Loss of poly(ADP-ribose) polymerase 1 attenuates renal fibrosis and inflammation during unilateral ureteral obstruction

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Kim J. Padanilam BJ. Loss of poly(ADP-ribose) polymerase 1 attenuates renal fibrosis and inflammation during unilateral ureteral obstruction. Am J Physiol Renal Physiol 301: F450–F459, 2011. First published May 25, 2011; doi:10.1152/ajprenal.00059.2011.—Poly(ADP-ribose) polymerase 1 (PARP1) contributes to necrotic cell death and inflammation in several disease models; however, the role of PARP1 in fibrogenesis remains to be defined. Here, we tested whether PARP1 was involved in the pathogenesis of renal fibrosis using the unilateral ureteral obstruction (UUO) mouse model. UUO was performed by ligation of the left ureter near the renal pelvis in Parp1-knockout (KO) and wild-type (WT) male mice. After 10 days of UUO, renal PARP1 expression and activation were strongly increased by 6- and 13-fold, respectively. Interstitial fibrosis induced by UUO was significantly attenuated in Parp1-KO kidneys compared with that in WT kidneys at 10 days, but not at 3 days, based on collagen deposition, α-smooth muscle actin (α-SMA), and fibronectin expression. Intriguingly, the UUO kidneys in Parp1-KO mice showed a dramatic decrease in infiltration of neutrophil and reduction in expression of proinflammatory proteins including intercellular adhesion molecule-1, tumor necrosis factor-α, inducible nitric oxide synthase, and toll-like receptor 4 as well as phosphorylation of nuclear factor-κB p65, but not transforming growth factor-β1 (TGF-β1) at both 3 and 10 days. Pharmacological inhibition of PARP1 in rat renal interstitial fibroblast (NRK-49F) cell line or genetic ablation in primary mouse embryonic fibroblast cells did not affect TGF-β1-induced de novo α-SMA expression. Parp1 deficiency significantly attenuated UUO-induced histological damage in the kidney tubular cells, but not apoptosis. These data suggest that PARP1 induces necrotic cell death and contributes to inflammatory signaling pathways that trigger fibrogenesis in obstructive nephropathy.

PARP1 inhibitor; mouse embryonic fibroblast; PARP1 knockout

A gap in our knowledge of key factors that facilitate fibrogenesis.

Poly(ADP-ribose) polymerase 1 (PARP1) is a nuclear protein that can regulate gene expression as a transcriptional coactivator and protein functions via poly(ADP-ribose)ylation. PARP1 has nucleosome binding properties and a transcription-related regulation of chromatin structure (26, 54). Poly(ADP-ribose)ylation can have different effects on various proteins including activation, downregulation, changes in protein conformation and promotion of protein-protein interactions (55), and is essential to embryonic development and chromosome stability (35). Additionally, the role of PARP1 as transcriptional regulator is confirmed by pharmacological inhibition and PARP1 inactivation studies demonstrating its influence on the expression of inflammatory cytokines including inducible nitric oxide synthase (iNOS), intercellular adhesion molecule-1 (ICAM-1), P-selectin, and E-selectin (49, 58, 59). The activation of PARP1 is important for DNA repair but its excessive activation leads to depletion of intracellular NAD+, resulting in subsequent necrotic cell death. In previous studies, we and others demonstrated pharmacological or genetic inhibition of PARP1 protected several tissues against ischemia, stroke, or diabetes (1, 32, 43, 57). Considering the complexity of renal interstitial fibrosis that includes aberrant expression of various cytokines and interstitial matrix molecules, infiltration of inflammatory cells, and tubular and vascular cell loss, we hypothesized that the interstitial fibrosis process may require regulation at the transcriptional and/or posttranslational level by a master regulatory gene such as PARP1. To gain further insight into the role of PARP1 in fibrosis, we performed UUO in Parp1-deficient mice and investigated whether PARP1 contributes to inflammation, matrix deposition, and cellular injury following UUO.

