Preconditional Activation of Hypoxia-Inducible Factors Ameliorates Ischemic Acute Renal Failure

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Activation of hypoxia-inducible transcription factor (HIF) has been identified as an important mechanism of cellular adaptation to low oxygen. Normoxic degradation of HIF is mediated by oxygen-dependent hydroxylation of specific prolyl residues of the regulative α-subunits by HIF prolyl hydroxylases (PHD). It was hypothesized that inhibition of HIF degradation by either hypoxia or pharmacologic inhibition of PHD would confer protection against subsequent ischemic injury. For testing this hypothesis ischemic acute renal failure was induced in rats by 40 min of clamping of the left renal artery after right-sided nephrectomy. Before surgery, pretreatment with either carbon monoxide, leading to tissue hypoxia, or the novel PHD inhibitor FG-4487 was applied. No toxic effects of FG-4487 were observed. Both pretreatments strongly induced the accumulation of HIF-1α and HIF-2α in tubular and peritubular cells, respectively, as well as HIF target gene expression. The course of subsequent ischemic injury was significantly ameliorated by both strategies of preconditioning, as evident from a significant improvement of serum creatinine and serum urea after 24 and 72 h. Furthermore, tissue injury and apoptosis were less severe, which were quantified by application of a standardized histologic scoring system in a blinded manner. In conclusion, the data provide proof of principle that preconditional activation of the HIF system protects against ischemic injury. Inhibiting the activity of HIF hydroxylases therefore seems to have considerable clinical perspectives.


Prolonged renal ischemia or hypoperfusion can cause acute renal failure, which still is associated with a high morbidity and mortality and prolonged hospitalization (1). To date, there are no specific therapeutic options to prevent or improve ischemic acute renal failure (iARF), and therapeutic efforts are merely supportive. Therefore, a strong necessity for novel interventional strategies exists.

High cellular oxygen demand and regionally reduced oxygen supply lead to physiologically low oxygen tensions within the renal cortex and the medulla (2). For this reason, proximal tubular cells and the outer medulla are particularly susceptible to hypoxic/ischemic injury (3). In fact, hypoxia is considered an important pathophysiologic mechanism in ARF as a result of consequences of inadequate energy supply on cytoskeletal architecture, membrane physiology, and protein synthesis.

There has been increasing interest in cellular effects of hypoxia since the discovery of a widespread system of hypoxia-inducible gene expression (4) mediated by hypoxia-inducible transcription factors (HIF). HIF are heterodimers that are composed of a constitutive β subunit (HIF-β) and one of two alternative α subunits, HIF-1α or HIF-2α. Under normoxic conditions, HIF-α is degraded rapidly via the ubiquitin-proteasomal pathway. To mark HIF-α for degradation, specific prolyl residues are hydroxylated by an enzyme family of HIF-prolyl hydroxylases (prolyl hydroxylase domain protein [PHD]), which require dioxygen and 2-oxoglutarate as co-substrates (reviewed in ref [5]). At least three different PHD have been identified so far (6,7), termed PHD1, PHD2, and PHD3, but their relative contribution to HIF-α regulation still remains to be clarified. Reduced activity of the PHD under hypoxic conditions, when oxygen is lacking as a substrate or after application of 2-oxoglutarate-analogues, results in HIF-α accumulation (8). Subsequently, HIF-α forms dimers with HIF-β and binds to a specific DNA motif in the hypoxia response elements, thus transactivating target genes. To date, more than 100 HIF target genes have been identified, including erythropoietin (EPO), vascular endothelial growth factor, glucose transporters and heme oxygenase-1 (HO-1), which have the potential to confer adaptation and reduced sensitivity to hypoxia (9). We previously showed that kidney cells have a widespread capacity to activate the HIF system under systemic hypoxia (10), under...
regional hypoxia (11,12), and in response to administration of PHD inhibitors (8). However, after total renal ischemia, HIF activation is limited and confined primarily to the collecting duct (10). In line with the concept of a tissue-protective effect of the HIF system, some single HIF target gene products have been shown to ameliorate renal ischemic injury, including EPO (13,14) and HO-1 (15,16). Given that HIF induction may be anatomically limited during iARF and the necessary time required to induce HIF and expression of its target gene products, we hypothesized that activating the HIF system before an ischemic insult, i.e., preconditional HIF activation, would achieve the most effective tissue protection. We tested this hypothesis using a rat model of renal ischemia/reperfusion with pretreatment of either inhaled CO to induce functional anemia or a novel PHD inhibitor given intraperitoneally.

