HIF Activation Protects From Acute Kidney Injury

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ABSTRACT

The contribution of hypoxia to cisplatin-induced renal tubular injury is controversial. Because the hypoxia-inducible factor (HIF) pathway is a master regulator of adaptation to hypoxia, we measured the effects of cisplatin on HIF accumulation in vitro and in vivo, and tested whether hypoxic preconditioning is protective against cisplatin-induced injury. We found that cisplatin did not stabilize HIF-1α protein in vitro or in vivo under normoxic conditions. However, hypoxic preconditioning of cisplatin-treated proximal tubular cells in culture reduced apoptosis in an HIF-1α-dependent fashion and increased cell proliferation as measured by BrdU incorporation. In vivo, rats preconditioned with carbon monoxide before cisplatin administration had significantly better renal function than rats kept in normoxic conditions throughout. Moreover, the histomorphological extent of renal damage and tubular apoptosis was reduced by the preconditional treatment. Therefore, development of pharmacologic agents to induce renal HIF might provide a new approach to ameliorate cisplatin-induced nephrotoxicity.

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Ischemia and toxicity are considered the main pathophysiological factors that lead to the development of acute kidney injury. A substance that is well known to induce toxic kidney injury is cis-diammino-dichloro-platinum (cisplatin).1 Cisplatin is widely used as a chemotherapeutic agent in the treatment of solid tumors, but its application is frequently limited because of nephrotoxic side effects, which occur even after a single dose of cisplatin in up to 28% to 36% of cancer patients.2 Renal cisplatin toxicity is associated with the induction of apoptotic cell death, inflammation, and necrosis predominantly of the S3 segment of the proximal tubule.3–5 In addition to the toxic damage of tubular cells, reduced renal blood flow6 with subsequent hypoxia of the outer medulla may contribute to cisplatin-induced renal injury.7 Because the S3 segment has a low capacity for anaerobic glycolysis despite high metabolic demands, a decline in oxygen tension in the outer medulla, where oxygen tensions are already physiologically low, could rapidly lead to energy deprivation and thereby contribute to cellular injury.8

A central cellular mechanism of adaptation to low oxygen tensions operates through activation of the hypoxia-inducible transcription factor (HIF). The oxygen-regulated α-subunit of HIF dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT) and transcriptionally induces a broad spectrum of target genes involved in glycolysis, pH regulation, vascular tone, or erythropoiesis.9,10 The HIF-α subunit is constitutively expressed but rapidly degraded by the ubiquitin-proteasome system in the presence of oxygen. Up-regulation of HIF in different models of acute renal injury, such as renal segmental infarction,11 ischemia-reperfusion,12,13 and radiocontrast-induced injury,14 suggests that the renal HIF system plays an important role in the protection against injury. In support of this concept, we have recently been able to show that induction of HIF before isch-
emia-reperfusion ameliorates renal injury and function. One important question in this context is whether the protective effect of the induction of HIF target genes is limited to hypoxic injuries or may also extend to different nonhypoxic mechanisms of cellular damage.

Cisplatin-induced renal injury appears to be an attractive and clinically important model to test this question, but conflicting data exist so far about the role of HIF and hypoxia in cisplatin-induced tubular toxicity. One report demonstrated HIF-independent protective effects of hypoxia when cells were simultaneously exposed to cisplatin. In contrast, Tanaka et al. showed that cisplatin itself increased HIF-1α activation in the rat kidney and injection of cobalt chloride, potentially through HIF-α, reduced the number of apoptotic cells. However, the effect of hypoxic preconditioning in cisplatin-induced renal injury has not yet been studied. We thus investigated in vitro and in vivo whether HIF is induced by cisplatin, if hypoxic preconditioning has protective effects on cellular injury induced by cisplatin and whether such an effect is mediated by HIF.

