

Nitric Oxide Regulates Cell Sensitivity to Cisplatin-Induced Apoptosis through S-Nitrosylation and Inhibition of Bcl-2 Ubiquitination

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Abstract

Cisplatin is a potent cytotoxic agent commonly used for the treatment of solid tumors. However, tumor cell-acquired resistance to cisplatin-induced apoptosis is a major limitation for efficient therapy, as frequently observed in human lung cancer. Nitric oxide (NO) is a key regulator of apoptosis, but its role in cisplatin-induced cell death and the underlying mechanism are largely unknown. Previous studies indicate increased NO synthase activity and elevated NO production in lung carcinomas, which correlate with the incidence of chemotherapeutic resistance. Here, we show that NO impairs the apoptotic function of cells and increases their resistance to cisplatin-induced cell death in human lung carcinoma H-460 cells. The NO donors sodium nitroprusside and dipropylene-triamine NONOate were able to inhibit cisplatin-induced cell death, whereas the NO inhibitors aminoguanidine and 2-(4-carboxyphenyl)-4,4,5,5-tetra-methylimidazole-1-oxyl-3-oxide had opposite effect. Cisplatin resistance in H-460 cells is mediated by Bcl-2, and NO up-regulates its expression by preventing the degradation of Bcl-2 via ubiquitin-proteasome pathway. Cisplatin-induced generation of reactive oxygen species causes dephosphorylation and degradation of Bcl-2. In contrast, generation of NO has no effect on Bcl-2 phosphorylation but induces S-nitrosylation of the protein, which inhibits its ubiquitination and subsequent proteasomal degradation. These findings indicate a novel pathway for NO regulation of Bcl-2, which provides a key mechanism for cisplatin resistance and its potential modulation for improved cancer chemotherapy. (Cancer Res 2006; 66(12): 6353-60)

Introduction

The platinum coordination complex *cis*-diamminedichloroplatinum (cisplatin) is a widely prescribed chemotherapeutic agent. It is generally used alone or in combination with other therapeutic agents for the treatment of solid tumors, such as testicular, ovarian, bladder, cervical head and neck, and small cell and non-small cell lung cancer (1, 2). The cytotoxic mode of action of cisplatin is still unclear, although it was suggested that the interaction of this compound with DNA forms DNA adducts, primarily intra-strand cross-link adducts, which activate several signal transduction

pathways, including ATR, p53, p73, and mitogen-activated protein kinase, and culminate in the activation of cell apoptosis (3). Cisplatin-induced cell death depends further on the generation of reactive oxygen species (ROS; refs. 4, 5). However, cisplatin-mediated cell death is frequently impaired and is a major limitation of cisplatin-based chemotherapy (1, 6).

One frequent mechanism of apoptosis resistance of tumor cells is the deregulated expression of proapoptotic and antiapoptotic proteins. Amplification and overexpression of the B-cell lymphoma-2 (Bcl-2) proto-oncogene occurs in many malignancies, including small cell lung carcinomas, and impairs the intrinsic apoptotic signaling by neutralizing proapoptotic Bcl-2 family members, such as Bax (7, 8). Bcl-2 plays a crucial role in cisplatin-induced apoptosis and is a key determining factor of cisplatin resistance (9–12). The expression of Bcl-2 is regulated by several mechanisms, including transcription, posttranslational modifications, dimerization, and degradation. Increasing evidence suggest that Bcl-2 expression is mainly regulated at the posttranscriptional level by protein stability. Numerous stimuli can induce the degradation of Bcl-2, including tumor necrosis factor- α (TNF- α), ROS, lipopolysaccharide, β -amyloid, and ischemia (9, 13–17). Degradation of Bcl-2 in response to certain stimuli, such as TNF- α and ROS, require its dephosphorylation (16, 17). Degradation of Bcl-2 is mainly mediated by the ubiquitin-dependent proteasome complex upon the covalent attachment of ubiquitin (18, 19).

Nitric oxide (NO) is a key cellular mediator synthesized from L-arginine in a reaction catalyzed by NO synthases (NOS; refs. 20, 21). NO has been shown to possess both proapoptotic and antiapoptotic functions, depending on the cell type and cellular redox state, as well as on the concentration and flux of NO (22, 23). Induction of apoptosis by NO was attributed to its ability to induce oxidative stress and caspase activation (24). In contrast, endogenous NO production or the exposure to appropriate amounts of NO reportedly inhibits apoptosis, which has been shown in various *in vivo* and *in vitro* experimental models (25, 26). NOS play a role in the production of NO in lung neoplasia and may thus influence NO-mediated functions in tumor tissues (27). Elevation of NOS activity has been reported in human lung adenocarcinomas, and increased tumor-associated NO production has been observed in lung cancer patients (25, 28, 29). Together with the high incidence of cisplatin resistance in lung cancer, NO may play a role in regulating lung carcinoma cell sensitivity to cisplatin-mediated cell death.

The mechanisms by which NO regulates apoptosis resistance to cisplatin have not been investigated. In the present study, we determined the role of NO in cisplatin-induced apoptosis and elucidated its regulatory mechanisms using pharmacologic and gene manipulation approaches. Our findings show an important

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role of NO in Bcl-2 regulation and its antiapoptotic function in cisplatin-induced cell death. The mechanism by which NO regulates Bcl-2 involves *S*-nitrosylation and inhibition of ubiquitin/proteasome-mediated degradation. Such regulation is independent of the dephosphorylation process, thus revealing the existence of a novel mechanism of cell death regulation, which might be exploited in cancer chemotherapy.