MATERIALS AND METHODS

Animal preparation. Mice were cared before and during the experimental procedures in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC), University of Nebraska Medical Center (UNMC), and the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals. All protocols had received prior approval from the UNMC-IACUC Parp1-knockout (KO) and wild-type (WT) male mice (8 to 10 wk old; Jackson Laboratories, Bar Harbor, ME) were anesthetized by intraperitoneal injection of a cocktail containing ketamine (200 mg/kg body wt) and xylazine (16 mg/kg body wt). After that, a left ureter was obstructed completely near the renal pelvis using a 6–0 silk tie (20, 42). Sham-operated mice underwent the same surgical procedure except for the ureter ligation. After 3 or 10 days of UUO or sham, kidneys were either fixed in 4% paraformaldehyde for histological studies or snap-frozen in liquid nitrogen for biochemical studies.

Hematoxylin
and eosin-stained sections were used for histological damage score as described previously (17, 22, 23).

Collagen deposition. Collagen deposition was assessed by both Sirius red staining (51) and hydroxyproline assay (45). To assess Sirius red staining, the paraffin section was stained with Sirius red solution (0.1% Direct Red 80 and 1.3% picric acid; Sigma, St. Louis, MO) and washed in acidified water (0.5% acetic acid; Sigma). The area of positive Sirius red staining was measured in at least five high-power fields (×200 magnification) per kidney using NIH Image software (Image J). For the hydroxyproline assay, the kidney was homogenized in 10 N HCl and hydrolyzed by autoclaving. The hydrolysate was incubated in a Chloramine T reagent [0.84% chloramines-T, 42 mM sodium acetate, 2.6 mM citric acid, and 39.5% isopropanol (pH 6.0)]. Next, the sample was incubated in a DMAB reagent [15% 4-(dimethylamino)benzaldehyde in isopropanol/piperidine (pH 6.0)]. After being boiled, the sections were stained with Sirius red staining (51) and hydroxyproline assay (45). To assess TUNEL assay, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was carried out using In Situ Cell Death Detection Kit, Fluorescein (Roche, Mannheim, Germany) following the manufacturer's protocol, as previously described (18, 20).

Cell culture and treatment. Normal rat kidney interstitial fibroblast (NRK-49F) cells (American Type Culture Collection, Manassas, VA) were maintained in DMEM/F-12 medium supplemented with 10% FBS (Invitrogen, Carlsbad, CA) at 37°C with 5% CO₂. Cells were initially grown until 70% confluence on culture plates and then changed to serum-free medium. After serum starvation for 24 h, recombinant human TGF-β1 (R&D Systems, Minneapolis, MN) was added to the culture at a final concentration of 1 ng/ml (15). Benzamide (BNZ) or 3-aminobenzamide (3AB), as PARP1 inhibitors, was also added at the same time at a final concentration of 10⁻⁴, 10⁻³, or 10⁻² M. After incubation for 48 h, the cells were harvested for Western blot analysis. For primary mouse embryonic fibroblast (MEF) isolation, uteri isolated from 13.5-day-pregnant Parp1-KO or WT mice were washed with PBS. After the head and visceral tissues were removed from the isolated embryos, the remaining bodies were broken by pipetting in 0.1 mM trypsin/1 mM EDTA solution (1 ml/embryo) and then incubated at 37°C for 20 min. After trypsinization, DMEM-high-

Immunofluorescent staining. Immunofluorescent staining was performed as we previously described (18, 21, 23). Briefly, to reveal antigen epitope, the sections were boiled in 10 mM sodium citrate buffer (pH 6.0). After being blocked, the section was incubated with polyclonal anti-α-SMA (Sigma) or polymorphonuclear neutrophil (PMN; Accurate, Westbury, NY) antibody and then incubated with FITC-conjugated horse anti-mouse or goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA).

Fig. 1. Poly(ADP-ribose) polymerase 1 (PARP1) expression and activation in mouse kidneys during unilateral ureteral obstruction (UUO). Kidneys in Parp1-knockout (KO) and wild-type (WT) male mice were harvested at 0, 3, or 10 days after UUO. A: PARP1 expression and activation were examined by Western blot analysis using anti-PARP1 and poly(ADP-ribose) (PAR) antibody, respectively. Anti-β-actin antibody was used as a loading control. B and C: protein bands were quantified using Lab Works analysis software. Data are expressed as means ± SE (n = 4 to 6). *P < 0.01, **P < 0.001 vs. respective day 0. #P < 0.05, ##P < 0.001 vs. respective day 3. D: PARP1 expression and activation in Parp1-KO and WT mice at 10 days after UUO or sham.
glucose medium containing 10% FBS (9 ml/embryo) was added, cultured on a 100-mm dish at 37°C with 5% CO₂, and used within three passages (52). When the cells were grown until 70% confluence on culture plates, they were starved in serum-free medium for 24 h. After that, recombinant human TGF-β1 was added to the culture at a final concentration of 1 ng/ml for 48 h, and the cells were harvested for Western blot analysis.