RESULTS

HIF-1α Protein Is Not Induced by Cisplatin in Proximal Tubular Cells

The effects of cisplatin on the HIF-system were tested in the immortalized human renal proximal tubular cell line HKC-8. No cellular accumulation of HIF-1α protein was observed under normoxic conditions and after 8 h incubation with increasing cisplatin concentrations (10 to 100 μM) (Figure 1, A). To test HIF-1α transactivation, HKC-8 cells were transfected with an HIF-1α-sensitive luciferase reporter construct. Increasing doses of cisplatin led to a dose-dependent decrease of reporter gene activity compared with untreated or vehicle-treated cells, which confirmed that cisplatin does not activate HIF (Figure 1, B). HKC-8 cells, exposed to the chemical HIF inducer 2,2′-dipyridyl (DP) for 8 h, showed a marked HIF-1α protein accumulation. Preincubation with increasing cisplatin concentrations reduced DP-induced HIF signals dose-dependently (Figure 1, C). Removal of cisplatin before DP stimulation led to decreased HIF protein levels that were comparable to those under continued cisplatin exposure (Figure 1, D). Thus, HIF-1α protein and HIF transactivation are not induced but rather suppressed by cisplatin in renal tubular cells in vitro.

Cisplatin Treatment of HKC-8 Cells Reduces HIF-1α Target Gene Expression

To test for effects of cisplatin on the transcriptional activity of HIF in HKC-8 cells, we performed RNAase protection assays for HIF-1α and target genes. mRNA levels of the HIF-1 target glucose transporter-1 (Glut-1) were induced by the chemical HIF-inducer DP. Increasing concentrations of cisplatin decreased Glut-1 mRNA expression as well as HIF-1α mRNA expression (Figure 2, A). Pretreatment with cisplatin for 8 h almost completely suppressed the Glut-1 mRNA induction by DP (Figure 2, B). Because cisplatin was removed before DP stimulation, this indicates that the inhibitory effect persisted beyond the exposure. The internal loading control (U6-small nuclear RNA) was also suppressed at high doses of cisplatin, which presumably reflected a reduced total transcriptional activity and/or a loss of viability of cells under these conditions. Taken together, these data indicate that in cultured renal cells HIF target gene activation is markedly suppressed by cisplatin.

Figure 1. Cisplatin does not induce HIF-1α protein and does not transactivate an HIF-1α responsive reporter construct. (A) No induction of HIF-1α protein in the renal proximal tubular cell line HKC-8 was detectable with different concentrations of cisplatin or vehicle (DMF). The chemical HIF-inducer 2,2′-dipyridyl (DP) was used as a positive control. (B) Accordingly, an HIF-1α responsive luciferase reporter construct was not activated but suppressed by different concentrations of cisplatin in transient transfection assays in HKC-8 cells. DP was used as a positive control for HIF-1α activation (normalized to β-galactosidase, mean ± SD, n = 3). (C) Pretreatment with cisplatin before HIF-1α stabilization with DP reduced HIF protein levels dose-dependently in HKC-8 cells. This reduction was irrespective of ongoing cisplatin exposure (w/o MC, without medium change; D, left 3 lanes) or of cisplatin withdrawal (w MC, with medium change; D, right 3 lanes). Representative immunoblots of 3 independent experiments are shown. β-actin was used as loading control.
Hypoxic Preconditioning Reduces Cisplatin-Induced Apoptosis in Proximal Tubular Cells

Preconditional HIF induction was recently demonstrated to be protective against ischemic renal injury. We therefore investigated whether this principle could also be applied to cisplatin toxicity. Cisplatin-induced apoptosis in proximal tubular cells was quantified by measuring cleavage of the fluorogenic substrate of caspase-3, DEVD-AMC. In HKC-8 cells, 50 μM cisplatin increased caspase-3 activity 6.96 ± 1.1-fold compared with untreated cells. Hypoxic preconditioning at 1% oxygen for 12 h before cisplatin treatment significantly reduced this increase to 4.81 ± 0.7-fold (n = 3, P < 0.05; Figure 3, B), whereas it had no effect in MEFHIF1α−/− cells (6.2 ± 0.6-fold versus 6.8 ± 1.1-fold; n = 3, P = not significant). Moreover, the increase in caspase-3 activity after cisplatin exposure was higher in HIF-deficient than in wild-type MEFs, suggesting that basal HIF activation is already protective against cisplatin. Simultaneous exposure to cisplatin and hypoxia also attenuated apoptosis, but independent of the HIF-1α genotype of the cells (Figure 3, B).

We conclude that a functional HIF pathway is required for the anti-apoptotic effects of hypoxic preconditioning in vitro.

