

Cytoprotective Effects of Hypoxia against Cisplatin-Induced Tubular Cell Apoptosis: Involvement of Mitochondrial Inhibition and p53 Suppression

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Hypoxia that is caused by vascular defects or disruption is commonly associated with renal diseases. During cisplatin nephrotoxicity, hypoxic regions are identified in the outer medulla and the renal cortex. However, the regulation of cisplatin injury by hypoxia is unclear. Previous work has demonstrated the cytoprotective effects of hypoxia against apoptotic injury. This study further examines the cytoprotective mechanisms in models of cisplatin-induced tubular cell apoptosis. In cultured renal tubular cells, 20 μ M cisplatin induced approximately 60% apoptosis within 16 h. The rate of apoptosis was suppressed to <20%, when the incubation was conducted under hypoxia (2% O₂). Mitochondrial events of apoptosis, namely Bax accumulation and cytochrome c release, also were ameliorated. During cisplatin treatment, cell ATP was maintained in both normoxic and hypoxic cells. Hypoxic incubation lowered extracellular pH, but prevention of the pH decrease did not restore cisplatin-induced apoptosis. The cytoprotective effects of hypoxia also were independent of hypoxia-inducible factor 1 (HIF-1). Cobalt, as hypoxia, activated HIF-1 yet did not suppress cisplatin-induced apoptosis. Moreover, hypoxia suppressed cisplatin-induced apoptosis in HIF-1-deficient mouse embryonic stem cells and renal proximal tubular cells. Conversely, mitochondrial inhibitors, particularly inhibitors of respiration complex III (antimycin A and myxothiazol), mimicked hypoxia in apoptosis suppression. The effects of hypoxia and mitochondrial inhibitors were not additive. It is interesting that both hypoxia and complex III inhibitors ameliorated cisplatin-induced p53 activation. Therefore, the cytoprotective effects of hypoxia are independent of changes in cell ATP, pH, or HIF but may involve mitochondrial inhibition and the suppression of p53.

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Hypoxia, a condition of decreased availability of oxygen, contributes to the regulation of pathophysiology in various kinds of cells and tissues (1). In the kidneys, the unique organization of the vascular system leads to low oxygen tensions in specific regions, resulting in a physiologic situation of persistent hypoxia (2). Although it is unclear how this affects renal physiology, it is widely recognized that hypoxia is a critical determinant of the development of renal pathology under disease conditions. Acute disruption of blood supply limits oxygen availability to the kidneys, which induces renal cell injury, culminating in ischemic renal failure (3). In addition, recent studies have suggested the involvement of hypoxia in chronic renal disease (2). As a result, strategies have been proposed (2) and tested to enhance hypoxic tolerance for renoprotection under various disease conditions (4–7).

In response to hypoxia, cells can be irreversibly injured. However, cells possess a finely regulated system to cope with

hypoxic stress (1). In this direction, hypoxia-inducible transcription factors have been identified, including HIF-1 and -2 (8–10). HIF-1 and -2 are expressed in different and sometimes partly overlapping cell populations. In the kidneys, HIF-1 and not HIF-2 is induced by hypoxia and relevant conditions in renal tubular cells (11). Upon activation, HIF-1 binds to hypoxia-responsive elements in specific genes to induce their expression. These genes promote oxygen delivery, enhance cellular capacity of anaerobic metabolism, or protect the cells from injury and death (8–10).

pH is another factor that is implicated in cell injury under hypoxia. In hypoxic cells, cellular metabolism is shifted to the anaerobic direction, resulting in the production of acidic metabolites such as lactate. As a consequence, acidosis frequently is associated with tissue hypoxia or ischemia. It remains controversial as to how acidic pH affects cell injury and death. In cultured cardiomyocytes, acidic pH seems to be the primary cause of cell death (12). On the contrary, lower pH protects various types of cells during hypoxic incubation or tissue ischemia (13). In cultured renal tubular cells, our recent work has demonstrated the protective effects of acidic pH against apoptosis after ATP depletion (14). Acidic pH inhibits the formation of apoptosome, the protein complex that is responsible for caspase activation.

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Cisplatin, a widely used chemotherapy drug, induces nephrotoxicity, which limits its therapeutic efficacy (15). Although cell death in renal tubules is widely recognized, cisplatin also is expected to disrupt vasculature in the kidneys, leading to hypoperfusion or hypoxia (16). This view is supported by direct evidence from recent studies that detected the staining of outer renal medulla and cortex of cisplatin-treated animals by pimonidazole, a hypoxia-specific dye (6). In addition, HIF transgene is activated in the kidneys during cisplatin treatment (6). Despite these observations, whether and how hypoxia regulates cisplatin-induced renal cell injury and death remain to be clarified.

Using cultured renal tubular cells, our previous work showed that cells become resistant to apoptosis when incubated under severe hypoxia or anoxia (17,18). Although a role for IAP-2, a caspase interacting and inhibitory protein, has been implicated in the cytoprotective effects of hypoxia, these studies also suggest the involvement of multiple factors (18). In this study, we extended these observations and examined the possible underlying mechanisms in model systems of cisplatin-induced tubular cell apoptosis. It is shown that the cytoprotective effects of hypoxia seem to be independent of cell ATP and pH changes during hypoxic incubation and, importantly, independent of HIF-1. However, pharmacologic inhibition of mitochondria, particularly at respiration complex III, can mimic hypoxia in cytoprotection. It is interesting that both hypoxia and mitochondrial inhibitors suppress p53 activation, a critical apoptotic event during cisplatin treatment (19–21). Therefore, the cytoprotective effects of hypoxia against cisplatin-induced tubular cell apoptosis may involve mitochondrial inhibition and p53 suppression.

Materials and Methods

Cells

The immortalized rat kidney proximal tubular cell (RPTC) line originally was obtained from Dr. Ulrich Hopfer (Case Western Reserve University, Cleveland, OH). The cells were maintained and plated for experiments as described previously (22). Wild-type (HIF-1 $\alpha^{+/+}$) and HIF-1 $\alpha^{-/-}$ mouse embryonic stem cells were kindly provided by Dr. Gregg Semenza at Johns Hopkins University, Baltimore, MD (23). Wild-type (HIF-1 $\alpha^{+/+}$) and HIF-1 $\alpha^{-/-}$ primary RPTC were prepared by a method detailed in previous publications (24,25). Briefly, proximal tubules were isolated from renal cortex of 3- to 4-wk-old *Hif-1 $\alpha^{2-lox/2-lox}$* mice. After *in vitro* culture and expansion, one group of cells was treated with doxycycline to inactivate *Hif-1 α* .

