The p53 inhibitor pifithrin-α can stimulate fibrosis in a rat model of ischemic acute kidney injury

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Dagher PC, Mai EM, Hato T, Lee SY, Anderson MD, Karozos SC, Mang HE, Knipe NL, Plotkin Z, Sutton TA. The p53 inhibitor pifithrin-α can stimulate fibrosis in a rat model of ischemic acute kidney injury. Am J Physiol Renal Physiol 302: F284–F291, 2012. First published November 2, 2011; doi:10.1152/ajprenal.00317.2011.—Inhibition of the tumor suppressor p53 diminishes tubular cell apoptosis and protects renal function in animal models of acute kidney injury (AKI). Therefore, targeting p53 has become an attractive therapeutic strategy in the approach to AKI. Although the acute protective effects of p53 inhibition in AKI have been examined, there is still relatively little known regarding the impact of acute p53 inhibition on the chronic sequelae of AKI. Consequently, we utilized the p53 inhibitor pifithrin-α to examine the long-term effects of p53 inhibition in a rodent model of ischemic AKI. Male Sprague-Dawley rats were subjected to bilateral renal artery clamping for 30 min followed by reperfusion for up to 8 wk. Pifithrin-α or vehicle control was administered at the time of surgery and then daily for 2 days [brief acute administration (BA)] or 7 days [prolonged acute administration (PA)]. Despite the acute protective effect of pifithrin-α in models of ischemic AKI, we found no protection in the microvascular rarefaction at 4 wk or development fibrosis at 8 wk with pifithrin-α administered on a PA schedule compared with vehicle control-treated animals. Furthermore, pifithrin-α administered on a PA schedule actually produced worse fibrosis compared with vehicle control animals after ischemic injury [21%/area (SD4.4) vs.16%/area (SD3.6)] as well as under sham conditions [2.6%/area (SD1.8) vs. 4.7%/area (SD1.3)]. The development of fibrosis with PA administration was independent of microvascular rarefaction. We identified enhanced extracellular matrix progression, epithelial-to-mesenchymal transition, and amplified inflammatory responses as potential contributors to the augmented fibrosis observed with PA administration of pifithrin-α.

Inflammatory cytokines, and a myriad of infiltrating cells all participate to shape the disease phenotype and its severity (38). Historically, each of these pathways has been investigated in isolation with therapeutic approaches resulting in variable success rates in the laboratory animal. More importantly, therapeutic interventions have predominantly focused on acute outcomes such as serum urea and creatinine measured over a period of few days following the insult. For many of these therapeutic interventions, whether the acute protective effects translated into beneficial long-term outcomes remains to be determined.

The tumor suppressor p53 is now recognized to be an important player in various forms of AKI. We and others showed that p53 inhibition confers functional protection in models of renal ischemia-reperfusion injury (IRI) and cisplatin nephrotoxicity (21, 24, 25, 49). While reduction in tubular cell apoptosis appears to underlie the protective effects of p53 inhibition, it is now recognized that p53 is a multifaceted protein and can modulate various mechanisms involved in AKI. These include the death receptor pathways, hypoxia-inducible factors (HIF), autophagy, reactive oxygen species, the cell cycle, and metabolism (44, 46). The involvement of p53 in these various and often opposing pathways can result in a dissociation of acute protection and long-term effects. Furthermore, the duration of p53 modulation may be another important variable. For example, we previously demonstrated that inhibition of p53 at the time of injury in a rodent model of renal IRI upregulates HIF-1α (42). While this mechanism may contribute to the protection of renal function acutely, it could have detrimental long-term consequences through the promotion of HIF-1 fibrogenic pathways (15). In contrast, recent work has demonstrated that acute inhibition of p53 3 days after injury may relieve epithelial cell cycle arrest at G2/M and prevent long-term fibrosis (50). Consequently, the appropriate timing and duration of p53 inhibition are an important problem to address viewing the progress of p53 inhibitors into phase 2 clinical trials of AKI.