Hypoxic Preconditioning Prevents Cisplatin-Induced Reduction of DNA Synthesis but Does Not Change DNA Content

To evaluate effects of hypoxic preconditioning before cisplatin administration on cellular proliferation rates, BrdU incorporation was investigated in HKC-8 cells. BrdU incorporation was not reduced by hypoxia (Figure 4, C), suggesting that hypoxia did not suppress proliferation. Distribution of the cells in G1, S, and G2 phase of the cell cycle was also not significantly altered by hypoxia alone (Figure 4, F). Hypoxic preconditioning significantly reduced cisplatin-induced caspase-3 activity in wild-type MEFs (from 3.1 ± 0.2-fold to 2.1 ± 0.4-fold; n = 3, P < 0.05; Figure 3, B), whereas it had no effect in MEFHIF1α−/− cells (6.2 ± 0.6-fold versus 6.8 ± 1.1-fold; n = 3, P = not significant). Moreover, the increase in caspase-3 activity after cisplatin exposure was higher in HIF-deficient than in wild-type MEFs, suggesting that basal HIF activation is already protective against cisplatin. Simultaneous exposure to cisplatin and hypoxia also attenuated apoptosis, but independent of the HIF-1α genotype of the cells (Figure 3, B). We conclude that a functional HIF pathway is required for the anti-apoptotic effects of hypoxic preconditioning in vitro.

Figure 2. HIF-1α and HIF-1α target gene mRNA levels are reduced by cisplatin. (A) RNase protection assays show that HIF-1α mRNA was reduced dose-dependently in HKC-8 cells after cisplatin treatment compared with controls or vehicle (DMF)-treated cells. Expression of the HIF target gene Glut-1 was also reduced by cisplatin. DP treatment demonstrated down-regulation of Glut-1 mRNA. (B) Up-regulation of Glut-1 is reduced dose-dependently after HKC-8 cells were pretreated with cisplatin, demonstrating the impaired responsiveness of the HIF system in the presence of cisplatin. Representative data of 3 independent experiments. U6sn mRNA was used as loading control.

Figure 3. Hypoxic preconditioning reduces cisplatin-induced apoptosis in tubular cells and embryonic fibroblasts. (A) HKC-8 cells (left) and mouse primary proximal tubular cells (mPT, right) were incubated with 1% O2 before stimulation with 50 μM cisplatin. In both cell types, hypoxic preconditioning reduced relative fluorometric caspase-3 activity significantly, indicating an anti-apoptotic effect. (B) No reduction in cisplatin-induced caspase-3 activity after hypoxic preconditioning was observed in HKC-8 cells (left) and MEFHIF1α−/− (MEF HIF1α−/−) compared with wild-type MEFs, which indicates that the observed cytoprotective effect was dependent on the presence of functional HIF-1α. When cisplatin was applied simultaneously to hypoxia, apoptosis rates were comparably attenuated, but effects were seen in both genotypes, indicating that HIF induction plays a role in preconditional but not in simultaneous application of hypoxia. Data are mean of 3 independent experiments ± SD. *P < 0.05.
ing significantly increased the number of BrdU positive cells after cisplatin treatment (33.8 ± 9.5% versus 12.2 ± 5%, n = 3, P < 0.05; Figure 4, B, D, and F), indicating increased proliferation and viability. Proportions of cells in G2/M phase were significantly reduced in cisplatin-treated samples independent of hypoxic preconditioning (Figure 4, F). Taken together, hypoxic preconditioning does not induce cell cycle arrest in HKC-8 cells and the protective effect of hypoxia becomes apparent in the increased ability of cells to proliferate after cisplatin exposure.

Renal Cisplatin Injury Is Not Associated With Marked Tissue Hypoxia and Does Not Induce HIF-1α in Rats

To corroborate the in vitro results that cisplatin does not induce HIF-1α in renal tubular cells, cisplatin (8 mg/kg body weight) was injected intraperitoneally into rats and HIF-protein was studied by immunohistochemistry after 1, 24, 72, and 120 h. In line with previous studies, little or no HIF-1α staining was observed in normoxic control animals. Treatment with 0.1% carbon monoxide (CO) led to a robust induction of HIF-1α (Figure 5, B). Corresponding to in vitro experiments, HIF-1α was not detectable in cisplatin injured rat kidneys at 120 h (Figure 5, C) and at all other time points examined (not shown).

