p53 regulates renal expression of HIF-1α and pVHL under physiological conditions and after ischemia-reperfusion injury

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Ischemia-reperfusion injury (IRI) is a common cause of acute kidney injury (AKI) and is characterized by widespread tubular and microvascular damage. The tumor suppressor p53 is upregulated after IRI and contributes to renal injury in part by promoting apoptosis. Acute, short-term inhibition of p53 with pifithrin-α conveys significant protection against IRI. The hypoxia-inducible factor-1 (HIF-1) pathway is also activated after IRI and has opposing effects to those promoted by p53. The balance between the HIF-1 and p53 responses can determine the outcome of IRI. In this manuscript, we investigate whether p53 regulates the HIF-1 pathway in a rodent model of IRI. HIF-1α is principally expressed in the collecting tubules (CT) and thick ascending limbs (TAL) under physiological conditions. However, inhibition of p53 with pifithrin-α increases the faint expression of HIF-1α in proximal tubules (PT) under physiological conditions. Twenty-four hours after IRI, HIF-1α expression is decreased in both CT and TAL. HIF-1α expression in the PT is not significantly altered after IRI. Acute inhibition of p53 significantly increases HIF-1α expression in the PT after IRI. Additionally, pifithrin-α prevents the IRI-induced decrease in HIF-1α in the CT and TAL. Parallel changes are observed in the HIF-1α transcriptional target, carbonic anhydrase-9. Finally, inhibition of p53 prevents the dramatic changes in Von Hippel-Lindau protein morphology and expression after IRI. We conclude that activation of p53 after IRI mitigates the concomitant activation of the protective HIF-1 pathway. Modulating the interactions between the p53 and HIF-1 pathway can provide novel options in the treatment of AKI.

acute kidney injury; acute renal failure; hypoxia-inducible factor-1α; Von Hippel-Lindau protein

ACUTE KIDNEY INJURY (AKI) is a frequently encountered clinical syndrome that is associated with considerable morbidity and mortality (24, 42). Although the cause of human AKI is frequently multifactorial, diminished renal perfusion due to hypotension or sepsis is the most common proximate factor (21, 24). Downstream of the inciting event, a complex series of interactions among injured tubules, inflammation, and microvascular alterations serves to extend the initial injury (3–5). Nonetheless, lethal and sublethal injury to the tubule remains centrally important to the overall pathophysiology of AKI, and significant strides have been made toward elucidating the molecular mechanisms involved in these processes.

The tumor suppressor protein p53 is a homotrameric transcription factor that is perhaps best known for regulating the cell cycle and the ultimate fate of a cell in response to DNA damage (44). A primary function of p53 in the setting of cellular DNA damage is to eliminate irreversibly injured and potentially neoplastic cells via promotion of apoptosis (25). Recently, p53 has been shown to be an important regulator of renal tubular cell viability in animal models of ischemic and nephrotoxic AKI (16, 45). Activation of p53 in these models of AKI induces proapoptotic pathways, which significantly contribute to renal tubular cell death and kidney dysfunction. Furthermore, pharmacological or genetic inhibition of p53 provides a substantial protective effect in these models of AKI (12, 13, 16, 17). Interestingly, evidence is accumulating that p53 is an important regulator of cellular stress pathways via mechanisms that do not rely solely on its role as a transcription factor. These nontranscriptional roles include diverse actions such as direct activation of apoptosis at the level of the mitochondria and interactions with hypoxia-inducible factor-1 (HIF-1) (8, 26).

The interaction between p53 and HIF-1 is particularly germane to acute ischemic injury of the kidney. HIF-1 is a heterodimeric transcription factor composed of a constitutively expressed β-subunit and a regulated α-subunit (36). HIF-1 is important for promoting a variety of cellular responses to hypoxia such as stimulating glycolysis, enhancing neovascularization, and inhibiting apoptosis (47). In addition, activation of HIF-1 has been demonstrated to be protective in animal models of ischemic AKI (1, 10, 23). It is of interest to point out that many of the effects induced by HIF-1 activation oppose those induced by p53 activation (8, 34). Consequently, when both pathways are simultaneously activated under appropriate stress conditions they can counter each other through complex interactions at various levels. Of particular relevance is that p53 has been shown to directly repress the transcriptional activity of HIF-1 through competition for the shared coactivator p300, (2, 35) and promote Von Hippel-Lindau protein (pVHL)-independent degradation of the HIF-1α subunit (28).