Reagents and Antibodies

For caspase activity measurement, carbobenzoxy-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (DEVD.AFC) and 7-amino-4-trifluoromethyl coumarin (AFC) were purchased from Enzyme Systems Products (Dublin, CA). Antibodies were from the following sources: Mouse monoclonal anti-cytochrome c (7H8.2C12) from BD Pharmingen (San Diego, CA); mouse monoclonal anti-Bax (1D1) from NeoMarkers (Fremont, CA); rabbit polyclonal anti-HIF-1 α from Novus Biologicals (Littleton, CO), rabbit polyclonal anti-p53 and anti-phospho-p53 (Ser15) antibodies from Cell Signaling Technology (Beverly, MA), and all secondary antibodies from Jackson ImmunoResearch (West Grove, PA). Other reagents, including cisplatin and mitochondrial inhibitors

(rotenone, antimycin A, myxothiazol, oligomycin, and azide) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cisplatin Treatment

RPTC and Mouse Embryonic Stem Cells. The cells were plated for experiment at a density to reach approximately 90% confluence after overnight growth. The cells then were washed once with PBS and incubated with 20 μ M cisplatin in fresh culture medium. A stock solution of cisplatin (100 mM in DMSO) was freshly prepared for each experiment. For incubation under normoxia, cells were incubated with cisplatin in a regular cell culture incubator that contained 21% oxygen. For hypoxia, cells were incubated with cisplatin in a hypoxia chamber (COY Laboratory Products, Ann Arbor, MI) with a computerized oxygen controller to maintain an atmosphere of 2 or 5% oxygen. For anoxia, cells were incubated with cisplatin in an anoxia chamber (COY Laboratory Products) with no oxygen; in addition, 1.2 unit/ml EC Oxyrase, a biocatalytic oxygen-reducing agent, was added to the cells to scavenge residual oxygen in the medium (17,18). The culture medium that was used for hypoxic and anoxic incubations was pre-equilibrated overnight in respective chambers. For studying the effects of cobalt and mitochondrial inhibitors, the chemicals were added at indicated concentrations along with cisplatin, and the incubation was conducted in a regular cell culture incubator with 21% oxygen.

HIF-1 $\alpha^{+/+}$ and HIF-1 $\alpha^{-/-}$ Primary RPTC. The cells were treated with 25, 50, or 100 μ M cisplatin for 24 h in glucose-containing medium in a cell culture incubator with normal oxygen or in a hypoxic chamber with 0.2% oxygen.

Examination of Apoptosis

Apoptosis was monitored by morphologic and biochemical methods as described in our previous work (18,20,26). Morphologically, at the end of experimental incubation, cells were stained with 10 μ g/ml Hoechst 33342 for 2 to 5 min. Cellular and nuclear morphology then was recorded by phase contrast and fluorescence microscopy. Typical apoptotic morphology that was examined included cellular shrinkage, nuclear condensation and fragmentation, and formation of apoptotic bodies. For quantification, four fields with approximately 200 cells per field were checked in each dish to estimate the percentage of apoptotic cells. Biochemically, caspase activity was measured as described previously (18,20,26,27). Briefly, cells were extracted with 1% Triton X-100 to collect cell lysate. The lysate of 25 μ g of protein was added to an enzymatic reaction that contained 50 μ M DEVD.AFC, a fluorogenic peptide substrate of caspases. After 60 min of reaction at 37°C, fluorescence was measured at excitation 360 nm/emission 530 nm. For each measurement, a standard curve was constructed using free AFC. On the basis of the standard curve, the fluorescence reading from each enzymatic reaction was translated into the nanomolar amount of liberated AFC to indicate caspase activity.

Measurement of Cell ATP

Cell ATP was determined with an ATP Bioluminescent Assay kit (Sigma). Briefly, cells were extracted with perchloric acid. The extract was neutralized with K₂CO₃ and mixed with the ATP Bioluminescence reagent. The reaction was recorded immediately on a luminometer. For each measurement, a standard curve was constructed using ATP freshly prepared in distilled water. On the basis of the standard curve, the signals of the samples were translated into molar amounts of ATP. Cell protein was determined in parallel dishes for the normalization of the ATP values.

Assay of Cell Viability

For assessment of cell viability, a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay (CellTiter96; Promega, Madison, WI) was used (28). The assay included a tetrazolium compound (MTS), which was reduced by metabolically active cells to become colorimetric with absorbance at 490 nm. The assay was conducted at a range at which the absorbance after MTS reduction was proportional to the number of active or living cells.

Analysis of Bax and Cytochrome C Redistribution during Cisplatin Treatment

Subcellular distribution of Bax and cytochrome c was analyzed by cellular fractionation, followed by immunoblot analysis (18,26). For fractionation, cells were exposed to 0.05% digitonin in an isotonic buffer (in mM: 250 sucrose, 10 Hepes, 10 KCl, 1.5 MgCl₂, 1 EDTA, and 1 EGTA [pH 7.1]). The soluble fraction was collected as cytosolic extract. The remaining digitonin insoluble part was washed once with isotonic buffer and then dissolved in a 2% SDS buffer to collect the membrane-bound organellar fraction that was enriched with mitochondria. The digitonin soluble and insoluble fractions were analyzed for Bax and cytochrome c by immunoblotting analysis.

Immunoblot Analysis

Protein concentration in cellular extracts was determined using the bicinchoninic acid reagent from Pierce Chemical Co. (Rockford, IL). The same amounts (usually 25 µg) of protein were loaded in each lane for electrophoresis under reducing conditions and subsequently electroblotted onto polyvinylidene difluoride membranes. The blots were incubated in a blocking buffer with 1% BSA and 2% fat-free milk and then exposed to the primary antibodies overnight at 4°C. Finally, the blots were incubated with the horseradish peroxidase-conjugated secondary antibody, and antigens on the blots were revealed using the enhanced chemiluminescence kit from Pierce.

Statistical Analyses

Data were expressed as means ± SD ($n \geq 3$). Statistical analysis was conducted using the GraphPad Prism software (GraphPad, San Diego, CA). Statistical differences within multiple groups were determined by multiple comparisons with Tukey posttest after ANOVA. Statistical differences between two groups were determined by *t* test. $P < 0.05$ was considered to indicate significant differences.

Results

Suppression of Cisplatin-Induced Apoptosis by Hypoxia

Our previous work established an *in vitro* model of cisplatin-induced apoptosis in cultured RPTC (20). In this model, 20 µM cisplatin induced typical apoptosis in a time-dependent manner. After 16 h of cisplatin treatment, the cells assumed a condensed morphology (Figure 1A, b). In addition, nuclei of these cells became condensed and fragmented (Figure 1A, f). It is interesting that when cisplatin incubation was conducted in a hypoxia chamber that contained only 2% oxygen, apoptosis was ameliorated (Figure 1A, d and h). The difference in apoptosis between the normoxic and hypoxic cells then was quantified by counting the cells with typical apoptotic morphology (Figure 1B). Under normoxia (21% oxygen), the earliest apoptosis was induced by 8 h of cisplatin incubation. By 16 h, approximately 60% of cells underwent apoptosis. At the end of 24 h of cisplatin incubation, the rate of apoptosis increased to 80%. Cisplatin-induced apoptosis clearly was suppressed by hypoxia

(2% oxygen). As a result, <20% apoptosis was detected in the cells that were incubated with cisplatin for 16 h under hypoxia (Figure 1B). The morphologic observations were confirmed by biochemical analysis of caspase activation, a central event of apoptosis (Figure 1C). For example, 16 h of cisplatin treatment increased caspase activity to 55 nmol/mg per h under normoxia but only to approximately 24 nmol/mg per h under hypoxia. We further determined the proteolytic cleavage of caspase-3 into active forms. As shown in Figure 1D, procaspase-3 was cleaved during 16 to 24 h of cisplatin incubation, releasing the 10-kD active fragment (lanes 2 and 3). This cleavage was suppressed when the cells were treated under hypoxia (Figure 1D, lanes 4 and 5). The cytoprotective effects of hypoxia also were shown for anoxia (0% oxygen) and, to lesser extent, for 5% oxygen (data not shown).

