Up-regulation of HIF in experimental acute renal failure: Evidence for a protective transcriptional response to hypoxia

CHRISTIAN ROSENBERGER, SAMUEL N.HEYMAN, SEYMOUR ROSEN, AHUVA SHINA, MARINA GOLDFARB, WANJA GRIETHE, ULRICH FREI, PETRA REINKE, SEBASTIAN BACHMANN, and KAI-UWE ECKARDT

Department of Nephrology and Medical Intensive Care, Charité University Medicine, Berlin, Germany; Department of Medicine, Hadassah Hospital, Mt. Scopus and the Hebrew University Medical School, Jerusalem, Israel; Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts; Nephrology Unit, Bikur Holim Hospital, Jerusalem, Israel; Institute of Anatomy, Humboldt-University, Berlin, Germany; and Division of Nephrology and Hypertension, University of Erlangen-Nuremberg, Germany

Up-regulation of HIF in experimental acute renal failure: Evidence for a protective transcriptional response to hypoxia.

Background. Medullary hypoxia is believed to play an important role in the pathogenesis of acute renal failure (ARF). Hypoxia-inducible transcription factors (HIF) are recognized as master regulators of hypoxic adaptation, but little is known about their role in renal disease.

Methods. A multi-insult rat model of ARF combining the application of contrast medium with nitric oxide synthase (NOS) and cyclooxygenase (COX) inhibition was used to study chronology and distribution of the oxygen regulated HIF isoforms HIF-1α and HIF-2α in comparison with the hypoxia-marker pimonidazole between 10 minutes and 48 hours after injury induction. Treatment with furosemide was used to study HIF expression under conditions of ameliorated tissue injury.

Results. Contrast medium in combination with NOS and COX inhibition resulted in widespread induction of HIF in the outer and inner medulla that was initiated within 10 minutes, reached the highest levels at 2 hours and diminished 8 hours to 24 hours thereafter. HIF isoforms were expressed in a cell type-specific fashion: HIF-1α in tubular and HIF-2α in interstitial and endothelial cells. The degree of HIF-1α accumulation varied between nephron segments, being much stronger in collecting ducts than in medullary thick ascending limb of the loop of Henle (mTAL). Comparison with pimonidazole staining and the effect of furosemide indicated that HIF induction in mTAL is maximal with moderate hypoxia and declines with increasing severity of hypoxia.

Conclusion. A complex pattern of HIF activation appears to play an important role in tissue preservation as a response to regional renal hypoxia. The limited capacity of mTAL cells for HIF activation may explain their susceptibility to injury.

Key words: HIF, pimonidazole, hypoxia, acute renal failure, radiocontrast, heme-oxygenase-1.

Accepted for publication August 26, 2004

© 2005 by the International Society of Nephrology

Acute renal failure (ARF) is a polyetiologic disease with persistingly high incidence, lack of specific therapy, and high mortality [1]. Concepts of the pathophysiology of ARF traditionally distinguish between ischemic and toxic mechanisms, but the interplay of these mechanisms is complex [2]. Moreover, the extent of structural damage and functional impairment depends on the activity of endogenous compensatory mechanisms.

Radiocontrast agents are prime examples in this respect (see review in [3]). Although primarily considered as “nephrotoxins” their application can impair renal perfusion [4]. Local synthesis of prostaglandins and nitric oxide usually prevents overt tissue damage [5–7], but inactivation of both protective mechanisms during dye administration in rats leads to acute tubular necrosis (ATN) associated with substantial functional derangement [8]. This established model of ARF, based on triple agent injury [contrast medium, combined with cyclooxygenase (COX) and nitric oxide synthase (NOS) inhibition] induces most prominent changes in medullary thick ascending limb of the loop of Henle (mTAL). Comparison with pimonidazole staining and the effect of furosemide indicated that HIF induction in mTAL is maximal with moderate hypoxia and declines with increasing severity of hypoxia.

Microelectrode and Doppler flow studies suggest that medullary hypoxia play a crucial role in the pathogenesis of tubular lesions under these conditions [4, 7, 11–13]. Thus, inactivation of prostaglandin and nitric oxide synthesis in addition to contrast medium application leads to a further reduction in medullary perfusion and oxygen tensions [8]. The severity of tubular damage in the outer medulla increases with increasing distance from vascular bundles, which is also consistent with an important role of local oxygen gradients [8, 13, 14]. Moreover, under these experimental conditions mTAL oxygen consumption appears to correlate with tubular damage, since furosemide,
which inhibits sodium transport in mTAL confers structural and functional protection [15]. On the other hand, when delivered alone, none of the three protocol drugs produce any substantial decline in kidney function or overt morphologic changes [8], even though each was found to independently reduce medullary oxygenation [7, 8, 13, 16].