The net result of the complex interaction between these two pathways can ultimately determine the degree of tissue injury in ischemic AKI, and so understanding this interaction is a critical step in our knowledge of the pathophysiology of AKI. Therefore, in this manuscript we examine the effects of acute p53 inhibition on HIF-1α in the kidney under physiological conditions and in an animal model of ischemic AKI. We

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demonstrate that inhibition of p53 dramatically increases HIF-1α expression in spatially distinct areas of the renal parenchyma under physiological conditions and following ischemic injury. In addition, we demonstrate that p53 inhibition affects pVHL expression in a tubule-specific manner. Our results suggest that the role of p53 in ischemic AKI clearly extends beyond the direct control of apoptosis. We propose that the regulation of HIF-1α and pVHL by p53 is an important determinant of the renal response to ischemic injury and that modulation of this interaction can provide novel options in the therapeutic approach to AKI.

METHODS

Animals and experimental model of AKI. Male Sprague-Dawley rats, weighing 150–200 g, were obtained from Harlan (Indianapolis, IN). All experiments were conducted in accordance with The Guide for the Care and Use of Laboratory Animals (Washington, DC: National Academy Press, 1996) and approved by the Institutional Animal Care and Use Committee. Animals were anesthetized with 5% halothane for induction followed by buprenorphine HCl (0.01 mg/kg) subcutaneously and 1.5% halothane for maintenance and then placed on a homeothermic table to maintain core body temperature at 37°C. A midline incision was made, the renal pedicles were isolated, and bilateral renal ischemia was induced by clamping the renal pedicles for 30 min with microserrifines. After removal of the microserrifines, reperfusion was monitored visually before closure of the abdominal surgical wound. Two milliliters of prewarmed (37°C) sterile saline containing either pifithrin-α (3 mg/kg dissolved in 24 μl of DMSO, Calbiochem, San Diego, CA) or an equal volume of DMSO was administered intraperitoneally just after closing of the surgical incision. Animals were allowed to recover on a homeothermic pad to maintain body temperature until the righting reflex was restored. Sham surgery consisted of an identical procedure with the exception of immediate release of the clamps. Reperfusion time varied between 0 and 24 h.

Tissue immunostaining and confocal microscopy. At the time of death, kidneys were perfused in situ with 4% paraformaldehyde. Tissues were subsequently processed for immunofluorescence staining or immunohistochemistry. Fifty-micrometer vibratome sections of fixed kidney tissue were obtained for immunofluorescence staining. Primary antibodies to HIF-1α, mouse monoclonal clone ESEE122 (Novus Biologicals, Littleton, CO) or goat polyclonal sc-8711 (Santa Cruz Biotechnology, Santa Cruz, CA), Von Hippel-Lindau protein (pVHL; rabbit polyclonal 2738, Cell Signaling Technology, Danvers, MA), Tamm-Horsfall protein (THP; rabbit polyclonal sc-16240, Santa Cruz Biotechnology), p53 (sheep polyclonal PC35, EMD Biosciences-Calbiochem, San Diego, CA), and carbonic anhydrase-9 (CA9; rabbit polyclonal sc-25600, Santa Cruz Biotechnology) were utilized for immunostaining. Appropriate secondary antibodies conjugated with Cy5, Alexa-555, or Alexa 647 were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA) or Invitrogen-Molecular Probes (Carlsbad, CA).

