Hypoxia-inducible factor modulates tubular cell survival in cisplatin nephrotoxicity

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Hypoxia-inducible factor modulates tubular cell survival in cisplatin nephrotoxicity. Am J Physiol Renal Physiol 289: F1123–F1133, 2005. First published June 14, 2005; doi:10.1152/ajprenal.00081.2005.—Hypoxia-inducible factor (HIF)-1 is a transcription factor mediating cellular response to hypoxia. Although it is expressed in tubular cells of the ischemic kidney, its functional role is not fully clarified in the pathological context. In this study, we investigated a role of HIF in tubular cell apoptosis induced by cisplatin. HIF-1 was expressed in tubular cells in the outer medulla 3 days after cisplatin administration. With the in vivo administration of cobalt to activate HIF, the number of apoptotic renal tubular cells became much smaller in the outer medulla, compared with the vehicle group. We also examined the functional role of HIF-1 in vitro using immortalized rat proximal tubular cells (IRPTC). In hypoxia, IRPTC that express dominant-negative (dn) HIF-1α showed impaired survival in cisplatin injury at variable doses (25–100 μM, 24 h), which was not obvious in normoxia. The observed difference in cell viability in hypoxia was associated with the increased number of apoptotic cells in dnHIF-1α clones (Hoechst 33258 staining). Studies on intracellular signaling revealed that the degree of cytochrome c release, dissipation of mitochondrial membrane potentials, and caspase-9 activity were all more prominent in dnHIF-1α clones than in control IRPTC, pointing to the accelerated signaling of mitochondrial pathways. We propose that HIF-1 mediates cytoprotection against cisplatin injury in hypoxic renal tubular cells, by reducing the number of apoptotic cells through stabilization of mitochondrial membrane integrity and suppression of apoptosis signaling. A possibility was suggested that activation of HIF-1 could be a new promising therapeutic target for hypoxic renal diseases.

apoptosis; mitochondria; caspase

MAMMALIAN CELLS POSSESS AN inborn mechanism by which they adapt to the hypoxic environment. Hypoxia-inducible factor (HIF)-1 is central to this mechanism, mediating angiogenesis, erythropoiesis, and glycolytic adaptation. It is a member of the basic helix-loop-helix-PAS period circadian protein (PER), aryl-hydrocarbon receptor (AHR), aryl-hydrocarbon-receptor nuclear translocator (ARNT), and single-minded protein (SIM) transcription family, which is composed of α- and β-subunits; the former is quickly degraded by prolyl hydroxylation, binding to von Hippel-Lindau protein and ubiquitin-proteasomal degradation in normoxia (12, 13), whereas the latter is constitutively expressed. In hypoxia, the α-subunit escapes ubiquiti-nation, forms a heterodimer with the β-subunit, binds to the enhancer sequence, and transactivates target genes, such as VEGF, erythropoietin, and glucose transporter 1 (GLUT1).

In the kidney, histochemical studies using a highly sensitive immunostaining method clarified the expression pattern of the HIF-1α and HIF-2α isoforms (21, 22). In response to hypoxia, HIF-1α was expressed in tubular cells, including proximal and distal segments, connecting tubules, and collecting ducts, whereas HIF-2α was localized in glomerular and peritubular endothelial cells and fibroblasts. In addition, our group (28) recently developed a transgenic rat model that allows us to detect cells with high transcriptional activity of HIF, thus extending these findings. It was clarified that tubular cells express HIF in two distinct models of chronic renal diseases: the remnant kidney and the puromycin-induced nephrotic syndrome. Undoubtedly, these findings suggest a functional role of HIF in the pathological progression of renal diseases. However, it is not fully understood what roles HIF plays during the pathogenesis.

cis-Diaminedichloroplatinum II (cisplatin) is a chemotherapeutic agent widely used for the treatment of solid tumors, but its clinical application is not infrequently limited by its nephrotoxicity, including tubular cell apoptosis. Pathological alterations are manifest mainly in the S3 segment of proximal tubules in the outer stripe of the outer medulla (4), the most susceptible portion of the kidney to ischemic insult, and previous studies have shown that, in some models of toxic acute renal failure, injury to this segment is associated with reduced renal blood flow (32). Therefore, it is highly plausible that the outer medulla is exposed to the hypoxic environment. Based on these backgrounds, we hypothesized that HIF-1 may be expressed in the hypoxic S3 segment in cisplatin injury and may play certain roles in the modulation of tubular cell apoptosis.

