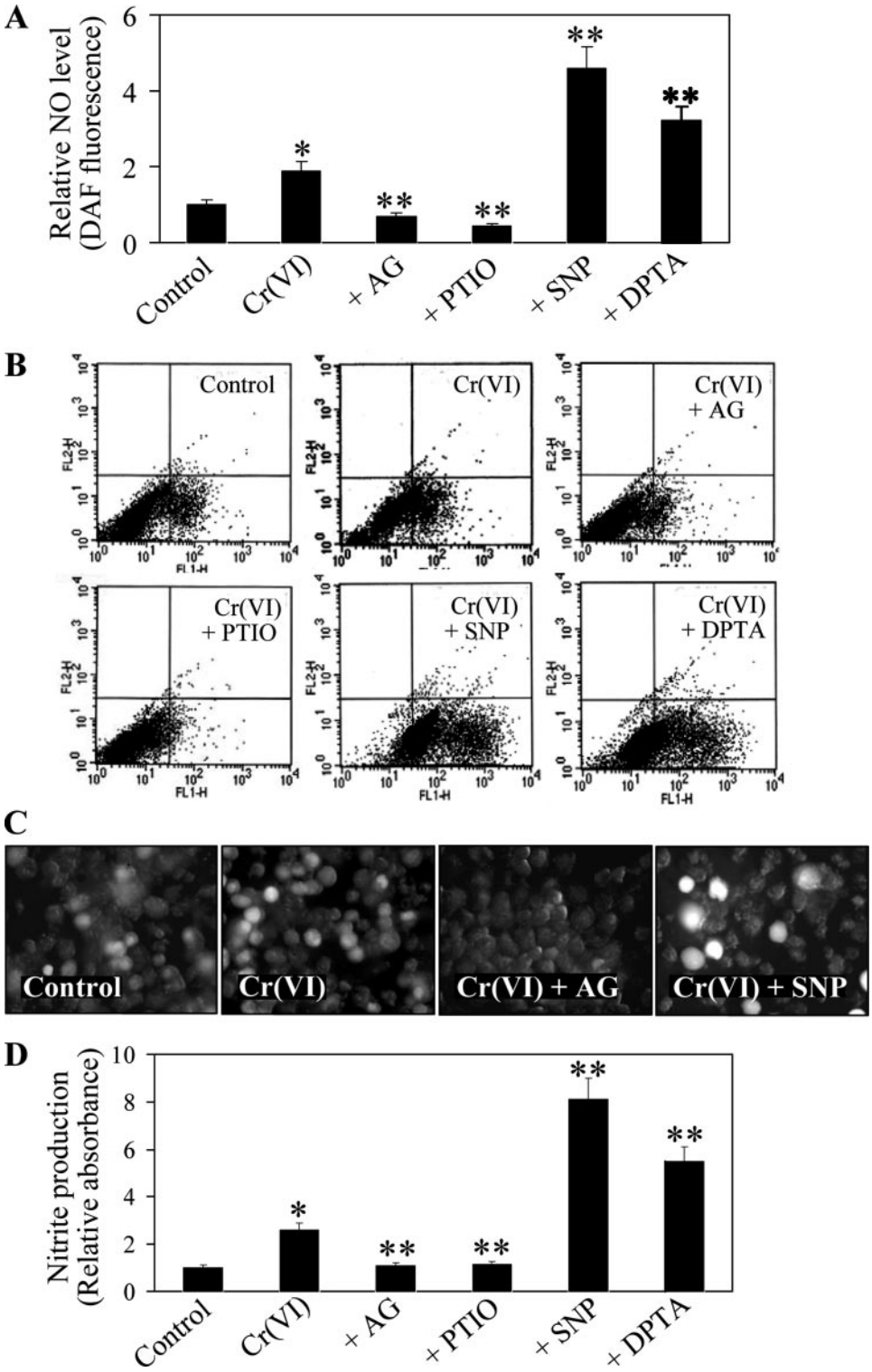

FIGURE 3. **Effect of NO modulators on cellular NO levels.** *A* and *B*, flow cytometric analysis of NO production in H-460 cells. Cells were treated with the NO inhibitor AG (300 μM) or PTIO (300 μM) or with the NO donor SNP (500 μg/ml) or DPTA NONOate (400 μM) for 1 h, after which they were treated 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. ($n \geq 3$). *, $p < 0.05$ *versus* nontreated control; **, $p < 0.05$ *versus* Cr(VI)-treated control. (original magnification, ×400).