Statistics. Results were expressed as means ± SE. Statistical differences among groups were calculated using ANOVA followed by a least significant difference post hoc comparison using the SPSS 12.0

Fig. 2. Reduction of collagen deposition induced by UUO in Parp1-KO mouse kidneys. Kidneys in Parp1-KO and WT male mice underwent UUO or sham operation for 3 or 10 days. Collagen deposition was evaluated by Sirius red staining (A and B) and hydroxyproline measurement (C). Scale bars = 50 μm. B: area of positive Sirius red staining was measured in at least 5 high-power fields (×200 magnification) per kidney using NIH Image software (Image J). Data are expressed as means ± SE (n = 4 to 6). *P < 0.01, **P < 0.001 vs. respective sham.

Fig. 3. Reduction of fibronectin and α-smooth muscle actin (α-SMA) expression by UUO in Parp1-KO mouse kidneys. Kidneys in Parp1-KO and WT male mice underwent UUO or sham operation for 3 or 10 days. A: fibronectin and α-SMA expression were examined by Western blot analysis. Anti-β-actin antibody was used as a loading control. B and C: protein bands were quantified using Lab Works analysis software. Data are expressed as means ± SE (n = 4 to 6). *P < 0.05, **P < 0.01, ***P < 0.001 vs. respective sham.
program. Differences between groups were considered statistically significant at a $P$ value of <0.05.

RESULTS

UUO induces PARP1 expression and activation. To study the role of PARP1 in the fibrotic kidney, we first assessed the expression and activation of PARP1 by Western blot analysis in WT kidneys. After 3 and 10 days of UUO, the expression of both full-length and cleaved form of PARP1 was gradually increased (Fig. 1, A and B). Because catalytic activation of PARP1 adds PAR polymers to itself, the assessment of PARP1 activation was accomplished using Western blot analysis of the total poly(ADP-ribosyl)ated PARP1 level. The PARP1 activation was also markedly increased in 3 and 10 days of obstructed kidneys (Fig. 1C), consistent with the PARP1 expression. In Parp1-KO kidneys, PARP1 expression and activation were not detectable 10 days after sham or UUO (Fig. 1D).

Loss of PARP1 reduces renal fibrosis during UUO. To explore whether PARP1 is involved in renal fibrosis, we measured collagen deposition in Parp1-KO and WT mouse kidneys using Sirius red staining and hydroxyproline content. Ten days after UUO, Parp1-WT mice showed a dramatic induction of Sirius red-positive area and hydroxyproline concentration compared with that in KO mice. However, their levels after 3 days of UUO showed no significant difference between KO and WT mice (Fig. 2, A–C). Similarly, fibronectin expression in Parp1-KO kidneys 10 days after UUO was significantly lower than that in WT kidneys, whereas its expression 3 days after UUO was not different between KO and WT mouse kidneys (Fig. 3, A and B). Because α-SMA-positive myofibroblast is one of the principle cells responsible for the accumulation of extracellular matrix in renal fibrosis, we investigated the magnitude of myofibroblast activation induced by UUO in Parp1-KO and WT kidneys. UUO markedly induced α-SMA expression in WT kidneys. However, the induction of α-SMA expression after 10 days of UUO was significantly reduced in Parp1-KO mice, but not at 3 days after UUO (Fig. 3, A and C). Consistent with the Western blot