Suppression of Mitochondrial Events of Apoptosis by Hypoxia

Cisplatin-induced apoptosis in renal tubular cells may involve multiple signaling pathways (15,29). Among them, a critical role has been suggested for the mitochondrial events of apoptosis, namely Bax accumulation in mitochondria and consequent cytochrome c release (30,31). To determine whether hypoxia affected these mitochondrial events, we fractionated the cells into cytosolic and mitochondrial fractions for immunoblot analysis. The results are shown in Figure 2. In control cells without cisplatin treatment, Bax was detected mainly in the cytosol (Figure 2, lane 1). After 16 h of cisplatin treatment under normoxia, Bax accumulated in the mitochondrial fraction, accompanied by the loss of Bax from the cytosol (Figure 2, lane 2). Importantly, when cisplatin treatment was conducted under hypoxia, Bax translocation was ameliorated (Figure 2, lane 3). In line with these observations, cisplatin-induced cytochrome c release from mitochondria into cytosol also was suppressed in hypoxic cells (Figure 2B). The results suggest that hypoxia may attenuate cisplatin-induced apoptosis at a level of or upstream of mitochondria.

Cell ATP during Cisplatin Treatment and the Effects of Hypoxia

Decreased availability of oxygen during hypoxia is expected to limit ATP production *via* oxidative phosphorylation. To determine whether this was involved in the cytoprotective effects of hypoxia, we measured cell ATP during cisplatin incubation under normoxia or hypoxia. The ATP level of control cells was approximately 40 nmol/mg protein. Consistent with previous studies (19), cisplatin treatment did not induce ATP depletion in RPTC (Figure 3). As a result, cell ATP after 4 and 16 h of cisplatin treatment was maintained at 42.5 and 44 nmol/mg protein, respectively. Notably, cisplatin treatment under hypoxia did not decrease cell ATP either (Figure 3). The metabolic shift to anaerobic glycolysis seems to have been sufficient to maintain ATP in hypoxic cells during the observation period of 16 h. The results suggest that the cytoprotective effects of hypoxia are not a result of ATP depletion or energetic defect of the cells.

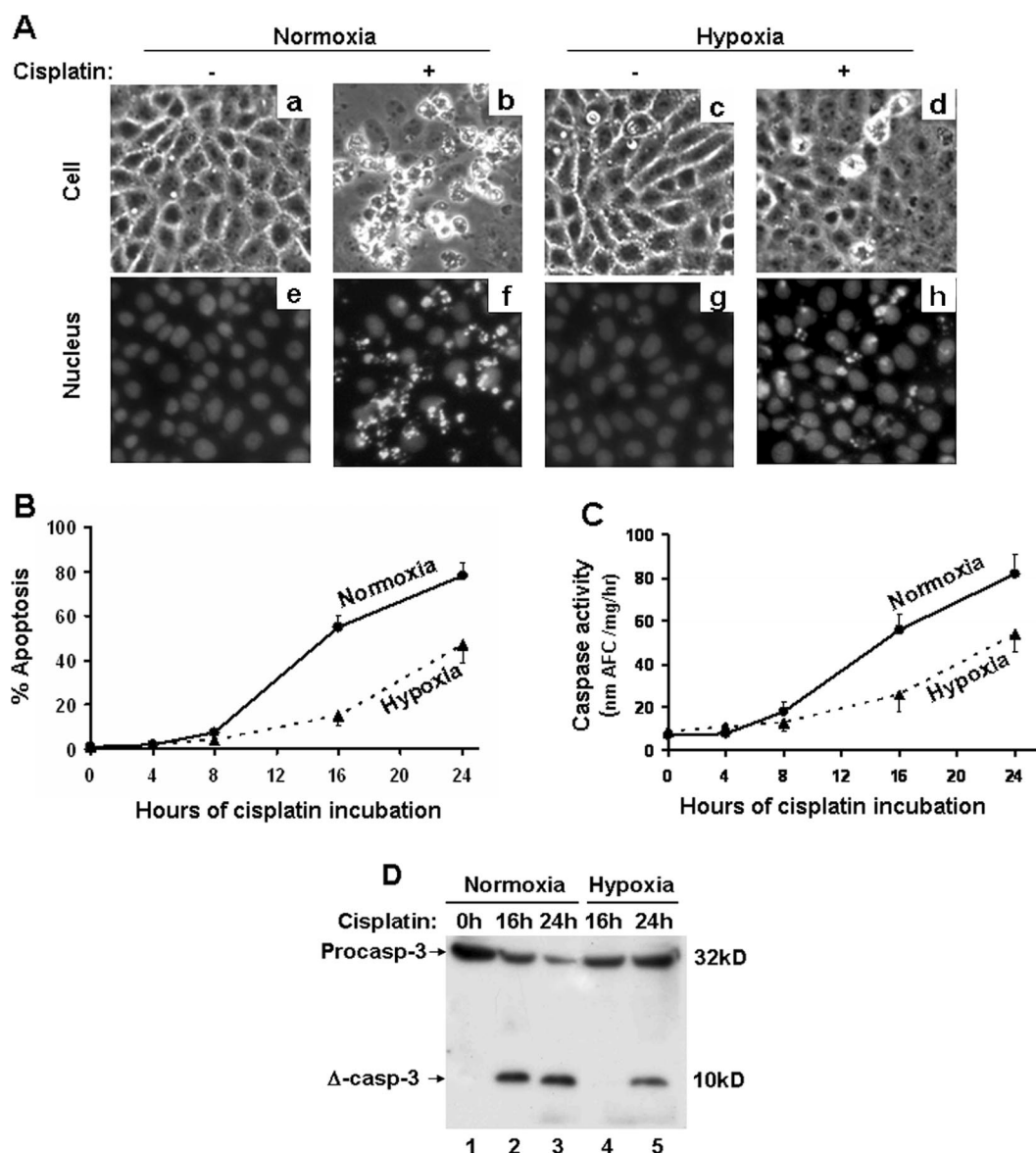


Figure 1. Suppression of cisplatin-induced apoptosis by hypoxia. (A) Morphology. Rat kidney proximal tubular cell (RPTC) were incubated for 16 h under normoxia or hypoxia (2% oxygen) in the absence (–) or presence (+) of 20 μ M cisplatin. The cells were stained with Hoechst 33342. Cell morphology and nuclear staining were recorded by phase contrast and fluorescence microscopy, respectively. (B) Time course of apoptosis. RPTC were incubated with 20 μ M cisplatin under normoxia or hypoxia for 0 to 24 h. The percentage of apoptosis was evaluated by counting the cells with typical apoptotic morphology. (C) Caspase activity. After cisplatin incubation under normoxia or hypoxia, cell lysate was collected to determine caspase activity by enzymatic assay using DEVD.AFC as the substrate. Data are expressed as mean \pm SD ($n = 4$). (D) Proteolytic processing of caspase-3 into active forms. Cell lysates were collected for immunoblot analysis using an antibody reactive to the proform and the 10-kD active fragment of caspase-3. The results show that cisplatin-induced apoptosis was suppressed under hypoxia.