Materials and Methods

Cells and reagents. NCI-H460 cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in RPMI 1640 containing 5% fetal bovine serum, 2 mmol/L L-glutamine, and 100 units/mL penicillin/streptomycin in a 5% CO₂ environment at 37°C. Sodium nitroprusside, aminoguanidine, 2-(4-carboxy-phenyl)-4,4,5,5-tetramethylimidazole-1-oxo-3-oxide (PTIO), *N*-acetylcysteine (NAC), DTT, benzoyloxycarbonyl-Val-Ala-Asp-(OMe) fluoromethyl ketone (zVAD-fmk), and cisplatin were obtained from Sigma Chemical, Inc. (St. Louis, MO). Dipropyleneamine NONOate was from Alexis Biochemical (San Diego, CA). Diaminofluorescein diacetate (DAF-DA), dichlorofluorescein diacetate (DCF-DA), and Hoechst 33342 were obtained from Molecular Probes, Inc. (Eugene, OR). Antibodies for Bcl-2, phospho-Bcl-2 (Ser⁸⁷), myc, and peroxidase-labeled secondary antibodies and protein A-agarose were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for ubiquitin, *S*-nitrosocysteine, and β-actin were from Sigma (St. Louis, MO). The transfecting agent LipofectAMINE was obtained from Invitrogen (Carlsbad, CA).

Plasmid and transfection. The Bcl-2 plasmid was generously provided by Dr. C. 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 the NH₂-terminal myc epitope tag. Authenticity of all constructs was verified by DNA sequencing. Transient transfection was done using LipofectAMINE reagent (Invitrogen), 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 a six-well plate until they reached 80% to 90% confluence. One microgram of cytomegalovirus-neo vector and 15 μL of LipofectAMINE reagent with 2 μg of myc-tagged Bcl-2 plasmid were used to transfect the cells in each well in the absence of serum. After 10 hours, the medium was replaced with culture medium containing 5% fetal bovine serum. Approximately 36 hours after the beginning of the transfection, cells were trypsinized, and the cell suspensions were plated onto 75-mL culture flasks and cultured for 24 to 28 days with G418 selection (400 μg/mL). The pooled stable transfectant was identified by Western blot analysis of Bcl-2 and was cultured in G418-free RPMI for at least two passages before each experiment.

Apoptosis and cytotoxicity assays. Apoptosis was determined by Hoechst 33342 assay (Molecular Probes) and by DNA fragmentation ELISA using a kit from Roche Molecular Biochemicals (Indianapolis, IN). For Hoechst assay, cells were incubated with 10 μg/mL Hoechst 33342 for 30 minutes and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei by UV microscopy using a Pixera software (Leica Microsystems, Bannockburn, IL). For ELISA assay, cells were lysed with 200 μL of lysis buffer, and the cell lysate (20 μL) was mixed with an antibody solution provided by the supplier (80 μL) at room temperature for 2 hours. The substrate was then added after the wells were washed. After incubation for 10 minutes at 37°C, the reaction was stopped, and absorbance was measured using a microplate reader at a wavelength of 405 nm. Cytotoxicity was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay using a kit from Roche Molecular Biochemicals (MTT-based Cell Proliferation kit 1). Cells in 96-well plates were incubated with 500 mg/mL of MTT for 4 hours at 37°C. The intensity of the MTT product was measured at 550 nm using a microplate

reader. The relative percentage of cell survival was calculated by dividing the absorbance of treated cells by that of the control in each experiment.

NO and ROS detection. Intracellular NO and ROS production was determined by flow cytometry using the NO-specific probe DAF-DA and the oxidative probe DCF-DA (Molecular Probes) according to the manufacturer's instructions. Briefly, cells were incubated with the probe (10 μmol/L) for 30 minutes at 37°C, after which they were washed, trypsinized, resuspended in PBS (1 × 10⁶/mL), and immediately analyzed by flow cytometry using a 488-nm excitation beam and a 538-nm band-pass filter (FACSort, Becton Dickinson, Rutherford, NJ) with CellQuest software (Becton Dickinson).

Western blotting. After specific treatments, cells were incubated in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.5), 1% Triton X-100, 150 mmol/L NaCl, 10% glycerol, 1 mmol/L Na₃VO₄, 50 mmol/L NaF, 100 mmol/L phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mixture (Roche Molecular Biochemicals) for 20 minutes on ice. After insoluble debris was pelleted by centrifugation at 14,000 × *g* for 15 minutes at 4°C, the supernatants were collected and determined for protein content using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Proteins (40 μg) were resolved under denaturing conditions by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Bio-Rad). The transferred membranes were blocked for 1 hour in 5% nonfat dry milk in TBST [25 mmol/L Tris-HCl (pH 7.4), 125 mmol/L NaCl, 0.05% Tween 20] and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed thrice with TBST for 10 minutes and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 hour at room temperature. The immune complexes were detected by enhanced chemiluminescence detection system (Amersham Biosciences, Piscataway, NJ) and quantified using analyst/PC densitometry software (Bio-Rad). Mean densitometry data from independent experiments were normalized to result in cells in the control. The data were presented as the mean ± SD and analyzed by the Student's *t* test.

Immunoprecipitation. Cells are washed after treatments and lysed in lysis buffer at 4°C for 20 minutes. After centrifugation at 14,000 × *g* for 15 minutes at 4°C, the supernatants were collected and determined for protein content. Cleared lysates were normalized, and 60 μg proteins were incubated with 12 μL of anti-myc agarose bead (Santa Cruz Biotechnology) diluted with 12 μL Sepharose for 4 hours at 4°C. The immune complexes were washed with 20 volumes of lysis buffer, resuspended in 2× Laemmli sample buffer, and boiled at 95°C for 5 minutes. Immune complexes were separated by 10% SDS-PAGE and analyzed by Western blot as described.