protein is involved in the regulation of Cr(VI)-induced apoptosis, which was earlier shown to be associated with mitochondrial caspase-9 activation, we stably transfected H-460 cells with Bcl-2 or control plasmid, and their effect on Cr(VI)-induced apoptosis was determined by Hoechst 33342 assay. Fig. 4*A* shows that overexpression of Bcl-2 significantly inhibited Cr(VI)-induced apoptosis over a wide concentration range as compared with the vector-transfected control. Western blot analysis of the transfected cells shows an increase in Bcl-2 protein expression in the Bcl-2-transfected cells but not in the control transfectant (Fig. 4*B*). These results indicate the role of Bcl-2 as a negative regulator of Cr(VI)-induced apoptosis and further support the role of the mitochondrial pathway in Cr(VI)-induced cell death.

Effect of NO Modulators on Bcl-2 Expression—Having demonstrated the role of Bcl-2 in Cr(VI)-induced apoptosis, we next examined the potential regulation of Bcl-2 by NO in H-460 cells. The effect of Cr(VI) treatment on Bcl-2 expression was first characterized in these cells by immunoblotting. Fig. 5*A* shows that treatment of the cells with Cr(VI) caused a dose-dependent decrease in the expression level of Bcl-2. Fig. 5*B* also shows that this effect of Cr(VI) was time-dependent with the effect clearly noticeable at 6 h post-treatment and thereafter. Next, 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. 6*A* 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. 6*B* 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-

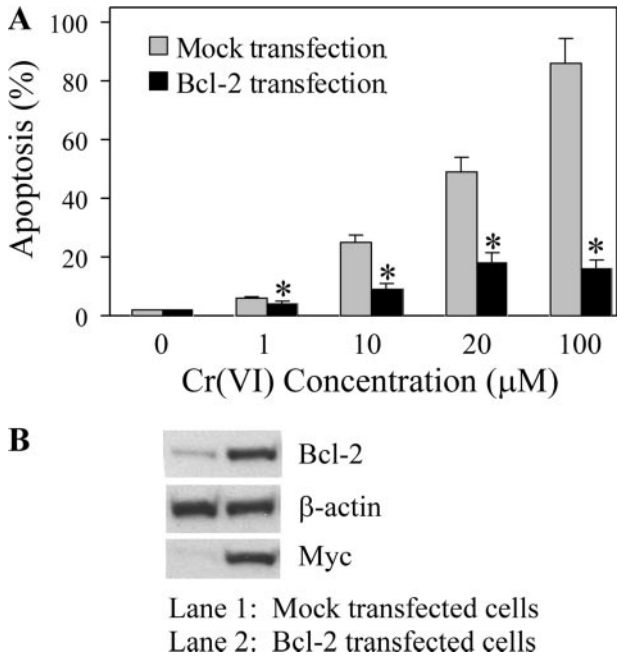

FIGURE 4. **Bcl-2 overexpression increases cell death resistance to Cr(VI).** *A*, H-460 cells were stably transfected with Myc-tagged Bcl-2 plasmid or control pcDNA3 plasmid. Transfected cells were treated with varying doses of Cr(VI) (0–100 μM) for 12 h and apoptosis was determined by Hoechst 33342 assay. Plots are mean ± S.D. ($n = 4$). *, $p < 0.05$ *versus* mock-transfected controls. *B*, Western blot analysis of Bcl-2 expression in mock- and Bcl-2-transfected cells. β-Actin was used as a loading control.

