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Peroxide Is a Key Mediator of Bcl-2 Down-Regulation and Apoptosis Induction by Cisplatin in Human Lung Cancer Cells

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ABSTRACT

Susceptibility to apoptosis is an essential prerequisite for successful eradication of tumor cells by chemotherapy. Consequently, resistance to apoptosis has been established as one of the mechanisms responsible for the failure of therapeutic approaches in many types of cancers. In the present study, we investigated the susceptibility of human lung cancer H460 cells to apoptotic cell death induced by cisplatin and determined its regulatory mechanisms. Treatment of the cells with cisplatin induced rapid generation of multiple oxidative species and a concomitant increase in apoptotic cell death. Apoptosis induced by cisplatin was mediated through the mitochondrial death pathway, which requires caspase-9 activation and is regulated by Bcl-2. Cisplatin induced down-regulation of Bcl-2 through a process that involves dephosphorylation and ubiquitination of the protein, which facilitates its degradation by proteasome. This down-regulation was inhibited by antioxidant enzymes catalase and glutathione peroxidase (H_2O_2 scavenger), but not by superoxide dismutase ($O_2^{\cdot-}$ scavenger) or deferoxamine ($OH^{\cdot}$ inhibitor). Electron spin resonance and flow cytometric analyses showed the formation of H_2O_2 along with $O_2^{\cdot-}$ and $OH^{\cdot}$ radicals after cisplatin treatment. H_2O_2 was generated in part by dismutation of $O_2^{\cdot-}$ and served as a precursor for $OH^{\cdot}$. Together, our results indicate an essential role of H_2O_2 in the regulation of Bcl-2 and apoptotic cell death induced by cisplatin. Because aberrant expression of Bcl-2 has been associated with death resistance of cancer cells to chemotherapy, the results of this study could be used to aid the design of more effective strategies for cancer treatment.

Cisplatin (*cis*-diamminedichloroplatinum) is a commonly used chemotherapeutic agent against a wide range of human cancers; however, cancer cell-acquired resistance to cisplatin-induced apoptosis is a major problem limiting its effective use (Kartalou and Essigmann, 2001). Induction of reactive oxygen species (ROS) generation by cisplatin is considered to be one of the key mechanisms of apoptotic cell death (Choi et al., 2004; Li et al., 2004). However, the mechanisms by which ROS mediate the apoptotic effect of cisplatin and the identity of specific ROS involved in the process are unclear. Cisplatin has been shown to induce apoptosis through the mitochondrial (intrinsic) death pathway, although the death receptor (extrinsic) pathway has been shown to be activated in some cell types (Kamarajan et al., 2003; Wu et al., 2005). The intrinsic death pathway involves the release of cytochrome *c* from the mitochondria, which triggers caspase activation through caspase-activating proteins such as Apaf-1 (Li et al., 1997; Zou et al., 1999). Bcl-2 expression has been shown to interfere with the release of cytochrome *c* or with its binding to Apaf-1, thus preventing apoptosome formation and downstream apoptosis signaling (Newmeyer and Ferguson-Miller, 2003). Bcl-2 expression is tightly regulated by different mechanisms, including transcription, heterodimerization, and degradation. Degradation of Bcl-2 is mediated primarily through the ubiquitin-proteasome pathway (Dimmeler et al., 1999; Chanvorachote et al., 2006), which plays an important role in apoptosis induced by various chemotherapeutic agents and pathological stimuli (Haendeler et al., 1996; Paradis et al., 1996; Ueta et al., 1999).

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ABBREVIATIONS: cisplatin, *cis*-diamminedichloroplatinum; ROS, reactive oxygen species; ESR, electron spin resonance; GPx, glutathione peroxidase; CAT, catalase; SOD, superoxide dismutase; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DCFH-DA, 2′,7′-dichlorodihydro-fluorescein diacetate; DHE, dihydroethidine; PBS, phosphate-buffered saline; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; ELISA, enzyme-linked immunosorbent assay; AMC, amino-4-methyl coumarin; z-LEHD-fmk, *N*-benzyloxycarbonyl-Leu-Glu-His-Asp-fluoromethyl ketone; z-IETD-fmk, *N*-benzyloxycarbonyl-Ile-Glu-Thr-Asp-fluoromethyl ketone.

Mitochondria are a major source of ROS production and are involved in the regulation of Bcl-2 and apoptotic cell death induced by various stimuli (Hildeman et al., 2003; Moungjaroen et al., 2006). Antioxidants and thiol reductants such as *N*-acetyl cysteine and thioredoxin have been shown to block or delay apoptosis by sustaining Bcl-2 levels (Iwata et al., 1997; Chanvorachote et al., 2006). Increased Bcl-2 expression levels have been also linked to cisplatin resistance in many cancer cell types (Yang et al., 2004; Kausch et al., 2005). These findings, along with the evidence that increased expression of antioxidant enzymes is correlated with reduced effectiveness of cisplatin therapy (Nishimura et al., 1998; Shiga et al., 1999), have led to the hypothesis that ROS may function as key mediators of cisplatin-induced apoptosis by down-regulating Bcl-2 levels. By detoxifying ROS, antioxidants may prevent the effect of ROS on Bcl-2 and inhibit apoptosis. However, the role of specific ROS and antioxidant systems in the regulation of Bcl-2 and apoptosis induced by cisplatin is unclear. Previous studies have shown that O_2^- plays a role in Bcl-2 regulation (Ueta et al., 1999; Li et al., 2004). Down-regulation of SOD by antisense gene inhibition decreases Bcl-2 expression and increases cell death induced by cisplatin (Ueta et al., 1999). Because SOD acts by dismutation of O_2^- to form H_2O_2 and because H_2O_2 is a major source of $OH^\cdot$ production (i.e., via Fenton-like reactions), it is possible that other oxidative species may play a role. Indeed, our study shows that H_2O_2 and $OH^\cdot$ are induced along with O_2^- in cisplatin-treated cells, supporting this possibility and the potential association between these oxidative species.

The present study was undertaken to clarify the role of specific ROS and antioxidant systems in Bcl-2 regulation and apoptosis induction by cisplatin. We also determined the mechanisms by which these ROS regulate Bcl-2 expression and apoptotic response to cisplatin treatment.