Results

NO inhibits cisplatin-induced apoptosis in H-460 cells. Cisplatin is frequently used as a chemotherapeutic agent for solid tumors and has been reported to induce apoptosis in sensitive cells, including lung epithelial cells (1, 2). To study the role of NO in cisplatin-induced apoptosis, we first characterized cell death response to cisplatin treatment in human lung epithelial carcinoma H-460 cells. Cells were treated with various concentrations of cisplatin (0-500 μmol/L), and apoptosis was determined after 12 hours by Hoechst 33342 and ELISA-based DNA fragmentation assays. Treatment of the cells with cisplatin caused a dose-dependent increase in cell apoptosis over control level, as indicated by increased nuclear fluorescence and chromatin condensation (Fig. 1A). At the treatment dose of 50 μmol/L, ~10% of the treated cells showed apoptotic nuclear morphology with the cell death response approaching 60% at the treatment dose of 500 μmol/L. ELISA results showed a similar dose effect of cisplatin on DNA fragmentation (Fig. 1B). To confirm the death-inducing effect of cisplatin, cells were similarly treated with cisplatin, and cell survival was determined by MTT assay. Consistent with the apoptosis assays, our results showed that cisplatin decreased cell survival in a dose-dependent manner (Fig. 1C).

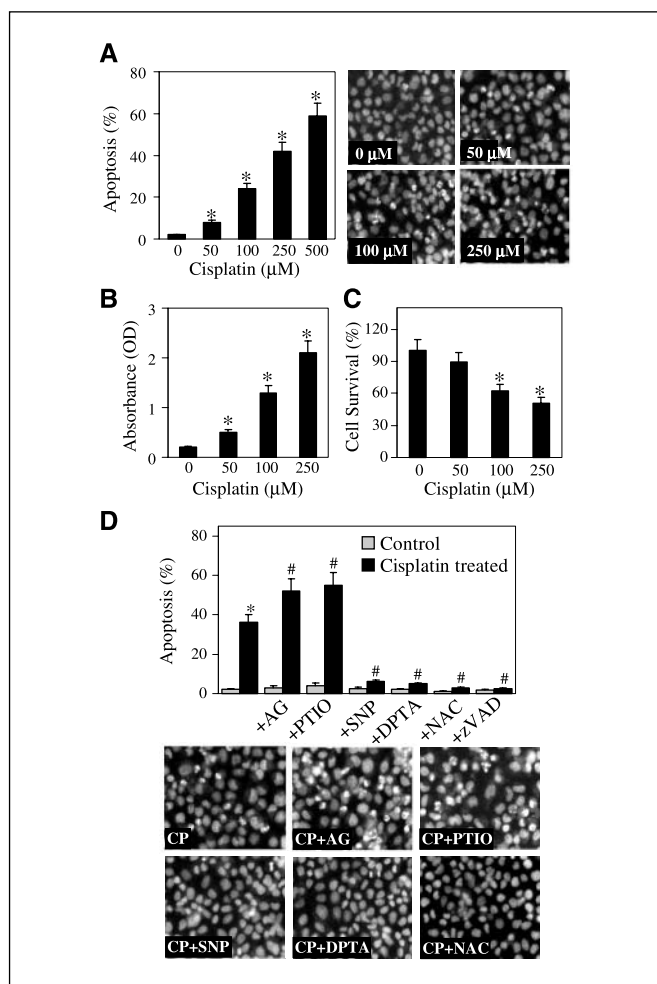


Figure 1. Cisplatin induces cell apoptosis and its inhibition by NO. Subconfluent (90%) monolayers of H-460 cells were treated with cisplatin (0-500 $\mu\text{mol/L}$) at 37°C for 12 hours. *A*, dose effect of cisplatin on cell apoptosis determined by Hoechst 33342 assay. *B*, effect of cisplatin on DNA nucleosomal fragmentation determined by ELISA. *C*, effect of cisplatin on cell survival determined by MTT assay. *D*, effect of NO/ROS modulators on cisplatin-induced apoptosis. H-460 cells were pretreated with sodium nitroprusside (SNP; 500 $\mu\text{g/mL}$), dipropylentriamine (DPTA) NONOate (200 $\mu\text{mol/L}$), aminoguanidine (AG; 300 $\mu\text{mol/L}$), PTIO (300 $\mu\text{mol/L}$), NAC (1 mmol/L), or zVAD-fmk (10 $\mu\text{mol/L}$) for 1 hour. The cells were then either left untreated or treated with cisplatin (200 $\mu\text{mol/L}$) for 12 hours and analyzed for apoptosis by Hoechst 33342 assay. Columns, mean ($n = 4$); bars, SD. *, $P < 0.05$ versus nontreated control; #, $P < 0.05$ versus cisplatin-treated control.

To investigate the potential role of NO in the regulation of cisplatin-induced apoptosis, cells were treated with various NO inhibitors and donors followed by cisplatin treatment. Figure 1D shows that the NO synthase inhibitor aminoguanidine and the NO scavenger PTIO effectively increased the cellular response to cisplatin-induced cell death, whereas the NO donors sodium nitroprusside and dipropylentriamine NONOate had an opposite effect. The NO-modulating agents, when used alone at the indicated concentrations, had no significant effect on cell apoptosis (Fig. 1D). These results indicate that NO plays a role as a negative regulator of cisplatin-induced apoptosis. Because previous studies have shown that cisplatin induces cell death via an ROS-dependent mechanism (9, 30), we also tested whether ROS inhibition by the antioxidant NAC could prevent cisplatin-induced apoptosis in our cell system. Figure 1D shows that cotreatment of the cells with

cisplatin and NAC completely inhibited the apoptotic effect of cisplatin. Likewise, cotreatment of the cells with caspase inhibitor zVAD-fmk completely inhibited the apoptotic effect of cisplatin, indicating the requirement of caspase activation in this process. These results suggest that both ROS and NO play an important role in cisplatin-induced apoptosis through a caspase-dependent mechanism. However, ROS serve as a positive regulator, whereas NO is a negative regulator of cisplatin-induced apoptosis.

