Poly(ADP-ribose) polymerase-1 gene ablation protects mice from ischemic renal injury

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Zheng, Jianfeng, Kishor Devalaraja-Narashimha, Kurinji Singaravelu, and Babu J. Padanilam. Poly(ADP-ribose) polymerase-1 gene ablation protects mice from ischemic renal injury. Am J Physiol Renal Physiol 288: F387–F398, 2005. First published October 19, 2004; doi:10.1152/ajprenal.00436.2003.—Increased generation of reactive oxygen species (ROS) and the subsequent DNA damage and excessive activation of poly(ADP-ribose) polymerase-1 (PARP-1) have been implicated in the pathogenesis of ischemic injury. We previously demonstrated that pharmacological inhibition of PARP protects against ischemic renal injury (IRI) in rats (Martin DR, Lewington AJ, Hammerman MR, and Padanilam BJ. Am J Physiol Renal Physiol 279: R1834–R1840, 2000). To further define the role of PARP-1 in IRI, we tested whether genetic ablation of PARP-1 attenuates tissue injury after renal ischemia. Twenty-four hours after reperfusion following 37 min of bilateral renal pedicle occlusion, the effects of the injury on renal functions in PARP−/− and PARP+/+ mice were assessed by determining glomerular filtration rate (GFR) and the plasma levels of creatinine. The levels of plasma creatinine were decreased and GFR was augmented in PARP−/− mice. Morphological evaluation of the kidney tissues showed that the extent of damage due to the injury in PARP−/− mice was less compared with their wild-type counterparts. The levels of ROS and DNA damage were comparable in the injured kidneys of PARP+/+ and PARP−/− mice. PARP activity was induced in ischemic kidneys of PARP+/+ mice at 6–24 h postinjury. At 6, 12, and 24 h after injury, ATP levels in the PARP+/+ mice kidney declined to 28, 26, and 43%, respectively, whereas it was preserved close to normal levels in PARP−/− mice. The inflammatory cascade was attenuated in PARP−/− mice as evidenced by decreased neutrophil infiltration and attenuated expression of inflammatory molecules such as TNF-α, IL-1β, and intercellular adhesion molecule-1. At 12 h postinjury, no apoptotic cell death was observed in PARP−/− mice kidneys. However, by 24 h postinjury, a comparable number of cells underwent apoptosis in both PARP−/− and PARP+/+ mice kidneys. Thus activation of PARP post-IRI contributes to cell death most likely by ATP depletion and augmentation of the inflammatory cascade in the mouse model. PARP ablation preserved ATP levels, renal functions, and attenuated inflammatory response in the setting of IRI in the mouse model. PARP inhibition may have clinical efficacy in preventing the progression of acute renal failure complications.

acute renal failure; necrosis; apoptosis; inflammation

ISCHEMIA-REPERFUSION of the kidney results in damage to the most distal (S3) segment of the proximal tubule (58). Depending on the severity of the injury, cells of the affected nephron segments undergo cell death by either apoptosis and/or necrosis (36). The molecular pathways adopted by the renal tissue that lead to necrotic and apoptotic cell death following an ischemic episode are not fully understood. Hypoxia resulting from decreased blood flow leads to a variety of secondary effects, including a breakdown in cellular energy metabolism and generation of reactive oxygen species (ROS) (33). The severe DNA damage resulting from increased ROS activity leads to excessive activation of poly(ADP-ribose) polymerase (PARP) and subsequent ATP depletion (3, 65).