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**Fig. 4.** Immunofluorescence study for α-SMA expression in the obstructed kidneys of Parp1-KO and WT mice. Kidneys in Parp1-KO and WT male mice underwent UUO or sham operation for 3 or 10 days. A: α-SMA was detected by immunofluorescent staining using anti-α-SMA antibody. Mouse IgG was used as a negative control (NC). Visible blue color indicates nucleus stained by DAPI. Scale bars = 50 μm. B: area of positive α-SMA staining was measured in at least 5 high-power fields (∗×200 magnification) per kidney using Image J. Data are expressed as means ± SE (n = 4 to 6). ∗$P < 0.01$, ∗∗$P < 0.001$ vs. respective sham.
results, immunofluorescence staining also revealed a suppressive effect of Parp1 deficiency on α-SMA expression after 10 days of UUO, but not at 3 days. As shown in Fig. 4A, the UUO kidneys derived from WT mice displayed the expression of α-SMA in the tubulointerstitium. Loss of Parp1 gene significantly attenuated the α-SMA-positive area 10 days after UUO, but its level was not changed at 3 days after UUO (Fig. 4B). In the sham-operated kidney, collagen deposition, fibronectin, and α-SMA expression were not significantly altered between Parp1-KO and WT mice (Figs. 2 and 3). The data indicate that loss of Parp1 gene attenuates renal fibrosis at the later stages of UUO.

Loss of PARP1 inhibits renal inflammation during UUO. To determine whether PARP1 contributes to proinflammatory response in renal fibrosis, we next examined neutrophil recruitment and proinflammatory gene expression. Three and 10 days after UUO, prominent infiltration of PMN-positive neutrophils occurred in WT kidneys. However, Parp1-KO mice showed a significant reduction of interstitial PMN-positive neutrophils in the UUO kidneys (Fig. 5, A and B). Additionally, WT mice showed an increase in the expression of proinflammatory cytokines including ICAM-1, TNF-α, iNOS, and TLR4 in the kidney 3 and 10 days after UUO, whereas loss of PARP1 dramatically inhibited their expressions 3 and 10 days after UUO (Fig. 6, A–E). In the sham-operated kidney, neutrophil infiltration and proinflammatory protein expressions were not significantly different between Parp1-KO and WT mice (Figs. 5 and 6). These results indicate that loss of PARP1 blocks renal inflammation induced by UUO.

Loss of PARP1 inhibits NF-κB activation during UUO. NF-κB controls many genes involved in inflammation. Here, we tested whether PARP1 contributes to the activation of NF-κB in renal interstitial fibrosis using Western blot analysis. As shown in Fig. 7, WT kidneys revealed an increase of the phosphorylation of NF-κB p65 (p-p65) 10 days after UUO, whereas Parp1-KO kidneys had a dramatic reduction in the UUO kidney. Thus, the data suggest that the proinflammatory effect of PARP1 may be linked to NF-κB activation, which may participate in interstitial fibrogenesis.

Loss of PARP1 reduces necrosis, but not apoptosis, during UUO. Because PARP1 contributes to necrotic cell death in various disease models, we examined histological damage, including necrosis, and apoptotic cell death induced by UUO in Parp1-KO and WT mouse kidneys. Ten days after UUO, the score of histological damage in kidney tubules of Parp1-KO mice was significantly lower than that in WT mice (Fig. 8, A and B). Similarly, the score of necrosis in tubular epithelia cells after UUO was significantly reduced in Parp1-KO mice compared with that in WT mice (1.68 ± 0.44 vs. 3.99 ± 0.19, P = 0.004, n = 6). Using TUNEL assay, apoptotic cell death at 10 days after UUO was markedly increased in both Parp1-KO and WT mice. No difference in apoptosis, however, was detected in the sham-operated kidneys of both genotype mice (Fig. 8, A and C). These results suggest that loss of PARP1 attenuates tubular cell necrosis, but not apoptosis, in renal fibrosis induced by UUO.
Loss of PARP1 does not affect TGF-β1 expression in vivo and α-SMA activation by TGF-β1 in vitro. Since excessive TGF-β1 plays an important role as a mediator in progressive fibrosis, we tested the hypothesis that altered TGF-β1 expression may account for the difference in interstitial fibrosis in Parp1-KO and WT mice. As demonstrated in Fig. 9, UUO caused a marked increase in renal expression of TGF-β1 and p-Smad3 in the kidneys of both Parp1-KO and WT mice. No significant difference in TGF-β1 and p-Smad3 level was found in the obstructed kidneys of Parp1-KO and WT mice at 10 days after UUO (Fig. 9, A-C), suggesting that the renal protection in Parp1-KO mice is not attributable to TGF-β1/Smad3 signaling pathway following UUO.