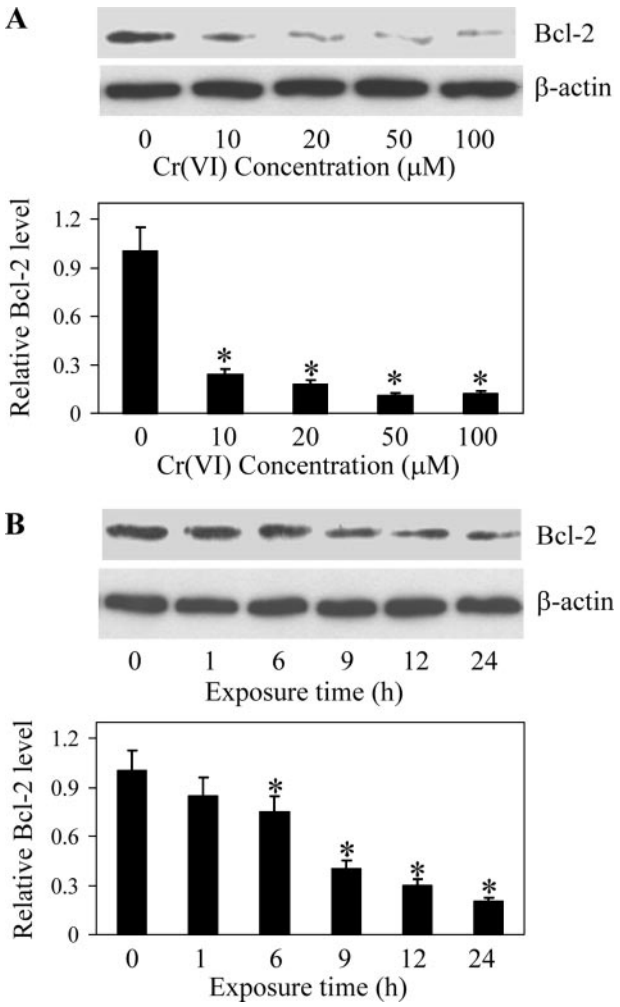

FIGURE 5. **Effect of Cr(VI) on Bcl-2 expression.** *A*, H-460 cells were treated with varying doses of Cr(VI) (0–100 μM) for 12 h, and cell lysates were prepared and analyzed for Bcl-2 by Western blotting. *B*, cells were treated for various times (0–24 h) with Cr(VI) (20 μM) and Bcl-2 expression was 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* nontreated control.

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. 6*A* 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). The result was confirmed by the observation that another proteasome inhibitor, MG132, also inhibited Bcl-2 down-regulation induced by Cr(VI) (data not shown).

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 (49–51). 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, 52). 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. 6*C*). Control experiments using the known inducer of Bcl-2 dephosphorylation TNF-α (10) demonstrated a substantial reduction in Bcl-2 phosphorylation in the treated cells (not shown). 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. 7). 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).