For immunohistochemistry, kidneys were paraffin embedded, sectioned at 4 μm, deparaffinized, and stained using the DakoCytomation Envision+ System, horseradish peroxidase (Dako North America, Carpinteria, CA), and primary antibody to HIF-1α (mouse monoclonal clone ESEE122, Novus Biologicals). Some tissues underwent antigen retrieval by boiling in sodium citrate buffer (pH 6.0) for 15 min in a pressure cooker before immunohistochemical staining. Negative controls were obtained by incubating kidney tissue sections from sham animals and animals undergoing renal ischemia with secondary antibodies in the absence of primary antibodies. Kidney tissue sections undergoing immunofluorescent staining were counterstained with fluorescein-labeled phalloidin (Molecular Probes, Eugene OR), and tissues undergoing immunohistochemistry were counterstained with hematoxylin. Confocal immunofluorescent images of kidney tissue sections were collected at ×40 magnification using a LSM-510 Zeiss confocal
microscope (Heidelberg, Germany) equipped with argon and helium/ neon lasers. Eight to ten images were collected from the cortex, outer stripe of the outer medulla, inner stripe of the outer medulla, and the inner medulla of the kidney from each animal. Regions of interest containing selected tubular segments in each image were analyzed with Metamorph software (Universal Imaging, West Chester, PA). Immunohistochemical images of kidney tissue sections were obtained at ×40 magnification with a Nikon Diaphot compound microscope (Melville, NY).

**Cell culture experiments.** LLC-PK1 cells were maintained in DMEM (GIBCO BRL, Grand Island, NY) containing 10% fetal bovine serum and penicillin-streptomycin. Cells were grown in a humidified atmosphere (95% air-5% CO2) at 37°C. Cells were fed 24 h before experiments and were 80% confluent at the time of the experiment. Hypoxia was induced by placing the cells into an atmosphere containing 0.1% O2 for 18 h. Chemical anoxia was induced by incubation of the cells in substrate-free media (amino acids and glucose omitted) containing 0.1 mM antimycin A as previously described (39), followed by three washes with warm PBS and recovery in DMEM for 4 h.

**Western blotting.** For cell culture experiments, LLC-PK1 monolayers under control and experimental conditions were quickly washed at the end of the experiment with PBS followed by the addition of 125 μl of SDS buffer (1% SDS, 10 mM Tris·HCl, 2 mM EDTA; pH 7.5) prewarmed to 100°C. The samples were scraped into a microfuge tube and kept at 100°C for 5 min. Subsequently, the samples were sonicated and then centrifuged at 4°C. The supernatants were carefully removed for protein determinations and Western blotting. Proteins were measured by a BCA assay (Pierce Chemical). Blots were scanned on a Bio-Rad Fluor-S Multimager to determine band densities.

**Statistical analysis.** All results represent n = 3 animals unless noted otherwise. Results are expressed as means ± SD. Band density data and immunofluorescence data were analyzed for significance by a nested ANOVA and then planned comparisons by a one-way ANOVA. P = 0.05 was utilized to determine significance.

**RESULTS**

*HIF-1α expression and localization in the kidney.* To evaluate the spatial distribution of HIF-1α in the kidney under physiological conditions, we used a monoclonal antibody to HIF-1α to immunostain rat kidney sections. We observed

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**Fig. 2. Colocalization of HIF-1α to the TALs with Tamm-Horsfall protein (THP) immunostaining.** In addition to identifying tubular segments by actin cytoskeleton morphology, we confirmed the identity of TALs (as opposed to collecting tubules) by staining for THP. A: red staining of HIF-1α in collecting tubules (arrow) and less intense staining localized to putative TALs in the outer stripe of the outer medulla (arrowheads). B: the same field is shown as in A, but the TALs are identified by colocalization of THP (blue), confirming the identity of TALs. C: red HIF-1α staining in collecting tubules (arrow) and TALs (arrowheads) of the inner stripe. D: the same field is shown as in C with colocalization of THP (blue). Red HIF-1α staining colocalized with blue THP results in a merged purple staining. Bar = 50 μm.
prominent HIF-1α immunostaining in the collecting tubules, as well as the thick ascending (TAL) and thin limbs of the loop of Henle (Fig. 1). Minimal HIF-1α immunostaining was detected in the proximal tubules. Counterstaining F-actin with FITC-phalloidin allowed the identification of various tubular segments; however, we confirmed localization of HIF-1α to the TAL by coimmunostaining with an antibody to Tamm-Horsfall protein (Fig. 2) as described previously (7). Control sections stained with secondary antibodies alone revealed no immunofluorescence (not shown).