Cellular Acidification during Hypoxic Incubation Is Not Involved in the Cytoprotective Effects of Hypoxia

During hypoxic incubation, mammalian cells tend to shift their metabolism to anaerobic glycolysis, as a result of the decreased availability of oxygen and oxidative phosphorylation. As a result, acidic metabolites such as lactate accumulate, leading to cellular acidification. Depending on the cellular context, acidic pH may promote or inhibit cell injury and death (12–14). In our experiments (Figure 4A), 16 h of hypoxic incubation lowered the pH of

the incubation medium from approximately 7.35 to approximately 7.0, regardless of whether cisplatin was present. To determine the role of pH decrease or acidification in the cytoprotective effects of hypoxia, 20 mM Hepes buffer at pH 7.4 was added during hypoxic incubation to maintain the pH at approximately 7.35 (Figure 4A). However, despite the maintenance of pH, cisplatin-induced apoptosis still was suppressed by hypoxia (Figure 4B). Therefore, cellular acidification was not critical to the cytoprotective effects of hypoxia during cisplatin treatment.

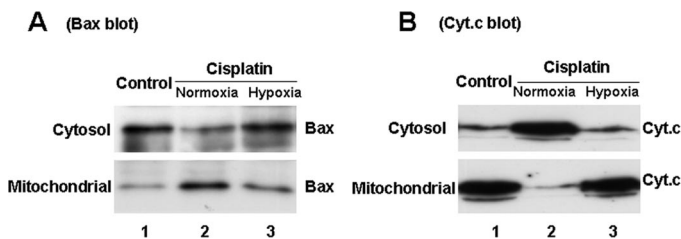


Figure 2. Suppression of cisplatin-induced Bax translocation and cytochrome c release by hypoxia. RPTC were incubated with 20 μ M cisplatin for 16 h under normoxia or hypoxia. Control group was cultured under normoxia without cisplatin exposure. After incubation, the cells were fractionated into the cytosolic fraction and the mitochondria-containing membrane fraction. The fractions were analyzed for Bax (A) and cytochrome c (B) by immunoblot analysis. The results show that Bax accumulated in the mitochondria after cisplatin treatment, whereas cytochrome c was released from the organelles. Redistribution of these two proteins was inhibited under hypoxia.

Cobalt Induces HIF-1 but Does Not Protect Tubular Cells against Cisplatin-Induced Apoptosis

Under hypoxia, mammalian cells can mount an adaptive response, which helps the cells to endure the stress and survive (32,33). Critical mechanisms that govern the adaptive response include HIF. HIF-1 is ubiquitously expressed, whereas HIF-2 expression is restricted to specific cells and tissues. In renal tubular cells, only HIF-1 is detected upon induction (11,25). In addition to hypoxia, HIF-1 can be induced by pharmacologic agents, including cobalt ion (8–10). To determine whether HIF-1 was a key to the observed cytoprotective effects of hypoxia, we initially examined the effects of cobalt. The results are shown in Figure 5. Both hypoxia and cobalt induced HIF-1 in RPTC (Figure 5A, lanes 2 through 6). At 200 μ M, cobalt consistently induced higher HIF-1 expression than hypoxia. Despite the induction of HIF-1, cobalt, unlike hypoxia, did not

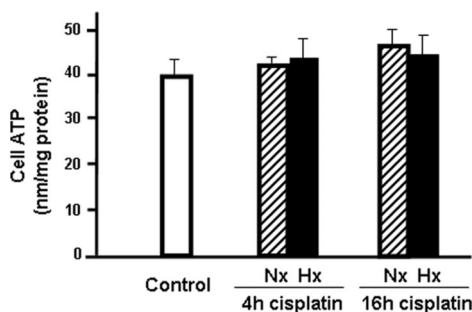


Figure 3. Cell ATP during cisplatin treatment under normoxia and hypoxia. RPTC were incubated with 20 μ M cisplatin under normoxia (Nx) or hypoxia (Hx) for 4 or 16 h. At the end of incubation, cells were extracted with perchloric acid for the measurement of ATP as described in Materials and Methods. Protein was determined in parallel dishes. Cell ATP was normalized with protein. Data are expressed as mean \pm SD ($n = 4$). The results show that cell ATP did not change significantly during cisplatin treatment under normal oxygen as well as hypoxia.

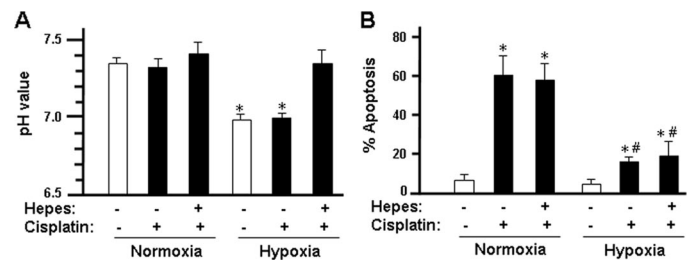


Figure 4. Acidification during hypoxic incubation and the effects of maintaining pH. RPTC were incubated for 16 h under normoxia or hypoxia in the absence (–) or presence (+) of 20 μ M cisplatin. For controlling pH, 20 mM Hepes (pH 7.4) was added during the incubation (+). (A) pH during cell incubation. After the incubation, medium was collected and immediately measured for pH. *Statistically significantly different from the pH values of the normoxia groups. (B) Apoptosis. Apoptotic cells were counted to estimate the percentage of apoptosis by morphologic methods. Data are expressed as mean \pm SD ($n = 3$). *Significantly different from control groups without cisplatin treatment; #significantly different from the cisplatin-treated normoxia groups. The results show that the incubation medium became weakly acidic during hypoxic incubation. The HEPES buffer maintained the medium pH at approximately 7.4 during hypoxic incubation but did not restore cisplatin-induced apoptosis.

show significant cytoprotective effects against cisplatin-induced apoptosis (Figure 5B). On the basis of these results, it is concluded that simply inducing HIF-1 cannot mimic the cytoprotective action of hypoxia.

Hypoxia Protects against Cisplatin-Induced Apoptosis in HIF-1–Deficient Mouse Embryonic Stem Cells

To determine further the role of HIF-1 in the observed cytoprotective effects of hypoxia, we examined mouse embryonic stem cells (ES) that were deficient of HIF-1 (23). As expected, when wild-type and HIF-1–deficient ES cells were subjected to hypoxic incubation, HIF-1 was induced in wild-type but not HIF-deficient cells (Figure 6A, lanes 4 and 2). Under normoxia, cisplatin induced approximately 70% apoptosis in the wild-type as well as the HIF-deficient cells (Figure 6B). Importantly, under hypoxia, cisplatin-induced apoptosis was suppressed to approximately 30% in both types of cells. These results not only demonstrate the cytoprotective effects of hypoxia against cisplatin injury in mouse ES cells but also negate a role of HIF-1 in the observed cytoprotection.