PARP activation is important in DNA repair but its excessive activation can result in a substantial depletion of the intracellular NAD+. This leads to impairment of several NAD+-dependent metabolic pathways such as glycolysis and mitochondrial respiration, resulting in reduced ATP production. Furthermore, NAD+ is replenished in reactions that consume ATP, exacerbating the ATP shortage resulting in “cell suicide” by energy depletion (3). Beneficial effects of PARP inhibition in conditions such as hemorrhagic shock, colitis, and cerebral ischemia, however, are not due to a decrease in NAD+ content and energy charge, posing a challenge to the suicide hypothesis (19, 25, 41). PARP activation also is shown to contribute to mitochondrial dysfunction, leading to the rapid deterioration of mitochondrial integrity (59, 64) and intracellular acidification (1), both processes having direct consequences for cell viability. A recent report using in vitro models indicates that PARP-1-mediated cell death also has many features in common with apoptotic forms of cell death (64).

Activation of PARP has been demonstrated in ischemic injury in the brain, heart, skeletal muscle, retina, gut, and the kidney. Several inhibitors of PARP including benzamide, 3-amino benzamide (3-AB), and several other chemically distinct molecules (2) have been used to inhibit its activity in various models of ischemic injury and neurotoxicity (24, 28, 55, 56). Mice deficient for PARP have been shown to be protected against cerebral (13) and cardiac ischemia (44), shock (26), glutamate toxicity (42), streptozotocin-induced diabetes (43), and Parkinsonism (29).

In the kidney, we and others showed that inhibition of PARP accelerated the recovery of normal renal function and increased ATP levels at 24 h postinjury (7, 30). Recent evidence indicates that active mechanisms such as activation of PARP play important roles in necrotic cell death following renal ischemia (30). Inhibition of PARP activity in renal proximal tubule cells subjected to oxidant injury protected against necrotic cell death but not from apoptosis (17).

The combination of the pharmacological PARP inhibitor data with the gene knockout findings strongly suggests that...
PARP activation is a key mediator of ischemic cell death. Whether PARP-deficient mice would be resistant to ischemic renal injury has not been investigated. In this study, we tested the hypothesis that PARP-null mice would be resistant to ischemic renal injury.

MATERIALS AND METHODS

Animal and surgical procedures. Mice were cared for 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 Guide for the Care and Use of Laboratory Animals. All protocols had received prior approval from the UNMC-IACUC. 129SV (wild-type) or PARP−/− male mice (~20 g, Jackson Laboratories) were anesthetized by intraperitoneal administration of a cocktail containing ketamine (200 mg) and xylazine (16 mg) per kilogram of body weight. Ischemic injury was induced in wild-type mice and PARP-deficient mice by bilateral renal pedicle clamping using microaneurysm clamps. After 37 min of occlusion, the clamps were removed, and reflow was verified visually. Sham-operated control animals underwent the same surgical procedure, except for the occlusion of the renal arteries.

Evaluation of renal function. Renal function was assessed at 1–5 days postsurgery. Blood samples (0.1 ml) were collected retro-orbitally under light halothane anesthesia into heparinized tubes, and plasma was obtained after centrifugation. Serum creatinine and blood urea nitrogen were measured at various time points following the injury using standard assays as we previously described (38).

Glomerular filtration rate measurement. Surgical and clearance procedures were performed as described by Pluznick et al. (45) with minor modifications. In brief, mice were anesthetized as described above and the body temperature was maintained near 37°C using a heated surgery table. As required, additional doses of the cocktail were used to maintain anesthesia. A tracheostomy was performed using polyethylene (PE-90) tubing to facilitate breathing. The left external jugular vein was cannulated with PE-10 tubing for arterial pressure measurements and blood sampling. Urine was collected and stored under mineral oil. Saline solution was added to sample and background wells, respectively. The background values were averaged and then subtracted from the average of sample wells. The results were expressed as counts per minute per microgram of protein.