Materials and Methods

Cells and Reagents. Human lung epithelial H460 cells were obtained from American Type Culture Collection (Manassas, VA). The cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37°C in a 5% CO_2/95% air incubator. Cisplatin, lactacystin, and deferoxamine were obtained from Sigma Chemical Co. (St. Louis, MO). Superoxide dismutase and catalase were from Roche Diagnostics (Indianapolis, IN). Dihydroethidium bromide (DHE), dichlorodihydrofluorescein diacetate (DCFH-DA), and Hoechst 33342 were from Invitrogen. Caspase-8/FADD-like IL-1β-converting enzyme fluorometric substrate (7-amino-4-trifluoromethyl coumarin), caspase-9/Mch6 fluorometric substrate (Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin), caspase-8 inhibitor (z-IETD-fmk), and caspase-9 inhibitor (z-LEHD-fmk) were from Alexis Biochemicals (San Diego, CA). Antibodies for Bcl-2, phospho-Bcl-2 (Ser87), myc, and peroxidase-labeled secondary antibodies and protein A-agarose were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for ubiquitin and β-actin were from Sigma. The transfecting agent Lipofectamine was obtained from Invitrogen.

Plasmids and Transfection. The Bcl-2, GPx, and SOD1 plasmids were generously provided by Dr. Christian Stehlik (Northwestern University, School of Medicine, Chicago, IL). Authenticity of all plasmid constructs was verified by DNA sequencing. Stable transfectants of Bcl-2, GPx, and SOD were generated by culturing H460 cells in a six-well plate until they reached 80% confluence. One microgram of cytomegalovirus-neo vector and 15 μl of Lipofectamine reagent with 2 μg of Bcl-2, GPx, SOD, or control pcDNA3 plasmid were used to transfect the cells in the absence of serum. After 10 h, the medium was replaced with culture medium containing 5% fetal bovine serum. Approximately 36 h after the beginning of the transfection, the cells were digested with 0.03% trypsin and the cell suspensions were plated onto 75-ml culture flasks and cultured for 24 to 28 days with G418 selection (400 μg/ml). Individual drug-resistant colonies were isolated using cloning cylinders (Bellco Glass, Vineland, NJ) and transferred for expansion and analysis by Western blotting. Stable transformants were grown in G418-free RPMI medium for at least two passages before each experiment.

Apoptosis and Caspase Activity Assays. Apoptosis was determined by Hoechst 33342 assay (Molecular Probes) and ELISA-based DNA fragmentation assay using a kit from Roche Molecular Biochemicals (Indianapolis, IN). For Hoechst assay, cells were incubated with 10 μg/ml Hoechst 33342 for 30 min, and scoring the percentage of cells having intensely condensed chromatin and/or fragmented nuclei was done by fluorescence microscopy (Axiovert; Carl Zeiss, Göttingen, Germany) using Pixera software. For ELISA assay, cells were lysed with DNA lysis buffer (200 μl), and the cell lysate (20 μl) was mixed with an antibody solution provided by the supplier (80 μl) in 96-well plates at room temperature for 2 h. After washing with the incubation buffer, the substrate buffer (100 μl) was added to each well and incubated for 10 min at 37°C. Optical density was then measured using a microplate reader at a wavelength of 405 nm.

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 aspartic acid residue to release the fluorescent leaving group, amino-4-methyl coumarin (AMC). Cell extracts containing 20 μg of protein were incubated with 100 mM HEPES containing 10% sucrose, 10 mM dithiothreitol, 0.1% CHAPS, and 50 μM caspase substrate in a total reaction volume of 250 μl. The reaction mixture was incubated for 2 h at 37°C. At the end of incubation, the liberated fluorescent group AMC was determined fluorometrically (RF-531PC spectrofluorometer; Shimadzu, Kyoto, Japan) at the excitation and emission wavelengths of 380 nm and 460 nm, respectively.

ROS Detection. The ESR spin trapping technique with DMPO as the spin trap was used to detect short-lived free radical generation. The intensity of the spin adduct signal was used to measure the amount of short-lived radicals trapped, and the hyperfine couplings of the spin adduct were generally characteristics of the original trapped radicals. All ESR measurements were conducted using a Varian E9 ESR spectrometer and a flat cell assembly (Varian Inc., Palo Alto, CA). Reactants were mixed in a test tube in a final volume of 500 μl. The reaction mixture was then transferred to a flat cell for measurement. Hyperfine couplings were measured (to 0.1 G) directly from magnetic field separation using potassium tetraperoxochromate and 1,1-diphenyl-2-picrylhydrazyl as reference standards. The software EPRDAP, version 2.0, was used for data acquisition and analysis.

Flow cytometric analyses of superoxide and hydroperoxide generation were performed using DHE and DCFH-DA as fluorescent probes, respectively. Cells (1×10^6/ml) were incubated with the probes (10 μM) for 30 min at 37°C, after which they were washed, resuspended in phosphate-buffered saline (PBS), and analyzed for fluorescence intensity using FACSCalibur (BD Biosciences, San Jose, CA) at the excitation and emission wavelengths of 488 nm and 538 nm for DCF measurements and at 488 nm and 610 nm for DHE measurements, respectively. The median fluorescence intensity was quantitated by CellQuest software (BD Biosciences, San Jose, CA) analysis of the recorded histograms.

Western Blot Analysis. Cell extracts were performed by incubating the cells in lysis buffer containing 20 mM Tris-HCl, pH 7.5, 1% Triton X-100, 10% glycerol, 150 mM sodium chloride, 1 mM sodium orthovanadate, 50 mM sodium fluoride, 100 mM phenylmethylsulfonyl fluoride, and a commercial protease inhibitor mix-

ture (Roche) for 20 min on ice. After insoluble debris was pelletted by centrifugation at 14,000*g* for 15 min at 4°C, the supernatants were collected and determined for protein content using bicinchoninic acid assay. Proteins (40 μg) were resolved on a 10% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). The transferred membranes were blocked for 1 h in 5% nonfat dry milk in 25 mM Tris-HCl, pH 7.4, 125 mM NaCl, and 0.05% Tween 20 and incubated with the appropriate primary antibodies at 4°C overnight. Membranes were washed three times with 25 mM Tris-HCl, pH 7.4, 125 mM NaCl, and 0.05% Tween 20 for 10 min and incubated with horseradish peroxidase-coupled isotype-specific secondary antibodies for 1 h at room temperature. The immune complexes were detected by enhanced chemiluminescence (Supersignal West Pico; Pierce, Rockford, IL) and quantified using analyst/PC densitometry software (Bio-Rad Laboratories). Mean densitometry data from independent experiments were normalized to the results in control cells. The data were presented as the mean ± S.D. and analyzed by the Student's *t* test.