We also utilized a polyclonal antibody directed against a different epitope of HIF-1α to validate the immunostaining pattern of HIF-1α obtained with the mouse monoclonal antibody. HIF-1α immunostaining with this polyclonal antibody revealed an identical distribution in the collecting tubules and the TAL and thin limbs of the loop of Henle (Fig. 3, B and D) to that obtained with the monoclonal antibody (Fig. 3, A and C). In addition, immunohistochemistry using the monoclonal antibody also demonstrated a similar distribution pattern for HIF-1α in the kidney under physiological conditions (Fig. 3, E and F). Interestingly, while nuclear staining was observed in these segments of the tubule, the more prominent staining was in the cytoplasm (Fig. 3, G and H). For example, the average immunofluorescence intensity for HIF-1α in collecting tubules was 88 ± 25 (arbitrary units/pixel) in the cytoplasm, 45 ± 17 in the nucleus, and 5 ± 1 in the tubular lumen (used as background control).

**Inhibition of p53 ameliorates alterations in HIF-1α expression following ischemia-reperfusion injury.** Given our findings for HIF-1α expression in specific segments of the kidney under physiological conditions, we next sought to examine the effects of ischemia-reperfusion injury on the spatial expression of HIF-1α in the kidney. Temporally, we focused our examination at 24 h of reperfusion following ischemic injury, since at this time point in our model we observe a peak in p53 levels and renal dysfunction as determined by serum creatinine as well as significant structural and functional alterations in the renal tubular epithelium and renal microvasculature. Interestingly, in both the collecting tube and TAL we observed a significant decrease in HIF-1α expression (Fig. 4 and see Fig. 7). We did not observe a change in the minimal HIF-1α signal of the proximal tubules following renal ischemia-reperfusion injury.

Given the protective effect that p53 inhibition provides in this model of ischemic AKI (16) and the mounting evidence that p53 can repress the HIF-1α response, we next sought to determine whether p53 inhibition with the small molecule inhibitor pifithrin-α (18) can augment a tubular HIF-1α response following ischemia-reperfusion injury. Inhibition of p53 conferred an overall protective effect on histology and the actin cytoskeleton of tubules (Fig. 4). Animals treated with pifithrin-α also had significantly lower serum creatinine 24 h post-ischemic-reperfusion injury, as we have previously reported (16). Moreover, p53 inhibition led to an increase in HIF-1α expression in the collecting tubules and TAL compared with vehicle-treated controls in this model of ischemic AKI (Fig. 4 and see Fig. 7). The level of HIF-1α expression in these tubular segments was similar to that observed in the vehicle-treated, sham-operated controls (see Fig. 7); thus p53 inhibition preserved HIF-1α expression in these tubular segments despite ischemia-reperfusion injury. Additionally, while ischemia-reperfusion alone had little effect on the minimal baseline HIF-1α expression in the proximal tubules, inhibition of p53 resulted in a significant increase in HIF-1α expression in the proximal tubules of the outer stripe of the medulla compared with vehicle-treated animals undergoing either sham surgery or surgery to induce ischemia-reperfusion injury (Fig. 4 and see Fig. 7).

Because p53 expression is maximal 24 h after reperfusion, we examined HIF-1α expression at 0 and 2 h postreperfusion.
Our previous studies have shown p53 expression to be minimal at these early time points (16). As shown in Fig. 4E, there was a significant increase in proximal tubular HIF-1α expression at 0 and 2 h postreperfusion. This early increase was no longer observed 24 h postreperfusion, as discussed above. The beneficial effects of pifithrin-α suggest that the loss of this early HIF-1α response by 24 h in untreated animals is secondary to increased p53 expression.