To test if cisplatin induces tissue hypoxia, pimonidazole was injected 30 min before death. Pimonidazole accumulates in hypoxic tissues and was detectable in the renal medulla of...
normoxic kidneys, which is known to have physiologically low oxygen tensions (not shown). Pimonidazole did not accumulate in the untreated kidney cortex (Figure 5, E) and not 1 h and 72 h after cisplatin. First after 120 h pimonidazole staining was slightly increased in perinecrotic regions of the tubuli (Figure 5, F), indicating some degree of regional tissue hypoxia at a late time point and possibly secondary to the toxic injury. Neither HIF-1α nor pimonidazole was detectable in animals preconditioned with CO for 10 h before cisplatin administration at the endpoint after 120 h (Figure 5, D and G).

Preconditioning With Carbon Monoxide Ameliorates Renal Morphology and Reduces Apoptosis in Cisplatin-Treated Rats

To investigate whether the protection by hypoxic preconditioning in vitro could be transferred to the in vivo situation, we exposed rats to 0.1% CO for 10 h before cisplatin injection. CO treatment reduces tissue oxygen availability by blocking the oxygen carrying capacity of hemoglobin and has previously been shown to robustly induce HIFα in renal tubular cells in vivo (Figure 5, B). After 120 h, HIFα levels were decreased. HIFα was analyzed in the kidneys 120 h after cisplatin injection. HIFα protein levels were significantly reduced in CO-treated animals compared to control animals (39.9 ± 14.7 mg/dl, P < 0.05) (Figure 7, A). Serum urea levels supported the creatinine data which were also reduced in the CO-treated group compared to control animals (39.9 ± 14.7 mg/dl, P < 0.05) (Figure 7, B). Thus, together with the morphologic data, these findings demonstrate a clear protection against cisplatin-induced kidney injury by CO preconditioning in vivo.

DISCUSSION

The main finding of this study is that preconditional HIF induction can protect renal tubular cells against cisplatin, which does not by itself induce but rather impairs the HIF system. This conclusion is based on the combined and consistent results of in vitro and in vivo experiments, showing a protective effect of preconditional HIF up-regulation, independent of whether this was induced by hypoxia in vitro or by functional anemia in vivo.

In previous studies, conflicting results were reported with regard to the effect of cisplatin on HIF accumulation in cells and tissues. For instance, in rat hepatoma cells, cisplatin has been described to induce HIF protein levels regardless of prevailing oxygen tensions. In contrast, Tanaka et al. did not observe any HIF activation in an immortalized rat proximal tubule cell line. In the present study, results of HIF immuno-
also been implicated in stabilization of HIF-1 activity. Thus, this study provides further insights into the interactions of cisplatin and the HIF system and implicates HIF in cisplatin-induced cellular injury. In MEFs, hypoxia significantly reduced proliferation, decreased cdk-2 activity, and increased p21 expression in an HIF-1α-dependent manner. Therefore, it could be argued that the protective effects of preconditioning only result from better cell survival and less cell damage or represent a causative factor for the protective role of hypoxia cannot clearly be answered.

Additional reports demonstrated beneficial effects of known HIF-downstream gene products such as vascular endothelial growth factor and heme-oxygenase 1 in cisplatin-induced injury. Daily administration of very high doses of the HIF target erythropoietin to cisplatin-treated rats improved creatinine clearance or increased tubular regeneration. Although it is possible that each of these HIF target genes play an important role in mediating the protective effect of preconditioning, whether the higher proliferation rates after hypoxic preconditioning only result from better cell survival and less cell damage or represent a causative factor for the protective role of hypoxia cannot clearly be answered.

In contrast to Tanaka et al.,17 we were unable to detect any HIF-1α activation or significant pimonidazole binding in the kidneys of cisplatin-treated animals. Differences in the experimental protocols, such as the doses of cisplatin used, the animal strains, pimonidazole application protocols (30 min before death in our versus 3 d in their study), and the techniques used for the detection of HIF activation (HIF immunostaining in our wild-type animals versus HRE reporter activation in transgenic rats) may contribute to these discrepancies.