In this study, the activation of HIF was identified in the outer medulla in rat cisplatin nephrotoxicity, using “HIF-sensing” transgenic rats. By administering cobalt to stabilize HIF chemically (7, 24), we found the number of apoptotic tubular cells to be significantly reduced in these areas. In vitro, the role of HIF-1 was investigated by introducing dominant-negative HIF-1α (dnHIF-1α) to immortalized rat proximal tubular cells (IRPTC). dnHIF-1α clones showed impaired survival in cisplatin injury in hypoxia, which was associated with the accelerated signaling of mitochondrial pathways, such as cytochrome c release and caspase-9 activation. These findings

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suggest that, in hypoxia, HIF-1 protects renal tubular cells from cisplatin toxicity through inhibition of mitochondrial signaling pathways in apoptosis.

MATERIALS AND METHODS

In vivo experiments. HIF-sensing transgenic rats, established recently in our laboratory, were enrolled in this study (28). They harbor a transgene composed of hypoxia-responsive elements (HRE, enhancer) of the rat VEGF gene (16) and FLAG-tagged luciferase reporter gene and allow us to identify loci of hypoxic tubules. Male rats weighing 250–300 g were injected with 6 mg/kg cisplatin (Sigma) or vehicle (saline) intraperitoneally (n = 4–8 each) and housed in a light- and temperature-controlled environment. At days 1, 3, 5, and 7, these rats were anesthetized with ketamine (50 mg/kg), blood samples were taken via cardiac puncture, and the left kidneys were removed for analyses. Blood urea nitrogen (BUN) and serum creatinine levels were measured with a conventional laboratory method (Wako, Osaka, Japan). All experiments conformed to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the committee on ethical animal care at University of Tokyo.

In another set of experiments, the role of HIF was investigated in vivo using cobalt. Additional rats (n = 6 each) were injected with cisplatin in combination with either cobalt (5 mg·kg⁻¹·day⁻¹·sec) or vehicle (PBS) from day –1 to day 3, to activate HIF. Kidneys were removed at day 3, and the number of apoptotic cells was counted by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining in the outer medulla and cortex.

The third set of experiments consisted of rats that were administered with pimonidazole, a chemical marker to detect hypoxia (Chemicon). Two hours after intravenous injection of pimonidazole (60 mg/kg), rats were subjected to either cisplatin or vehicle (n = 3 each). At day 3, kidneys were removed and hypoxic tubules were detected immunohistochemically with Hypoxyprobe-1Mab1 (Chemicon), according to the supplier’s protocol.

Histopathology and immunohistochemistry. Methyl-Carnoy’s or buffered formalin-fixed, paraffin-embedded sections (3-μm thick) were deparaffinized and brought to water through graded ethanol. Periodic acid-Schiff (PAS) staining was used for routine histological examination. HIF-expressing renal tubules, damaged tubular cells, and vascular endothelial cells were detected with an indirect immunoperoxidase method, using anti-FLAG (M2, 1/350; Sigma), V9 (vimentin, 1/500; Dako, Carpinteria, CA), and JG12 (1/100; Bender MedSystems, Vienna, Austria) antibodies, respectively. After endogenous peroxidase was quenched with 3% H₂O₂ in PBS for 5 min, sections were incubated with the corresponding primary antibodies, biotinylated anti-mouse IgG (1/400; Vector) and Vectastain Elite ABC reagent (Vector Laboratories). Color development was made with H₂O₂ and diaminobenidine. Negative controls were served by the omission of the incubation step with the primary antibodies. For the detection of specific HIF-α isoforms, tissues were perfusion fixed and probed with anti-HIF-1α (1/100; Novus Biologicals) or anti-HIF-2α (PM9, 1/3000) antibodies (22), and sites of HIF-α-expressing nuclei were detected using TSA amplification (TSA biotin system; Perkin Elmer).

Apoptotic cells were detected by TUNEL staining. Buffered formalin-fixed sections were processed with cytolin. After a 5 min quenching with endogenous peroxidase, sections were reacted with terminal deoxynucleotidyl transferase and biotinylated dUTP in terminal deoxynucleotidyl transferase reaction buffer (30 mM Tris·HCl, pH 7.2, 140 mM sodium cacodylate, 1 mM Mn²⁺) for 60 min at 37°C. Biotinylated nuclei were detected using horseradish peroxidase-avidin (Vector Laboratories), H₂O₂, diaminobenzidine, and nickel chloride. The number of TUNEL-positive cells was counted under ×200 magnification, in both the outer medulla and cortex. At least 10 nonoverlapping fields were evaluated per section and averaged.