Cytoprotective Effects of Hypoxia in HIF-1–Deficient Primary Culture of Mouse Proximal Tubular Cells

The results shown above are discrepant with a recent report (6) that suggested a role for HIF-1 in hypoxia protection of renal tubular cells. To resolve this issue, we examined primary cultures of wild-type and HIF-1–deficient mouse renal tubular cells. The absence of HIF-1 in the deficient cells was confirmed (data not shown [24,25]). The primary cultures were subjected to 24 h of incubation with 25, 50, and 100 μ M cisplatin under normoxia or hypoxia. Cisplatin at these concentrations induced

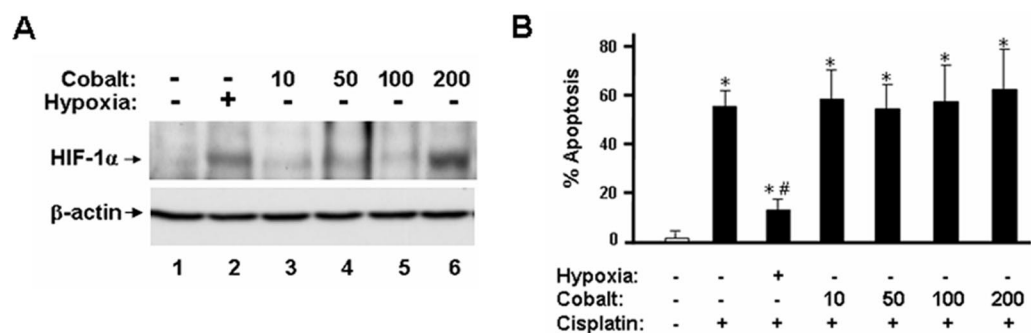


Figure 5. Effects of cobalt on hypoxia-inducible factor 1α (HIF-1α) and cisplatin-induced apoptosis. (A) Immunoblot of HIF-1α. RPTC were subjected to 16 h of incubation under hypoxia (lane 2) or under normoxia in the absence (lane 1) or presence of 10 to 200 μM cobalt chloride (lanes 3 through 6). Whole-cell lysate was collected for immunoblot analysis of HIF-1α. The blot was reprobed for β-actin to control protein loading and transferring. Shown is a representative blot of three separate experiments. (B) Apoptosis. RPTC were incubated with 20 μM cisplatin for 16 h under hypoxia or under normoxia in the presence of 10 to 200 μM cobalt chloride. Apoptosis was analyzed by morphologic examination. The results show that both cobalt and hypoxia induced HIF-1; however, cobalt did not mimic hypoxia to protect against cisplatin-induced apoptosis.

apoptosis and not necrosis in the primary cultures (data not shown). To determine cell injury and death, we examined cell viability using an MTS assay, which analyzed the metabolic activity of the cells (Figure 7). Under normoxia, 25 μM cisplatin treatment decreased cell viability to approximately 80% in both wild-type and HIF-1–deficient renal tubular cells. Hypoxia had cytoprotective effects, and, as a result, cell viability was maintained at virtually 100% of control. In the presence of 50 μM cisplatin, cell viability decreased to approximately 50% under normoxia, whereas approximately 70% of cells remained viable under hypoxia. Cisplatin at 100 μM led to further decrease of cell viability to approximately 30% under normoxia, and, again, hypoxia protected the cells, showing a viability of approximately 60%. To corroborate with the viability assay, we analyzed apoptosis. The results are shown in Figure 7B. Clearly, hypoxia inhibited apoptosis that was induced by 25, 50, and 100 μM cisplatin. Again, the effects of hypoxia were similar in the wild-type and HIF-deficient cells (Figure 7B). Therefore, hypoxia protected the primary cultures of renal tubular cells at every tested concentration of cisplatin. Importantly, the cytoprotective effects of hypoxia were comparable in the wild-type and the HIF-1–deficient renal tubular cells. Together with the cobalt (Figure 5) and mouse ES cell (Figure 6) experiments, the results strongly suggest that HIF-1 is not the key to the cytoprotective effects of hypoxia under our experimental conditions.

Mitochondrial Inhibitors Can Mimic Hypoxia in Protection against Cisplatin-Induced Apoptosis

The lack of oxygen during hypoxic incubation is expected to inhibit mitochondrial activity. To determine whether mitochondrial inhibition was involved in the cytoprotective effects of hypoxia, we examined the effects of several pharmacologic inhibitors of mitochondria. The mitochondrial inhibitors tested included four respiration inhibitors (rotenone for complex I, antimycin A and myxothiazol for complex III, and azide for complex IV), one F1-F0 ATPase inhibitor (oligomycin), and a mitochondrial uncoupler (CCCP). The results are shown in

Figure 8A. Clearly, the complex III inhibitors (antimycin A and myxothiazol) were most effective in blocking cisplatin-induced apoptosis (from approximately 60 to 10%). The F1-F0 ATPase inhibitor oligomycin also was effective, whereas rotenone and azide had marginal effects (Figure 8). No effects were shown for CCCP, a mitochondrial uncoupler that dissipates inner membrane potential and blocks ATP production in the organelles (34). Together, it is suggested that mitochondrial inhibition at specific sites might be involved in the cytoprotective effects of hypoxia against cisplatin-induced tubular cell apoptosis.

Our subsequent experiment determined whether the cytoprotective effects of hypoxia and mitochondrial inhibitors were additive (Figure 8B). Consistent with earlier results, both hyp-

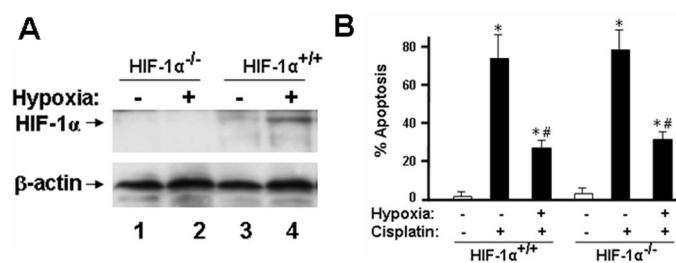


Figure 6. Cytoprotective effects of hypoxia in HIF-1α–deficient mouse embryonic stem (ES) cells. (A) Immunoblot of HIF-1α. Wild-type (HIF-1α^{+/+}) and HIF-1α–deficient (HIF-1α^{-/-}) mouse ES cells were incubated under normoxia or hypoxia for 16 h for collection of whole-cell lysate for immunoblot analysis of HIF-1α. The blot was reprobed for β-actin to control protein loading and transferring. (B) Wild-type and HIF-1α–deficient cells were incubated with 20 μM cisplatin under normoxia or hypoxia for 16 h. Apoptosis was examined by morphologic methods. Data are expressed as mean ± SD (n = 4). *Statistically significantly different from the control groups without cisplatin treatment; #significantly different from the cisplatin-treated normoxia groups. The results show that hypoxia suppressed cisplatin-induced apoptosis in wild-type as well as HIF-1α–deficient cells.