With respect to the possible mechanisms by which HIF target genes apparently provide protection, various pathophysiological mechanisms of cisplatin have to be considered, which may contribute to its tubular toxicity. In addition to a direct DNA damage, these include generation of free oxygen radicals, caspase activation, or activation of TNFα-dependent apoptosis pathways. Recently, inhibition of the cyclin-dependent kinase-2 (cdk-2) has been identified to be protective in cisplatin-induced cellular injury. In MEFs, hypoxia significantly reduced proliferation, decreased cdk-2 activity, and increased p21 expression in an HIF-1α-dependent manner. Therefore, it could be argued that the protective effects of preconditional HIF activation in cisplatin-induced renal injury described here may also be mediated by the cessation of cell growth. However, BrdU incorporation assays in HKC-8 cells indicated that hypoxic preconditioning did not alter cell proliferation and cell cycle distribution compared with normoxia. Moreover, it significantly increased the percentage of proliferating cells compared with cisplatin treatment without preconditioning. Whether the higher proliferation rates after hypoxic preconditioning only result from better cell survival and less cell damage or represent a causative factor for the protective role of hypoxia cannot clearly be answered.

Additional reports demonstrated beneficial effects of known HIF-downstream gene products such as vascular endothelial growth factor and heme-oxygenase 1 in cisplatin-induced injury. Daily administration of very high doses of the HIF target erythropoietin to cisplatin-treated rats improved creatinine clearance or increased tubular regeneration. Although it is possible that each of these HIF target genes play an important role in mediating the protective effect of preconditional HIF induction, we hypothesize that activating both isoforms of the transcription factor HIF itself is superior to the activation of HIF-1α and HIF-2α contributes to the induction of a broad array of different adaptive genes, although the role of the latter in the hypoxic response is less completely understood.

HIF is considered as a central mechanism of adaptation to hypoxia, but reduced oxygen availability affects cellular function in multiple ways. In the present study, we used mouse embryonic fibroblasts lacking functional HIF-1α as a model to investigate its contribution to the protective effects of hypoxic preconditioning. Because only cells that express HIF-1α protein were protected against cisplatin injury, we conclude that HIF is critical for the observed effects. Wang et al. observed an HIF-independent suppression of apoptosis when cisplatin-exposed immortalized rat kidney proximal tubular cells were simultaneously kept under hypoxic conditions (2% oxygen). The protective effect of this experimental design was attributed to suppressed p53 levels resulting from inhibition of the respiratory chain complex III. In keeping with their results, we also...
observed reduced caspase-3 levels independent of HIF-1α expression when cells were exposed to cisplatin under simultaneous hypoxia (Figure 3, B). Importantly, we observed an HIF-dependent effect of hypoxia when applied preconditionally. This demonstrates that both HIF-dependent and HIF-independent effects of hypoxia can mitigate toxic injury. Preliminary experiments, studying the effect of hypoxic preconditioning in a model of gentamycin toxicity, are suggesting that this protective effect might not be not specific for cisplatin.

One of the limitations of the present study is the lack of specific compounds for preconditional HIF stabilization. New prolyl-hydroxylase inhibitors are presently under investigation, which inhibit HIF degradation under normoxic conditions and thereby induce HIF target genes.34 We have unfortunately not been able so far to prove the protective effect of a prolyl hydroxylase inhibitor in the cisplatin model in vivo because of the restricted availability of such compounds. Because functional anemia induced by CO has previously been demonstrated to stabilize HIF, we used CO for preconditional experiments. In ischemia reperfusion experiments, using the same protocol for CO exposure as in this study, CO preconditioning and a new prolyl hydroxylase inhibitor led to comparable levels of HIF induction and preservation of renal function.15 A recent study suggested that HIF-1α, induced by CO in macrophages, is a critical factor for the protective response of CO.35 On the other hand, CO has also been implicated to be involved in cytoprotection at low concentrations by mechanisms other than HIF activation, such as MAPK36 and PPAR-γ activation.37 In addition, CO releasing molecules have also been successfully tested for cytoprotection in cisplatin-mediated cell injury.38 Therefore, we cannot definitely rule out that effects other than HIF stabilization contribute to the in the protective effect of CO observed in our experiments.