Recently the pathogenetic role of cellular hypoxia in different diseases has received growing attention, following the discovery of a widespread system of cellular oxygen sensing that controls gene expression (reviewed in [17, 18]). It has long been a general belief that cellular consequences of hypoxia are secondary to a critical decline in adenosine triphosphate (ATP) levels and changes in pericellular oxygen tensions were not considered to be important as long as ATP production is maintained. In fact, inhibitors of mitochondrial respiration and glycolysis have frequently been used to mimic insufficient oxygen supply and study its cellular consequences in renal tubules [2]. This paradigm about the cellular consequences of hypoxia, however, needs to be revised, since there is growing evidence that oxygen is also an important regulator of cellular functions independent of energy generation. Hypoxia-inducible transcription factors (HIF) have been recognized as master gene regulators under oxygen deprivation and control genes involved in red blood cell production, iron metabolism, vascular tone and architecture, energy metabolism, cell proliferation, differentiation, and viability [17, 18]. Given this broad spectrum of responses, it is highly likely that the overall effect of HIF expression promotes the adaptation of cells to oxygen deprivation and is thus tissue protective under hypoxic conditions.

HIF is a heterodimer, composed of a constitutive β subunit and one of two alternative α subunits (HIF-1α and HIF-2α), which are regulated through oxygen-dependent proteolysis. We have recently shown that both HIF-α isoforms are inducible in different cell populations of the kidney, and colocalize with HIF target genes under systemic hypoxia and after renal infarction [19, 20]. Moreover, marked transcriptomic changes, including a cluster of genes identified as HIF targets, have recently been observed in a human proximal tubular epithelial cell line in response to hypoxia [21]. These findings illustrate that the HIF system is operating in the kidney and raise the possibility that the study of HIF expression allows identification of hypoxic cells and assessment of the relationship between a hypoxic response and cell morphology under the complex conditions of renal injury.

In order to obtain direct evidence for the extent of cellular hypoxia and a subsequent transcriptional response in acute renal injury we have therefore investigated the tissue distribution and chronology of the oxygen-regulated HIF-α subunits in the rat model of radiocontrast-induced ARF described beforehand. To correlate the findings with the degree of regional hypoxia, we have also studied the formation of pimonidazole adducts, an acknowledged marker of severe tissue hypoxia [22]. This approach allows us for the first time to demonstrate extensive single cell hypoxia, hypoxic adaptation and its relationship to cell damage in a clinically relevant model of ARF.

**METHODS**

**Animal experiments**

The study was approved by the institutional review board for the care of animal subjects and was performed in accordance with National Institutes of Health guidelines. Fifty-five male Sprague-Dawley rats (200 to 250 g, purchased from Herlan Co. (Jerusalem, Israel) were allowed free access to standard rat chow and water. Under ketamine (100 mg/kg) polyethylene tubes were inserted into the femoral artery and vein for drug administration, as detailed below (four to six animals per group) and the animals were allowed to recover until harvesting of the kidneys.

**Single drug protocol**

To test the induction of subclinical renal hypoxia, the following agents were administered alone in doses that were proven not to cause renal functional or morphologic changes [8], and the kidneys were harvested at 2 hours after injection: (1) indomethacin (Sigma Chemical Co., St. Louis, MO, USA) was dissolved in phosphate buffer (pH 8.4) and administered intravenously at the dose of 10 mg/kg; (2) NO₃-nitro-L-arginine methyl ester (L-NAME) (Sigma Chemical Co.) was dissolved in 0.9% saline and administered intravenously at the dose of 10 mg/kg; and (3) iothalamate meglumine (Angio-Conray Mallinckrodt Specialty Chemicals, St. Louis, MO, USA) 60% was injected through the arterial line at the dose of 6 mL/kg over 2 to 3 minutes.

**Complete ARF protocol**

To induce ARF, a combination of the three agents in the order mentioned above was sequentially administered at 15-minute intervals as previously detailed [8].

To assess the time course of tissue hypoxia, the animals were subjected to the complete ARF protocol for 40 minutes (10 minutes after the last injection with iothalamate), 2 hours, 4 hours, 8 hours, 24 hours, and 48 hours.

In control animals L-NAME, iothalamate and indomethacin were substituted by equal volumes of saline or phosphate buffer, respectively.
Effect of furosemide on HIF-induction and tissue hypoxia in ARF model

Furosemide reduces oxygen consumption in the renal medulla by blocking salt reabsorption in thick ascending limbs, and improves functional and morphologic outcome in rat radiocontrast nephropathy [15]. Furosemide was added to the complete ARF protocol mentioned above, and delivered intraperitoneally (20 mg/kg) 15 minutes before indomethacin, with an additional intravenous dose of 2 mg/kg 3 minutes before the administration of the radiocontrast.

Detection of tissue hypoxia by pimonidazole adducts

Pimonidazole, delivered in vivo, reliably binds to tissue at oxygen tensions below 10 mm Hg [22], where it can be detected by immunohistochemistry with help of specific antipimonidazole antibodies. Pimonidazole (Hy- poxyprobe) (Natural Pharmacia International, Belmont, MA, USA) was administered intravenously at the dose of 60 mg/kg 1 hour prior to sacrifice in animals subjected to complete ARF protocol.