In this paper, we examine the long-term effects of the p53 inhibitor pifithrin-α on fibrosis and microvascular rarefaction in a model of renal IRI in the rat. In particular, we provide evidence that some but not all pifithrin-α regimens can result in renal fibrosis. We also show potential roles for fibronectin deposition, epithelial-to-mesenchymal transition (EMT), and macrophage survival in mediating the long-term profibrotic effects of pifithrin-α administration. Finally, our results do not support a role for microvascular rarefaction as a prerequisite for fibrosis in the setting of pifithrin-α administration. These data are the first to underscore potential detrimental long-term...
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 microscrews. After removal of the microscrews, reperfusion was monitored visually before closure of the abdominal surgical wound. Two milliliters of prewarmed (37°C) sterile saline containing either the p53 inhibitor 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 the surgical incision was closed. 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. Since the p53 level was not significantly different between ischemic AKI (24, 34) and p53 activity may persist for the duration of pifithrin-α treatment, we did not perform sham surgery for this treatment group. In addition, the acute inhibition of p53 by a variety of strategies is protective in animal models of ischemic AKI (24, 34). However, the long-term consequences of acute p53 inhibition following renal IRI are not fully appreciated. As mentioned previously, given the increase in p53 expression and activity following ischemic kidney injury we utilized two dosing regimens of pifithrin-α to examine the long-term consequences of acute p53 inhibition following renal IRI.

RESULTS

Duration of pifithrin-α administration alters the fibrotic response in the kidney. The acute inhibition of p53 by a variety of strategies is protective in animal models of ischemic AKI (24, 34). However, the long-term consequences of acute p53 inhibition following renal IRI are not fully appreciated. As mentioned previously, given the increase in p53 expression and activity following ischemic kidney injury we utilized two dosing regimens of pifithrin-α to examine the long-term consequences of acute p53 inhibition following renal IRI.

Tissue staining and microscopy. At the time of death, kidneys were rapidly perfused fixed with either 100% methanol or 4% paraformaldehyde. Tissues were subsequently processed for immunofluorescence staining or standard histochemistry. Fifty-micrometer vibratome sections of methanol-fixed kidney tissue were obtained for immunofluorescent staining. Primary antibodies to cablin (rabbit anti-human polyclonal IgG), fibronectin (rabbit anti-rat polyclonal IgG; MD Biosciences, St. Paul, MN), S100A4 (rabbit anti-human polyclonal IgG; Daco, Carpinteria, CA), and CD68 (goat anti-human polyclonal IgG; Santa Cruz Biotechnology, Santa Cruz, CA) were utilized for immunostaining. Appropriate secondary antibodies conjugated with Texas Red or Cy5 were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). 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. Leukocyte esterase staining of paraffin-embedded kidney tissue was performed using a naphthol AS-D chloroacetate (specific esterase) kit (Sigma, St. Louis, MO) as per the manufacturer’s instructions. Trichrome staining of kidney tissues was performed by the Indiana University Histopathology Lab utilizing standard histochemistry procedures.

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. Trichrome-stained images of kidney tissue sections were obtained at ×40 magnification with a Nikon Diaphot compound microscope (Melville, NY). Five to seven images were collected of a kidney from each animal. Percent of total area staining positive for fibrosis (trichrome), cavin, or fibronectin was determined with Metamorph software (Universal Imaging, West Chester, PA). Cells staining positive for S100A4, CD68, or leukocyte esterase in each field were manually counted.

Macrophase viability and activation assays. RAW 264.7 macrophages (ATCC, Manassas, VA) were cultured and maintained as per ATCC recommendations. Approximately 10 × 10^3 cells/well were plated into a 96-well plate and incubated in media containing 10 μM pifithrin-α or vehicle control. After a 24-h incubation period, the wells were washed three times with PBS. Cell viability in each well was determined with a CellTiter-Glo luminescent cell viability assay (Promega Biosciences, Madison, WI) as per the manufacturer’s instructions.

Statistical analysis. Results are expressed as means ± SD. Data were analyzed by ANOVA and then planned comparisons by a pairwise t-test with a Holm correction. A P = 0.05 was utilized to determine significance.
hypoxia (4) that is hypothesized to usher in the development and progression of fibrosis and CKD (11, 12, 23, 35). Consequently, we examined the effect of prolonged acute pifithrin-α administration after injury on microvascular density to determine whether the profibrotic response of this dosing regimen was related to microvascular rarefaction. Microvascular structures identified by cablin immunostaining (7) were quantified 4 wk following injury and before the development of fibrosis observed at 8 wk. Consistent with earlier reports by us (18, 29) and others (3), we observed a significant 30–40% loss of microvascular density 4 wk after ischemic injury in animals that occurred primarily in the outer medullary region of the kidney (Fig. 2). However, microvascular density was not significantly different in animals treated with pifithrin-α after ischemic injury compared with vehicle-treated animals (Fig. 2).