Our findings may have significant clinical implications. While hypoxic exposure per se appears unfeasible and cobalt chloride, another inducer of the HIF system used in experimental models,17 is too toxic for application in humans, the prolyl hydroxylase inhibitors offer significant potential for organ protection and could be used to mitigate kidney injury during subsequent cisplatin administration. A potential caveat of such an approach is that up-regulation of the HIF system would not be confined to the kidney and might also interfere with the desired chemotherapeutic effects of cisplatin on tumor cells. A future challenge may therefore be to develop compounds that preferentially induce HIF in the kidney. Taken together, our data provide evidence that HIF is an attractive target for protection against not only hypoxic but also nonhypoxic kidney injuries.

CONCISE METHODS

Cells and Reagents
The human proximal tubular cell line HKC-8 was generously provided by L. Racusen (Baltimore, MD). MEFs were a gift of G. Semenza (Baltimore, MD). Cell culture reagents were from Invitrogen (Karlsruhe, Germany) and Biochrom (Berlin, Germany). All other chemicals were from Sigma (Taufkirchen, Germany). The reporter plasmid 6xHRE/tk/luc was a kind gift from P. Ratcliffe (Oxford, United Kingdom).

Cell Culture
HKC-8 cells were cultured in DMEM/Ham’s F-12 supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium. MEFs were grown in Dulbecco’s modified Eagle’s medium with 5% fetal calf serum. Cisplatin was dissolved in dimethylsulfoxide (DMSO).

Isolation of Murine Proximal Tubular Cells
Primary murine proximal tubular cells were isolated from the kidney cortex of 4-wk-old mice as described39 and cultured in serum-free DMEM/Ham’s F-12 supplemented with 5 µg/ml insulin, 5 µg/ml transferrin, and 5 ng/ml selenium.

HIF Protein Extraction and Immunoblotting
For HIF protein expression, HKC-8 cells were treated with various concentrations of cisplatin (5 to 100 µM) or vehicle (DMSO) for 8 h in the absence or presence of the iron chelator 2,2’-dipyridyl (100 µM) (ICN, Costa Mesa, CA). Preparation of cell lysates and immunoblotting were performed as described previously40 with antibodies against HIF-1α (Alexis Biochemicals, Loerrach, Germany).

Luciferase Reporter Gene Assays
HKC-8 cells were transfected with the HIF-1α-responsive 6xHRE/tk/luc reporter plasmid, which contains six HRE binding motifs of the mouse phosphoglycerate kinase, which control the expression of firefly luciferase19 and with a pCMV-β-galactosidase expression vector using Fugene (Roche, Mannheim, Germany). After stimulation with cisplatin, vehicle or 100 µM DP for 18 h, luciferase activities were determined using the luciferase assay system (Promega, Mannheim, Germany) and were normalized to the respective β-galactosidase expression.

RNA Preparation and RNase Protection Assay
Total RNA was extracted with RNAzol-B (Biotzol, Eching, Germany) and analyzed by RNase protection assay as described previously41; 40 µg of total RNA (1 µg for U6-snRNA) was hybridized to [32P]-labeled antisense RNA probes of HIF-1α, glucose transporter-1, and U6-small nuclear RNA.

Measurement of Caspase-3 Activity
After hypoxic preconditioning for 12 h with 1% O2 in a Jouan IG 750 incubator (Thermo Electron, Dreieich, Germany), cells were incubated with 50 µM cisplatin for 12 h in serum containing medium. They were lysed in a buffer containing 100 mM HEPES, 10% sucrose, 0.1% Chaps, and 1 mM EDTA; 30 µg of protein was incubated with 13 µM DEVD-AMC (Biomol, Hamburg, Germany), the fluorogenic substrate of caspase-3. Cleaved AMC substrate was detected by a fluorometer (Tecan, Graslitz, Germany) using 360 nm excitation and 465 nm emission wavelength.
BrdU Incorporation and Cell Cycle Analysis