Modulation of NO and ROS levels in H-460 cells. To provide a relationship between the apoptotic response and NO/ROS modulation by the test agents, we treated the cells with cisplatin and NO/ROS modulators and studied their effects on intracellular NO/ROS levels by flow cytometry using the NO fluorescent probe DAF-DA and the ROS probe DCF-DA. Figure 2A shows that the NO donor sodium nitroprusside significantly increased the cellular level of NO, whereas the NO inhibitor aminoguanidine inhibited it,

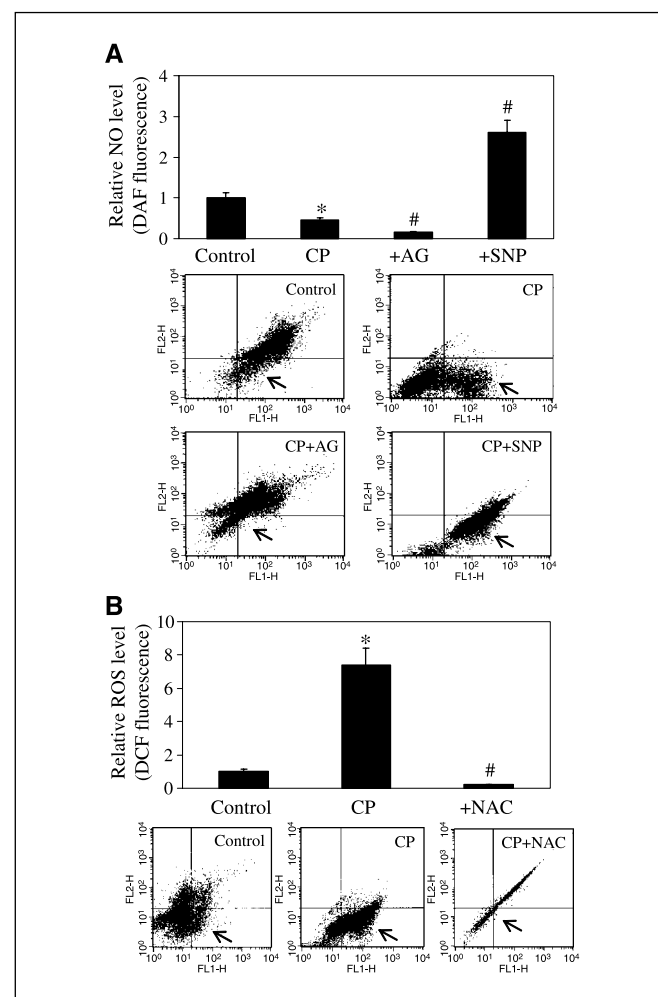


Figure 2. Effects of cisplatin and NO/ROS modulators on cellular NO and ROS levels. *A*, subconfluent (90%) monolayers of H-460 cells were pretreated with aminoguanidine (AG; 300 $\mu\text{mol/L}$) or sodium nitroprusside (SNP; 500 $\mu\text{g/mL}$) for 1 hour, after which they were treated with cisplatin (CP; 200 $\mu\text{mol/L}$) and analyzed for NO levels by flow cytometry using the fluorescent probe DAF-DA as described in Materials and Methods. The plots show peak NO response at 1 hours post-cisplatin treatment. Representative results with the corresponding region indicated by an arrow. *B*, cells were pretreated with the ROS scavenger NAC (1 mmol/L) for 1 hour and then exposed to cisplatin (200 $\mu\text{mol/L}$) for 1 hour. ROS production was determined by flow cytometry using the fluorescent oxidative probe DCF-DA. Columns, mean ($n = 4$); bars, SD. *, $P < 0.05$ versus nontreated control; #, $P < 0.05$ versus cisplatin-treated control.

as indicated by the corresponding changes in DAF fluorescence intensity. The antioxidant NAC completely inhibited cisplatin-induced ROS generation as shown by the decrease in DCF fluorescence intensity (Fig. 2B). Treatment of the cells with cisplatin alone had an inhibitory effect on cellular NO level but increased the ROS level over nontreated controls (Fig. 2A and B). These results support our earlier finding and indicate the regulatory role of NO and ROS in cisplatin-induced cell death.

Bcl-2 expression determines cell death resistance to cisplatin in H-460 cells. Aberrant expression of the antiapoptotic Bcl-2 protein has been shown to mediate cisplatin resistance in many cell types (9–12). To test whether Bcl-2 is involved in apoptosis resistance to cisplatin in H460 cells, we stably transfected the cells with Bcl-2 or control plasmid, and their effect on cisplatin-induced apoptosis was determined. Figure 3A shows that overexpression of Bcl-2 significantly inhibited apoptosis over a wide concentration range of cisplatin treatment compared with mock transfection controls. Western blot analysis of Bcl-2 shows an increased expression of the protein in Bcl-2-transfected cells but not in mock-transfected cells (Fig. 3B). These results indicate the role of Bcl-2 in cell death resistance to cisplatin.

NO modulates Bcl-2 expression in H-460 cells. Having shown the role of NO and Bcl-2 in cisplatin-induced apoptosis, we next investigated the potential regulation of Bcl-2 by NO in H-460 cells. Cells were treated with cisplatin in the presence or absence of NO modulators and Bcl-2 expression was determined by immunoblotting after 12 hours. Figure 4A shows that cisplatin treatment caused a significant decrease in Bcl-2 expression, which was further decreased upon the addition of the NO inhibitors aminoguanidine and PTIO. In contrast, treatment of the cells with the NO donor sodium nitroprusside or dipropylentriamine NONOate completely inhibited cisplatin-induced Bcl-2 down-regulation and further increased the Bcl-2 expression over nontreated control level. The modulating effect