Paradoxically, we noted that pifithrin tended to reduce the HIF-1α response at 0 and 2 h postreperfusion (data not shown), an effect opposite to what is observed at 24 h. It is possible that inhibition of p53 conveys overall tissue protection that minimizes the need for a HIF-1α response in the early postreperfusion phase. However, in the late postreperfusion phase (the 24-h time point investigated in this paper), p53 expression is maximal and directly antagonizes an increase in HIF-1α expression despite stimuli to HIF-1α such as diminished perfusion and inflammation that gradually develop 24 h post-ischemia-reperfusion (during the extension phase of AKI) (38). As expected, it is at this time point that inhibition of p53 increases HIF-1α expression.

p53 expression increases in all tubular segments in an ischemic AKI model. We have previously demonstrated that proximal tubular p53 expression is increased in this model of ischemic AKI (16). Because we detected an effect of p53 inhibition on HIF-1α expression in many tubular segments, we

Fig. 4. Effect of ischemia-reperfusion injury and pifithrin-α on renal HIF-1α expression. A: representative section from the outer stripe 24 h after ischemic injury. Proximal tubules (P) have thin and dilated brush borders and show minimal HIF-1α staining (red). Cortical collecting tubules have reduced HIF-1α staining (arrow, red) compared with sham (Fig. 2B). B: representative section from the inner stripe of the outer medulla and shows significantly reduced HIF-1α staining in collecting tubules (arrow) and thick ascending limbs (arrowheads; compare with Fig. 2C). C and D: representative sections from the outer and inner stripes of the outer medulla, respectively, taken from animals treated with pifithrin-α at the time of ischemic injury. Note the intense HIF-1α staining (red) in collecting tubules (arrows) and thick limbs (arrowheads) and the detectable HIF-1α staining in proximal tubules (P in C). This was absent in animals not treated with pifithrin-α (A). Bar = 50 μm. E: time course of HIF-1α expression in proximal tubules from the outer medulla post-ischemia-reperfusion (I-R). Values are means ± SD and represent average fluorescence intensity/pixel in arbitrary units (n = 3 animals).
examined the spatial expression of p53 in the kidney. We observed upregulation of p53 in all segments of the tubule 24 h after ischemic injury (Fig. 5).

Inhibition of p53 augments proximal tubular HIF-1α expression in the outer stripe of the medulla under physiological conditions. In view of the fact that we observed an increase in proximal tubular HIF-1α expression following ischemia-reperfusion injury in animals treated with pifithrin-α to levels that were above those detected under baseline conditions, we subsequently investigated the effect of p53 inhibition on HIF-1α levels in tubular segments under physiological conditions. Intriguingly, we found that pifithrin-α treatment significantly increased HIF-1α expression in the proximal tubules of the outer medullary stripe under physiological con-

Fig. 5. Effect of I-R on renal p53 expression. Sections are stained for p53 (yellow), FITC-phalloidin (green), and a nuclear stain (DAPI; blue). Note the increase in both cortical and medullary p53 following I-R injury. Many cells with p53 staining show condensed fragmented nuclei typical of apoptosis (arrowheads). The increase in p53 with I-R is seen in all tubule segments. Bar = 50 μm.
conditions (Figs. 6 and 7). In contrast, pifithrin-α treatment did not significantly increase HIF-1α expression in the collecting tubule or TAL under physiological conditions (Fig. 7).

**Immunoblot analysis of renal HIF-1α changes after ischemia or pifithrin-α treatment confirms fluorescence microscopic data.** We thought to complement the fluorescence microscopic data with Western blot analysis utilizing yet another antibody to HIF-1α, the H1α67 clone. As shown in Fig. 8, a band was detected in sham kidneys and probably represents the baseline staining seen in collecting duct and TAL segments (Figs. 1 and 2). This band decreased significantly after ischemia, a change prevented by pifithrin-α (compare with Fig. 4). Finally, kidneys from sham animals treated with pifithrin-α showed an increase in HIF-1α compared with control kidneys, probably representing the induction of HIF-1 expression in proximal tubules (compare with Fig. 6). These findings strongly support the data obtained by fluorescence microscopy and further validate the immunostaining data.