Materials and Methods

If not otherwise stated, all chemicals used were from Sigma-Aldrich (Taukirchen, Germany).

Cell Culture

HKC-8 cells, an SV40-transformed human proximal tubular cell line (provided by Prof. L. Racusen, Johns Hopkins University School of Medicine, Baltimore, MD) were cultured in DMEM/Ham’s F12 (Seromed, Biochrom, Berlin, Germany) in a 1:1 ratio that contained 10% FCS (PAA Laboratories GmbH, Pasching, Austria), 2 mM L-glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, and 50 μg/ml gentamicin. Primary human proximal tubular epithelial cells were isolated as described (17), after informed consent of patients who were undergoing tumor nephrectomy. In brief, tissue of healthy kidney cortex was dissected mechanically. Monolayer cell cultures were generated by outgrowth from 1-mm³ pieces of the normal cortex on a matrix of FCS. For maintenance and experiments, cells were cultured in serum-free DMEM/F12 supplemented with 2 mM L-glutamine, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 36 ng/ml hydrocortisone, 4 μg/ml triiodo-L-thyronine, 10 ng/ml EGF, and 50 μg/ml gentamicin. Passages 3 to 4 were used for the study, approximately 5 to 6 wk after tissue preparation.

HIF Protein Extraction and Immunoblotting

Protein extraction and blotting were performed essentially as described previously (8). Cells were grown to subconfluence and stimulated with 100 μM FG-4487 or exposed to hypoxia (1% O₂) for 6 h. Cells were washed with PBS, and cell lysis was performed using 8 M urea, 10% glycerol, 10 mM Tris–HCl (pH 6.8), 1% SDS, 5 mM dithiothreitol, and Complete Mini Protease-Inhibitor Cocktail (Roche, Mannheim, Germany). A total of 100 μg of each extract was separated on 8% polyacrylamide gels. Proteins were transferred onto polyvinylidene difluoride membranes and probed with monoclonal mouse anti-HIF-1α antibody (clone MGC-3, 1 μg/ml; ALEXIS Biochemicals, Lausen, Switzerland) or a polyclonal rabbit anti–HIF-2α antibody (PM9, 1:400 [18]) and horseradish peroxidase–conjugated secondary antibodies (DAKO, Hamburg, Germany). Signals were visualized by chemiluminescence (SuperSignal West Dura Extended; Pierce, Rockford, IL). After analysis, membranes were stained with Coomassie Blue to verify equal protein loading and transfer.

Animals

The study was approved by the institutional review board for the care of animal subjects and was performed in accordance with the Declaration of Helsinki. Male Sprague-Dawley rats (Winkelmann, Borchen, Germany) were used at weights of 180 to 230 g. Rats were housed in a standard cage, were fed a standard diet, and had free access to water.

Experimental Groups and Protocol

Preconditioning. The PHD-I FG-4487 was identified from a large library of PHD-I for its capacity to activate the HIF pathway in vitro. It inhibits all PHD isoforms at low nanomolar concentrations. For testing in vivo potency, FG-4487 was dissolved in 100 μl of DMSO and 900 μl of NaCl 0.9%, and independent of dosage, a total volume of 1 ml was injected intraperitoneally. Various dosages (25, 50, and 100 mg/kg body wt) were given, and renal HIF-α accumulation was analyzed at 6, 12, and 24 h as described next (n = 2 animals for each time point and dosage). On the basis of the results of these pilot experiments, a dose of 25 mg/kg body wt was chosen used for subsequent experiments and given 6 h before the ischemic insult. As an alternative method of HIF induction animals were exposed to 0.1% CO as described (10).