Morphologic studies

Kidneys were perfusion-fixed with 3% paraformaldehyde via the abdominal aorta exactly as previously reported [23], stored in ice-cooled phosphate-buffered saline (PBS), and processed for paraffin embedding. Two micron paraffin sections were processed for routine histology or immunohistochemistry. Hematoxylin and eosin staining was performed according to standard procedures. Counterstaining with Richardson's reagent served for a better discrimination of interstitial compartments. Immunohistochemistry was performed with the following primary antibodies exactly as previously reported [19, 20]: mouse antihuman HIF-1α (a67) (Novus Biologicals, Littleton, CO, USA) (1:10,000), rabbit antimouse HIF-2α (PM9) (gift from Patrick Maxwell, Welcome Trust Center for Human Genetics, Oxford, UK) (1:10,000), mouse-anti-rat CD31 (marker for endothelial cells, Serotec, Oxford, UK, 1:500), rabbit anti rat heme oxygenase-1 (HO-1) (Stressgen, Victoria, Canada) (1:60,000), mouse anti pimonidazole (1:1000 Hypoxyprobe) (Natural Pharmacia International, Belmont, MA, USA).

Semiquantitative analysis of tubular HIF-1α response in comparison to pimonidazole staining

The relationship between the degree of hypoxia (pimonidazole staining) and tubular HIF-1α induction was assessed semiquantitatively in the inner stripe of the outer medulla at 2 hours after complete ARF protocol. Core areas of hypoxia (10× power field) were determined on the basis of strong pimonidazole staining. Parallel sections were stained for hematoxylin and eosin and HIF-1α, and each tubular profile in the area was evaluated. Pimonidazole staining was classified as absent, moderate, or strong, according to the intensity of immunohistochemical signals. HIF-1α staining was classified according to signal abundance as absent (no nuclear signal per tubular section), moderate (one to two nuclear signals per tubular cross-section), or strong (three and more nuclear signals per tubular cross-section). Collecting ducts and mTAL were counted separately and stratified according to the staining intensity for both pimonidazole and HIF-1α. The average counts of two 10× power fields from each of five animals were used.

Signal analysis

Signals were analyzed with a Leica DMRB microscope (Leica, Bensheim, Germany), using differential interference contrast. Photographs were digitally recorded by means of a Visitron system (Visitron, Puchheim, Germany).

Statistics

Data are presented as mean percentage ± SD of tubules. All collecting ducts and TALs were stratified according to the degree of concordance of the two immunostaining techniques. The nonpaired Student t test was used to compare collecting ducts and mTAL regarding co-staining with pimonidazole adducts and HIF-1α. The concordant tubular staining by the two assays for each tubular segment and the comparisons of staining discordance between collecting ducts and TALs were also assessed by Pearson chi-square analysis. The difference was considered significant at P < 0.05.

RESULTS

Detection of tissue hypoxia by pimonidazole adducts in experimental ARF (complete ARF protocol)

To assess regional hypoxia in the kidney, we first assessed the binding of pimonidazole adducts in animals injected with the bioreductive dye under control conditions and after induction of ARF using the triple agent protocol. No signals were identified in control animals (not shown) or in the cortex of treated animals. In contrast, strong signals were detected in the medulla at 2 hours after induction of ARF by the combined delivery of indomethacin, iothalamate, and L-NAME (Fig. 1A, D, and G). Signal abundance increased from the outer medulla to the papillary tip. In general, signal distribution coincided with known renal oxygen profiles. In the outer stripe only some tubules at the base of medullary rays were positive (Fig. 1A), whereas in the inner stripe signals were strongest in the deep portion and with increasing distance from vascular bundles (Fig. 1D).
At 8 hours after induction of ARF, signals had disappeared in the outer stripe (Fig. 1B), and decreased with respect to 2 hours in the inner stripe (Fig. 1E). Strong papillary signals persisted, but distribution changed over time, moving from the papillary edge inwards at 8 hours (Fig. 1H).

At 24 hours after induction of ARF, pimonidazole adducts were no longer detectable in all kidney regions (Fig. 1C, F, and I).

**Distribution of HIF-α expression 2 hours after delivery of indomethacin, iothalamate, or L-NAME alone, or in combination (complete ARF protocol)**