Real-time PCR. The transgene expression was quantified by real-time PCR as a marker of HIF activation. RNA was isolated from renal cortex with ISOGEN (Nippon Gene, Tokyo, Japan). cDNA was synthesized with iPrime reverse transcription system (Promega), and 1/20 (vol/vol) was used as a template for PCR quantification. The reaction was run on an iCycler (Bio-Rad) using iQ SYBR green PCR supermix (Bio-Rad), according to the supplier’s protocol; the relative amount of target genes was then corrected for that of β-actin. Primers for the transgene and VEGF have been described previously (28).

Cell culture. IRPTC is a cultured cell line originating from proximal tubular cells of male Wistar rats, immortalized by transformation with origin-defective SV40 DNA (10). Cells were cultured in DMEM (Nissui, Tokyo, Japan), buffered with 25 mM HEPES at pH 7.4, supplemented with 5% FBS (JRH Biosciences), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.01 mM nonessential amino acids at 37°C under a humidified atmosphere of 5% CO₂-95% air. At 80% confluence, medium was changed to serum-free DMEM, and cells were subjected to cisplatin treatment. Hypoxic atmosphere was provided by placing cells in a multi-gas incubator (APM-3D; ASTEC, Fukusha, Japan), in which 1% O₂-5% CO₂ was constructed with nitrogen as the balance. Cell death induced by hypoxia (1% O₂) per se was negligible at this time frame (up to 24 h, not shown).

IRPTC clones with the forced expression of dnHIF-1α have been established previously in our laboratory (27). A mammalian expression vector, pcDNA3.1—(Invitrogen), expressing a 1.1-kb fragment coding for the dnHIF-1α lacking both the DNA binding and transactivation domain, was stably expressed in IRPTC. This method offsets the reporter activity of HRE-driven luciferase reporter vector (HRE-Luc) from 3.8 ± 0.4- to 0.7 ± 0.1-fold in hypoxia. These dominant-negative clones were further characterized by quantifying mRNA of HIF-1 target genes, such as VEGF, heme oxygenase-1 (HO-1), and GLUT1, as previously described (18).

The specific role of HIF-1α in IRPTC was corroborated by silencing of HIF-1α by RNA interference, using an siRNA expression vector, pSilencer2.0-U6 (Ambion). The siRNA sequence targeting rat HIF-1α corresponds to the coding region 407–427 (accession no. NM024359.1), relative to the start codon.

Cell viability assays and detection of apoptosis. Cell viability after cisplatin treatment was quantified by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reduction to formazan (Promega). IRPTC was seeded in 96-well culture dishes (TPP) at 1.0 × 10⁵ cells per well. Approximately 40–48 h later, cells were stimulated with 0, 25, 50, and 100 μM cisplatin for 24 h. After treatment, 1/5 (vol/vol) MTS reagent was added in each well, and cells were incubated for another hour. By measuring the optic absorbance at 450 nm with a microtiter plate reader, we calculated the relative cell viability in each treatment group.

Apoptosis in these cells was detected by Hoechest 33258 staining (Dojin Chemical, Kumanomoto, Japan). Cells with characteristic nuclear morphology, such as shrinkage and blebbing, and those with apoptotic bodies were categorized as being in apoptosis and counted with a microscope equipped with fluorescence filters (Olympus, Tokyo, Japan).

Immunoblotting. Release of cytochrome c from mitochondria to the cytosol was compared in both clones by Western blotting. Control IRPTC and dnHIF-1α clones were exposed to 50 μM cisplatin for 3, 6, 12, and 24 h. Cytosol fractions were obtained by digitonin permeabilization of plasma membranes. In brief, cells were collected and washed in PBS, permeabilized with 0.5% digitonin buffer (in mM: 250 sucrose, 10 HEPES, 10 KCl, 1.5 MgCl₂, 1 EDTA, and 1 EGTA; pH 7.1) for 1 min and centrifuged at 3,000 g for 15 min. Pellets including nuclei and mitochondria were set aside, and supernatants were used as cytosol fractions. Negative staining with anti-cytochrome oxidase antibody (subunit IV; Molecular Probes, Eugene OR) confirmed no recognizable contamination of this fraction with mitochondrial components.