Ischemia/Reperfusion Injury. Animals were anesthetized with ketamine (100 mg/kg body wt) and pentobarbital sodium (50 mg/kg body wt, Nembutal; Abbott, Wiesbaden, Germany). To maintain a constant body temperature of 37°C, we used a heated table with temperature feedback by a rectal probe (Heating Controller type 861; Hugo Sachs Elektronik-Harvard Apparatus, March, Germany). A blood sample of 300 μl was taken via tail-vein puncture (time point 0 h). The abdomen was shaved, and a midline incision was made. After removal of the right kidney, the left renal artery was prepared and clamped with an arterial clamp (B-2; S&T, Neuhausen, Switzerland) to induce ischemia, which was verified by the change of the renal color. During ischemia, the abdomen was closed. After an ischemic period of 40 min, the clamp was removed and reperfusion was started. The abdomen was closed in two layers, and the rat was kept on the heating table until awakening after anesthesia. At 24 h, the animals were anesthetized shortly with isoflurane (Forene; Abbott) and a second blood sample of 300 μl was drawn (24 h). After 72 h, the animals were anesthetized and a third blood sample (72 h) was drawn. Thereafter, animals were killed by cervical dislocation, and the left kidney was removed for analysis (Figure 1).

Groups. Animals were divided into five groups (n = 10 each) as follows:

- Group 1 (Sham): Sham-operated animals without induction of iARF
- Group 2 (CO): Animals pretreated with carbon monoxide 0.1% before induction of iARF
- Group 3 (UnT): Untreated animals with induction of iARF; control for group 2
- Group 4 (FG-4487): Animals pretreated with the specific PHD-I inhibitor given intraperitoneally
- Group 5 (Veh): Vehicle-treated animals with injection of 100 μl of DMSO + 900 μl of NaCl 0.9% 6 h before induction of iARF; control for group 4.

Tissue Preparation

One half of the kidneys were fixed by immersion in 3% paraformaldehyde in PBS (pH 7.4) for 24 h and processed for paraffin embedding. The second half were snap-frozen in liquid nitrogen for RNA analysis.

Immunohistochemistry

Paraffin sections (2 to 4 μm) were dewaxed in xylene and rehydrated in a series of ethanol washes. Slides were coated with 3-aminopropyl-triethoxysilane. For detection of HIF isoforms, monoclonal mouse anti-human HIF-1α antibody (a67; Novus Biologicals, Littleton, CO) and a...
polyclonal rabbit anti-mouse HIF-2α antibody (PM-9) were used as described previously (10,18). Additional primary antibody was monoclonal mouse anti-rat ED-1 (Serotec, Duesseldorf, Germany). Biotinylated secondary anti-mouse or anti-rabbit antibodies (Dako, Hamburg, Germany) were used. For signal amplification and visualization, a catalyzed signal amplification system (CSA-Kit; Dako) based on a streptavidin-biotin-peroxidase reaction was used according to the manufacturer’s instructions. Antigen retrieval was performed for 6 min in preheated target retrieval solution (Dako), using a pressure cooker. Between incubations, specimens were washed two to three times in buffer (50 mM Tris-HCl, 300 mM NaCl, and 0.1% Tween-20 [pH 7.6]). As chromogen for peroxidase reaction diaminobenzidine was used. Control samples were from normoxic adult rat kidneys, in which HIF-α is not detectable (10), or samples that were prepared with preimmune serum from animals that were immunized against HIF-2α. Terminal deoxynucleotidyl transferase-mediated dUTP nick-end (TUNEL) staining was performed using In Situ Cell Death Detection Kit, Flourescein (Roche, Penzberg, Germany) according to the manufacturer’s instructions.