The accumulation of HIF transcription factors was determined using antibodies specific for the HIF-1α and HIF-2α subunits. As described previously [19, 20], no signals were detectable in vehicle-treated animals (not shown). Also in treated animals signals were absent in the cortex (not shown), but marked nuclear accumulation of HIF-1α and -2α occurred in the medulla. If detectable, staining always appeared in the deep papilla, where it was strongest. Additional staining preponderantly occurred in the inner stripe, followed by the outer stripe and inner medulla. The distribution of HIF-α staining in general paralleled regions with extreme tissue hypoxia as assessed by pimonidazole adducts. The expression of the two distinct HIF-α subunits occurred largely in parallel, but there were subtle differences in their regional distribution. HIF-1α expression was nearly undetectable after indomethacin alone (Fig. 2A, E, and I), appeared in the papilla after iothalamate only (Fig. 2J), spread toward the inner and outer stripe after L-NAME alone (Fig. 2C, G, and K), and was yet more abundant and intense after complete ARF protocol (Fig. 2D, H, and L). In general, signal intensity and abundance increased from the outer medulla to the deep papilla. HIF-2α expression appeared in the papilla following indomethacin (Fig. 3I),
spread toward the outer medulla after iothalamate alone (Fig. 3B, F, and J), and was most pronounced after L-NAME alone (Fig. 3C, G, and K) and in the complete ARF protocol (Fig. 3D, H, and L), respectively. As with HIF-1α, signal abundance and intensity increased from the outer stripe to the deep papilla. However, HIF-2α expression after single agents was overall stronger when compared to HIF-1α.

**Time course of HIF-α expression after complete ARF protocol**

To assess the kinetics of HIF-α accumulation HIF expression was then determined in animals between 10 minutes and 48 hours following the last injection in the triple agent protocol. Already at 10 minutes after administration of the last of three protocol drugs, some signals were detectable in the low papilla (not shown). At 2 hours strong signals were detectable in the whole renal medulla (Table 1). Thereafter, staining gradually decreased over time and was no longer detectable after 48 hours. Remarkably, papillary HIF-1α signals also exhibited spatial redistribution (Fig. 4), shifting from the rim of the papillary tip (2 hours) toward the core of the papilla (8 hours). This redistribution paralleled tissue hypoxia as assessed by pimonidazole binding (compare with Fig. 1) and resembled previous observations in animals subjected to renal artery clamping [20].
Fig. 3. Expression of hypoxia-inducible transcription factor (HIF)-2α at 2 hours after either indomethacin, sodium iothalamate, or Nω-nitro-L-arginine methyl ester (L-NAME) alone, or the combination of all three agents [complete acute renal failure (ARF) protocol]. Only weak signals are detectable in the papilla with indomethacin (J). With iothalamate signals are detectable in papilla (J), inner stripe (F) and in the base of medullary rays in the outer stripe (B). With L-NAME signals show similar distribution, but are stronger and more abundant (C, G, and K). With all three agents signals are even more intense (D, H, and L) than with L-NAME (magnification: 300×).

Cell type localization of HIF-α signals

Although the regional distribution of HIF-1α and HIF-2α were similar, the cell types expressing both HIF isoforms were clearly different.

HIF-1α. Signals were detectable in all medullary tubular cell types, but were strongest in medullary collecting ducts (Figs. 2C, D, G, H, K, and L; 4; 6D; and 8A, C, and E). mTAL, which exhibited the most intense injury (area outlined in Fig. 6A and B) only rarely expressed HIF-1α. Additionally, interstitial cells were frequently positive as well in the papilla (Figs. 2J to L; 4A and B; and 8E), and occasionally in the inner stripe (Fig. 8C). Vascular endothelial cells were negative, as assessed in parallel sections stained for the endothelial marker CD31 (data not shown).

HIF-2α. Tubular profiles were constantly negative (Figs. 3 and 5). After counterstaining with Richardson’s reagent signals could be attributed to capillaries (Fig. 5B and D) or interstitial cells (Fig. 5A and C). In the papilla, where capillaries and thin limbs cannot be distinguished by routine morphology, parallel sections counterstained for the endothelial marker CD31 proved capillary location (Fig. 5E and F).

Influence of furosemide on tissue preservation and on HIF-α expression after complete ARF protocol

As expected on the basis of previous experiments [15], furosemide treatment attenuated mTAL injury at 2 hours after induction of the combined ARF protocol (Fig. 6C).
Table 1. Time course of hypoxia-inducible transcription factors (HIF)-1α/2α signals after complete acute renal failure (ARF) protocol

<table>
<thead>
<tr>
<th>Renal zone</th>
<th>10 minutes</th>
<th>2 hours</th>
<th>4 hours</th>
<th>8 hours</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Outer stripe</td>
<td>—</td>
<td>+</td>
<td>(+)</td>
<td>(+)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Inner stripe</td>
<td>—</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Inner medulla</td>
<td>—</td>
<td>(+)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Papilla</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>(+)</td>
<td>—</td>
</tr>
</tbody>
</table>

In general, both signal intensity and abundance developed concordantly, but abundance (HIF-1α in tubular cells, and HIF-2α in interstitial/endothelial cells) was the main scoring criterion. Symbols are: (+), <5% of cells; +, 5% to 10% of cells; ++, 10% to 25% of cells; ++++, more than 25% of cells.

Furosemide did not induce HIF-α when delivered alone (data not shown). In complete ARF protocol, however, furosemide unexpectedly increased HIF-1α signals, which were clearly more abundant in mTALs in areas with preserved tissue (Fig. 6D). On the contrary, interstitial HIF-2α signals remained unchanged (not shown).