For immunoblotting analysis, proteins were resolved with 12% SDS-PAGE under reducing conditions and transferred onto polyvi-
nylidene difluoride membranes (Amersham, Piscataway, NJ). After blocking with 2.5% skim milk, we probed membranes with anti-cytochrome c antibody (1/500; Santa Cruz Biochemistry) followed by alkaline phosphatase-conjugated anti-mouse IgG (Promega). Specific bands were detected with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma). Coomassie brilliant blue staining of the membranes confirmed equal loading and transfer.

**Measurement of mitochondrial membrane potentials and caspase-9 activity.** Changes in mitochondrial membrane potentials were measured semiquantitatively by rhodamine 123 uptake. Control IRPTC and dnHIF-1α clones were challenged with cisplatin and loaded with 10 μM rhodamine 123 (Wako) at 37°C for 15 min. Fluorescence intensity was analyzed with flow cytometry (FACScan and CellQuest Pro software; Beckton Dickinson).

The relative activity of caspase-9 was measured with a colorimetric assay kit (Medical and Biological Laboratories, Nagoya, Japan). Cells were collected and lysed in the cell lysis buffer provided with the kit. Samples of 100 μg of protein were then reacted in the reaction buffer containing 200 μM p-nitroaniline-conjugated LEHD and 10 mM DTT at 37°C for 1 h. By measuring the optical absorbance at 405 nm, we calculated the relative caspase-9 activity.

Another set of measurements were carried out by adding exogenous cytochrome c to cell lysates of both control IRPTC and dnHIF-1α clones, to reconstitute caspase-9 activity (5). Two microliters of 1 mg/ml exogenous cytochrome c (bovine; Sigma) and 4 μl of 10 mM dATP (Takara, Osaka, Japan) were added to 100 μg of cytosol fractions and incubated at 30°C for 1 h. The mixture was then processed for caspase measurement.

**Statistical analyses.** Data are expressed as means ± SE, unless otherwise noted. Analyses were carried out with StatView software (version 5.0; SAS Institute, Cary, NC). Differences among groups were compared by unpaired Student’s t-tests with the correction of Bonferroni or Dunn’s method. P values below 0.05 were considered statistically significant.

**RESULTS**

**Time course of cisplatin nephrotoxicity.** A single intraperitoneal injection of 6 mg/kg cisplatin induced significant nephrotoxicity in rats (Table 1). BUN and serum creatinine levels were elevated from 13.0 ± 0.6 mg/dl and 0.72 ± 0.08 mg/dl to 29.8 ± 4.0 mg/dl and 1.78 ± 0.20 mg/dl at day 3 (P < 0.01 for both), which returned nearly to the baseline by day 5. Histochemical analyses using PAS sections revealed that the pathological changes were most evident in the S3 segment of the proximal tubules at day 3 and consisted of brush-border loss, flattening, nuclear condensation, and foci of sloughing (not shown), in keeping with previous observations (4).

**Cisplatin induces HIF activation in the outer medulla.** A previous study demonstrated that the renal blood flow is reduced significantly in the early stage of cisplatin nephrotoxicity (32). Therefore, we hypothesized that renal tubules might be exposed to hypoxia and express HIF protein (Fig. 1). Immunostaining with anti-FLAG antibody revealed a much stronger expression of the transgene in the outer medulla of the cisplatin-treated kidney at day 3 (Fig. 1, A and B), reflecting HIF activation. Probing for specific HIF-α isoforms clarified that HIF-1α was expressed in proximal tubular cells in the outer medulla, as well as in tubules in the inner medulla and papilla. HIF-2α, on the other hand, was expressed exclusively in the peritubular (interstitial and endothelial) areas in the medulla and cortex adjacent to the outer medulla and medullary rays (Fig. 1, C–F). Considering also that the HIF-1α isoform is expressed exclusively in the tubular compartment (21), the upregulation of the transgene most likely reflected the activity of HIF-1 protein. A theoretical background for HIF activation was obtained by pimonidazole staining, which identified hypoxic tubules in the outer medulla (Fig. 1, G and H). Hypoxia in these areas was associated with narrowed peritubular capillary structures, as confirmed by immunostaining with JG12 antibody (Fig. 1, I and J). Furthermore, serial staining for pimonidazole and Phaseolus vulgaris agglutinin-E lectin, a specific marker of proximal tubules, identified a substantial portion of hypoxic tubules in these areas as proximal tubules (Fig. 1, K and L). Although hypoxia and HIF activation were most evident in the outer medulla, cortical tubules also stained positive for pimonidazole and anti-FLAG antibody, although to a lesser extent (not shown).