RNase Protection Assay
Total RNA was extracted from frozen tissue samples with RNAzol B, according to the manufacturer’s instructions (Biogenesis, Poole, UK). RNase protection assays were performed essentially as described previously (19). 32P-labeled riboprobes were synthesized using SP6 or T7 RNA polymerase (Roche Diagnostics, Mannheim, Germany), generating the fragments EPO, 193 bp (accession no. L10608); HO-1, 186 bp (accession no. BC091164); and U6 small nuclear RNA, 106 bp (U6sn; accession no. X01366). Radiolabeled riboprobes were protected from RNase digestion by hybridizing to the following amounts of total RNA: EPO, 50 μg; HO-1, 50 μg, U6sn, 1 μg. The last was used as internal control for each sample, adding one tenth (i.e., 100 ng) of each reaction to its appropriate sample after RNase digestion. After resolution on polyacrylamide gels, signals were quantified using a phosphor imager (Fujix, BAS 2000; Fuji, Japan). Values were normalized to the control (U6sn).

Morphologic Analysis
Renal cross-sections were stained with hematoxylin and eosin (H&E), and the entire sample was analyzed for tubular cell necrosis, tubular dilatation, intratubular cell detachment, interstitial edema, interstitial cellular infiltrate, hemorrhages, and intratubular calcification in a ×200 magnification. Slides were evaluated in a blinded manner by a nephropathologist. Abnormalities were graded by use of a semiquantitative score from 0 to 4+: 0, denotes no abnormalities; 1+, changes affecting <25% of the sample; 2+, changes affecting 25 to 50% of the sample; 3+, changes affecting 50 to 75% of the sample; and 4+, changes affecting >75% of the sample.

Macrophage Infiltration and Apoptosis
The number of ED-1– and TUNEL-positive cells in each section was calculated by counting the number of positive cells in 20 randomly selected fields of the entire sample. Quantification was made blinded by a nephropathologist.

Statistical Analyses
All results are expressed as mean ± SD. P < 0.05 was considered significant. Statistical analyses were performed using covariate analysis and Mann-Whitney U and Kruskal-Wallis tests with utilization of SPSS Software for Windows (Version 13.0; SPSS, Inc., Chicago, IL).

Results
HIF-α Accumulates In Vitro and In Vivo after Systemic Hypoxia and FG-4487 Treatment
For evaluation of the potency of FG-4487 to stabilize HIF, it was applied in vitro and in vivo and compared with the capacity of established hypoxic activation of HIF. Immunoblotting of protein extracts of HKC-8 cells (immortalized human tubular cells) and primary human tubular cells (hPT) showed stabilization of both HIF-α isoforms after hypoxia and FG-4487 (100 μM) treatment (Figure 2).

Moreover, both stimuli, CO and FG-4487, were capable of stabilizing HIF-α in vivo (Figure 3). Six hours after treatment
with FG-4487 at a dosage of 25 mg/kg body wt intraperitoneally, HIF-1α accumulated in the nuclei of tubular epithelial cells of virtually all nephron segments except for the distal convoluted tubule (Figure 3C), whereas HIF-1α staining after CO treatment was predominant in the proximal tubule (Figure 3B) as described previously (10). Both FG-4487 and CO led to an accumulation of HIF-2α in interstitial and glomerular cells; in the latter, FG-4487 seemed to be more potent than CO (Figure 3, E and F).

Independent of dosage (25, 50, or 100 mg/kg body wt), HIF-1α accumulation decreased 12 h after application of FG-4487 and disappeared 24 h after injection (data not shown), as compared with 6 h. Untreated (Figure 3, A and D) and vehicle-treated animals (data not shown) were HIF-1α negative. Importantly, identical to hypoxic conditions, this novel PHD inhibitor also led to specific activation of HIF-1α in distinct cell populations, namely epithelial cells for HIF-1α and interstitial cells for HIF-2α.

**HIF Activation by CO and FG-4487 Results in Target Gene Activation**

The right kidneys, which were removed before ischemia/reperfusion of the contralateral kidney, were used to obtain information about the efficacy of preconditioning. RNase protection assays for the two HIF target genes EPO and HO-1 showed strong and comparable mRNA induction after treatment with either CO or FG-4487 (Figure 4).