**Relationship between the extent of hypoxia and tubular expression of HIF-1α at 2 hours after complete ARF protocol**

For a direct comparison between the degree of tissue hypoxia and HIF induction serial sections of animals pretreated with pimonidazole were stained for pimonidazole adducts, HIF and hematoxylin and eosin. Figure 7 shows an example where corresponding tubular profiles are marked with dots (mTAL) or with asterisks (collecting duct). In general, mTAL showed stronger pimonidazole-staining (and thus deeper hypoxia) than adjacent collecting duct, but occasionally also collecting duct exhibited highest degree of pimonidazole-staining. The colors of the symbols indicate the degree of pimonidazole binding, ranging from absent (black), to medium (blue), and strong (red).

Although areas of pimonidazole and HIF staining were overlapping, a direct comparison revealed that HIF accumulation was not entirely proportional to pimonidazole staining. Thus, statistical analysis of sections in five animals revealed that in mTAL the highest degree of HIF-1α staining occurred at medium and not at strongest pimonidazole staining (Table 2). Moreover, the relationship between HIF and pimonidazole staining varied between different tubular segments. Collecting ducts expressed HIF-1α over a much wider range of oxygen tensions (judged by the degree of pimonidazole staining) than mTAL. In the latter tubular segment, 92% of tubules intensely stained for pimonidazole adducts were HIF-1α negative, as compared with only 9% of intensely-stained collecting duct (P < 0.001) (Student t test). A 3 × 3 chi-square analysis for the extent of frequency of concordant pimonidazole and HIF-1α staining revealed that while in TALs Pearson chi-square value was 25.8 (DF4) (P < 0.0001), indicating a high degree of unmatched pimonidazole and HIF-1α scores, in collecting duct Pearson chi-square value was 5.6, only (P = 0.23), meaning that both parameters were matched at a statistical significance. Moreover, the two tubular segments were found to differ significantly regarding the incidence of full concordance of staining intensity (2 × 2 chi-square value 8.3) (P < 0.001).

Considering that all pimonidazole-positive tubules were hypoxic, hypoxic induction of HIF-1α was around 90% in collecting ducts. On the contrary, only 40% to 10% of mTAL staining for pimonidazole responded with HIF accumulation (at a medium and strong pimonidazole binding, respectively).

Noteworthy, within a distance of three to five tubular diameters from pimonidazole-positivity, collecting duct frequently stain for HIF-1α, indicating in this tubular segment an additional cellular response to hypoxia at...
Fig. 5. Cellular localization of hypoxia-inducible transcription factor (HIF)-2α at 2 hours after complete acute renal failure (ARF) protocol [combined delivery of indomethacin, iothalamate and Nω-nitro-L-arginine methyl ester (L-NAME)]. Blue counterstaining with Richardson’s reagent was performed for a better identification of interstitial compartments (F) is a threshold image for the endothelial marker CD31 (E and F) are parallel sections. Arrowheads are interstitial cells, arrows are capillary endothelial cells. HIF-2α–positive cells can be identified as both interstitial (A and C) and capillary endothelial cells (B and D). In the papilla parallel sections allow identification of HIF-2α signals in capillary endothelial cells (E and F) [magnification (A to D) 450 ×; (E and F) 400 ×].

DISCUSSION

The present study, by using two independent techniques, is the first to provide direct immunohistochemical evidence for hypoxia in cells of the renal medulla in a radiocontrast ARF model. In comparison with previous work demonstrating selective necrosis of mTAL segments after contrast medium application [8], there are three main novel findings. First, hypoxia is not confined to tubules that show morphological signs of injury, but occurs throughout large parts of the outer and inner medulla. Second, within the hypoxic area a widespread transcriptional response is initiated through the activation of HIF in tubular, interstitial and endothelial cells. Third, the degree of HIF induction inversely correlates with tissue damage, suggesting a role in tissue preservation.