Immunoprecipitation. Cells were washed after treatment with ice-cold PBS and lysed in lysis buffer at 4°C for 30 min. After centrifugation at 14,000*g* for 15 min at 4°C, the supernatants were collected and determined for protein content. Cell lysates containing 60 μg of protein were incubated with 12 μl of anti-myc agarose bead (Santa Cruz Biotechnology) diluted with 12 μl of G-protein bead for 6 h at 4°C. The immune complexes were then washed three times with 20 volumes of lysis buffer, resuspended in 2× Laemmli sample buffer, and heated at 95°C for 5 min. Immunoprecipitates containing 20 μg of protein were separated by 10% SDS-polyacrylamide gel electrophoresis and analyzed by Western blot as described.

Results

Cisplatin Induced Apoptosis of H460 Cells through Mitochondrial Death Pathway. To study the apoptosis pathway and cell death response to cisplatin treatment in human lung epithelial H460 cells, we treated the cells with various concentrations of cisplatin (0–100 μM) and determined their caspase activities and apoptotic response by fluorescence and ELISA-based assays. Caspase activity assays show that cisplatin was able to induce caspase-9 activation in a dose-dependent manner but had no significant effect on caspase-8 activity (Fig. 1A). At 100 μM, cisplatin induced approximately 8-fold increase in caspase-9 activity and a parallel increase in apoptotic cell death as indicated by Hoechst nuclear fluorescence (Fig. 1B). The apoptotic cells exhibited shrunken nuclei and chromatin condensation with intense nuclear fluorescence (Fig. 1C). ELISA results showed a similar effect of cisplatin on nuclear fragmentation (Fig. 1D). Because caspase-9 serves as the apical caspase of the intrinsic (mitochondrial) pathway of apoptosis whereas caspase-8 represents the apical caspase of the extrinsic (death receptor) pathway (Green and Reed, 1998; Wallach et al., 1999), the results of this study suggest that the mitochondrial pathway is the major pathway of apoptosis induced by cisplatin. The requirement of mitochondrial death pathway in the induction of apoptosis by cisplatin was confirmed by the observation that caspase-9 inhibitor (z-LEHD-fmk) effectively inhibited the apoptotic effect of cisplatin, whereas caspase-8 inhibitor (z-IETD-fmk) was ineffective (Fig. 1, B–D).

Overexpression of Bcl-2 Protected Cells from Cisplatin-Induced Apoptosis. The mitochondrial death pathway is known to be regulated by the antiapoptotic Bcl-2 protein (Green and Reed, 1998). To test whether this protein is also involved in the regulation of apoptosis induced by cisplatin, cells were stably transfected with Bcl-2 or control plasmid, and its effect on cisplatin-induced apoptosis was determined. Western blot analysis of Bcl-2 transfected cells showed an increase in Bcl-2 expression over vector-transfected control (Fig. 2A). Apoptosis assay showed a strong reduction in apoptotic response to cisplatin treatment in Bcl-2-transfected cells compared with vector-transfected cells (Fig. 2B). Overexpression of Bcl-2 also caused a substantial reduction in caspase-9 activity induced by cisplatin (Fig. 3C). These results indicate the role of Bcl-2 as a negative regulator of cisplatin-induced apoptosis and further support the role of mitochondria in the death signaling process.

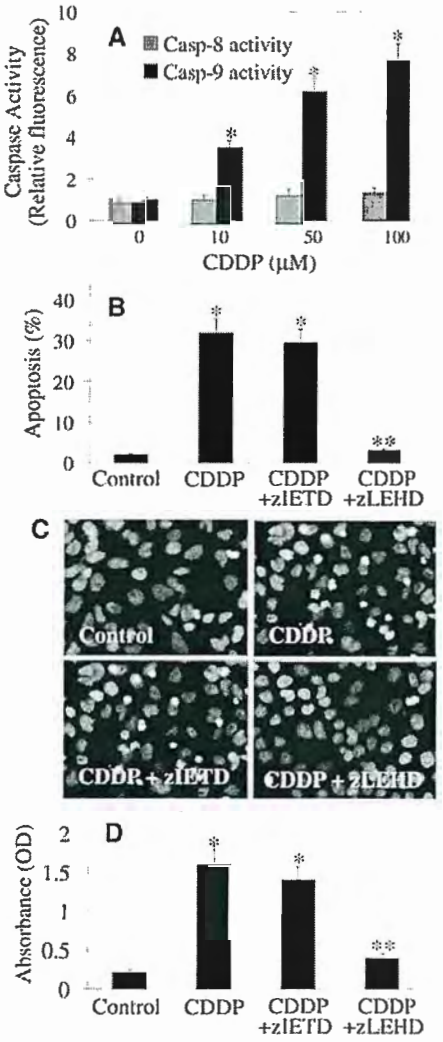

Fig. 1. Cisplatin induces caspase activation and apoptosis in human lung epithelial H460 cells. A, subconfluent (90%) monolayers of H460 cells were treated with varying concentrations of cisplatin (*cis*-diamminedichloroplatinum) (0–100 μM) for 12 h, and cell lysates (50 μg of protein) were prepared and analyzed for caspase-8 and -9 activity using the fluorometric substrate IETD-AMC and LEHD-AMC, respectively. B, cells were similarly treated with cisplatin (100 μM) in the presence or absence of caspase-8 inhibitor (z-IETD-fmk; 10 μM) or caspase-9 inhibitor (z-LEHD-fmk; 10 μM) and analyzed for apoptosis by Hoechst 33342 assay. C, fluorescence micrographs of treated cells stained with the Hoechst dye. Apoptotic cells exhibited condensed nuclei with bright nuclear fluorescence. D, cells were treated with cisplatin with or without caspase inhibitors as described in B and were analyzed for DNA nucleosomal fragmentation by ELISA. Data are mean ± S.D. ($n = 4$). *, $p < 0.05$ versus untreated control. **, $p < 0.05$ versus cisplatin-treated control.

Cisplatin Induced Bcl-2 Down-regulation through Hydrogen Peroxide. To determine the potential role of ROS in Bcl-2 regulation, the expression levels of Bcl-2 after cisplatin treatment were determined in the presence and absence of various known antioxidants. Western blot analysis of Bcl-2 showed a substantial down-regulation of the protein by cisplatin treatment (Fig. 3A). This down-regulation was completely inhibited by pretreatment of the cells with catalase (H_2O_2 scavenger) but not with SOD (O_2^- scavenger) or deferoxamine (a metal chelator that inhibits $OH^\cdot$ production via Fenton reaction) (Fig. 3B). Apoptosis assays similarly indicate that catalase was effective in inhibiting cisplatin-induced apoptosis, whereas SOD and deferoxamine were less effective (Fig. 3C). These results suggest that H_2O_2 is the primary oxidative species responsible for Bcl-2 down-regulation and apoptosis induction by cisplatin.