The upregulation of the transgene was quantified as a surrogate marker of HIF activation (Fig. 2A). Cisplatin treatment in vivo induced a significant increase in the transgene expression at day 3, reaching 2.9-fold (P < 0.05). The upregulation appeared to persist even at day 5 but was no longer significant and returned to the baseline at day 7 (not shown). To exclude the possibility that the upregulated HIF in renal tubules was caused by the direct action of cisplatin, rather than by hypoxia, the transgene vector (HRE-Luc) was transiently transfected to IRPTC and subjected to cisplatin treatment in vitro (Fig. 2B). Cisplatin did not induce any recognizable increase in the reporter gene activity at doses of 25–100 μM, which supports our notion that HIF is activated in vivo primarily by hypoxia.

**Roles of HIF activation by cobalt.** On the basis of the above findings that HIF was upregulated in cisplatin nephrotoxicity, a possible role of HIF was investigated by systemically activating HIF with cobalt. Representative figures of vehicle- or cobalt-treated kidneys are shown in Fig. 3. At day 3, the number of TUNEL-positive tubular cells was significantly reduced by cobalt in the outer medulla (Fig. 3, A and B). Vimentin staining, a marker of tubular damage, was also reduced in cobalt-treated kidneys (Fig. 3, C and D). The number of TUNEL-positive tubular cells was counted both in the outer medulla and cortex (Fig. 4). In both areas, the number of apoptotic tubular cells was significantly reduced by activating HIF with cobalt [Fig. 4, A and B; counting in outer medulla: 18.1 ± 1.9 (vehicle) vs. 9.9 ± 1.6 (cobalt); in cortex: 13.5 ± 1.4 (vehicle) vs. 8.7 ± 0.5 (cobalt); P < 0.01 for both]. Likewise, BUN and serum creatinine levels tended to be lower in the cobalt-treated group (Fig. 4, C and D). These findings further led us to investigate a role of HIF in cisplatin injury in vitro.

**Hypoxia induces the HIF-1α isoform in IRPTC.** The expression of HIF-1α and HIF-2α isoforms was examined in IRPTC (Fig. 5). Immunoblotting using anti-HIF-1α and anti-HIF-2α antibodies revealed an obvious increase of the HIF-1α subunit by hypoxia, suggesting the functional operation of this isoform. HIF-2α, on the other hand, was undetectable in our experimental conditions. Therefore, is appears likely that HIF-1α is the
Fig. 1. Cisplatin induces hypoxia-inducible factor (HIF) activation in the outer medulla. At day 3, an increase in the transgene expression was observed in the outer medulla of the cisplatin-treated kidney (A and B), signifying HIF activation. Immunostaining for HIF-1α isoforms revealed that HIF-1α was expressed in proximal tubular cells in the outer medulla (C and D; inset shows magnified view), whereas HIF-2α was restricted to the peritubular areas in the medulla and the cortex adjacent to it (E and F; inset shows magnified view). Pimonidazole staining showed that these areas are exposed to hypoxia in cisplatin nephrotoxicity (G and H). Immunostaining with JG12 antibody revealed narrowed peritubular capillaries in these areas (I and J). Serial staining for pimonidazole and Phaseolus vulgaris agglutinin-E (PHA-E) lectin revealed that a substantial portion of hypoxic tubules in the outer medulla are proximal tubules. Original magnification = ×200 (A–D, G, and H) and ×400 (E, F, and I–L). *Hypoxic, PHA-E-positive tubules; #hypoxic, PHA-E-negative tubules.

Fig. 2. Transgene expression. Expression of the transgene was quantified as a marker of HIF-activation. At day 3, the expression level of transgene mRNA reached 2.9-fold and persisted until day 5 (A). Parallel quantification of VEGF mRNA revealed no significant changes, although they harbor hypoxia-responsive elements (HRE) in their promoters in common. Transgene expression was analyzed in cultured immortalized rat proximal tubular cells (IRPTC) cell line (B). The transgene vector (HRE-Luc) was transiently transfected to IRPTC, and cells were exposed to cisplatin treatment for 8 h. Transgene activity, as measured by dual-luciferase assay, was not elevated by cisplatin at 25–100 μM, excluding the possibility that the observed transgene expression in vivo was mediated by the direct action of cisplatin. Cobalt chloride (Co; 100 μM) was used as positive control. A: real-time PCR; n = 6 rats. *P < 0.05 vs. day 0. B: dual-luciferase assay. Data are expressed as means ± SD; n = 4. **P < 0.01 vs. control.