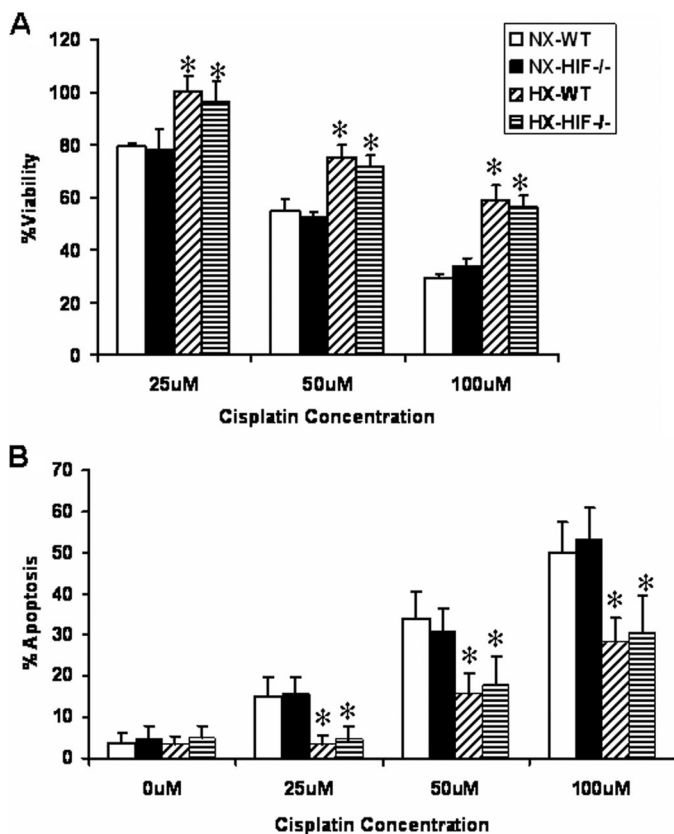


Figure 7. Cytoprotective effects of hypoxia in HIF-1 α ^{-/-} primary renal tubular cells. Proximal tubular cells were isolated from Hif-1 α ^{2-lox/2-lox} mice. The cells were cultured *in vitro* without treatment (wild-type [WT]) or treated with doxycycline to inactivate Hif-1 α (HIF^{-/-}), as described in Materials and Methods. These two groups of cells were incubated for 24 h with 25, 50, or 100 μ M cisplatin under normoxia (NX) or hypoxia (HX). (A) Cell viability was measured by MTS assay, as described in Materials and Methods. (B) Apoptosis was estimated by cell counting based on morphologic criteria. Data collected from three separate experiments in triplicate are expressed as mean \pm SD. *Statistically significantly different from the normoxic groups. The results show that hypoxia protected the WT as well as the HIF-1 α ^{-/-} primary tubular cells against cisplatin-induced apoptosis.

oxia and antimycin A decreased apoptosis during cisplatin incubation. The effects of hypoxia plus antimycin A were not different from that of hypoxia alone (Figure 8B). Similar results were shown for myxothiazol (data not shown). On the contrary, additive effects were shown for hypoxia and carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone, a general peptide inhibitor of caspases. The results support the possibility that the protective effects of hypoxia and mitochondrial inhibitors might be related.

Effects of Hypoxia and Mitochondrial Inhibitors on p53 Activation during Cisplatin Treatment

The signaling pathways that lead to tubular cell apoptosis during cisplatin treatment are complex and remain to be clarified (15,29). Nevertheless, recent studies have suggested the

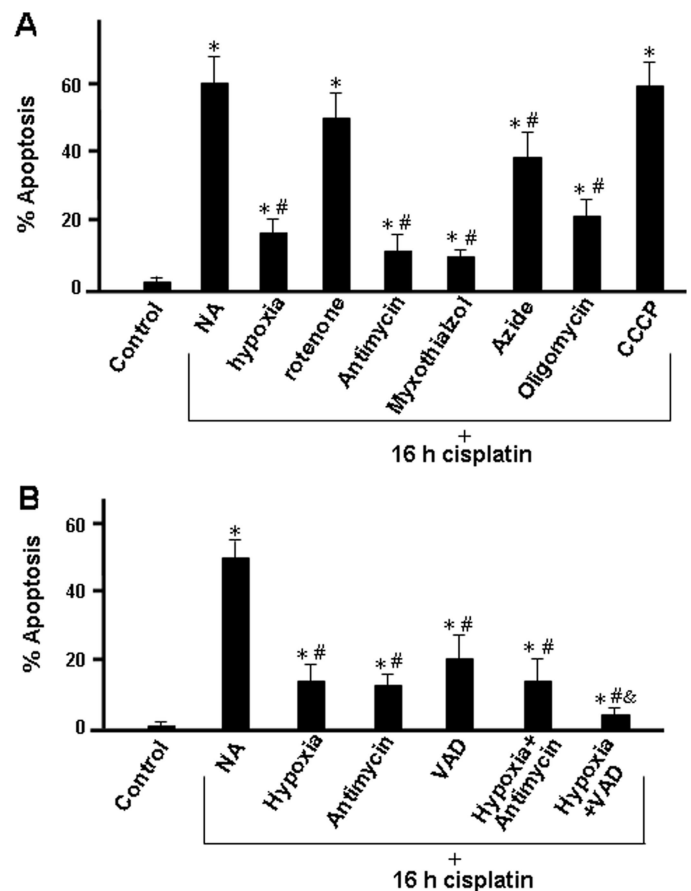


Figure 8. Effects of mitochondrial inhibitors on cisplatin-induced apoptosis. (A) RPTC were incubated with 20 μ M cisplatin for 16 h under normoxia or hypoxia. In the groups with mitochondrial inhibitors, cells were incubated with cisplatin under normoxia in full culture medium that contained 10 μ g/ml rotenone, 10 μ M antimycin A, 10 μ M myxothiazol, 10 mM azide, or 10 μ M oligomycin. (B) Cells were incubated with 20 μ M cisplatin for 16 h under normoxia (NA), hypoxia, normoxia with 10 μ M antimycin A (Antimycin), normoxia with 100 μ M carbobenzoxy-Val-Ala-Asp-fluoromethyl ketone (VAD), hypoxia with 10 μ M antimycin A (Hypoxia+Antimycin), or normoxia with 100 μ M VAD (Hypoxia+VAD). Apoptosis was assessed by morphologic methods. Data are expressed as mean \pm SD ($n = 4$). *Statistically significantly different from the NA group; #significantly different from the hypoxia group; &significantly different from the control group. The results show that mitochondrial inhibitors could mimic hypoxia to suppress cisplatin-induced apoptosis. In addition, the cytoprotective effects of hypoxia and antimycin A were not additive.

involvement of p53 (19–21,31). In RPTC cells, p53 is activated during cisplatin treatment, and the inhibition of p53 by pharmacologic as well as molecular approaches ameliorates cisplatin-induced apoptosis (20). Our latest work further suggested p53 regulation of p53 upregulated modulator of apoptosis α and its role in tubular cell apoptosis during cisplatin treatment (31). To determine whether p53 was involved in the cytoprotective effects of hypoxia and mitochondrial inhibitors, we examined p53 induction and phosphorylation under the experi-

mental conditions. p53 was induced and phosphorylated after 8 to 24 h of cisplatin treatment under normoxia (Figure 9A, lanes 2 through 4). Both p53 induction and phosphorylation were suppressed when the cells were treated with cisplatin under hypoxia (Figure 9A, lanes 5 through 8). The conclusion was supported further by densitometric analysis of blots from separate experiments (Figure 9, B and C). It is interesting that cisplatin-induced p53 activation also was suppressed by antimycin A and myxothiazol (Figure 9D, lanes 4 and 5), two inhibitors of mitochondrial respiration complex III that were shown to be most effective in blocking cisplatin-induced apoptosis (Figure 8). Much less effects were shown for oligomycin, azide, rotenone, and CCCP (Figure 9D, lanes 3, 6, 7, and 8). Collectively, the results suggest that apoptosis suppression by hypoxia, antimycin A, and myxothiazol may involve the suppression of p53 during cisplatin treatment.