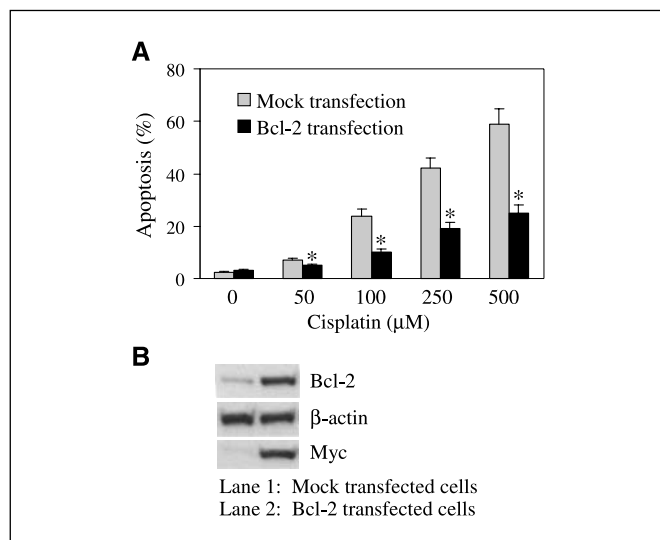


Figure 3. Bcl-2 overexpression increases cell death resistance to cisplatin. *A*, H-460 cells were stably transfected with a myc-tagged Bcl-2 plasmid or a control pcDNA3 plasmid as described in Materials and Methods. Transfected cells were treated with increasing doses of cisplatin (0–500 µmol/L) for 12 hours, and apoptosis was determined by Hoechst 33342 assay. *, $P < 0.05$ versus mock transfection. *B*, Western blot analysis of Bcl-2 expression in mock and Bcl-2 transfected H-460 cells. Cell extracts were prepared and separated on 10% polyacrylamide-SDS gels, transferred, and probed with Bcl-2 and myc antibodies. β-Actin was used as a loading control.

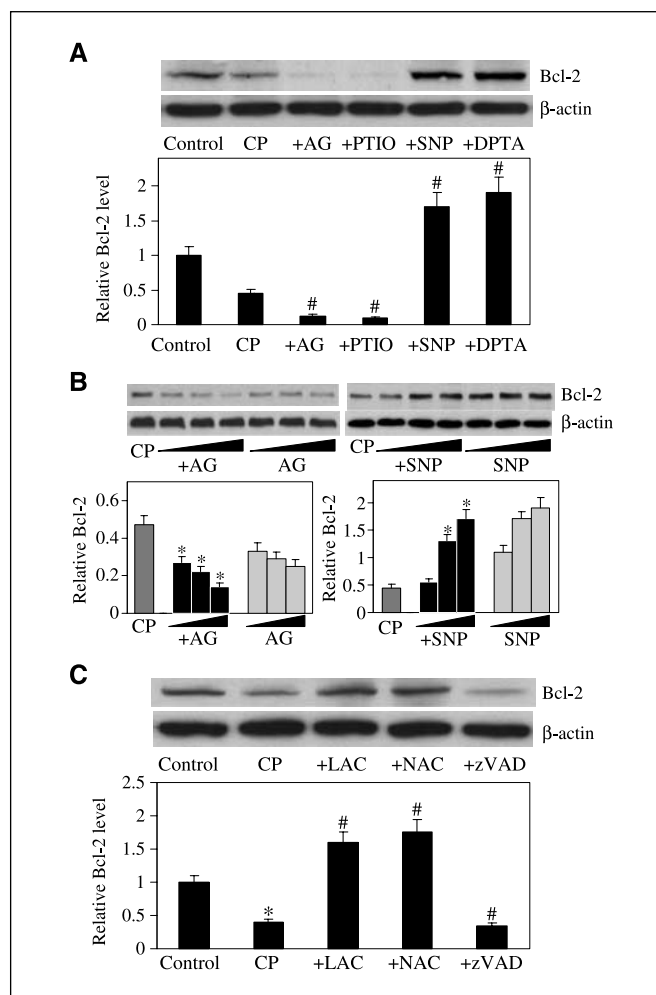


Figure 4. NO inhibits cisplatin-induced Bcl-2 down-regulation. *A*, subconfluent monolayers of H-460 cells were pretreated for 1 hour with aminoguanidine (AG; 300 µmol/L), PTIO (300 µmol/L), sodium nitroprusside (SNP; 500 µg/ml), or dipropylentriamine (DPTA) NONOate (200 µmol/L). The cells were then treated with cisplatin (CP; 200 µmol/L) for 12 hours, and cell extracts were prepared and analyzed for Bcl-2 by immunoblotting. 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 cisplatin (control). *B*, dose effect of NO modulators on Bcl-2 expression. Cells were pretreated with varying doses of aminoguanidine (100, 200, and 300 µmol/L) or sodium nitroprusside (100, 250, and 500 µg/mL) for 1 hour, after which they were either left untreated or treated with cisplatin (200 µmol/L) for 12 hours. Cell lysates were prepared and analyzed for Bcl-2 expression by immunoblotting. *C*, cells were pretreated with lactacystin (20 µmol/L), NAC (1 mmol/L), or zVAD-fmk (10 µmol/L) for 1 hour followed by cisplatin treatment (200 µmol/L) for 12 hours. Columns, mean ($n = 4$); bars, SD. *, $P < 0.05$ versus nontreated control; #, $P < 0.05$ versus cisplatin-treated control.

of NO on Bcl-2 expression was dose dependent as shown by the gradual decrease and increase in Bcl-2 levels upon treatment with the increasing doses of the NO inhibitor aminoguanidine and NO donor sodium nitroprusside, respectively (Fig. 4B).

Because previous studies have shown that Bcl-2 is rapidly down-regulated by proteasomal degradation via an ROS-dependent pathway (9, 17), we therefore investigated whether Bcl-2 down-regulation by cisplatin is also mediated by this pathway. Cells were treated with lactacystin, a highly specific proteasome inhibitor, and its effect on cisplatin-induced Bcl-2 down-regulation was examined by immunoblotting. Figure 4C shows that lactacystin completely

inhibited Bcl-2 down-regulation by cisplatin, indicating the dominant role of proteasomal degradation in the down-regulation process. The result was confirmed by the observation that another proteasome inhibitor (MG132) also inhibited the decrease in Bcl-2 expression caused by cisplatin (data not shown). Figure 4B also shows that the antioxidant NAC was able to inhibit Bcl-2 down-regulation by cisplatin, supporting the role of ROS in the degradation process.

Bcl-2 has also been reported to be enzymatically cleaved by caspase-3 in some cells (31). To test whether this process might be involved in cisplatin-induced Bcl-2 degradation in H-460 cells, we treated the cells with caspase inhibitor zVAD-fmk and studied its effect on Bcl-2 expression by immunoblotting. Figure 4C shows that the caspase inhibitor was unable to inhibit Bcl-2 down-regulation induced by cisplatin, indicating the insignificant role of this enzyme in cisplatin-induced Bcl-2 degradation.