Oxygen dependency of HIF accumulation

Considerable insight has recently been gained into the mechanisms regulating HIF [17, 18]. In the presence of oxygen two prolyl residues of the HIF-α subunits are hydroxylated, which induces binding to a ubiquitin ligase complex and subsequent proteasomal degradation. If oxygen is insufficiently available for enzymatic hydroxylation, the HIF-α subunits are stabilized, bind to constitutively expressed HIF-β and translocate into the nucleus. Here we used high amplification immunohistochemistry to demonstrate the nuclear accumulation of HIF-α, but directly correlating HIF induction with cellular oxygen tensions remains difficult. In isolated cells, tonometry indicated a half-maximal induction of HIF at 1.5% to 2% O2 (corresponding to pO2 values of about 10 to 15 mm Hg) [26]. These levels are lower than the values of around 15 to 18 mm Hg which were measured in the outer medulla after contrast medium application [11, 12]. Since even electrodes with a small tip indicate an average value of a tissue sample, lowest values may be significantly below those recorded [11]. Moreover, tissue oxygen tensions are presumably lower if contrast medium...
Fig. 6. Influence of furosemide on inner stripe morphology and hypoxia-inducible transcription factor (HIF)-1α expression 2 hours after complete acute renal failure (ARF) protocol [combined delivery of indomethacin, iothalamate, and N⁵-nitro-L-arginine methyl ester (L-NAME)]. Hematoxylin and eosin staining. (A and C). (A and B) and (C and D) are pairs of parallel sections. 2 is thin limbs, 3 is thick ascending limb of the loop of Henle (mTAL), 4 is collecting duct, and VB is vascular bundle. Complete ARF protocol induces cellular damage consisting of cytoplasmic fragmentation, nuclear pyknosis, and tubular collapse [area outlined in (A)]. HIF-1α appears mainly in collecting ducts, but damaged areas are almost void of signals [area outlined in (B)]. Furosemide leads to tubular preservation (C), and to an increased HIF-1α staining, especially in mTAL (magnification 400×).

Fig. 7. Relationship between tissue hypoxia and hypoxia-inducible transcription factor (HIF)-1α expression in the inner stripe at 2 hours after complete acute renal failure (ARF) protocol [combined delivery of indomethacin, iothalamate, and N⁵-nitro-L-arginine methyl ester (L-NAME)]. Consecutive sections stained with hematoxylin and eosin (A), for HIF-1α (B), and for pimonidazole adducts as tissue markers of hypoxia (C). Corresponding tubular profiles are labeled with colored dots [medullary thick limb of the loop of Henle (mTAL)] or with colored asterisks (collecting ducts). The color of the labels defines the degree of hypoxia assessed by pimonidazole-staining (C). Black is no detectable hypoxia, blue is moderate hypoxia, and red is strong hypoxia. Strongest HIF-1α staining occurs at moderate degrees of hypoxia. The mTALs with strongest hypoxia are negative for HIF-1α. However, some collecting ducts void of pimonidazole staining are positive for HIF-1α, indicating hypoxia above the threshold for pimonidazole adduct formation (magnification 100×).

There is clear evidence also that in vivo the relationship between HIF protein levels and pO₂ is variable and depends on intrinsic cellular properties. We have previously shown that there are marked differences in the ability of different tubular cell types to induce HIF in response to systemic hypoxia [19]. Consistent with these findings the application is combined with NOS and COX inhibition, as in the present protocol. Renal oxygen tensions widely below those measured by electrodes after isolated contrast medium application are also suggested by the staining for pimonidazole adducts, which are considered to form at pO₂ values below 10 mm Hg [27].
HIF response to contrast medium application was much more marked in collecting ducts than mTAL. The collecting duct epithelium therefore seems to usefully up-regulate HIF, while the mTAL epithelium fails to mount a comparable cellular preservation response.

A further aspect of cellular specificity for HIF induction relates to the induction of the two different HIF\(\alpha\) isoforms, HIF-1\(\alpha\) and HIF-2\(\alpha\). Under systemic hypoxia HIF-1\(\alpha\) is expressed in tubular cells and HIF-2\(\alpha\) in peritubular cells [28] and this complementary pattern was also consistently seen in this study after application of the different agents alone or in combination.

**Hypoxia in radiocontrast-induced ARF**

Two main mechanisms have been implicated in the reduction of renal oxygen tensions following contrast medium application: increased tubular oxygen consumption and decreased renal perfusion [3]. The former is considered to result mainly from enhanced distal tubular sodium load due to the osmotic diuresis, while the latter is attributed to vasoconstriction, as well as possible compression of peritubular capillaries resulting from enhanced intratubular pressure. Renal vasoconstriction in response to contrast medium appears to be unique, as other vascular beds vasodilate, and the molecular basis of this difference has not been resolved [4]. Differences in the vascular response to contrast medium exist even within the kidney and in contrast to cortical and papillary vasoconstriction [29], outer medullary regional blood flow is enhanced by contrast medium [8]. NOS inhibition prior to contrast medium application abolishes the medullary vasodilation and augments hypoxic tubular injury [8]. Further aggravation of tubular damage occurs in the presence of inhibitors of prostaglandin synthesis [8]. Interestingly we found some induction of HIF after application of L-NAME or indomethacin only (Figs. 2 and 3), which is consistent with reductions in medullary oxygen tensions [13] and indicates that both systems play a permissive role in maintaining renal oxygenation. These findings support the view that cells in the renal medulla normally operate at the edge of adequate oxygenation and thus at continuous risk of hypoxia [31].

**Comparison between staining for HIF and pimonidazole adducts**

Although in general we observed a remarkable overlap between areas staining for pimonidazole adducts and HIF, comparing both patterns also revealed potentially important differences. Thus tubules which stained most extensively for pimonidazole did not have strongest HIF signals and vice versa (Fig. 7) (Table 2).