**Preconditional HIF Activation by CO or FG-4487 Ameliorates Renal Function in iARF**

Rats that were pretreated with either CO for 10 h or FG-4487 6 h before renal ischemia showed improved renal function compared with their respective controls (UnT/Veh). Before initiation of renal ischemia, there were no differences in serum creatinine and urea between groups (0 h; Figure 5, Table 1). During follow-up, 24 and 72 h after onset of renal ischemia, both serum creatinine and urea were significantly lower in the CO group versus UnT (p < 0.05; n = 10; Figure 5A, Table 1) and

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Figure 2. Induction of HIF-α by hypoxia and FG-4487 in human proximal tubular cells. HKC-8 (immortalized renal proximal tubular cells) and primary human proximal tubular cells (hPT) were exposed to hypoxia (1% O2) or FG-4487 (100 μM) for 6 h. Immunoblotting for HIF-α protein shows strong accumulation of HIF-1α (top) and HIF-2α (bottom) by both treatments.

Figure 3. Induction of HIF-α by CO and FG-4487 in rat kidneys. Specimens of the cortex from right kidneys that were removed before ischemia of the left kidney in untreated rats (A and D), animals that were exposed to CO (B and E), and animals that were treated with FG-4487 (C and F). Both preconditioning procedures led to a HIF-α accumulation in rat kidneys with nuclear HIF-1α staining predominantly in epithelial cells of the proximal tubule and the collecting ducts (B and C) and HIF-2α staining of peritubular interstitial cells (E and F). In comparison with CO, application of FG-4487 revealed additional positive HIF-1α staining in the medullary thick ascending limb (C) and stronger staining for HIF-2α in glomerular cells (F).

Figure 4. Induction of HIF target genes in rat kidneys after exposure to CO or FG-4487. Total RNA from right kidneys after hypoxia were subjected to RNase protection after hybridization with probes for the HIF target genes rat EPO (rEPO), rat heme oxygenase 1 (rHO-1), and U6 small nuclear RNA (U6sn) as control. Representative assay demonstrating that CO and FG-4487 resulted in strong induction of EPO and HO-1 mRNA levels as compared with sham-operated (sham), untreated (UnT), and vehicle-treated (Veh) animals. Complete digestion of radiolabeled probes was verified by taking the probes through the procedure (no RNA).
in the FG group compared with the Veh group ($P < 0.05$; $n = 10$; Figure 5B, Table 1). There were no significant differences between the CO and FG group and between the Veh and UnT group, respectively. After 72 h, serum creatinine of CO- and FG-4487–treated animals were not significantly higher than in the sham group (Figure 5, A and B), whereas serum urea remained moderately elevated in all groups after 72 h of reperfusion ($P < 0.05$; $n = 10$; Table 1).

Preconditional HIF Activation by CO or FG-4487 Leads to Reduced Tissue Damage

After 72 h of reperfusion, the left kidney was removed and underwent blinded histomorphologic analysis by a nephropathologist. Semiquantitative scoring of H&E stains ($n = 10$ per group) demonstrated significant morphologic differences in the CO and FG-4487 group compared with their respective controls ($P < 0.05$; Figure 6). Representative H&E-stained sections showed reduced cast formation and less extensive tubular necrosis after CO or FG-4487 treatment in comparison with the respective controls (UnT and Veh, respectively), whereas kidneys of sham animals exhibited normal renal morphology (Figure 6). Subanalysis of the inner stripe of the outer medulla, the region with the most pronounced differences in HIF-1α expression between CO- and FG-4487–treated animals, revealed a clear trend for a stronger reduction in acute tubular necrosis for FG-4487 compared with CO, although the difference did not reach statistical significance (Table 2).

Preconditional HIF Activation by CO or FG-4487 Reduces the Extent of Apoptosis

Apoptosis of renal cells was assessed by TUNEL staining (Figure 7). TUNEL-positive epithelial cells rarely were ob-

### Table 1. Time course of serum urea after ischemia/reperfusion

<table>
<thead>
<tr>
<th></th>
<th>0 h</th>
<th>24 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham</td>
<td>37 (±7)</td>
<td>46 (±6)</td>
<td>46 (±5)</td>
</tr>
<tr>
<td>UnT</td>
<td>39 (±10)</td>
<td>257 (±40)</td>
<td>202 (±70)</td>
</tr>
<tr>
<td>CO</td>
<td>52 (±8)</td>
<td>158 (±78)</td>
<td>120 (±78)</td>
</tr>
<tr>
<td>Veh</td>
<td>35 (±10)</td>
<td>319 (±135)</td>
<td>265 (±113)</td>
</tr>
<tr>
<td>FG-4487</td>
<td>41 (±10)</td>
<td>179 (±61)</td>
<td>91 (±36)</td>
</tr>
</tbody>
</table>