NO does not affect Bcl-2 phosphorylation in H-460 cells. Bcl-2 stability is mainly regulated by phosphorylation with the Ser⁸⁷ phosphorylation playing a dominant role in this process (16, 17). Apoptotic stimuli, such as TNF- α and ROS, induce dephosphorylation of Ser⁸⁷, which promotes proteasomal degradation of Bcl-2 (9, 16, 17). To investigate the potential mechanism of NO regulation of Bcl-2, we tested the effect of NO modulators on Bcl-2 phosphorylation in cisplatin-treated cells. As control, cells were treated with the ROS scavenger NAC, and its effect on Bcl-2 phosphorylation was determined by Western blotting. The results show that cisplatin strongly induced Bcl-2 dephosphorylation at Ser⁸⁷, and NAC completely inhibited this dephosphorylation (Fig. 5A). In contrast, neither of the NO donors (sodium nitroprusside and dipropyleneetriamine NONOate) nor NO inhibitors (aminoguanidine and PTIO) was able to significantly affect this dephosphorylation (Fig. 5A). These results suggest that NO regulates Bcl-2 stability via a different mechanism from ROS, and that this regulation is phosphorylation-independent.

NO prevents cisplatin-induced ubiquitination of Bcl-2. To further investigate the mechanism by which NO protects cisplatin-induced Bcl-2 degradation, we analyzed ubiquitination of Bcl-2 in response to cisplatin treatment by immunoprecipitation. Cells were cotransfected with myc-tagged Bcl-2 and ubiquitin expression plasmids, and 36 hours later, they were treated with cisplatin in the presence or absence of NO donors or inhibitors. After the treatments, cell lysates were prepared and immunoprecipitated using an anti-myc antibody. The resulting immune complexes were then analyzed for ubiquitin by Western blot using an anti-ubiquitin antibody. The results show that cisplatin was able to induce ubiquitination of Bcl-2 in a dose-dependent manner, and that the NO inhibitors aminoguanidine and PTIO increased this ubiquitination (Fig. 5B). In contrast, the NO donors sodium nitroprusside and dipropyleneetriamine NONOate completely inhibited ubiquitination of Bcl-2 (Fig. 5C). These results along with our earlier phosphorylation results indicate that NO inhibited ubiquitination of Bcl-2 through a phosphorylation-independent process.

NO induces S-nitrosylation of Bcl-2 and inhibits its ubiquitination. Increasing evidence indicates that NO plays an important role in apoptosis through S-nitrosylation of several key proteins that regulate the apoptosis pathways (32, 33). To determine whether NO could nitrosylate Bcl-2, which has not been shown, and whether this process could affect Bcl-2 stability, we did immunoprecipitation experiments evaluating the effect NO on Bcl-2 S-nitrosylation. Cells expressing ectopic myc-Bcl-2 were treated with cisplatin and NO modulators as described, and cell lysates

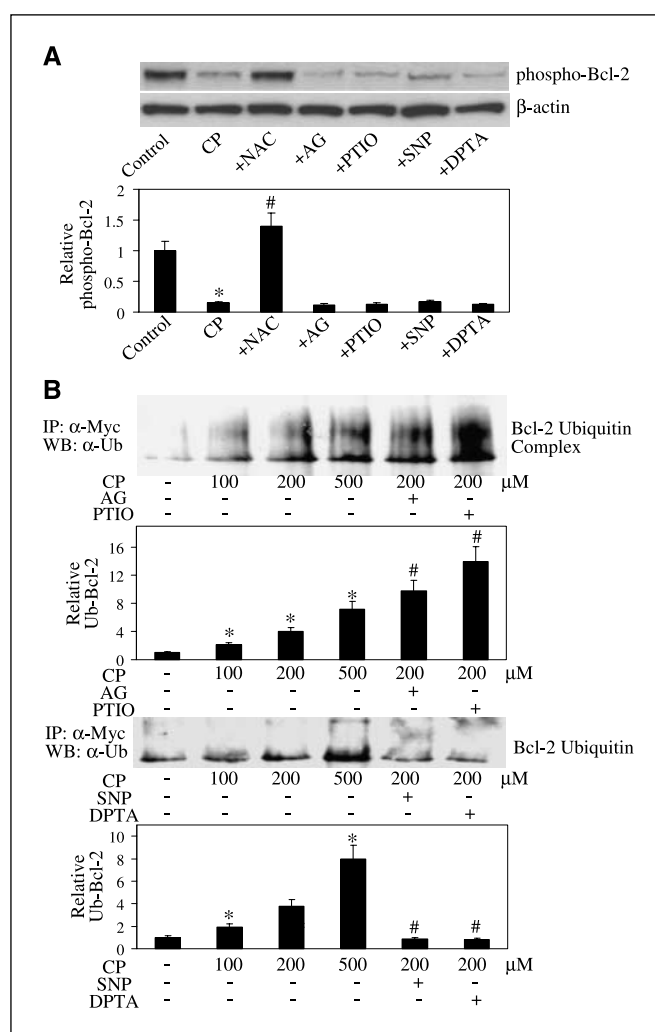


Figure 5. Effects of NO modulators on Bcl-2 phosphorylation and ubiquitination. **A**, subconfluent monolayers of H-460 cells were pretreated for 1 hour with sodium nitroprusside (SNP; 500 μ g/mL), dipropyleneetriamine (DPTA) NONOate (200 μ mol/L), aminoguanidine (AG; 300 μ mol/L), PTIO (300 μ mol/L), or NAC (1 mmol/L). The cells were then treated with cisplatin (CP; 200 μ mol/L) for 12 hours, and cell lysates were prepared and analyzed for Bcl-2 phosphorylation using phospho-specific Bcl-2 (Ser⁸⁷) antibody. Densitometry was done to determine the relative levels of Bcl-2 phosphorylation after reprobing with β -actin antibody. **B**, H-460 cells were transiently transfected with ubiquitin and myc-tagged Bcl-2 plasmids. Thirty-six hours later, the cells were pretreated with the same concentrations of NO modulators for 1 hour followed by cisplatin treatment in the presence of lactacystin (10 μ mol/L). 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 2 hours post-cisplatin treatment, where ubiquitination was found to be maximal. Columns, mean ($n = 4$); bars, SD. *, $P < 0.05$ versus nontreated control; #, $P < 0.05$ versus cisplatin-treated control.