**Expression of CA9 parallels changes in HIF-1α levels in ischemic AKI and following p53 inhibition.** CA9 is a transmembrane carbonic anhydrase isoform that is tightly regulated by a HIF-responsive element close to its transcriptional start site (48). We evaluated CA9 expression as a read-out of HIF activity in this model of AKI. As anticipated, the expression of CA9 mirrored HIF-1α expression. Following renal ischemia-reperfusion injury, we observed a decrease in the collecting tubule and TAL expression of CA9; however, we saw little change in proximal tubular CA9 expression (Fig. 9). Treatment with pifithrin-α mitigated the decrease in CA9 expression following ischemic injury in the distal tubule (collecting tubule and TAL) and produced an increase in CA9 expression in the proximal tubules of the outer stripe of the medulla compared with vehicle-treated animals undergoing either sham surgery or surgery to induce ischemia-reperfusion injury (Fig. 9). Treatment with pifithrin-α had minimal effect on CA9 expression in the collecting tubule and TAL under physiological conditions.

**Inhibition of p53 enhances CA9 expression in cell culture models of ischemia.** To advance our understanding into the regulation of the HIF-1α pathway by p53, we examined the effect of pifithrin-α on CA9 in a porcine proximal tubular cell line (LLC-PK1) utilizing two models of ischemia/ischemia-reperfusion injury: hypoxia and substrate depletion. In both model systems, inhibition of p53 resulted in enhanced expression of CA9, thus supporting the suppressive effect of p53 on CA9 activity in these model systems (Fig. 10A). Moreover, treatment of LLC-PK1 cells with pifithrin-α under standard cell culture conditions also enhanced the expression of CA9 (Fig. 10B).

**Inhibition of p53 augments proximal tubular pVHL expression and averts alterations in pVHL following ischemia-reperfusion injury.** One of the major roles for pVHL is to operate as the substrate recognition component of an E3-ubiquitin-ligase complex which targets HIF-1α that has been hydroxylated on proline residues in an oxygen-dependent fashion. This complex rapidly directs HIF-1α to proteosomal degradation under normoxic conditions. Due to the important role pVHL plays in the regulation of HIF-1α, we examined the spatial expression of pVHL in the kidney following ischemia-reperfusion injury and during inhibition of p53. Under physiological conditions, we found that pVHL was essentially restricted to the cytoplasm of proximal tubules and the staining was more prominent in the proximal tubules of the outer medullary stripe than in the cortex. Very minimal and inconsistent pVHL immunostaining was observed in medullary
tubules. Inhibition of p53 resulted in a significant increase in pVHL in the proximal tubule that was ~50% greater than that observed in vehicle-treated animals (Figs. 11 and 12). Ischemia-reperfusion injury to the kidney caused a significant increase in pVHL in the proximal tubule that was almost 3-fold greater than that detected in vehicle-treated, sham-operated animals (Figs. 11 and 12). In addition ischemia-reperfusion injury resulted in the formation of dense pVHL aggregates in the cytoplasm of proximal tubular cells (Fig. 11). No change in the very minimal pVHL immunostaining was observed in other areas of the kidney. Of interest, inhibition of p53 prevented the morphological alterations of pVHL and the dramatic increase in expression seen in the proximal tubules following ischemia-reperfusion injury.

DISCUSSION

The tumor suppressor p53 is emerging as a multifaceted molecule involved in the control of diverse cellular functions (44). We have previously shown that inhibition of p53 attenuated renal injury in a model of ischemic AKI predominantly through reduction of apoptotic cell death (16, 17). In those studies, inhibition of p53 with pifithrin-α decreased the expression of Bax and prevented the translocation of p53 to mitochondria. In this paper, we examined the effect of p53 inhibition on another proposed regulator of injury in AKI, the pVHL/HIF-1 pathway.

Numerous studies have documented the upregulation of HIF-1α in kidney tissues following different types of injury (6). These maneuvers have included hypoxia, carbon monoxide treatment, no-reflow ischemia, and frank infarction (1, 10, 30–32, 43, 46). These different injurious stimuli likely explain the varied spatial and temporal patterns of renal HIF-1α expression reported in the literature. In this paper, we utilize a commonly used model of ischemic AKI, a 30-min bilateral renal artery clamp followed by reperfusion. Our findings focus on alterations of HIF-1α and pVHL expression after 24 h of reperfusion because this is a time when p53 levels in the kidney peak (16) and it is the time point most commonly investigated for tissue histology, inflammation, apoptosis, and function in animal models of AKI.