*Compared with their respective controls, both preconditioning procedures, CO and FG-4487, led to a significant and comparable decrease in serum urea in comparison with the respective controls. Data shown are mean (±SD) ($n = 10$). UnT, untreated; Veh, vehicle.

\[ P < 0.05 \text{ versus sham.} \]

\[ P < 0.05 \text{ versus UnT or Veh, respectively.} \]

### Table 2. Effect of pretreatment with CO or FG-4487 on after ischemia/reperfusion

<table>
<thead>
<tr>
<th>ATN Score</th>
<th>Cortex</th>
<th>Outer Stripe of Outer Medulla</th>
<th>Inner Stripe of Outer Medulla</th>
<th>Inner Medulla</th>
</tr>
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<tbody>
<tr>
<td>CO</td>
<td>0.60 (±0.97)</td>
<td>1.40 (±1.57)</td>
<td>1.70 (±1.49)</td>
<td>0.30 (±0.95)</td>
</tr>
<tr>
<td>FG-4487</td>
<td>0.60 (±0.97)</td>
<td>1.90 (±1.10)</td>
<td>0.80 (±1.23)</td>
<td>0.00 (±0.00)</td>
</tr>
<tr>
<td>$P$</td>
<td>0.50</td>
<td>0.21</td>
<td>0.08</td>
<td>0.17</td>
</tr>
</tbody>
</table>

In the inner stripe of the outer medulla, hypoxia-inducible transcription factor-1α stained positive after FG-4487, whereas it was virtually absent after CO treatment. Histologic scoring for acute tubular necrosis (ATN) of the inner stripe (boldface) clearly was lower in FG-4487–treated animals compared with CO-treated animals; also differences were NS. Data are mean (±SD) ($n = 10$).
served in kidneys of sham-operated animals (Figure 7). In contrast, ischemia caused a marked increase of apoptotic cells. Both CO and FG-4487 significantly reduced the number of apoptotic cells compared with UnT and Veh-treated animals, respectively ($P < 0.05$; Figure 7).

**CO but Not FG-4487 Reduces Macrophage Infiltration (ED-1–Positive Cells)**

Renal ischemia/reperfusion was associated with an increased tubulointerstitial macrophage infiltration, as indicated by the number of ED-1–positive cells (Figure 8). Preconditioning with CO significantly reduced macrophage infiltration after 72 h of reperfusion compared with untreated animals (UnT; $P < 0.05$; $n = 10$; Figure 8). However, pretreatment with FG-4487 did not alter the number of infiltrating macrophages in comparison with vehicle-treated animals (Veh; NS; $n = 10$; Figure 8).

**Discussion**

iARF is associated with severe hypoxic tubular damage predominantly of the proximal tubular cells. A large body of evidence demonstrates that the cellular consequences that are induced by repetitive hypoxia provide immediate (early phase) and long-lasting (late phase) endogenous protection against a subsequent, more severe hypoxic insult, a phenomenon termed “ischemic preconditioning,” which first was described for the heart (20). Although the molecular mechanisms that are responsible for this protective effect remain incompletely understood, the late-phase protection seems to result from adaptive gene regulation in response to hypoxia and ischemia. In particular with respect to the kidney, different experimental protocols of ischemic preconditioning were shown to be protective against a subsequent injury (reviewed in ref [21]).

The evidence that is available so far suggests that the generation of oxygen radicals as a result of the sequence of ischemia and reoxygenation is of crucial importance. Our study provides evidence for an additional, fundamentally different mechanism of “hypoxic preconditioning,” which depends on HIF and the subsequent induction of its target genes. This conclusion is based on the fact that an equal degree of structural and functional preservation of kidneys in response to ischemia/reper-
also have contributed to reduced macrophage infiltration have been described (23).