were immunoprecipitated and analyzed by Western blot using an anti-S-nitrosocysteine antibody. Figure 6A shows that treatment of the cells with cisplatin resulted in a substantial reduction in S-nitrosylated Bcl-2 levels, and cotreatment of the cells with the NO donor sodium nitroprusside or dipropyleneetriamine NONOate reversed this effect. Because S-nitrosylation by NO has been reported to be inhibited by strong reducing agents, such as DTT (34, 35), we further tested whether DTT could prevent the effect of NO on Bcl-2 S-nitrosylation and ubiquitination. The results show that DTT was able to prevent S-nitrosylation of Bcl-2 (Fig. 6A) and the ubiquitination effect of NO donors in cisplatin-treated cells

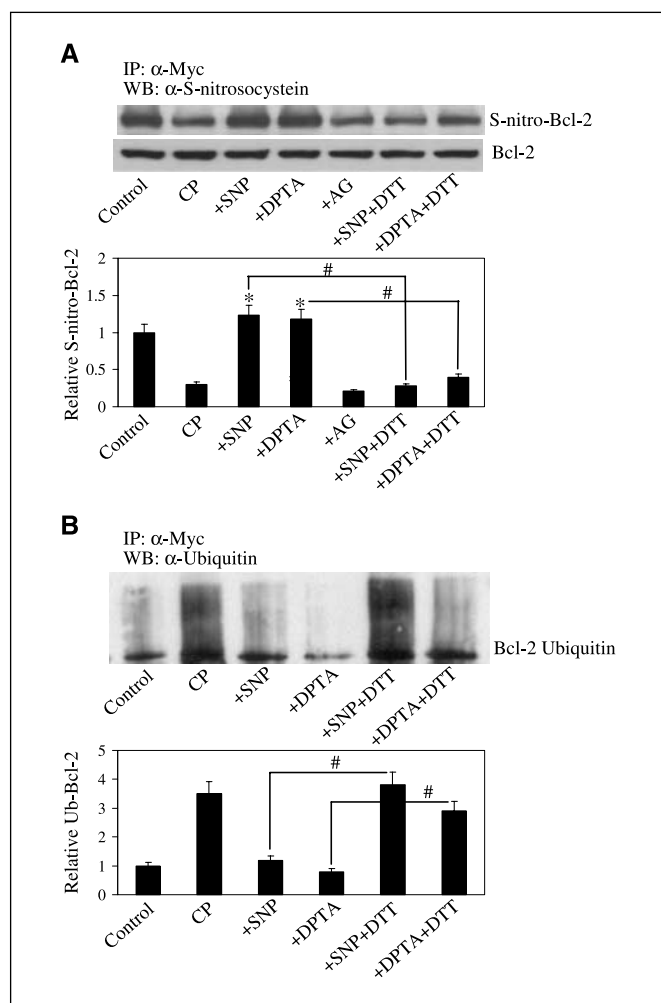


Figure 6. NO induces S-nitrosylation of Bcl-2 and inhibits its ubiquitination by cisplatin. **A**, effect of NO modulators on cisplatin-induced S-nitrosylation of Bcl-2. H-460 cells were transiently transfected with myc-tagged Bcl-2 plasmid. Thirty-six hours later, the cells were pretreated with sodium nitroprusside (SNP; 500 μ g/mL), dipropyleneetriamine (DPTA) NONOate (200 μ mol/L), or aminoguanidine (AG; 300 μ mol/L) in the presence or absence of DTT (10 mmol/L) for 1 hour. The cells were then treated with cisplatin (CP; 200 μ mol/L) for 2 hours, 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 done to determine the relative S-nitrosocysteine levels after reprobing of the membranes with Bcl-2 antibody. **B**, effect of NO modulators on cisplatin-induced Bcl-2 ubiquitination. Cells were cotransfected with ubiquitin and myc-Bcl-2 plasmids. Thirty-six hours later, they were treated with the indicated test agents in the presence of lactacystin (10 μ mol/L). Cell lysates were then immunoprecipitated with anti-myc antibody, and the immune complexes were analyzed for ubiquitin. Columns, mean ($n = 4$); bars, SD. *, $P < 0.05$ versus cisplatin-treated control; #, $P < 0.05$ versus NO-modulated controls.

(Fig. 6B). These results indicate that S-nitrosylation might be a key mechanism used by NO to regulate ubiquitination and proteasomal degradation of Bcl-2.

Discussion

The antiapoptotic function of Bcl-2 is tightly associated with its expression levels, and amplification of this protein has been linked to resistance to cell death induced by various DNA-damaging agents, including those used in cancer chemotherapy. Aberrant expression of Bcl-2 has been shown to mediate cisplatin resistance

in many types of tumor cells (9–12). Although the importance of gene expression in regulating apoptotic signal transduction has been emphasized in numerous studies, posttranslational modifications, such as ubiquitination and phosphorylation, have emerged as important regulators of Bcl-2 function (9, 16, 17, 36). However, the mechanisms underlying this regulation and in particular those relevant to cisplatin resistance have not been clearly elucidated. Our results show that treatment of human lung carcinoma H-460 cells with cisplatin resulted in a down-regulation of Bcl-2 and concomitant increase in apoptotic cell death (Figs. 1A and 4A). Down-regulation of Bcl-2 was associated with an increase in ubiquitination and proteasomal degradation of the protein (Figs. 4B and 5B). Overexpression of Bcl-2 protected the cells from cisplatin-induced cell death (Fig. 3), supporting the role of Bcl-2 in cisplatin resistance.