Induction of HIF-1-regulated genes by hypoxia in control IRPTC and dnHIF-1α clones. To investigate the functional role of HIF-1 in IRPTC, clones carrying the dnHIF-1α expression vector (dnHIF-1α clones 2.17 and 1.14) or those with the empty vector (control IRPTC) were constructed. These clones

dominant isoform in IRPTC. Immunocytochemistry corroborated these findings (not shown).

**ROLE OF HIF IN CISPLATIN INJURY**

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were characterized by measuring relative increases in HIF-1-regulated gene mRNAs at 6 h of hypoxia using real-time PCR. The relative increase in mRNA of VEGF, HO-1, and GLUT1 was 5.9-, 3.3-, and 3.5-fold in control IRPTC, respectively, whereas the increase was inhibited significantly to 3.3-, 1.2-, and 1.9-fold in the dnHIF-1/H9251 clone (clone 2.17). These results indicate the functional blockade of HIF-1 by the forced expression of dnHIF-1α.

Role of HIF manipulation on IRPTC survival. The effect of cobalt on IRPTC viability was examined by adding 25–50 μM cobalt to the culture medium (Fig. 6A). At these doses, cobalt improved IRPTC survival against cisplatin injury, consistent with in vivo findings, further suggesting a role of HIF in this type of injury. We then determined the specific role of HIF-1 by comparing cell viability among control IRPTC and dnHIF-1α clones. In hypoxia (Fig. 6B), two distinct clones...
Similar loss of cell viability was observed by silencing of HIF-1α by RNA interference. Cell viability was significantly impaired by this method (50 μM cisplatin, under hypoxia: 37.9 ± 1.4% (control IRPTC), 39.9 ± 1.2% (empty vector control), and 32.2 ± 1.3% (clones silenced for HIF-1α); P < 0.05), with 67% breakdown of the target gene mRNA and protein (not shown). These clones showed similar cell growth at this time frame (Fig. 6D), in keeping with previous findings in pancreatic cancer cells (2). When these clones (control IRPTC and dnHIF-1α IRPTC) were challenged for cisplatin in normoxia (Fig. 6C), there was no recognizable difference in viability.

The apoptotic cell death was detected by Hoechst 33258 staining under hypoxic conditions (Fig. 7). In both IRPTC clones, cisplatin induced alterations in nuclear morphology such as shrinkage, blebbing, and condensation, which are characteristic features of apoptosis (Fig. 7A). The number of nuclei with such morphology was apparently larger in dnHIF-1α clones than in control IRPTC (Fig. 7B; counting: 23.4 ± 1.8% vs. 16.2 ± 1.1%; P < 0.05). Parallel treatment of both clones in hypoxia only (without cisplatin) led to no recognizable nuclear changes. On the basis of the above findings that dnHIF-1α renders IRPTC susceptible to cisplatin injury in hypoxia and that it parallels the number of apoptotic cells, the intracellular signaling was compared between control IRPTC and dnHIF-1α clones, focusing on those responsible for mitochondrial signaling pathways.

Cytochrome c release and dissipation of mitochondrial membrane potentials are modulated by the HIF-1 activity. Cisplatin is a chemical agent that induces apoptosis in a number of epithelial cells through the mitochondrial pathways, and mitochondria are a crucial checkpoint determining the fate of cells. Therefore, we postulated that the difference in the proportion of cell death might be reflected by the degree of cytochrome c release and dissipation of mitochondrial membrane potentials (Fig. 8). Western blotting (Fig. 8A) using cytosol fractions showed that in dnHIF-1α clones a larger amount of cytochrome c was released from mitochondria to the cytosol than in control IRPTC, which was apparent at as early as 3 h after treatment. Similarly, dissipation of mitochondrial membrane potentials was more evident in the dnHIF-1α clones than in control IRPTC at 6 and 12 h (Fig. 8B). These findings imply that the difference in cisplatin injury between control IRPTC and dnHIF-1α clones is associated with the distinct degrees of cytochrome c release and dissipation of mitochondrial membrane potentials.