Prolonged acute pifithrin-α administration increases fibronectin deposition in the kidney. We previously demonstrated that inhibition of p53 with pifithrin-α upregulates von Hippel-Lindau tumor suppressor protein (pVHL) (42). Upregulation of pVHL can stimulate fibronectin deposition (5, 6, 28, 43), which in turn serves as a nidus for extracellular matrix (ECM) formation and fibrosis in the kidney (9, 36, 45, 53, 54). Accordingly, we examined the effect of administration of pifithrin-α for 7 days on the extent of fibronectin deposition in the kidney 4 wk after ischemic injury compared with vehicle-treated animals (Fig. 2).

Table 1. Eight-week functional data (n = 3)

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<th>Creatinine, mg/dl (SD)</th>
<th>Albuminuria, Ualb/Ucr (SD), mg/mg</th>
<th>Urinary Sodium, UNa/Ucr(SD), mmol/mg</th>
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<tbody>
<tr>
<td>Sham vehicle</td>
<td>0.34 (0.02)</td>
<td>2.1 (1.1)</td>
<td>0.05 (0.02)</td>
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<tr>
<td>Sham-pifithrin α</td>
<td>0.34 (0.05)</td>
<td>2.6 (1.1)</td>
<td>0.06 (0.03)</td>
</tr>
<tr>
<td>Ischemia vehicle</td>
<td>0.39 (0.03)</td>
<td>2.9 (0.5)</td>
<td>0.05 (0.02)</td>
</tr>
<tr>
<td>Ischemia-pifithrin α</td>
<td>0.37 (0.04)</td>
<td>5.0 (1.4)*</td>
<td>0.10 (0.05)</td>
</tr>
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*P < 0.05.
fibrosis following tubular injury (22, 31, 41). To further investigate the potential pathways contributing to increased fibrosis following the administration of pifithrin-α for 7 days after injury, we immunostained kidneys 4 wk after injury with an antibody to FSP1/S100A4 as a marker of EMT. We observed a significant increase in the number of cells staining positive for FSP1/S100A4 following ischemic injury in animals receiving pifithrin-α for 7 days compared with animals receiving vehicle after injury (Fig. 4). In addition, the majority of the cells staining positive for FSP1/S100A4 localized to tubules (Fig. 4D). While on average there were more cells staining positive for FSP1/S100A4 in kidney sections from sham-operated animals treated with pifithrin-α compared with vehicle control-treated animals, the difference did not achieve significance.

Administration of pifithrin-α increases tissue neutrophils and macrophages following renal injury. Since leukocytes can contribute to injury and fibrosis following renal IRI, we examined the effect of prolonged acute pifithrin-α administration on leukocyte accumulation in the kidney following injury. Neutrophils were identified in tissue sections by esterase staining and macrophages were identified in tissue sections by immunostaining for CD68 (17, 26). The number of tissue neutrophils was significantly increased 2 days after renal IRI in animals treated with pifithrin compared with vehicle-treated animals (Fig. 5A); however, this difference was no longer present at 7 days following injury. In contrast, the number of tissue macrophages was not different 2 days after injury but was significantly increased 7 days after ischemic injury in animals treated with pifithrin compared with vehicle control (Fig. 5B). To further examine the consequence of pifithrin-α administration on macrophages, we examined the in vitro viability of RAW 264.7 macrophages following treatment with pifithrin-α. Consistent with our observations in vivo, macrophages treated with...
pifithrin-α had a two-fold increase in cell viability compared with macrophages treated with vehicle alone (Fig. 5C).