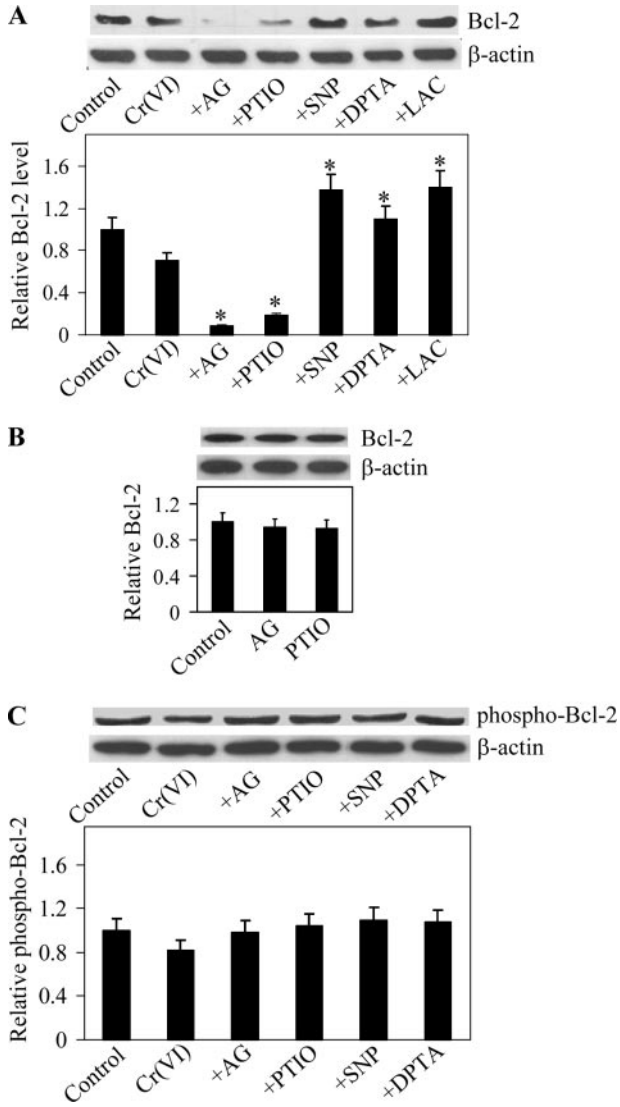

FIGURE 6. **Effect of NO modulators on Bcl-2 expression and phosphorylation.** *A*, H-460 cells were pretreated for 1 h with one of the following agents: SNP (500 μg/ml), DPTA NONOate (400 μM), AG (300 μM), PTIO (300 μM), and lactacystin (*LAC*) (10 μM). The cells were then treated with Cr(VI) (20 μM) for 6 h, and cell lysates were prepared and analyzed for Bcl-2 expression by Western blots using anti-Bcl-2 antibody. *B*, H-460 cells were treated with AG (300 μM) and PTIO (300 μM) for 6 h, and cell lysates were prepared and analyzed for Bcl-2 expression by Western blots using anti-Bcl-2 antibody. *C*, Bcl-2 phosphorylation was determined in the treated cells using phospho-specific Bcl-2 (Ser^{87}) antibody and cell lysates from above. 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* nontreated control.

NO-mediated S-Nitrosylation of Bcl-2 Inhibits Its Ubiquitination—NO can control the function of several proteins by an *S*-nitrosylation process (53–55). 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 using anti-*S*-nitrosocysteine antibody and by spectrofluorometry. Fig. 8, *A* and *B*, shows that treatment of the cells with Cr(VI) induced *S*-nitrosylation of Bcl-2, and this effect was enhanced by NO donors SNP and

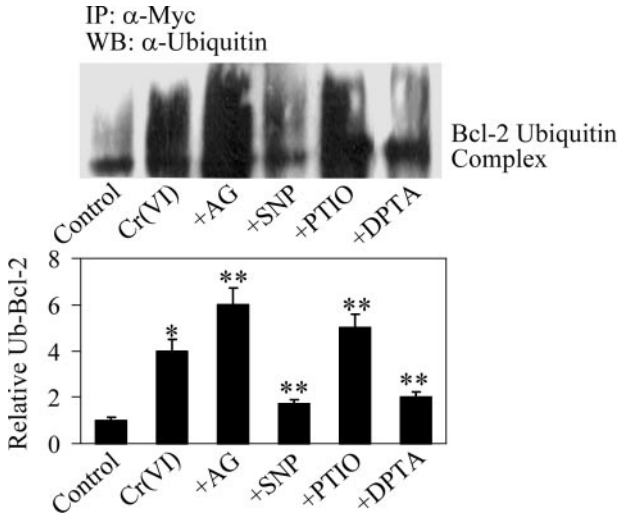

FIGURE 7. **Effect of NO on Bcl-2 ubiquitination.** Subconfluent monolayers of H-460 cells overexpressing Bcl-2 were pretreated with the NO inhibitor AG (300 μM) or PTIO (300 μM) or with the NO donor SNP (500 μg/ml) or DPTA NONOate (400 μM) for 1 h. Subsequently, the cells were treated with Cr(VI) (20 μM) for 3 h in the presence of lactacystin (10 μM) to prevent proteasome-mediated Bcl-2 degradation. Cell lysates were immunoprecipitated (*IP*) with anti-Myc antibody, and the immune complexes were analyzed for ubiquitin by Western blotting (*WB*). 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* nontreated control. **, $p < 0.05$ *versus* Cr(VI)-treated control.