One might speculate that pimonidazole uptake is impaired in the most hypoxic areas, but the observations in animals treated with furosemide favor a different interpretation. Since renal oxygen tensions are closely correlated with work load and hence oxygen consumption, inhibition of salt reabsorption by furosemide increases mTAL oxygen tensions. Previous studies have demonstrated the efficacy of furosemide to improve medullary oxygenation in healthy volunteers [31], to reverse radiocontrast-induced aggravated medullary hypoxia in rats [13], and to attenuate medullary hypoxemia in a related multi-insult rat model of radiocontrast nephropathy [32]. Somewhat surprisingly, therefore, the amelioration of tissue injury following the application of furosemide was associated with enhanced staining for HIF (Fig. 6). Together with the discordant staining intensity for HIF and pimonidazole we interpret these findings to indicate that HIF expression does not increase continuously with increasing severity of cellular hypoxia, but requires a critical level of maintained oxygenation. According to this interpretation the most severely hypoxic cells (i.e., those staining most intensively for pimonidazole) were unable to maximally induce HIF. Furosemide probably preserved a reduced level of oxygen availability which kept cells still hypoxic, but allowed HIF to be formed and stabilized. In vitro experiments in which cells were exposed to increasingly severe hypoxia support this notion, in that HIF protein levels and DNA binding were maximal at a certain oxygen concentration and declined more marked in collecting ducts than mTAL. The collecting duct epithelium therefore seems to usefully up-regulate HIF, while the mTAL epithelium fails to mount a comparable cellular preservation response.

A further aspect of cellular specificity for HIF induction relates to the induction of the two different HIF\(\alpha\) isoforms, HIF-1\(\alpha\) and HIF-2\(\alpha\). Under systemic hypoxia HIF-1\(\alpha\) is expressed in tubular cells and HIF-2\(\alpha\) in peritubular cells [28] and this complementary pattern was also consistently seen in this study after application of the different agents alone or in combination.

**Hypoxia in radiocontrast-induced ARF**

Two main mechanisms have been implicated in the reduction of renal oxygen tensions following contrast medium application: increased tubular oxygen consumption and decreased renal perfusion [3]. The former is considered to result mainly from enhanced distal tubular sodium load due to the osmotic diuresis, while the latter is attributed to vasoconstriction, as well as possible compression of peritubular capillaries resulting from enhanced intratubular pressure. Renal vasoconstriction in response to contrast medium appears to be unique, as other vascular beds vasodilate, and the molecular basis of this difference has not been resolved [4]. Differences in the vascular response to contrast medium exist even within the kidney and in contrast to cortical and papillary vasoconstriction [29], outer medullary regional blood flow is enhanced by contrast medium [8]. NOS inhibition prior to contrast medium application abolishes the medullary vasodilation and augments hypoxic tubular injury [8]. Further aggravation of tubular damage occurs in the presence of inhibitors of prostaglandin synthesis [8]. Interestingly we found some induction of HIF after application of L-NAME or indomethacin only (Figs. 2 and 3), which is consistent with reductions in medullary oxygen tensions [13] and indicates that both systems play a permissive role in maintaining renal oxygenation. These findings support the view that cells in the renal medulla normally operate at the edge of adequate oxygenation and thus at continuous risk of hypoxia [31].

**Comparison between staining for HIF and pimonidazole adducts**

Although in general we observed a remarkable overlap between areas staining for pimonidazole adducts and HIF, comparing both patterns also revealed potentially important differences. Thus tubules which stained most extensively for pimonidazole did not have strongest HIF signals and vice versa (Fig. 7) (Table 2).

One might speculate that pimonidazole uptake is impaired in the most hypoxic areas, but the observations in animals treated with furosemide favor a different interpretation. Since renal oxygen tensions are closely correlated with work load and hence oxygen consumption, inhibition of salt reabsorption by furosemide increases mTAL oxygen tensions. Previous studies have demonstrated the efficacy of furosemide to improve medullary oxygenation in healthy volunteers [31], to reverse radiocontrast-induced aggravated medullary hypoxia in rats [13], and to attenuate medullary hypoxemia in a related multi-insult rat model of radiocontrast nephropathy [32]. Somewhat surprisingly, therefore, the amelioration of tissue injury following the application of furosemide was associated with enhanced staining for HIF (Fig. 6). Together with the discordant staining intensity for HIF and pimonidazole we interpret these findings to indicate that HIF expression does not increase continuously with increasing severity of cellular hypoxia, but requires a critical level of maintained oxygenation. According to this interpretation the most severely hypoxic cells (i.e., those staining most intensively for pimonidazole) were unable to maximally induce HIF. Furosemide probably preserved a reduced level of oxygen availability which kept cells still hypoxic, but allowed HIF to be formed and stabilized. In vitro experiments in which cells were exposed to increasingly severe hypoxia support this notion, in that HIF protein levels and DNA binding were maximal at a certain oxygen concentration and declined