Cell cycle analysis was performed with a modified protocol for flow cytometry.\(^{42}\) DNA content was detected by propidium iodide staining, and DNA synthesis by BrdU incorporation. Cells were either incubated at normoxia or 1% O\(_2\) for 12 h before cisplatin (50 \(\mu\)M) was added to the medium for 6 h. A BrdU pulse (50 \(\mu\)M) was performed for 30 min, then cells were harvested and fixed overnight in 65% methanol. After washing with PAB buffer (2% bovine serum albumin, 0.01% sodium azide in phosphate-buffered saline) RNase A was added (81 U/ml) for 12 min at 37°C and 40 min at 20°C. Then cells were incubated for 4 min in pepsin-HCl solution (0.0093N HCl and 0.33 mg/ml pepsin) at 37°C. The enzyme reaction was stopped with ice cold PAB buffer, and cells were washed and resuspended in 2N HCl for 10 min at room temperature. Cells were incubated with anti-BrdU antibody (DAKO, Glostrop, Denmark), followed by incubation with an FITC-conjugated rabbit anti-mouse IgG antibody (DAKO). BrdU antibody (DAKO, Glostrop, Denmark), followed by incubation with an FITC-conjugated rabbit anti-mouse IgG antibody (DAKO). BrdU antibody (DAKO, Glostrop, Denmark), followed by incubation with an FITC-conjugated rabbit anti-mouse IgG antibody (DAKO). BrdU antibody (DAKO, Glostrop, Denmark), followed by incubation with an FITC-conjugated rabbit anti-mouse IgG antibody (DAKO). BrdU antibody (DAKO, Glostrop, Denmark), followed by incubation with an FITC-conjugated rabbit anti-mouse IgG antibody (DAKO).

Animals

Animal experiments were approved by the institutional review board for the care of animal subjects and were performed in accordance with National Institutes of Health guidelines. Male Sprague-Dawley rats (Charles River, Sulzfeld, Germany) were used at weights of 200 to 230 g. For the isolation of proximal tubular cells, C57BL/6 mice were purchased from Jackson Laboratories, Sulzfeld, Germany.

Experimental Groups and Protocol of Cisplatin-Induced Acute Renal Injury

To induce acute renal injury in rats, 8 mg/kg bw cisplatin was injected intraperitoneally. Serum creatinine and urea were determined at baseline (0 h) and at 24 h, 72 h, and at the end of the experiments (120 h). Animals (\(n = 10\)) were either preconditioned with 0.1% CO for 10 h to activate HIF and HIF target genes as described previously\(^{15}\) or were breathing room air before cisplatin injection. For immunohistochemistry, animals were killed at 1, 24, 72, and 120 h after cisplatin administration.

Immunohistochemistry and Pimonidazole Detection

Immunohistochemistry was essentially performed as described\(^{15}\) with a monoclonal antibody against HIF-1\(\alpha\) (Novus Biologicals, Littleton, CO). For signal amplification, a catalyzed signal amplification system (CSA-Kit, Dako, Hamburg, Germany) was used. To detect tissue hypoxia,\(^{20}\) pimonidazole was injected intravenously (60 mg/kg body weight) 30 min before death. Signal detection was performed with an anti-pimonidazole antibody (Natural Pharmacia International, Belmont, MA).\(^{13}\)

Histomorphological Scoring of Acute Tubular Injury

Renal cross sections were stained with hematoxylin and eosin and periodic acid-Schiff. Samples were analyzed for tubular cell necrosis, tubular dilation, and intratubular cell detachment (\(\times 2000\) magnification) and were all evaluated in a blinded manner by a nephropathologist. Abnormalities were graded by a semiquantitative score from 0 to 4+: 0, no abnormalities; 1+, changes affecting less than 25% of the sample; 2+, changes affecting 25% to 50%; 3+, changes affecting 50% to 75%; 4+, changes affecting more than 75%.

TUNEL Assay

Apoptotic cells were detected by the TUNEL assay as described previously.\(^{43}\) Cells were regarded as TUNEL-positive if their nuclei stained black and displayed typical apoptotic morphology with chromatin condensation. The number of apoptotic cells was counted in 10 randomly selected visual fields of blinded samples, using \(\times 400\) magnification.

Statistics

Unless indicated otherwise, data represent the means of 3 independent experiments \(\pm SD\). A \(P\) value \(< 0.05\) was considered significant. Statistical analyses were performed using \(t\) test, covariate analysis, Mann-Whitney-U test, and Kruskal-Wallis test calculated with SPSS Software for Windows (version 13.0).

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DISCLOSURES

None.

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