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 (56, 57), 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. 8*A*). Furthermore, it negated the effect of NO donors SNP and DPTA NONOate on Bcl-2 ubiquitination in Cr(VI)-treated cells (Fig. 8*C*). 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) (58, 59). 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. 8*D*), 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 (Fig. 9), suggesting

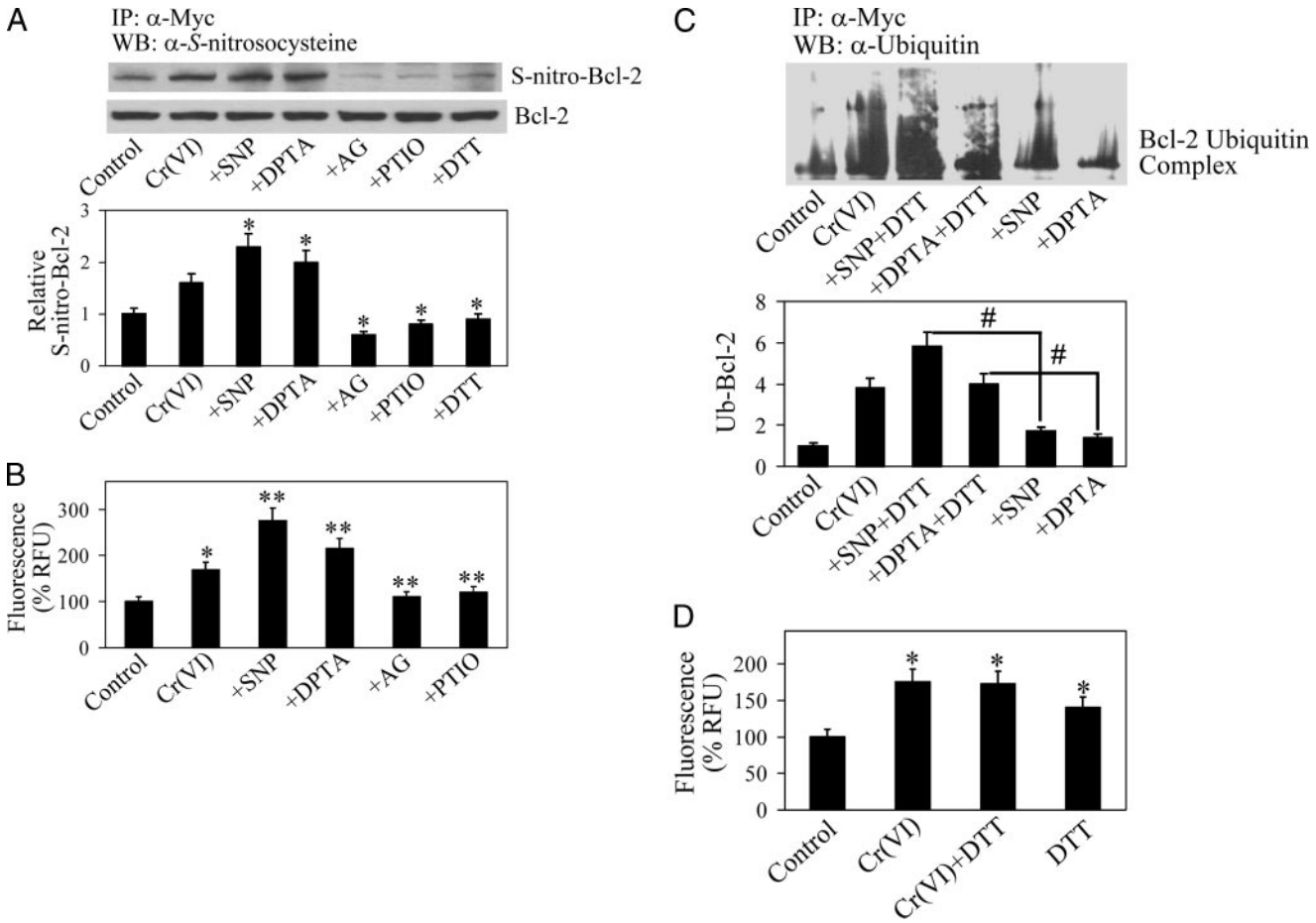

FIGURE 8. **Effects of NO on *S*-nitrosylation and ubiquitination of Bcl-2.** *A*, effect of NO modulators on Bcl-2 *S*-nitrosylation in Cr(VI)-treated cells determined by immunoblotting. Subconfluent monolayers of H-460 cells overexpressing Bcl-2 were pretreated with SNP (500 μg/ml), DPTA NONOate (400 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 (*IP*) using anti-Myc antibody. The resulting immune complexes were analyzed for *S*-nitrosocysteine by Western blotting (*WB*). 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 $HgCl_2$ and 200 μM diaminonaphthalene. 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* nontreated control. **, $p < 0.05$ *versus* Cr(VI)-treated controls. #, $p < 0.05$ *versus* NO-modulated controls.