To confirm the role of H_2O_2 in cisplatin-induced Bcl-2 down-regulation, cells were stably transfected with the peroxide-scavenging enzyme GPx or control plasmid, and their effects on Bcl-2 expression were examined. For comparison, cells were also transfected with SOD plasmid, and its effect on Bcl-2 expression was similarly determined. The results show that transfection with GPx and SOD resulted in a corresponding increase in the antioxidant enzyme expression levels compared with vector-transfected control (Fig. 4A). GPx overexpression completely inhibited Bcl-2 down-regulation and apoptosis induced by cisplatin (Fig. 4, B and C). It also inhibited caspase-9 activation induced by the apoptogen (Fig. 4D). Overexpression of SOD had no inhibitory effect on Bcl-2 down-regulation, and exhibited a much less pronounced effect on apoptosis and caspase-9 activation compared with the GPx overexpression. These results are consistent with the antioxidant enzyme treatment studies and indicate the role of H_2O_2 as a key mediator of cisplatin-induced Bcl-2 down-regulation and apoptosis.

Because Bcl-2 has been reported to be regulated by protea-

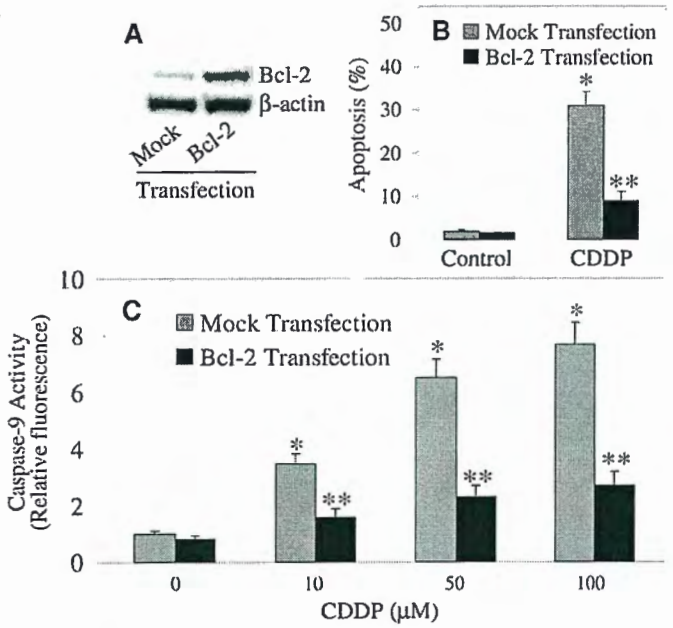

Fig. 2. Bcl-2 overexpression inhibits cisplatin-induced apoptosis and caspase activation. A, H460 cells were stably transfected with Bcl-2 or control plasmid as described under *Materials and Methods*. Cell extracts were prepared and separated on a 10% polyacrylamide-SDS gel, transferred, and probed with Bcl-2 antibody. Blots were reprobed with β-actin antibody to confirm equal loading of samples. B, transfected cells were treated with cisplatin (100 μM) for 12 h, and apoptosis was determined by Hoechst 33342 assay. C, dose effect of cisplatin on caspase-9 activation in mock and Bcl-2 transfected cells. Plots are mean ± S.D. (n = 4). *, $p < 0.05$ versus untreated control. **, $p < 0.05$ versus cisplatin-treated mock transfectant.

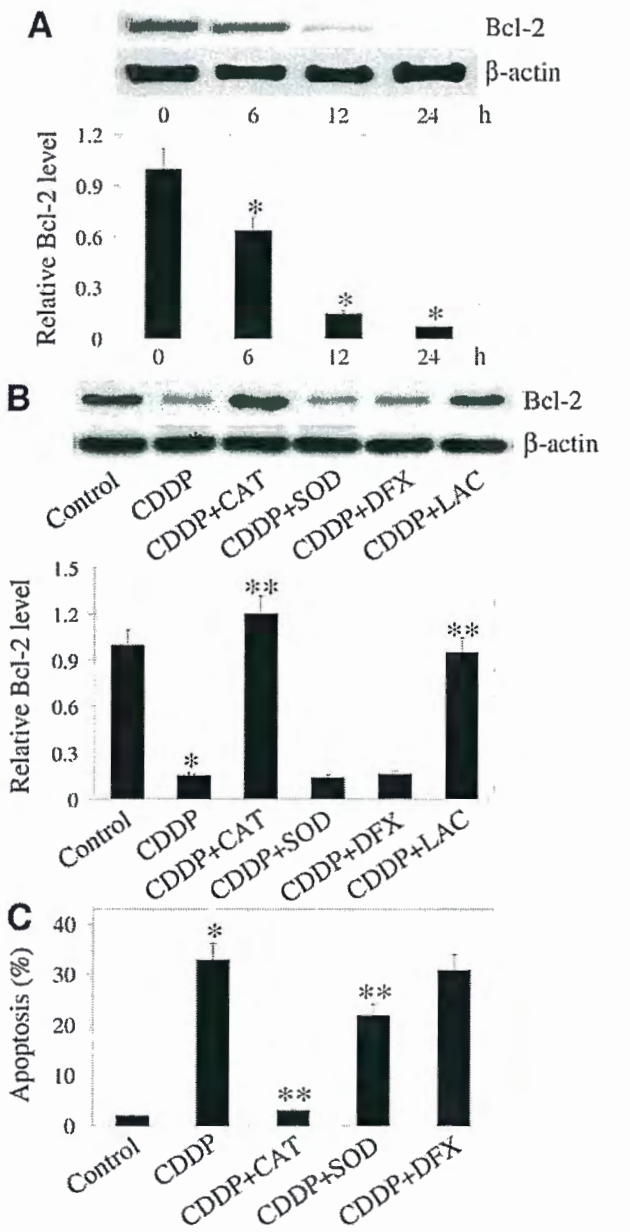

Fig. 3. Effect of cisplatin and antioxidants on Bcl-2 expression and apoptosis. A, H460 cells were treated with cisplatin (100 μM) for various times and analyzed for Bcl-2 expression by Western blots using anti-Bcl-2 antibody. B, effect of various antioxidants and proteasome inhibitor on cisplatin-induced Bcl-2 down-regulation. Cells were pretreated for 1 h with catalase (1000 U/ml), SOD (1000 U/ml), deferoxamine (1 mM), or lactacystin (10 μM) followed by cisplatin treatment (100 μM) for 12 h. Cell lysates were prepared and analyzed for Bcl-2 expression by Western blotting. Immunoblot signals were quantified by densitometry, and mean data from independent experiments were normalized to the result obtained in cells without cisplatin treatment (control). C, cells were treated with cisplatin and antioxidants as described above for 12 h and analyzed for apoptosis by Hoechst 33342 assay. Plots are mean ± S.D. (n = 3). *, $p < 0.05$ versus untreated control; **, $p < 0.05$ versus cisplatin-treated control.