To demonstrate whether the fibrogenetic activity of PARP1 is independent of inflammation, we further investigated the effects of PARP1 on TGF-β1-mediated α-SMA expression, an indicative marker for the myofibroblastic activation, in quiescent renal interstitial fibroblasts (27). The expression of α-SMA was markedly increased by exogenous TGF-β1 treatment in normal rat renal interstitial fibroblast NRK-49F cells, and simultaneous treatment with respective 3AB and BNZ (representative inhibitors for PARP1) did not affect TGF-β1 mediated α-SMA expression (Fig. 10, A and B). Similarly, the expression of α-SMA induced by TGF-β1 treatment was not changed by genetic deletion of PARP1 in primary MEF cells (Fig. 10C). These results suggest that the fibrogenetic effect of PARP1 may be associated with inflammatory responses.
In this study, we showed that PARP1 plays a role in renal fibrosis in the UUO model of renal injury, as indicated by the appearance of 6- and 13-fold increased PARP1 expression and activation, respectively. In addition, Parp1-KO mice display dramatically attenuated renal inflammation after 3 and 10 days of UUO, whereas Parp1-KO mice show attenuated renal fibrosis at the late stage of UUO. Together, these results indicate that the inhibition of renal inflammation in Parp1-KO mice is the primary pathway leading to the attenuated renal fibrosis.

Inflammatory response results in overproduction and deposition of collagen, which cause tissue fibrosis. Recent evidence indicates upregulation of a variety of chemokines and cytokines including TNF-α, angiotensin II, TGF-β1, monocyte chemotactrant protein-1 (MCP-1), ICAM-1, and vascular cell adhesion molecule-1 following UUO (3, 6, 24, 33, 47). In studies from several laboratories including ours, PARP1 is shown to be involved in the regulation of the inflammatory processes, being functionally associated with recruiting neutrophils and proinflammatory proteins; for example, genetic deletion or pharmacological inhibition of PARP1 suppresses the neutrophil accumulation and upregulation of ICAM-1, TNF-α, iNOS, and TLR4 in an exacerbated tissue or systemic inflammatory disorder (28, 31, 40, 56). Previously, we also noted the attenuated neutrophil infiltration and

DISCUSSION

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TNF-α expression in renal ischemia and reperfusion model of Parp1-KO mice (57). Consistent with previous reports in other disease and tissue models, our present data demonstrated that the absence of Parp1 gene resulted in the inhibition of the neutrophil accumulation and the reduced expression of inflammatory proteins (such as TLR4, TNF-α, ICAM-1, and iNOS) after 3 and 10 days of UUO, suggesting that endogenous PARP1 actually accelerates renal inflammation from the early stage of renal fibrosis.

A recent report using Tlr4-deficient mice demonstrated that TLR4 promotes fibrosis despite no change in myofibroblast activation, but attenuates tubular damage (2, 44). The inflammatory response was not different in the obstructed kidneys of Tlr4-deficient and WT mice, as reflected by the expression of chemokines (keratinocyte chemoattractant and MCP-1) and infiltrating macrophages. Our data reveal that Parp1 deficiency inhibits the expression of TLR4 in UUO-induced mice kidneys and attenuates fibrosis, inflammatory response, and tubular damage. These data suggest that the net effect of Parp1 deficiency on suppressing the inflammatory response overcomes the negative impact of Tlr4 deficiency on inflammation.

TNF-α is a proinflammatory, cytotoxic cytokine produced by renal tubular cells in response to UUO and is an important mediator of renal fibrosis and cellular apoptosis (33, 36). TNF-α stimulates renal fibrosis by increasing TGF-β1 activity and collagen deposition and induces apoptotic renal tubular cell death via death receptor signaling and caspase activation in the tubulointerstitial compartment of the kidney (11, 33, 36). Tnfr1/2-deficient mouse is shown to have decreased expression of fibrotic markers after UUO (10). The role of TNF-α in promoting renal fibrosis is further supported by a recent study demonstrating that neutralization of TNF-α with a soluble receptor ameliorates fibrosis in the UUO model (36). However, the role of TNF-α in the UUO model is recently challenged by a report demonstrating that Tnfa deficiency had no effect on renal fibrosis at 2 wk and markedly increased fibrosis at 4 wk after UUO (37). Our data demonstrate that in Parp1-deficient mice, TNF-α expression is significantly reduced and fibrosis, inflammatory response, and tubular damage are attenuated. Interestingly, our data also demonstrate that the two mechanisms proposed to be activated by TNF-α in promoting renal fibrosis, increased TGF-β1 activity and apoptosis, are not affected in the Parp1-deficient mice after UUO.