that this process is a general phenomenon that regulates the antiapoptotic 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 H-460 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 blot 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. 10*A*). 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. 10*B*), indicating that *S*-nitrosylation of the protein prevents its ubiquitination and subsequent degradation.

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 (42–44); 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 regulates Bcl-2 protein via *S*-nitrosylation and that this mechanism stabilizes the protein, maintaining its antiapoptotic function. Down-regulation of Bcl-2 was observed in

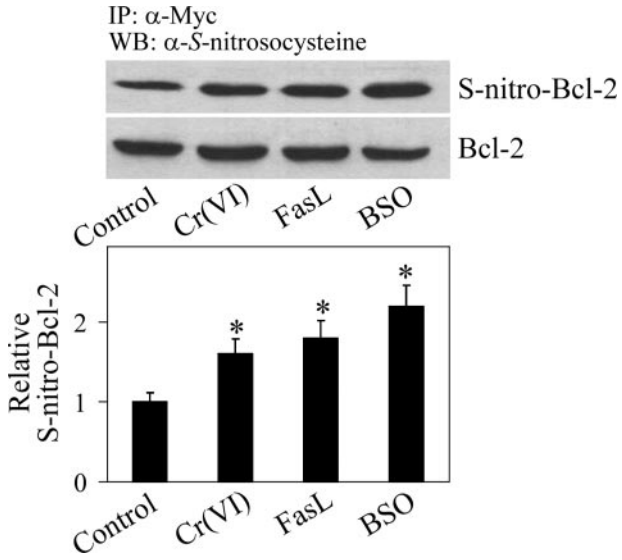

FIGURE 9. **Effect of stress inducers on Bcl-2 *S*-nitrosylation.** Subconfluent monolayers of H-460 cells overexpressing Bcl-2 were treated with FasL (200 ng/ml), BSO (100 μM), or Cr(VI) (20 μM) for 3 h, and cell lysates were prepared for immunoprecipitation (*IP*) using anti-Myc antibody. The resulting immune complexes were analyzed for *S*-nitrosocysteine by Western blotting (*WB*). Densitometry was performed to determine the relative *S*-nitrosocysteine levels after reprobing of the membranes with anti-Bcl-2 antibody. Data are mean ± S.D. ($n = 4$). *, $p < 0.05$ *versus* non-treated control.