An unexpected finding was the strong expression of HIF-1α in collecting tubules and TAL under physiological conditions. While this has been reported by some investigators (23, 37), other reports have failed to detect HIF-1α in kidneys under normal physiological conditions. This lack of detection might be related to differences in the methodology of tissue preparation or the antibodies utilized for immunostaining. In support of our results, Zou and colleagues (50) were able to detect HIF-1α in medullary collecting tubules of sham kidneys using both Northern and Western blot analysis. Our own immunoblot analysis with a different antibody also confirms the microscopy findings. Furthermore, we show HIF-1α expression in these tubules to have a strong cytoplasmic component in addition to the nuclear one. A cytoplasmic location of HIF-1α has also been reported by others in both normal and neoplastic tissues and might represent stabilization and accumulation of the protein before its nuclear translocation (22, 37). The presence of HIF-1α under physiological conditions cannot be ascribed solely to the diminished levels of tissue oxygenation in the medulla of the kidney because we also detected a strong HIF-1α signal in the collecting tubules of the cortex under physiological conditions.

Another finding of interest under physiological conditions was the restricted localization of pVHL to the proximal tubule.
VHL protein plays an important role in the canonical pathway for regulating HIF-1α (14). The lack of pVHL expression in the collecting tubules and other portions of the distal tubule suggests that regulation of HIF-1α in these tubular segments does not occur via this canonical pathway. Non-pVHL regulatory pathways of HIF-1α have been described and may be operative in these portions of the tubule although further investigation will be needed to verify the activation of these noncanonical pathways in this experimental system (9, 19).

Twenty-four hours after ischemic injury, the robust HIF-1α staining present in the collecting tubules and TAL under physiological conditions was significantly diminished. Conversely, there appeared to be a small increase in the minimal HIF-1α expression of the proximal tubule although this difference was not statistically significant compared with sham-operated rats. This finding in the proximal tubule was predominantly in the S3 segment, which is a region keenly susceptible to ischemic injury. Previous studies have provided mixed findings in regards to HIF-1α expression following ischemic injury. Some investigators have demonstrated an increase in HIF-1α expression of the proximal tubule during ischemia with a rapid disappearance during the reperfusion phase (1, 32, 46), while Villanueva et al. (43) demonstrated a sustained increase in HIF-1α during the first 24 h of reperfusion. Given that many areas of the kidney demonstrate a significant reduction in perfusion and altered vascular function at 24 h after ischemic injury, continued expression of HIF-1 into the reperfusion phase might be the predicted response in this model of ischemic AKI (38). Therefore, our finding that HIF-1α expression did not increase and in fact decreased in the cells of the collecting tubule and TAL was unanticipated to some extent. However, the dramatic preservation of HIF-1α expression following ischemia in animals treated with pifithrin-α suggests that p53 upregulation plays an important role in suppressing the HIF-1 response following ischemic injury.

While it is possible that the protective effect of p53 inhibition on the HIF-1α response may in part be an indirect effect
due to the overall tissue-protective effect of p53 inhibition that has been previously demonstrated (16, 17), three lines of argument support a direct effect of p53 on HIF-1 at 24 h postischemia. First, the tissue-protective effect of p53 inhibition should diminish the stimuli for increased HIF-1 expression. However, we observe an increase in HIF-1 above baseline in proximal tubular cells of the S3 segment following ischemic injury in animals treated with pifithrin-α. This increase above baseline in the setting of tissue protection implies a direct stimulatory effect of p53 inhibition on HIF-1. Second, we observe that p53 inhibition results in the upregulation of HIF-1 in the proximal tubule of sham-operated animals. This finding implies that p53 might play a direct role in the modulation of HIF-1 under physiological as well as pathological conditions. Furthermore, this finding suggests that p53 inhibition may have relevance in preconditioning the kidney before ischemic insults via upregulation of HIF-1 (1, 10), although this was not investigated further in this study. Third, we observe a protective effect of p53 inhibition in the collecting tubules and TAL following ischemic injury. The very low level of pVHL in these tubules suggests that p53 may play a direct role in the regulation of HIF-1 by noncanonical regulatory mechanisms described above.