some-mediated degradation (Breitschopf et al., 2000), we tested whether this regulation is responsible for Bcl-2 reduction induced by cisplatin. Cells were treated with lactacystin, a highly specific proteasome inhibitor, and its effect on cisplatin-induced Bcl-2 down-regulation was examined by Western blotting. Figure 3B shows that lactacystin strongly inhibited the down-regulating effect of cisplatin, supporting the role of proteasomal degradation in cisplatin-induced down-regulation of Bcl-2 (Li et al., 2004; Chanvorachote et al., 2006).

Cisplatin Induced Multiple ROS in H460 Cells. To provide a relationship between Bcl-2 and ROS levels in cisplatin-treated cells, ESR studies using the spin trapping agent DMPO were carried out. The ESR technique was used because it allows more accurate identification of the specific ROS involved. Cells were treated with cisplatin in the presence or absence of specific ROS scavengers and analyzed for ROS production. Nontreated cells with DMPO were used as a negative control. In the presence of added cisplatin, a clear positive signal was observed (Fig. 5A). The ESR spectrum consists of a 1:2:2:1 quartet with hyperfine splitting of $a_H = a_N = 14.9$ G, where a_N and a_H denote hyperfine splitting of nitroxyl nitrogens and α-hydrogen, respectively. Based on the line shape and hyperfine splitting, the spectrum was assigned to the DMPO-OH$^{\cdot}$ adduct, which is indicative of OH$^{\cdot}$ generation. The formation of DMPO-OH$^{\cdot}$ adduct was detectable as early as 5 min and peaked at approximately 30 min after treatment, at which time it gradually decreased to the baseline level (data not shown).

The addition of deferoxamine, a metal chelator that inhibits its OH$^{\cdot}$ radical production via the Fenton reaction, completely inhibited the ESR signal, indicating the specificity

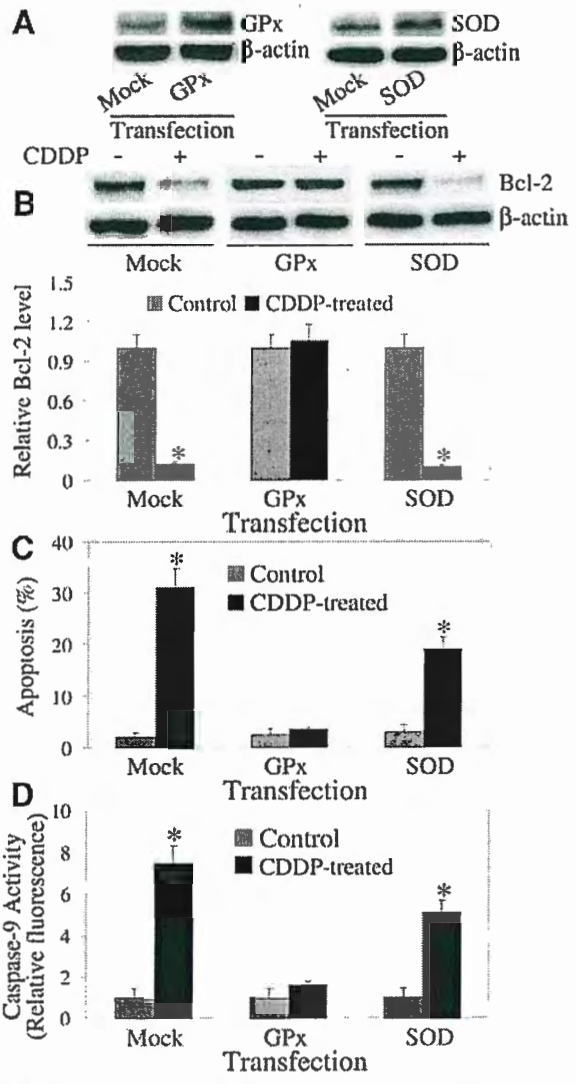

Fig. 4. Effects of GPx and SOD transfection on cisplatin-induced Bcl-2 down-regulation and apoptosis. A, H460 cells were stably transfected with GPx, SOD, or control pcDNA3 plasmid as described under *Materials and Methods*. Cell extracts were prepared and separated on 10% polyacrylamide-SDS gels, transferred, and probed with GPx or SOD antibody. β-actin was used as a loading control. B, transfected cells were treated with cisplatin (100 μM) for 12 h and analyzed for Bcl-2 expression by Western blotting. C and D, cells were similarly treated with cisplatin and analyzed for apoptosis and caspase-9 activity by Hoechst 33342 and fluorometric assays, respectively. Plots are mean ± S.D. ($n = 3$). *, $p < 0.05$ versus untreated control.