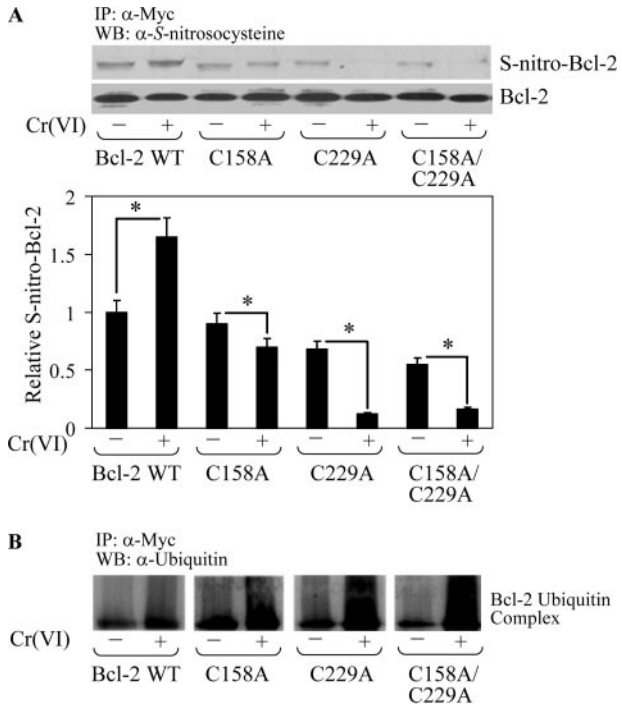

FIGURE 10. **Effect of cysteine mutations on *S*-nitrosylation and ubiquitination of Bcl-2.** *A*, H-460 cells were transiently transfected with Myc-tagged original Bcl-2 plasmid (*WT*) or with Myc-tagged C158A, C229A, or C158A/C229A mutant plasmid. 36 h later, the cells were treated with or without Cr(VI) (20 μM) for 3 h, and cell lysates were prepared for immunoprecipitation (*IP*) using anti-Myc antibody. The immune complexes were analyzed for *S*-nitrosocysteine by Western blotting (*WB*). Densitometry was performed to determine the relative *S*-nitrosocysteine levels after reprobing of the membranes with anti-Bcl-2 antibody. *B*, immunoprecipitates were prepared as described above and analyzed for ubiquitin by Western blotting. Data are mean ± S.D. ($n = 4$). *, $p < 0.05$ *versus* non-treated controls.

cells treated with Cr(VI), the effect that was accompanied by an increase in apoptotic cell death (Figs. 1 and 5). The addition of NO donors SNP and DPTA NONOate effectively inhibited this down-regulation and decreased the cell death (Figs. 2 and 6). In contrast, NO inhibitors AG and PTIO showed opposite effects, indicating the antiapoptotic 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 (60). In this study, we further showed that both mitochondrial and death receptor pathways of apoptosis were activated by Cr(VI), with the former playing a more dominant role (Fig. 1). The mitochondrial death pathway is regulated by Bcl-2, which prevents apoptosis by preserving mitochondrial permeability transition (51). The observation that Bcl-2 overexpression inhibited Cr(VI)-induced apoptosis (Fig. 4) substantiates the role of the mitochondrial death pathway in Cr(VI)-induced apoptosis.

The antiapoptotic 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. 6 and 7). The ability of the proteasome inhibitors lactacystin and MG132 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. 7). 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. 6), 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 (22, 53–55). 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. 8). Inhibition of *S*-nitrosylation by the known inhibitor DTT completely inhibited the effect of NO on Bcl-2 ubiquitination (Fig. 8), thus confirming the protective role of *S*-nitrosylation on Bcl-2 ubiquitination.

Recent evidence suggests that NO can nitrosylate E3 ubiquitin ligase (parkin, Mdm2) (58, 59). *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. 8). 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 (Cys^{158} and Cys^{229}) were mutated to alanines. Such mutations resulted in a complete inhibition of Bcl-2 *S*-nitrosylation by Cr(VI) (Fig. 10). The stronger inhibitory effect of the Cys^{229} mutation indicates that this cysteine is the major site of Bcl-2 *S*-nitrosylation. Further, cysteine mutations led to increased ubiquitination, confirming that *S*-nitrosylation of Bcl-2 prevents its ubiquitination and subsequent degradation.

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 (61). FasL is a death ligand that is known to induce apoptosis via the death receptor pathway (46, 47). 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.

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 its ability to nitrosylate 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.

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