Multiple mechanisms of cisplatin resistance in tumor cells have been proposed, including impaired cellular uptake and increased cellular efflux of cisplatin (37), increased DNA lesion repair (38, 39), and defects in mismatch repair that fail to trigger cell death (40–42). Intracellular cisplatin inactivation by redox reactions has also been proposed as a mechanism of cisplatin resistance (43, 44). Increased ROS generation by glutathione depletion or by superoxide dismutase antisense transfection has been shown to prevent Bcl-2-mediated cisplatin resistance (9, 44). Conversely, inhibition of ROS by antioxidants has been shown to increase cisplatin resistance (45). Glutathione can also detoxify cisplatin through the formation of glutathione adducts (43). Cellular glutathione levels can be augmented by NAC, which is converted intracellularly to the rate-limiting substrate for glutathione synthesis. Increased cisplatin resistance by NAC may involve glutathione-cisplatin adduct formation and a corresponding decrease in cisplatin-mediated DNA damage. Alternatively, glutathione may interfere with the apoptotic process through its ability to act as a redox buffer. Indeed, increased glutathione levels have been shown to increase apoptosis resistance to cisplatin through an ROS scavenging mechanism that is independent of cisplatin-DNA adduct formation or repair (44).

Increasing evidence has also shown that NO plays a role in apoptosis regulation through its ability to modulate ROS; that is, NO can interact with superoxide anion to form peroxynitrite (46, 47) and to modify key apoptosis-regulatory proteins through S-nitrosylation (32, 33). However, whether or not NO can nitrosylate Bcl-2 and whether it plays a role in cisplatin resistance have not been shown. In this study, we found that NO can nitrosylate Bcl-2 and prevent its degradation through the ubiquitin-proteasomal pathway. Addition of the NO donors sodium nitroprusside and dipropyleneetriamine NONOate increased Bcl-2 S-nitrosylation (Fig. 6A), decreased its ubiquitination and proteasomal degradation (Figs. 4 and 5), and increased cell death resistance to cisplatin (Fig. 1B). In contrast, the NO inhibitors aminoguanidine and PTIO showed opposite effects, thus confirming the role of NO in Bcl-2-mediated cisplatin resistance. It should be noted that although our results indicate the nitrosylation of Bcl-2 by NO, such nitrosylation may not be the only essential NO-mediated modification of apoptosis regulatory proteins that suppresses cisplatin-induced cell death.

The mechanism by which S-nitrosylation prevents ubiquitination of Bcl-2 is unclear but may involve conformational change of S-nitrosylated Bcl-2 protein, which prevents its recognition and subsequent attachment of ubiquitin by the enzyme ubiquitin ligases. Conformational changes of Bcl-2 by phosphorylation have been reported to affect its ubiquitination and stability (48). Moreover,

dephosphorylation of Bcl-2 by ROS and TNF- α has been shown to be required for the ubiquitination and degradation of Bcl-2 (9, 16, 17). Our results on the inhibitory effects of the ROS scavenger NAC on Bcl-2 dephosphorylation and degradation (Figs. 4 and 5) support this finding and further indicate the involvement of ROS in cisplatin-induced Bcl-2 instability. Unlike ROS, however, we found that NO had no effect on Bcl-2 phosphorylation (Fig. 5), whereas it exhibited strong effects on ubiquitination and degradation (Figs. 4 and 5). These results indicate that dephosphorylation of Bcl-2 might not be a necessary event for the triggering of ubiquitination, and that NO and ROS may regulate Bcl-2 stability via different mechanisms. Alternatively, ROS, through its ability to interact with NO, may decrease the availability and thus nitrosylation activity of NO, which results in increased Bcl-2 ubiquitination. The ability of the reducing agent DTT to inhibit both *S*-nitrosylation and ubiquitination of Bcl-2 (Fig. 5) further supports the role of *S*-nitrosylation and its regulation by ROS in the ubiquitination process.

Bcl-2 has been shown to be enzymatically cleaved by caspase-3 in some cells (31), and NO could inhibit this process by *S*-nitrosylation of the enzyme (49). However, our caspase inhibition study failed to detect the inhibitory effect of the caspase inhibitor zVAD-fmk on Bcl-2 expression (Fig. 4B), although this inhibitor completely inhibited cisplatin-induced apoptosis (Fig. 1D). The likely explanation for the observed discrepancy may be the difference in experimental conditions and cell type used. In Jurkat cells and prostate cancer PC-3 cells, caspase-3 inhibitor was also unable to prevent Bcl-2 cleavage, whereas proteasome inhibitors were found to prevent Bcl-2 degradation (36). In H-460 cells, caspase-mediated Bcl-2 cleavage was observed but only after 12 hours of drug treatment, whereas proteasome-mediated cleavage was also observed at earlier times (48). It was suggested that during

the early apoptosis process, caspase activity was still low and insufficient to affect Bcl-2 stability, whereas in late apoptosis, caspase activity was elevated and was able to induce Bcl-2 cleavage. Our results on the ubiquitination and proteasomal degradation of Bcl-2 and the lack of caspase-mediated cleavage at the early time period (>12 hours) support the previous finding and indicate that proteasomal degradation is a key early event in controlling Bcl-2 stability.

In summary, our data provide evidence that NO plays an important role in regulating cell death resistance to cisplatin. Cisplatin induces down-regulation of Bcl-2 through proteasome-mediated degradation. NO negatively regulates this process through its ability to nitrosylate the protein and inhibit its ubiquitination. In showing the *S*-nitrosylation of Bcl-2, we document a novel layer of regulation that links NO signaling with Bcl-2-mediated chemoresistance, which represents an important mechanism in the control of tumor development and progression. 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 tumor growth through *S*-nitrosylation. This finding on the novel function of NO in Bcl-2 regulation may have important implications in cancer chemotherapy and prevention.

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