NF-κB is an inducible transcription factor that is activated in response to UUO, with consequent tubulointerstitial cellular proliferation and interstitial fibrosis (39, 53). NF-κB activation upregulates the expression of a number of chemokines, cytokines, and cell adhesion molecules that have been implicated in the fibrinolytic changes of renal obstruction (34, 41, 46). In
addition, specific NF-κB inhibition with a proteasome inhibitor has been shown to attenuate obstruction-induced renal fibrosis (53). NF-κB encompasses a family of inducible transcription factors including NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB, and c-Rel (8, 13). Both p65 and p50 subunits of NF-κB directly bind to PARP1 in response to a stressor, and the formed complex induces iNOS, TLR4, and TNF-α expression in several diverse cell types (13, 16, 56). Furthermore, PARP1 inhibitors can reduce the DNA-binding activity of NF-κB and subsequently reduce its transcriptional activity (4), suggesting that PARP1 participates in augmenting the transcriptional activity of NF-κB. In this study, NF-κB activation induced by p65 phosphorylation after UUO has been reduced in Parp1-KO mice, suggesting that endogenous PARP1 regulates NF-κB activity. The reduced expression of iNOS, TLR4, and TNF-α molecules that are activated downstream of NF-κB signaling pathways also suggests that transcriptional activity of NF-κB is attenuated in Parp1-KO mice after UUO.

Profibrotic TGF-β1 activates interstitial fibroblasts in progression of chronic renal disease and induces epithelial-mesenchymal transition in cultured tubular epithelial cells (29). TGF-β1 transduces its signal via the transcription factors, Smad2 and Smad3, which oligomerize with Smad4 and bind to gene regulatory regions to orchestrate transcription. To determine whether Parp1 deficiency alters the TGF-β1/Smad3 signaling pathway, the level of expression of TGF-β1 and p-Smad3 in the UUO kidneys was determined. Intriguingly, both TGF-β1 and p-Smad3 expression after UUO were not significantly different between Parp1-KO and WT mice. This result contrasts with the data demonstrating decreased α-SMA expression and collagen deposition in the obstructed kidneys of Parp1-KO mice. This result suggests that the attenuation of renal myofibroblast activation and interstitial fibrosis is independent of TGF-β1/Smad3 signaling pathway and may explain the incomplete protection observed in the Parp1-KO mouse after UUO.

Necrosis has generally been considered an unregulated form of cell death, resulting in a loss of plasma membrane integrity and escape of intracellular contents to stimulate inflammatory responses. Conversely, apoptosis leads to a proteolytic degradation of intracellular contents for phagocytosis without induction of inflammation (5, 14). The blockage of urine flow by ureteral obstruction is known to induce both apoptotic and necrotic cell death in tubular epithelial cells (20, 25). In this study, the histological damage score including necrosis index in the UUO kidney of Parp1-KO mice has been less than that in WT mice. Interestingly, TUNEL-positive cells indicating apoptotic cell death were not significantly different in Parp1-KO and WT mice. These data suggest that PARP1 contributes to morphological consequences by necrotic cell death, but not apoptosis, in renal fibrosis. These data are consistent with the data reported from our laboratory and others that the pharmacological and/or genetic inhibition of PARP1 activity protects renal epithelial cells against necrotic cell death, but not apoptotic injury after chemical toxicity, oxidative stress, or ischemia (7, 9, 12, 38).

Collectively, the results of this study show that PARP1 activation in the UUO-induced kidney may integrate several pathways that converge to induce cellular necrosis and inflammation. Inhibition of renal inflammation in Parp1-KO mice may be the primary pathway leading to attenuated renal fibrosis after UUO. Targeting PARP1 may constitute a viable strategy for the treatment of fibrotic kidney diseases.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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