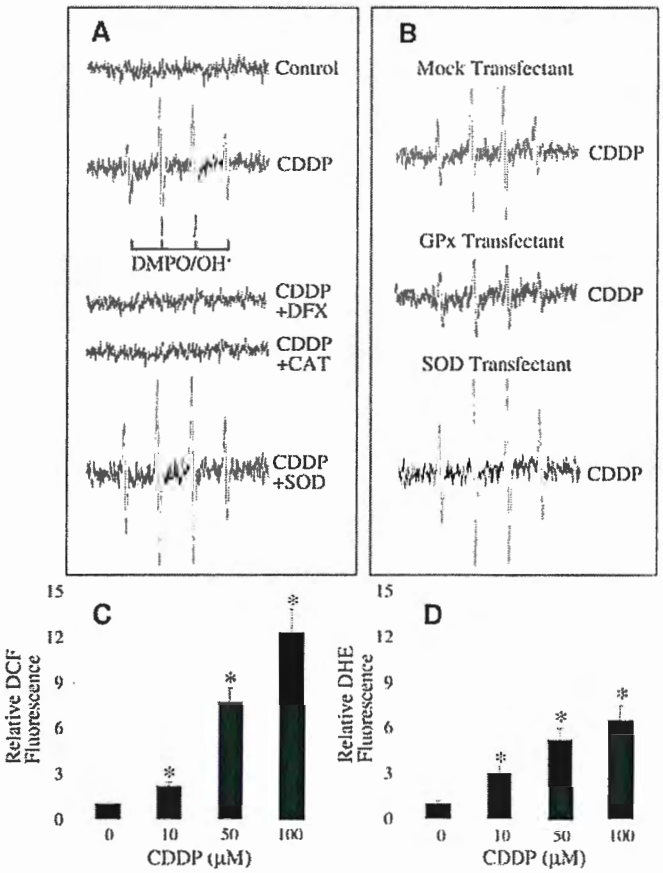

Fig. 5. Effect of cisplatin and antioxidants on cellular ROS generation. A, H460 cells (1×10^6/ml) were incubated in PBS containing the spin trapper DMPO (100 mM) with or without cisplatin (100 μM), catalase (1000 U/ml), SOD (1000 U/ml), and deferoxamine (1 mM). ESR spectra were then recorded 30 min after the addition of the test agents. The spectrometer settings were as follows: receiver gain at 1.5×10^5, time constants at 0.3 s, modulation amplitude at 1.0 G, scan time at 4 min, and magnetic field at 3470 ± 100 G. B, GPx, SOD, or vector-transfected cells were treated with cisplatin (100 μM) in the presence of DMPO (100 mM), and ESR signals were recorded as described in A. C and D, cells were treated with varying concentrations of cisplatin (0–100 μM) for 30 min, after which they were analyzed for peroxide and superoxide generation by flow cytometry using DCFH-DA and DHE as fluorescent probes as described under *Materials and Methods*. Plots are mean ± S.D. ($n = 4$). *, $p < 0.05$ versus untreated control.

of $OH^{\cdot}$ detection. The addition of catalase also inhibited the signal intensity, indicating that H_2O_2 was generated in cisplatin-treated cells and that this oxidative species was a precursor for $OH^{\cdot}$ generation (i.e., through a Fenton-like reaction). SOD showed no inhibitory effect and slightly increased the ESR signal, which is probably due to its ability to dismutate $O_2^{\cdot-}$ to H_2O_2. Similar results were observed in GPx and SOD-transfected cells in response to cisplatin treatment (Fig. 5B). The formation of H_2O_2 and $O_2^{\cdot-}$ in cisplatin-treated cells was confirmed by flow cytometric analysis using DCFH-DA and DHE as fluorescent probes, respectively. Upon treatment of the cells with cisplatin, cellular DCF and DHE fluorescence intensities were enhanced in a dose-dependent manner, supporting the generation of H_2O_2 and $O_2^{\cdot-}$ in cisplatin-treated cells (Fig. 5, C and D).

Cisplatin Induced Bcl-2 Ubiquitination through Hydrogen Peroxide. Ubiquitination is a major cellular process for selective protein removal via proteasomal degradation. It also provides rapid down-regulation of several key proteins involved in apoptotic cell death, including Bcl-2 (Hershko and Ciechanover, 1998; Breitschopf et al., 2000). To study the role of ubiquitination in cisplatin-induced Bcl-2 down-regulation and its regulation by ROS, immunoprecipitation studies were performed in cells transfected with ubiquitin and Bcl-2 plasmids. Transfected cells were treated with cisplatin in the presence or absence of various ROS scavengers, and the resulting immune complexes were analyzed for ubiquitination by Western blotting. Figure 6A shows that cisplatin treatment significantly induced ubiquitination of Bcl-2. This effect of cisplatin was observed as early as 1 h and peaked at approximately 2 h after treatment. Pretreatment of the cells with catalase completely inhibited the ubiquitination of Bcl-2 by cisplatin, whereas deferoxamine and SOD showed no significant inhibitory effect (Fig. 6A). These results indicate that H_2O_2 is the major oxidative species responsible for Bcl-2 ubiquitination induced by cisplatin. This finding is supported by the observation that treatment of the cells with H_2O_2 similarly induced ubiquitination of Bcl-2 and that this effect can be inhibited by catalase (Fig. 6B).

Hydrogen Peroxide Mediated Dephosphorylation of Bcl-2 by Cisplatin. Ubiquitination of Bcl-2 has been reported to be regulated by its phosphorylation (Breitschopf et al., 2000). Apoptotic stimuli such as tumor necrosis factor-α and lipopolysaccharide induce dephosphorylation of Bcl-2 at Ser87, which promotes its degradation by the proteasome (Dimmeler et al., 1999; Breitschopf et al., 2000). To test whether this regulation may be responsible for the ubiquitination of Bcl-2 by cisplatin via an ROS-dependent mechanism, cells were treated with cisplatin in the presence or absence of specific ROS inhibitors, and their effect on Bcl-2 phosphorylation was determined by Western blotting. Figure 7A shows that cisplatin strongly induced Bcl-2 dephosphorylation and that catalase but not SOD or deferoxamine inhibited this effect. These results indicate that cisplatin-induced Bcl-2 dephosphorylation is mediated through H_2O_2. Direct treatment of the cells with H_2O_2 similarly induced Bcl-2 dephosphorylation, which was inhibited by catalase (Fig. 7B). Previous studies have shown that dephosphorylation of Bcl-2 occurs before its ubiquitination and proteasomal degradation (Dimmeler et al., 1999; Breitschopf et al., 2000). Consistent with this finding, we found that the proteasome inhibitor lactacystin was unable to inhibit Bcl-2 dephosphorylation induced by cisplatin.

Discussion

The present study demonstrated the role of Bcl-2 in cisplatin-induced apoptosis and its regulation by ROS in human lung cancer epithelial H460 cells. Cell death induced by cisplatin was mediated through the mitochondrial death pathway via caspase-9 activation (Fig. 1). Overexpression of the mitochondrial Bcl-2 protein inhibited caspase-9 activation and apoptosis induction by cisplatin (Fig. 2), indicating that the mitochondrial pathway