Caspase-9 activity of control IRPTC and dnHIF-1α clones. Caspase-9 is a cysteine protease located downstream of mitochondrial regulatory mechanisms of apoptosis. Therefore, we examined whether the distinct amount of cytochrome c released into the cytosol might have contributed to the distinct...
degree of caspase-9 activity (Fig. 8C). Cisplatin induced caspase-9 activation in both control IRPTC and dnHIF-1α clones (241.5 ± 28.7% and 460.0 ± 64.2%, respectively), with the latter being much higher than the former (P < 0.05 between control IRPTC and dnHIF-1α clones). Considering the possibility that the degree of caspase-9 activity might be reflected by the amount of cytochrome c released from mitochondria, another set of experiments were made to reconstitute caspase-9 activity. Addition of exogenous cytochrome c and dATP into the reaction mixture of both control and dnHIF-1α clones reconstituted caspase-9 activity to a similar degree (471 ± 196.6% and 429 ± 103.6%). These data indicate that caspase-9 is activated in cisplatin injury in both control IRPTC and dnHIF-1α clones and that the degree is reflected by the amount of cytochrome c released from mitochondria. Together, the difference in the rate of cell death in control IRPTC and dnHIF-1α clones can be traced back to the distinct degrees of activation of mitochondrial signaling pathways.

Reduced cellular ATP levels in dnHIF-1α clones. One of the possible pathological links among cellular adaptation to hypoxia, cell viability, and the mitochondrial function can be found in alterations in energy generation and its storage. In this regard, we examined cellular ATP levels in both control and dnHIF-1α clones (Fig. 9A). At baseline, the ATP level in dnHIF-1α clones was 92.9 ± 4.2% of the control IRPTC. Hypoxia alone did not change cellular ATP storage levels either in control or in dnHIF-1α clones, at this time frame (111.0 ± 10.6% and 90.6 ± 2.7% at 24 h; P = not significant). By cisplatin treatment, however, a significant fall in cellular ATP content was observed in both control IRPTC and dnHIF-1α clones at 24 h. The dissipation in cellular ATP storage became much more prominent by abrogating the function of HIF (31.1 ± 7.4% in dnHIF-1α clones; P < 0.05 vs. control IRPTC in the cisplatin group).

Because a marked decrease in ATP levels was observed in dnHIF-1α clones treated with hypoxia and cisplatin, a possibility was raised that supplementation of energy sources exogenously might serve to protect cells from cisplatin injury in hypoxia (Fig. 9B). However, supplementation of neither 1–10 mM pyruvate nor additional 5–10 mM glucose in the culture medium contributed to the improvement in cell viability.

**DISCUSSION**

This study demonstrated that HIF, most likely HIF-1, was activated in renal tubules in the outer medulla in cisplatin nephropathy. By systemic stabilization of HIF in rats using cobalt, the number of TUNEL-positive tubular cells became significantly
smaller, suggesting the anti-apoptotic roles of HIF. Studies with a cultured proximal tubular cell line clarified that clones with the forced expression of dnHIF-1α showed impaired survival against this insult in hypoxia, which was associated with accelerated cytochrome c release, dissipation of mitochondrial membrane potentials, and caspase-9 activation. Collectively, these data suggest that HIF-1 expressed in hypoxic renal tubular cells plays a cytoprotective role in cisplatin injury.

Tubulointerstitial hypoxia has been recognized as a hallmark of, and a mediator to, progressive renal diseases (6, 8, 15, 19). In the kidney, tubular epithelial cells have been reported to express HIF-1 in response to hypoxia (22). A previous study
from our group had predicted renoprotective roles of HIF in the ischemia-reperfusion injury model (18). It remained as yet unclear, however, what the responsible mechanisms were and whether HIF acts in a disease-specific manner or it confers a renoprotective role in a broader array of renal diseases. This study attempted to address the role of HIF in a nephrotoxic disease model and identified an anti-apoptotic role as one of the responsible mechanisms for renoprotection.

Classically, hypoxia has been thought to play important roles in not only ischemic but also toxic acute renal failure because cellular injury occurs predominantly in the outer medulla, an area most susceptible to an ischemic insult. The S3 segment and the medullary thick ascending limb are located at the most distant portion from vascular beds and have very limited potential for glycolytic adaptation, which would provide a rationale to look for a role for HIF in a model of toxic acute renal failure.

In terms of renal circulation, a previous study reported reduced glomerular filtration in the early phase of cisplatin-induced acute renal failure in rats (32), thus suggesting the presence of hypoxia. Nevertheless, it was of significance to provide direct proof of it because hypoxia at the cellular level is to be determined not only by oxygen supply but also from a viewpoint of oxygen consumption. When glomerular filtration is reduced, it not only impairs oxygen supply but also affects tubular oxygen consumption. Therefore, a reduction of glomerular filtration alone does not prove to be evidence of tubular cell hypoxia (17). In this study, we conducted pimonidazole staining and identified hypoxic tubules in the outer medulla in cisplatin nephrotoxicity. Further analyses using serial sections clarified that a significant portion of hypoxic cells are indeed proximal tubular cells.