Discussion

This study has extended our previous observation of the cytoprotective effects of hypoxia against apoptotic injury. Importantly, it has examined the possible underlying mechanisms using model systems of cisplatin-induced tubular cell apoptosis. In the experimental models, cytoprotection by hypoxia does not seem to involve the changes of cell ATP or pH during hypoxia incubation. Importantly, HIF-1, a “master” regulator of cellular adaptive response under hypoxia, does not play a critical role either. Conversely, inhibition of mitochondria, particularly at the respiration complex III, can mimic hypoxia to

protect against cisplatin-induced apoptosis. Although the underlying mechanisms remain to be clarified, both hypoxia and complex III inhibitors ameliorate p53 activation during cisplatin treatment. Considering the involvement of p53 in cisplatin-induced apoptosis, it is suggested that hypoxia may protect renal tubular cells *via* mitochondrial inhibition and p53 suppression.

Cellular acidification during hypoxic incubation has been recognized for a long time. This is due mainly to the shift of metabolism to the anaerobic direction (*i.e.*, glycolysis), leading to the accumulation of acidic metabolites. However, it has been controversial regarding the role of the lowered pH in cell injury and death under pathologic conditions (12,13). Our recent work has shown that acidic pH of 6.5 or lower suppresses tubular cell apoptosis after ATP depletion. A critical step in the apoptotic cascade that is blocked by pH 6 to 6.5 is the assembly of apoptosome, a cytochrome c/Apaf-1/caspase-9 protein complex that is responsible for the initiation of caspase activation (14). In our study, pH decreased from 7.4 to approximately 7.0 during hypoxic incubation. According to our earlier results (14), this pH decrease was not sufficient to block apoptosome formation and apoptosis. Consistently, when the incubation pH was maintained at approximately 7.4 by Hepes buffer, cisplatin-induced apoptosis in hypoxic cells was not increased or restored to the level of normoxic cells (Figure 4). On the basis of these results, it is suggested that pH decrease or cellular acidification is not critical to the observed cytoprotective effects of hypoxia.

This study, somewhat to our surprise, indicates that HIF-1

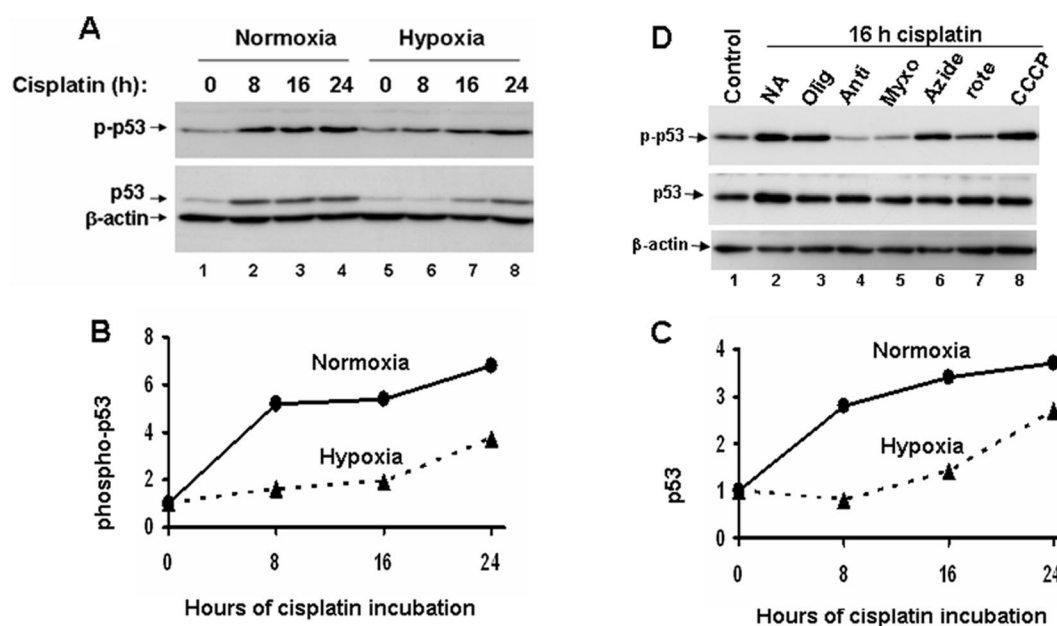


Figure 9. Effects of hypoxia and mitochondrial inhibitors on cisplatin-induced p53 activation. (A) RPTC were incubated with 20 μ M cisplatin under normoxia or hypoxia for 0 to 24 h. Whole-cell lysate was collected for immunoblot analysis of p53 and phospho-p53(serine 15). (B) Densitometry of p53 blots from three separate experiments. (C) Densitometry of phospho-p53 blots from three separate experiments. (D) RPTC were incubated for 16 h with 20 μ M cisplatin in the absence or presence of mitochondrial inhibitors. Whole-cell lysate was collected for immunoblot analysis of p53 and phospho-p53. Shown are representative blots of three separate experiments. The results indicate that hypoxia, antimycin A, and myxothiazol suppressed p53 activation during cisplatin treatment.

does not play an essential role in the protective effects of hypoxia. HIF, including HIF-1 and -2, are important regulators of the adaptive response of mammalian cells under hypoxic stress (8–10). In renal tubular cells, HIF-1 but not HIF-2 is expressed under hypoxia-related pathologic conditions, which is consistent with the ubiquitous regulatory role of HIF-1 (11). In our experiments, hypoxia induced HIF-1 but not HIF-2 (Figure 5 and data not shown). Cobalt also induced HIF-1; nevertheless, cobalt could not mimic hypoxia to protect the cells against cisplatin-induced cell death (Figure 5), suggesting that simply upregulating HIF-1 is not cytoprotective under the experimental condition. Importantly, hypoxia protected mouse ES cells regardless of the HIF-1 status (its presence or absence; Figure 6). In line with these observations, hypoxia protected primary cultures of HIF^{+/+} as well as HIF^{-/-} renal tubular cells (Figure 7). Therefore, it is concluded that HIF-1 and HIF-associated adaptive gene expression is not a key to the observed cytoprotective effects of hypoxia. It is interesting that recent work has demonstrated that, in the absence of glucose, HIF-1-deficient renal tubular cells are equally sensitive to hypoxic injury (24). In the presence of glucose, however, cell death is delayed in these primary cultures, suggesting that HIF-1 does not regulate the cell death machinery *per se*; rather, it is involved mainly in the regulation of glucose metabolism and energy production in these cells (24).