The assumption that HIF activation protects tissues against hypoxic damage so far mainly has been indirect, on the basis of the characteristics of HIF target genes. However, some in vivo studies further support our results. It has been shown that FG-0041, another 2-oxoglutarate analogue, protects against myocardial ischemia reperfusion injury (24). Although the antifibrotic properties of the compound were claimed to be responsible for the protective effect, activation of the HIF system also may have contributed. In isolated hearts of heterozygously HIF-1α-deficient (+/−) mice, the effect of delayed hypoxic preconditioning was abolished in comparison with hearts of HIF-1α (+/+ ) mice (25). In the kidney, Matsumoto et al. (26) previously demonstrated protection against ischemia/reperfusion after treatment with cobalt, which also activates HIF. However, in our hands, cobalt induces HIF-1α almost exclusively in the distal tubule (10), which raises the possibility that HIF-2α or factors other than HIF may have contributed to the observed protection of the proximal epithelium. It also should be noted that the effect of HIF may not be uniform in different models of injury. Helton et al. (27) recently reported that mice with inducible gene deletion of HIF-1α in the brain are more protected against severe ischemia compared with their wild-type littermates, which demonstrates a negative role for HIF-1α in the case of stroke in a certain experimental setting. In contrast, protective effects of PHD inhibitors in case of brain ischemia have been reported (28).

Further evidence for the protective capacity of the HIF system comes from observations of the effects of single target genes. In particular, preischemic EPO treatment was shown to reduce ischemia/reperfusion injury in rodent kidney (29). Even if administered upon reperfusion, a protective effect of EPO still was verifiable in iARF of the rat (14) and the mouse (13). Protection of rodent kidney by EPO is achieved with the use of very high dosages up to 1000 IU/kg body wt (13), which probably leads to serum EPO levels that are well above the level that is induced by endogenous HIF stabilization. We showed previously that in vitro EPO expression is predominantly HIF-2α and not HIF-1α dependent (30). Furthermore, interstitial fibroblasts are the major site of renal EPO production (19,31). After CO and FG-4487 treatment, these cells strongly stain positive for HIF-2α, which may lead to an increased local EPO production with potentially paracrine protective effects on the adjacent proximal tubular cells. EPO activation therefore may have contributed to the observed reduction of apoptosis in CO- and FG-4487–treated animals.

Also, preconditional activation of HO-1 via heat preconditioning (16), cobaltous chloride (32), or gene transfer (15) was shown to attenuate ischemia/reperfusion injury of the kidney and to ameliorate renal allograft survival in transplantation. Accordingly, iARF was enhanced in HO-1−/− deficient mice (33) and after inhibition of HO-1 activity by tin mesoporphyrin (34).

Although it is possible that the effect of one or few target gene products may play a dominant role in tissue protection that is exerted by HIF stabilization, activation of this “master switch” as an important physiologic response mechanism is

**Figure 8.** Effect of pretreatment with CO or FG-4487 on macrophage infiltration after ischemia/reperfusion. Macrophages that infiltrated the left kidney were stained by use of an ED-1 antibody. As shown in the quantitative blinded analysis (top right), the number of ED-1–positive cells unexpectedly was reduced only in the CO group to the level of sham-operated animals, whereas the number of ED-1–positive cells in the FG-4487 group was approximately the same level as in the UnT and Veh group (*P < 0.05 versus UnT or Veh, respectively).
likely to result in more reproducible and widely applicable effects than intervention that is based on a single gene. Importantly, the activation of the whole spectrum of HIF target genes may be synergistic, e.g., increasing glucose uptake into cells, as well subsequent steps of anaerobic glycolysis by transcriptional activation of almost every glycolytic enzyme (reviewed in ref [9]). In addition, the pharmacologic approach of HIF induction with 2-oxoglutarate analogues is easily applicable, and clinical trials in which an orally active PHD inhibitor is being used for induction of endogenous EPO production are already under way in humans (35). On the basis of our results, we propose that PHD inhibitors have a significant potential for clinical benefit under conditions of organ injury that deserves further exploration.

Acknowledgments
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