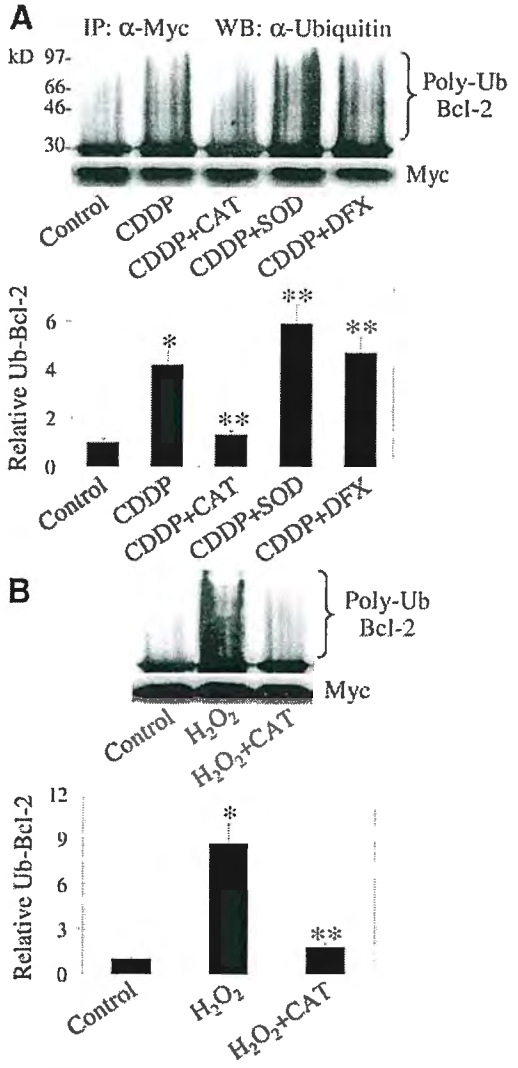

Fig. 6. Effect of cisplatin and antioxidants on Bcl-2 ubiquitination. A, H460 cells were transiently transfected with ubiquitin and myc-tagged Bcl-2 plasmids. Thirty-six hours later, the cells were treated with cisplatin (100 μM) in the presence or absence of catalase (1000 U/ml), SOD (1000 U/ml), or deferoxamine (1 mM). 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 h after treatment in which ubiquitination was found to be maximal. Blots were also probed with myc antibody to confirm equal loading of samples. B, transfected cells were treated with H_2O_2 (100 μM) with or without catalase (1000 U/ml), and Bcl-2 ubiquitination was similarly determined. Data are mean ± S.D. (n = 3). *, $p < 0.05$ versus untreated control; **, $p < 0.05$ versus treated control.

regulated by Bcl-2 is a major pathway for cisplatin-induced apoptosis. This finding is consistent with previous reports showing increased resistance to cisplatin-induced apoptosis in cells expressing high levels of Bcl-2 (Gerano et al., 1995; Bello et al., 2001). Because Bcl-2 is overexpressed in many cancer cell types and tumor specimens (Yang et al., 2004; Kausch et al., 2005), the results of these studies suggest that increased Bcl-2 expression may be a key determinant of cancer cell resistance to cisplatin.

Several mechanisms of cisplatin resistance have been proposed, including impaired cellular uptake of cisplatin (Kartalou and Essigmann, 2001), increased DNA lesion repair (Koberle et al., 1999), and defects in mismatch repair that fail to trigger cell death (Aebi et al., 1996; Fink et al., 1996). Intracellular cisplatin inactivation by redox regulation has also been proposed as a mechanism of cisplatin resistance (Ishikawa and Ali-Osman, 1993; Rudin et al., 2003). Inhibition of ROS by antioxidants has been shown to increase cisplatin resistance (Yang et al., 2000), whereas increased generation of ROS by glutathione depletion reverses the resistance via Bcl-2 down-regulation (Rudin et al., 2003). Bcl-2 has been shown to possess several independent functions, including abrogation of mitochondrial release of caspase-activating factors such as cytochrome *c* (Kluck et al., 1997) and modulation of antioxidant pathways (Voerhinger et al., 1998). Bcl-2 is regulated at different levels. Transcriptional regulation is a key step in controlling its expression, whereas post-translational modifications such as dephosphorylation and ubiquitination are essential to its stability and function under various pathological conditions (Fink et al., 1996; Haendeler et al., 1996). Pathological stimuli such as endotoxin (Haendeler et al., 1996), β-amyloid (Paradis et al., 1996), ischemia (Krajewski et al., 1995), and tumor necrosis factor-α (Breitschopf et al., 2000) have been shown to induce ubiquitin-proteasomal degradation of Bcl-2. Studies have also shown that such regulation may be mediated through ROS signaling, although the precise mechanism of regulation and the specific ROS involved are unclear.

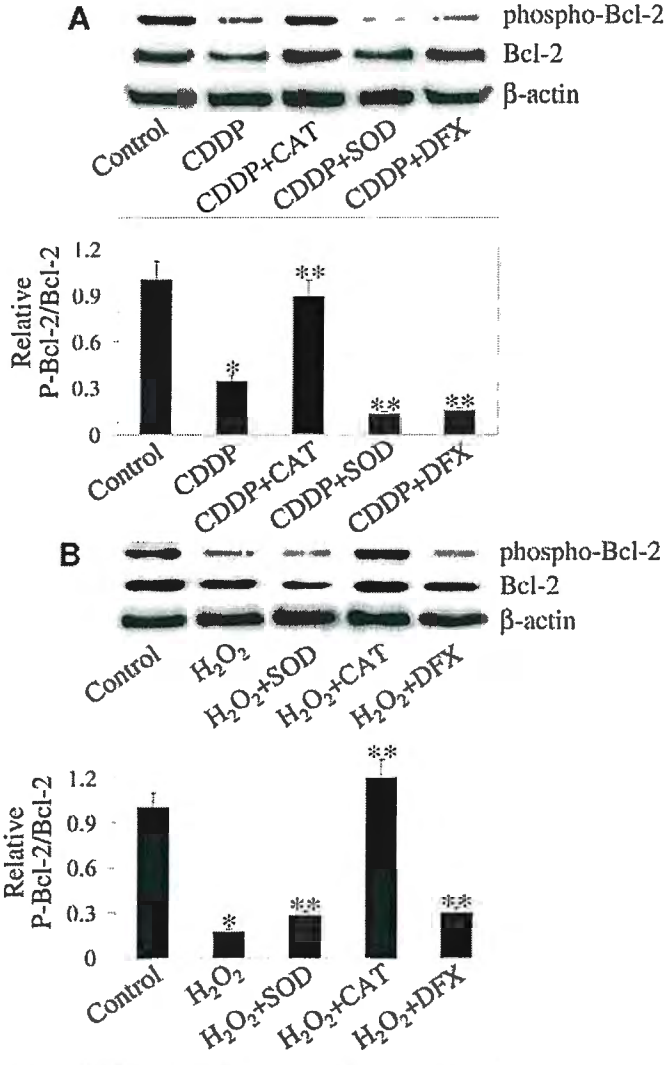

Fig. 7. Effect of cisplatin and antioxidants on Bcl-2 phosphorylation. A, H460 cells were treated with cisplatin (100 μM) in the presence or absence of catalase (1000 U/ml), SOD (1000 U/ml), or deferoxamine (1 mM) for 3 h. Cell lysates were prepared and analyzed for Bcl-2 phosphorylation by Western blotting using phosphospecific Bcl-2 (Ser87) antibody. Total Bcl-2 was also analyzed and used to determine the relative levels of Bcl-2 phosphorylation. β-Actin was used as a loading control. B, cells were treated with H_2O_2 (100 μM) in the presence or absence of antioxidants, and cell lysates were prepared and analyzed for phospho- and total Bcl-2 as described. Data are mean ± S.D. ($n = 3$). *$p < 0.05$ versus untreated control; **, $p < 0.05$ versus treated control.