Our results regarding the role of HIF-1 are discrepant with a recent study by Tanaka *et al.* (6). In that study, cobalt was shown to be cytoprotective against cisplatin-induced cell death in an immortalized RPTC line. In addition, expression of dominant negative HIF-1 enhanced cisplatin-induced apoptosis under hypoxia. The exact reason underlying the discrepancy between our results and the earlier study is unknown. However, it was noticed that the protective effects of cobalt in cultured tubular cells were marginal even in the earlier study. Also, it was unclear whether the effects of the dominant negative mutant of HIF-1 were specific; in other words, after being overexpressed, the mutant might have effects other than antagonizing HIF-1. *In vivo*, however, cobalt showed an obviously better protection against cisplatin-induced renal injury. Apoptosis was reduced in both the outer medulla and the cortex. Importantly, this was associated with the protection of renal function (6). Of note, the *in vivo* situation is far more complex than the *in vitro* cell culture model system. Cisplatin injury *in vivo* is not restricted to the tubular cells but may extend to the vascular system, including endothelial cells. Moreover, cisplatin triggers an intense inflammatory response, which may have long-lasting effects on the parenchyma and renal function (15,29). It remains unclear whether the renoprotective effects of cobalt that are seen *in vivo* are derived partly from cobalt's interference with these injury-related responses. Regardless of the underlying mechanisms, it is important to recognize the *in vivo* beneficial effects of cobalt, which may offer a novel renoprotective strategy.

In response to hypoxia, mammalian cells also may activate HIF-independent mechanisms for adaptation. For example, our previous work identified IAP-2, an apoptosis inhibitory protein that is upregulated by severe hypoxia or anoxia and antago-

nizes cell death in several apoptosis models (17,18). It is noteworthy that IAP-2 is induced only by severe hypoxia or anoxia (near 0% oxygen) (17,18). In our study, however, cytoprotective effects were shown for hypoxia of 2% oxygen, a degree of hypoxia that is insufficient to induce IAP-2. In addition, IAP-2, as a caspase interacting and inhibitory protein, is expected to block caspase activation without affecting upstream apoptotic events, yet mitochondrial events of apoptosis, including Bax accumulation and cytochrome c release, were suppressed by 2% oxygen hypoxia in our study (Figure 2). Together, it is suggested that IAP-2 is not involved in the observed cytoprotective effects of hypoxia against cisplatin-induced apoptosis.

It is interesting that our results show that several pharmacologic inhibitors of mitochondria can mimic hypoxia to protect renal tubular cells during cisplatin treatment (Figure 8). Among the mitochondrial inhibitors, oligomycin, an inhibitor of F(0)F(1)-ATPase, has been shown to suppress apoptosis in other experimental models (35). The effects of oligomycin may be related to the proton movement across the mitochondrial inner membrane (35). For the other mitochondrial inhibitors tested in our study, antimycin A and myxothiazol consistently showed the best cytoprotective effects against cisplatin-induced apoptosis (Figure 8). Antimycin A and myxothiazol are inhibitors of complex III in the respiratory chain, whereas rotenone and azide inhibit complex I and IV, respectively. CCCP, conversely, uncouples mitochondria *via* the dissipation of inner membrane potential. Therefore, the results suggest that inhibition of mitochondria at complex III may be particularly effective in suppressing tubular cell apoptosis during cisplatin treatment. Altogether, the results suggest that hypoxia may suppress apoptosis partly through its inhibition of mitochondria.

The effects of antimycin A and myxothiazol also provide circumstantial evidence regarding the role of free-radical production in the observed cytoprotection. Although both target respiration complex III, antimycin A and myxothiazol are drastically different in triggering free-radical production in mitochondria. Whereas antimycin A treatment induces mitochondrial free radicals, myxothiazol inhibits them (36–38), yet in our study, these two complex III inhibitors show comparable cytoprotective effects against cisplatin-induced apoptosis. On the basis of these observations, it is suggested that free-radical production or oxidative stress may not play a critical role in the observed cytoprotective effects of hypoxia and mitochondrial inhibitors.

The cytoprotective effects of hypoxia are shown for 2% oxygen and also for anoxia (0% oxygen) and, to a lesser extent, for 5% oxygen. When oxygen tension is raised to 10%, it does not protect any more. Nevertheless, our results do not suggest that the cytoprotection is caused mainly by respiration inhibition. First, it is unknown whether hypoxia of 2 to 5% oxygen is severe enough to cease respiration in these cells. Second and more important, cytoprotective effects are not shown for all respiration inhibitors (Figure 8). The complex III inhibitors antimycin A and myxothiazol are very protective, whereas other respiration inhibitors, including the complex I inhibitor rotenone, obviously are less effective.

It is intriguing that both hypoxia and complex III inhibitors (antimycin A and myxothiazol) suppress p53 activation during cisplatin incubation. It is noteworthy that hypoxia suppresses p53 at 8 h of cisplatin treatment (Figure 9), a time point with minimal apoptosis (Figure 1). Therefore, the hypoxic effects on p53 are not secondary to cytoprotection. Our recent work indicates that p53 is phosphorylated and induced early after cisplatin treatment (20). Importantly, inhibition of p53 by pharmacologic as well as molecular approaches diminishes cisplatin-induced tubular cell apoptosis, suggesting a role for p53 in cisplatin nephrotoxicity (19,20). These studies further indicate that p53 mediates cisplatin-induced apoptosis *via* the induction of p53 upregulated modulator of apoptosis α , an apical proapoptotic Bcl-2 family protein (31). In addition, recent work by Seth *et al.* (21) suggested a role for p53 in the regulation of caspase-2 and the release of apoptosis-inducing factor during cisplatin-induced tubular cell apoptosis. On the basis of these observations, it is concluded the suppression of p53 by hypoxia and antimycin A/myxothiazol may contribute to their cytoprotective effects during cisplatin treatment.

Although we believe that p53 suppression contributes to the observed cytoprotective effects of hypoxia and antimycin A/myxothiazol, it is unclear how p53 activation during cisplatin treatment is suppressed by these maneuvers. p53 regulation involves a complex signaling network (39,40). Normally, p53 is under the control of MDM2, which targets p53 to degradation *via* proteasome. Upon DNA damage, for example by cisplatin, an array of protein kinases, such as ATM and ATR, are activated, leading to p53 phosphorylation. Being phosphorylated, p53 does not interact with MDM2 for degradation; instead, it accumulates in the nucleus, resulting in the transcription of various genes, including critical regulators of apoptosis (41–43). This seems to be a simplistic view of p53 signaling in response to DNA damage. Of note, different types of DNA damage may activate different signaling pathways for p53 activation (41–43). In our study, etoposide-induced p53 activation is not affected by hypoxia (data not shown). It is known that cisplatin induces mainly inter- and intrastrand DNA cross-linking, whereas etoposide induces double-strand breaks. Therefore, hypoxia may specifically interfere with cross-linking-initiated p53 activation.

Conclusion

This study has demonstrated the cytoprotective effects of hypoxia against cisplatin-induced tubular cell apoptosis. The cytoprotective effects do not seem to involve changes of cell ATP or pH during hypoxic incubation or the activation of HIF-1. Conversely, mitochondrial inhibition and p53 suppression may have a role.

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