The mechanism by which p53 inhibits HIF-1 cannot be determined with certainty from our experiments. Pifithrin-α inhibits the transcriptional activity of p53, and this might reduce the competition between HIF-1α and p53 for their shared coactivator, p300 (2, 11, 35). However, p53 protein accumulates in the cytoplasm after ischemia-reperfusion injury (16) and can therefore directly interact with HIF-1α or participate in its degradation (28, 33). Since p53 inhibition results in higher levels of HIF-1α protein, it is possible that this mechanism is operational in our system as well. Finally, the specificity of the inhibitory effect of pifithrin-α on p53 can be of concern. Indeed, it was suggested that pifithrin-α can also suppress signaling through the heat shock transcription factor HSF1 and the glucocorticoid receptor, both of which share regulation with p53 by heat shock protein 90. However, subsequent reports reconfirmed the high specificity of pifithrin-α for p53 inhibition, particularly downstream of heat shock protein 90 (27).

Inhibition of p53 also caused dramatic changes in pVHL expression. Under sham conditions, pVHL localized primarily to proximal tubules close to or within the outer stripe of the outer medulla. The immunostaining pattern of pVHL had a
fine, granular cytoplasmic distribution. After ischemic injury, pVHL levels increased and the staining pattern assumed a dense globular morphology within the cytoplasm. Although the significance of these changes is not clear at this point, it is possible that these changes indicate modified localization, which contribute to increased HIF-1α degradation (15, 49) and thus prevent increased levels of HIF-1α in the proximal tubule following ischemic injury. It is also possible that these changes are secondary to disrupted cytoskeletal proteins known to be associated with pVHL (41). Inhibition by p53 significantly reduced the ischemia-induced alterations of pVHL morphology. Under physiological conditions, p53 inhibition also increased pVHL expression; however, the morphology of pVHL remained finely granular throughout the cytoplasm. It is possible that this increase in pVHL is in response to upregulation of HIF-1α by p53 under sham conditions as pVHL is known to be upregulated by HIF-1α as part of a negative feedback loop to normalize HIF-1α levels (15). Alternatively, this could represent direct regulation of pVHL by p53. While previous studies have demonstrated that pVHL can stabilize p53 (29), our findings suggest a potential reciprocal regulatory effect.

Although pVHL, a tumor suppressor, is best known for its role in oxygen-dependent degradation of HIF-1α, recent data also support functions for pVHL that are independent of this role. These include the modulation of pathways involved in the formation and deposition of extracellular matrix (20, 40). These functions ascribed to pVHL have importance in regard to the metastatic potential or invasiveness of tumors. These include the modulation of pathways involved in the formation and deposition of extracellular matrix (20, 40). Under physiological conditions, p53 inhibition also increased pVHL expression; however, the morphology of pVHL remained finely granular throughout the cytoplasm. It is possible that this increase in pVHL is in response to upregulation of HIF-1α by p53 under sham conditions as pVHL is known to be upregulated by HIF-1α as part of a negative feedback loop to normalize HIF-1α levels (15). Alternatively, this could represent direct regulation of pVHL by p53. While previous studies have demonstrated that pVHL can stabilize p53 (29), our findings suggest a potential reciprocal regulatory effect.

In conclusion, we report for the first time an important role for p53 in modulating the pVHL-HIF-1α system in the kidney. We show that p53 negatively regulates HIF-1α, a process operational both under sham conditions and after ischemic injury. Thus the protective role of p53 inhibition clearly extends beyond its effect on apoptosis. The interactions between p53 and the pVHL-HIF-1α pathway will likely offer novel avenues to understanding and treating ischemic or other forms of renal injury.

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