The expression and activation of HIF in the outer medulla appear primarily due to the reduced oxygen concentration because pimonidazole staining clearly identified hypoxia in these areas. Recently it was reported that cisplatin increased HIF-1α protein in a rat hepatoma cell line regardless of the ambient oxygen concentrations (33), suggesting the direct action of cisplatin in modulating HIF-1α expression. However, our reporter gene assay using IRPTC revealed no recognizable upregulation of the transgene (HRE-Luc) in response to cisplatin alone, making it less likely that the upregulation of the transgene reflected the direct action of cisplatin. In addition, the transgene construct has been shown not to respond to inflammatory cytokines such as TNF-α (28). Collectively, these findings suggest that the transgene, HRE-Luc, was activated primarily by hypoxia in cisplatin injury in vivo.

The early event in cisplatin injury in vitro is characterized by mitochondrial injury (1) and apoptosis (20). Our experiments using control IRPTC and dnHIF-1α clones showed clear differences in the degree of cytochrome c release, dissipation of mitochondrial membrane potentials, caspase-9 activity, and the resultant apoptosis under hypoxic conditions. Considering that caspase-9 activity was reconstituted to a comparable degree by exogenous supplementation of cytochrome c, it appears likely that the responsible regulatory checkpoint of HIF-1 on its anti-apoptotic properties can be traced back to the preservation of mitochondrial membrane stability.

Impaired cell viability of dnHIF-1α clones against cisplatin injury was also associated with a marked decrease in cellular ATP content. In view of recent reports elucidating the contributory roles of accelerated glycolysis in HIF-1 mediated cellular defense mechanisms (2, 3), it was of interest to address whether such was the case in renal tubular cells. Unfortunately, however, supplementation of neither pyruvate nor glucose into the culture medium contributed to cell survival in cisplatin injury. Parallel treatment with glycerol served as osmotic controls. For A, n = 3, *P < 0.05 vs. control IRPTC in the cisplatin group. B (MTS assay) is representative of 3 independent experiments. Data are expressed as means ± SD, n = 8.

Fig. 9. Cellular ATP levels during cisplatin treatment and the effect of supplementation of glycolytic substrates. A: cellular ATP levels were measured using a bioluminescent assay (Promega). At baseline, ATP level in dnHIF-1α clones was 92.9 ± 4.2% of control IRPTC. Hypoxia alone did not change cellular ATP levels either in control IRPTC or in dnHIF-1α clones significantly. By the combination of cisplatin treatment with hypoxia, however, a significant fall in cellular ATP content was observed in both clones at 24 h, which was more prominent in dnHIF-1α clones. B: effect of external supplementation of glycolytic substrates on cell viability was examined in hypoxia. Neither 1–10 mM pyruvate nor additional 5–10 mM glucose in the culture medium contributed to cell survival in cisplatin injury. Parallel treatment with glycerol served as osmotic controls. For A, n = 3, *P < 0.05 vs. control IRPTC in the cisplatin group. B (MTS assay) is representative of 3 independent experiments. Data are expressed as means ± SD, n = 8.
remains unclear, however, which of the HIF-regulated genes plays a central role in ameliorating cisplatin injury.

In summary, this study demonstrated that HIF mediates cytoprotection against cisplatin injury in hypoxic renal tubular cells. HIF, likely HIF-1, was activated in the S3 segment of proximal tubules in cisplatin injury in vivo, and the loss-of-function experiments in vitro showed that dnHIF-1α rendered IRPTC susceptible to cisplatin injury and apoptosis, which was associated with the increased release of cytochrome c, loss of mitochondrial membrane potential, and caspase-9 activity, all indicative of the acceleration of mitochondrial pathways. These results strongly suggest that HIF works as a positive factor for the survival of tubular cells in cisplatin nephrotoxicity. It remains still unclear, however, whether the renoprotection of HIF is disease specific or common to all types of ischemic renal diseases because HIF is obviously a multifactorial target affecting glycolysis, angiogenesis, erythropoiesis, and other synergistically and may exert divergent roles according to each pathological context, which warrants further research. Nevertheless, a possibility has been suggested here that maneuvers to activate HIF (9), including a method of silencing prolyl hydroxylase activities (11, 31), may become a new therapeutic option in treating ischemic renal diseases.

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