**DISCUSSION**

AKI is a commonly encountered clinical syndrome that is not only associated with considerable morbidity and mortality (19, 47) but an increased risk for development of CKD and progression to end-stage renal disease (1, 20, 32, 48). Several studies have validated the role that p53 plays in the pathophysiology of ischemic and nephrotoxic AKI (21, 24, 34, 49, 52). Indeed, a variety of genetic and pharmacologic (including pifithrin-α and siRNA) strategies to inhibit p53 has been shown to be protective in models of ischemic and nephrotoxic injury to the kidney (21, 24, 34, 49, 52), which underscores the specificity of p53 inhibition. Consequently, targeting p53 as a much needed therapeutic intervention in AKI is an attractive proposition. However, there is little known in regards to the optimal therapeutic window or the long-term consequences of acute p53 inhibition. It was the goal of this study to examine the long-term renal sequelae of acute p53 inhibition and to begin to delineate the optimal therapeutic window in a model.

Fig. 4. FSP1/S100A4 immunostaining. Rats underwent bilateral RAC for 30 min or sham operation and were administered vehicle control (A, C) or pifithrin-α (B, D) on the day of surgery and then daily for 7 consecutive days. Representative images of FSP1/S100A4 immunostaining (yellow; blue = DAPI) in the outer medullary region of the kidney from kidneys harvested 4 wk after sham operation (A, B) or RAC (C, D). E: average number of cells/field staining positive for FSP1/S100A4. *P < 0.05 vs. sham, n = 3 and 4–6 images per animal.

Fig. 5. Leukocyte infiltration and viability. A, B: rats underwent bilateral RAC for 30 min or sham operation and were administered vehicle control (□, ○) or pifithrin-α (●, ●) on the day of surgery and then daily until death. Rats were killed 2 and 7 days after ischemia and total polymorphonuclear cell (leukocyte esterase, A) and macrophage (CD68, B) counts were performed on kidney cross-sections. *P < 0.05 vs. sham, n = 3. C: equal numbers of RAW 264.7 macrophages were plated and then incubated in the presence of 10 μM pifithrin-α or vehicle control. Cell viability was measured after incubation for 24 h with a Cell Titer-Glo cell viability assay. *P < 0.05 vs. sham, n = 3.
of ischemic AKI. Our finding that the acute protective effect of the p53 inhibitor pifithrin-α in the setting of ischemic AKI does not translate into protection from microvascular rarefaction and fibrosis has therapeutic implications. Moreover, our finding that prolonged acute pifithrin-α administration increases kidney fibrosis following ischemia and in sham animals may be of significant clinical importance for the development of p53 inhibition as a therapeutic strategy.

The fact that brief acute pifithrin-α administration did not translate into preserved microvascular stability and less fibrosis is initially unexpected. If the a priori assumption is that the severity of initial kidney injury is the primary driving force in the ultimate development of microvascular rarefaction and fibrosis, then it would be expected that therapeutic strategies that limit the severity of the initial injury would translate into limiting these chronic sequelae. However, previous work by us demonstrated that acute inhibition of p53 in ischemic AKI does have divergent immediate effects that may impact the chronic structural sequelae (24). As we demonstrated, renal function and cellular apoptosis are protected by acute inhibition of p53 but indicators of necrotic tissue injury are not protected. This dichotomy may partially explain the lack of safeguarding from microvascular rarefaction and fibrosis by brief acute pifithrin-α administration in our current study.

On the other hand, the increased fibrosis we observed with the prolonged acute pifithrin-α administration and a recent study by Yang and co-workers (50) would suggest inhibition of p53 has duration-dependent effects that directly alter the fibrogenic process. In the study by Yang and co-workers, they provide evidence that administering pifithrin-α in a single dose 3 and 14 days after unilateral IRI in mice relieved epithelial cell cycle arrest which decreased the production of profibrotic factors and limited the formation of fibrosis. In our study, administering pifithrin-α daily to starting at the time of bilateral IRI in rats and continuing for 7 days ultimately increased renal fibrosis. Although these findings may highlight a fundamental difference between mice and rats, they are very suggestive of the timing of p53 inhibition is important to consider in the approach to AKI. Even more surprising was our finding that daily administration of pifithrin-α for 7 days increased fibrosis in sham-operated animals. However, there is growing appreciation that basal levels of p53 have important housekeeping functions in the unstressed cell (46) that evolutionarily predate its cancer surveillance role. Indeed, basal levels of p53 protein and mRNA have been detected in the kidney by immunostaining (42), immunoblotting (49), and PCR (8). Taken together, our findings suggest that prolonged acute inhibition of p53 intrinsically promotes the fibrogenic process in the kidney.