---

**Table 2.** Relationship between the intensity of tubular pimonidazole staining and hypoxia-inducible transcription factor (HIF)-1\(\alpha\) expression in the inner stripe at 2 hours after complete acute renal failure (ARF) protocol

<table>
<thead>
<tr>
<th>Pimonidazole</th>
<th>Absent</th>
<th>Moderate</th>
<th>Strong</th>
<th>Total N</th>
<th>Absent</th>
<th>Moderate</th>
<th>Strong</th>
<th>Total N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>% of total</td>
<td>% of total</td>
<td>% of total</td>
<td></td>
<td>% of total</td>
<td>% of total</td>
<td>% of total</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>63 ± 19</td>
<td>31 ± 16</td>
<td>4 ± 0.1</td>
<td>70</td>
<td>0 ± 0.1</td>
<td>80 ± 30</td>
<td>20 ± 29</td>
<td>12</td>
</tr>
<tr>
<td>Moderate</td>
<td>60 ± 14(a)</td>
<td>35 ± 7</td>
<td>5 ± 7</td>
<td>50</td>
<td>9 ± 6(a)</td>
<td>60 ± 17</td>
<td>31 ± 18</td>
<td>47</td>
</tr>
<tr>
<td>Strong</td>
<td>92 ± 15(b)</td>
<td>6 ± 9</td>
<td>3 ± 6</td>
<td>47</td>
<td>9 ± 9(b)</td>
<td>65 ± 13</td>
<td>28 ± 14</td>
<td>9</td>
</tr>
</tbody>
</table>

Data are presented as mean percentage ± SD from all tubules of the same nephron segment and same degree of pimonidazole staining. Total number (N) is the absolute number of tubules in all animals together.

HIF-1\(\alpha\) staining of mTAL “underestimates” hypoxia, being frequently negative even in the presence of pimonidazole staining. On the contrary, HIF-1\(\alpha\) staining of collecting duct shows 90% correlation with pimonidazole staining. The difference between mTAL and collecting duct hypoxic response is highly significant (\(P < 0.001\)) for both moderate hypoxia\(a\) and strong hypoxia\(b\).
Fig. 8. Expression of the HIF-1α-target-gene heme oxygenase-1 (HO-1) at 8h after complete ARF protocol (combined delivery of indomethacin, iothalamate and Nω-nitro-arginine methyl ester (L-NAME) L-NAME). (C and D) and (E and F) are pairs of parallel sections. 3 is thick ascending limb of the loop of Henle (mTAL), 4 is collecting duct, VB is vascular bundle, asterisks are tubuli, and with further reduction in oxygen availability [26]. In other words, there appears to be a “window of opportunity” for a HIF-dependent transcriptional response. When the severity of hypoxia exceeds this range, this response fails and injury may become irreversible. The functional role of HIF induction was exemplified in this study by demonstrating the expression of HO-1 in tubules staining positive for HIF, but the spectrum of HIF-responsive genes goes far beyond this single example [17, 21].

Potential therapeutic implications

This study also shows that the HIF response in the kidney is rapid, with some nuclear signals in the papilla detectable already 10 minutes after contrast medium application, the earliest time point investigated. Nevertheless, HIF induction was not instantaneous and after 2 hours its activation was much more prominent and widespread. Even when HIF is rapidly induced, transcription and translation of target genes and effective function of their products is likely to require much longer. It is intriguing to hypothesize therefore that greater protection against the cellular consequences of hypoxia might be achieved if HIF could be activated prior to a hypoxic insult, for a prolonged time period and to increased levels. Strategies for pharmacologic activation of the HIF system are currently under active investigation [33, 34], and could offer novel opportunities for protecting the kidney against the adverse consequences of radiocontrast and other insults.

CONCLUSION

Hypoxia and hypoxic adaptation of tubular and peritubular cells are detectable early during the development of radiocontrast-induced ATN, reach maximal levels in 2 hours and essentially disappear at 24 hours. The highest levels of HIF are present in collecting duct epithelium and these cells are preserved. A limited capacity of mTAL epithelium to mount a HIF response, as compared to the collecting duct, may contribute importantly to its susceptibility to hypoxic injury.

ACKNOWLEDGMENTS

The study was supported by the German Research Foundation (DFG Ec 873/2-2), and by funds from Harvard Medical Faculty Physicians at Beth Israel Deaconess Medical Center, Boston, MA.

---

arrowheads are interstitial cells. In control animals no staining was evident at the employed antibody concentration (A). In the outer stripe occasional staining of collecting ducts in the base of medullary rays (B). In the inner stripe and papilla heme oxygenase 1 (HO-1) appeared mainly in tubular profiles, and in some interstitial cells, as well (C and E). Parallel sections (D and F) stained for HIF-1α prove colocation on tubular profiles (magnification 150×).
REFERENCES


Reprint requests to Christian Rosenberger, M.D., Nephrology and Medical Intensive Care, Charité University Medicine, Augustenburger Platz 1, D-13353 Berlin, Germany.
E-mail: chrosenbe@aol.com