We have shown that multiple ROS are generated in cells in response to cisplatin treatment (Fig. 5). Generation of ROS occurred at the treatment doses that induce apoptosis and Bcl-2 down-regulation (Figs. 1 and 3). The antioxidant enzyme catalase and glutathione peroxidase inhibited this down-regulation (Fig. 3), indicating that H_2O_2 is the major oxidative species responsible for the down-regulation of Bcl-2 by cisplatin. This finding is consistent with previous reports identifying H_2O_2 as a key mediator of apoptosis in response to various stimuli ranging from xenobiotics to cytokines (Bai and Cederbaum, 2001). Inhibition of H_2O_2 by catalase also resulted in decreased cell death and increased Bcl-2 expression in activated T cells (Hildeman et al., 2003). In contrast to a previous report showing the role of $O_2^{\cdot-}$ in Bcl-2 regulation (Li et al., 2004), we found no inhibitory effect of SOD in our system. Although $O_2^{\cdot-}$ was induced by cisplatin in our cell system, its inhibition by SOD failed to inhibit Bcl-2 down-regulation by cisplatin (Fig. 3). This is probably a reflection of the differential expression of antioxidant enzymes in the test cell systems and the methods used to detect ROS. ESR is a preferred method for identifying specific ROS, offering a high degree of specificity in contrast to dye-based assay used in previous studies. Our ESR studies showed a slight but significant increase in the ESR signal after SOD treatment, suggesting catalytic conversion of $O_2^{\cdot-}$ to H_2O_2. It is possible that a phenotypic difference in the antioxidant enzyme expression determines the degree of sensitivity to specific stimuli. Should the level of SOD be high, most of the $O_2^{\cdot-}$ radicals would be efficiently converted to H_2O_2. It is also conceivable that potential suboptimal expression of catalase and glutathione peroxidase could have resulted in increased H_2O_2 presence. Such increase in H_2O_2, observed in our study, could in turn initiate a multitude of signaling cascades, including caspase activation and down-regulation of Bcl-2, leading to cell apoptosis.

Because H_2O_2 can be converted to $OH^{\cdot}$, which was observed in our ESR experiments, and because $OH^{\cdot}$ is known to be highly reactive, it is possible that this oxidative species may be a key regulator of Bcl-2 expression. However, our

inhibition studies using a metal chelator, deferoxamine, which inhibits $OH^{\cdot}$ production via the Fenton reaction, showed minimal protective effect of the inhibitor on Bcl-2 down-regulation induced by cisplatin (Fig. 3). A similar result was also observed with another known $OH^{\cdot}$ inhibitor, sodium formate, indicating its less important role in Bcl-2 regulation (data not shown).

The mechanism by which cisplatin induces ROS generation is incompletely understood but may involve the activation of NADPH oxidase and mitochondrial ROS generating system. Cisplatin has been shown to induce NOX-3, an isoform of NADPH oxidase that increases O_2^- production (Rybak et al., 2007). Superoxide generated by cisplatin leads to the formation of H_2O_2, which is catalyzed to form OH^- radicals. Cisplatin also causes a depletion of glutathione and antioxidant enzymes that are crucial to the elimination of intracellular ROS (Somani et al., 2001).

Our results also demonstrated that down-regulation of Bcl-2 by cisplatin is mediated through the ubiquitin-proteasomal degradation pathway, which is dependent on H_2O_2. Inhibition of ROS by the antioxidant enzyme catalase but not by SOD or deferoxamine inhibited the ubiquitination of Bcl-2 by cisplatin, indicating the dominant role of H_2O_2 in this process (Fig. 6). Direct treatment of the cells with H_2O_2 similarly induced Bcl-2 ubiquitination, supporting the above finding and indicating that H_2O_2 may be a common regulator of Bcl-2 under oxidative stress conditions. The mechanism by which H_2O_2 induces Bcl-2 ubiquitination involves dephosphorylation of the protein. Dephosphorylation of Bcl-2 has been shown to be an essential step for Bcl-2 ubiquitination and degradation (Breitschopf et al., 2000). Our results on the inhibitory effect of catalase on Bcl-2 dephosphorylation and degradation (Figs. 3 and 7) support this finding.

The mechanism by which peroxide induces Bcl-2 dephosphorylation is unclear but may involve its effect on the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway, which has been shown to be a key regulator of Bcl-2 dephosphorylation (Horiuchi et al., 1997; Dimmeler et al., 1999). Peroxide has also been shown to activate extracellular signal-regulated kinase (Guyton et al., 1996), and such activation is required for cisplatin-induced apoptosis (Choi et al., 2004). Peroxide may also exert a direct effect on Bcl-2 (i.e., by oxidizing specific cysteine residues on the protein that could alter its conformation and recognition by the phosphorylating enzymes).

Although the results of this study indicate that down-regulation of Bcl-2 is an important event in cisplatin-induced apoptosis, such down-regulation by itself may be insufficient to induce the apoptosis and that other mechanisms may be involved. Cisplatin has been shown to induce up-regulation of several key proapoptotic proteins such as Bax and Bak (Li et al., 2004). This up-regulation along with the reduced presence of the antiapoptotic Bcl-2 protein would facilitate the mitochondrial permeability transition, leading to cytochrome *c* release and apoptosis.

In summary, our data provide evidence that H_2O_2 plays an important role in regulating Bcl-2 expression and apoptotic cell death induced by cisplatin. H_2O_2 is a major oxidative species induced by cisplatin in human lung cancer H460 cells. H_2O_2 mediates Bcl-2 down-regulation by cisplatin through its ability to dephosphorylate the protein, which facilitates its ubiquitination and subsequent degradation by the proteasome. H_2O_2 may be a common regulator of Bcl-2 expression under diverse pathological and treatment conditions. Because aberrant expression of Bcl-2 has been associated with several human cancers, our findings on the regulatory mechanism of Bcl-2 by ROS could be important in the understanding of carcinogenesis and in the development of novel therapeutic strategies that could overcome cancer chemoresistance.

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