The mechanism by which prolonged acute administration of pifithrin-α promotes fibrosis in the kidney is not fully elucidated; however, our study provides some insight. First, our study suggests that the mechanism may be independent of tissue perfusion since we did not observe differences in microvascular rarefaction between animals treated with pifithrin-α or vehicle control in either the injury group or the sham-operated group. Thus worsening fibrosis was dissociated from loss of microvascular support in animals treated with pifithrin-α. This at first seems contrary to the prevailing notion that diminished microvascular reserve following renal IRI leads to persistent renal hypoxia (4), which in turn stimulates HIF-1-dependent pathways (14, 15) of organ fibrosis. However, we previously demonstrated that administration of pifithrin-α during renal IRI stimulates tubular HIF-1α expression (42), thus providing a mechanism for stimulating HIF-1 that would be independent of tissue perfusion. The augmented fibronectin deposition and tubular epithelial cell FSP1/S100A4 immunostaining following injury that we observed in this study in animals treated with pifithrin are consistent with subsequent HIF-1-mediated increases in ECM deposition and EMT that can contribute to renal fibrosis as previously described (14, 15). In addition, we previously demonstrated that inhibition of p53 significantly reduces alterations in cellular distribution of pVHL following ischemia and increases pVHL expression under physiologic conditions (42). Interestingly, prior studies have revealed that pVHL can directly modulate fibronectin deposition and ECM assembly in a fashion that is independent of HIF-1 (37, 40). Consequently, this interaction between p53 inhibition and pVHL warrants further investigation as a prospective mechanism that can impact fibrosis following injury and under physiologic conditions.

Our observation that animals treated with pifithrin have a significantly increased tissue neutrophil count 2 days after injury is another result that initially seems contrary to the acute protective effect of p53 inhibition. However, this contrast appears to again underscore the importance of tubular cell apoptosis during the acute phase of injury in this model of AKI. Our finding that treatment with pifithrin increases macrophage infiltration following ischemia and macrophage viability in vivo provides another potential mechanism for fibrosis and the differential effect of this dosing regimen on chronic sequelae. Macrophages can contribute to the fibrogenic process in the kidney and other organs following injury by a variety of possible mechanisms (10). Inhibition of p53 in macrophages has been demonstrated to sustain the survival and proinflammatory function of macrophages (30, 33) and our findings are consistent with this observation. Given the current interest in how different macrophage subtypes contribute to injury and repair processes, the impact of p53 inhibition on macrophage subtype populations following injury could be a fruitful area of future investigation and further expand the many facets of p53 functions in AKI. Our finding also suggests that developing pharmacological strategies to selectively inhibit p53 in tubules as opposed to leukocytes may be an important consideration in the approach to patient studies in AKI.

In this study, we used pifithrin-α as an inhibitor of p53. Previous work demonstrated that pifithrin-α interferes with the transcriptional function of p53 as well as the transcription-independent mitochondrial translocation of p53 in the kidney (24). Although the acute protective effects of p53 inhibition in models of AKI by pifithrin-α have been confirmed by siRNA strategies to inhibit p53, possible p53-independent effects of pifithrin-α cannot be excluded as contributing to the findings we report. Indeed, while there is general consensus that pifithrin-α does inhibit p53 and subsequent p53-regulated gene expression, it is also known that pifithrin-α can have p53-independent effects. These p53-independent effects include activation of the aryl-hydrocarbon receptor, suppression of heat shock and glucocorticoid inhibition signaling pathways, and modulation of cell cycle pathways (16, 27, 39). Subsequent studies using additional strategies to inhibit p53, such as
siRNA, will be helpful in determining the specificity of p53 inhibition in the findings that we report.

In conclusion, we demonstrated that administration of the p53 inhibitor pifithrin-α can promote kidney fibrosis both after ischemic injury and under physiologic conditions and that this outcome is dependent on the timing and duration of pifithrin-α administration. Potential mechanisms identified for this pro-fibrotic effect of pifithrin-α administration include augmented fibronectin deposition, enhanced EMT, and increased macrophage viability. In light of the emergence of p53 inhibition as an important therapeutic approach to AKI, these findings provide potentially important insight into the appropriate timing and duration of dosing p53 inhibitors in the setting of AKI.

REFERENCES