Analysis of DNA strand breaks. DNA damage at the cellular level in ischemic renal tissues was determined using the in situ nick translation method as previously described (15, 16). Briefly, cryostat sections were fixed in acetone followed by quenching of the endogenous peroxidase activity by treating with 0.3% H2O2 in methanol. To block nonspecific antibody binding, the slides were incubated in serum blocking solution (Reagent 1A, Histostain SP, Zymed Laboratories, San Francisco, CA) for 10 min. The in situ nick translation reaction was carried out for 25 min at room temperature in 50 mM Tris-HCl, pH 7.5, containing 5 U/100 μl of Kornberg polynucleotide, 0.1 mM diithiothreitol, 5 mM MgCl2, and 3 μM each of biotin-dUTP, dCTP, dGTP, and dATP. Slides were washed with 1× PBS and then processed for immunohistochemical detection of biotin using horse-radish peroxidase (HRP)-coupled streptavidin (Histostain SP, Zymed Laboratories).

PARP activity assay. The enzymatic activity of PARP was measured in kidney cell homogenates using methods previously described (32). The assay measures the amount of radioactive poly (ADP-ribose) that is incorporated into the proteins. Briefly, kidneys were dissected at 6 h, 12 h, 24 h, and 5 days after 37 min of ischemia and homogenized in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 25 mM MgCl2, and 0.1 mM phenylmethylsulfonyl fluoride. A fixed volume of sample was added to 500 μl of 56 mM HEPES buffer (pH 7.5) containing 28 mM KCl, 28 mM NaCl, 2 mM MgCl2, 0.01% digitonin, 125 nmol of NAD, and 0.25 μCi of [adenosine-2,8-3H]NAD (specific activity, 3.8 Ci/mmol) and incubated for 5 min at 37°C. The reaction was terminated by the addition of 200 μl of 50% ice-cold trichloroacetic acid (TCA) and centrifugation. The pellet was washed two additional times with TCA, resuspended in 2% SDS in 0.1 M NaOH, and incubated at 37°C overnight. The incorporation of radioactivity was determined by scintillation counting. The results were expressed as counts per minute per milligram of protein.

ATP assay. Kidney lysates were prepared by homogenizing the kidney in 5% TCA buffer followed by neutralization using 1 M Tris, pH 8.0. The cellular debris was removed by centrifugation at 1,000 g for 5 min. The ATP assay was performed using the Enliten ATP assay system (Promega, Madison, WI). Briefly, the ATP content was assayed by adding various concentrations of the cell extract into the Luciferase/Luciferin (L/L) reagent and determining the relative light units (RLU) with a 5-s delay and a 10-s signal integration time. The concentration of ATP corresponding to the RLU is then determined from an ATP standard curve. The protein concentration in the cell extract was estimated using the Bio-Rad protein assay reagents. Cellular ATP levels were expressed as nanomoles per milligram of cell protein.

Immunohistochemistry for neutrophils. Neutrophil infiltration was quantified by immunostaining for neutrophils followed by counting the number of stained cells. Bouin’s fixed mouse kidney sections were processed for immunostaining as described previously (37). The slides were stained for neutrophils by sequential incubation with rat anti-mouse neutrophils antibody (mAb 7/4; Serotec, Raleigh, NC) at a 1:100 dilution for 1 h at RT followed by HRP-conjugated rabbit antibody.
PARP-deficient sham-operated animals were not altered. KO, knockout.

anti-rat IgG at a 1:1,000 dilution for 1 h. The color development

PARP-deficient mice compared with that in wild-type animals. The values at
each time represent means ± SE. The values at days 2–3 are significantly
different (*P < 0.005; n = 8). The levels of creatinine in wild-type or
PARP-deficient sham-operated animals were not altered. KO, knockout.

Fig. 1. Plasma levels of creatinine were measured at various time points
post-renal ischemia in wild-type and poly (ADP-ribose) polymerase (PARP)-
deficient mice. The concentrations of creatinine were significantly reduced in
PARP-deficient mice compared with wild-type animals. The values at
days 1–3 postischemia in PARP-deficient mice compared with wild-type mice (Fig. 1). The mortality rate among both groups was identical. In both cases, one animal of
eight died within 24 h postinjury. There was no mortality among the five sham-operated mice. No change in the levels of creatinine was detected over a 5-day time period among sham-
operated mice.

GFR was measured in sham-operated and ischemia-induced wild-type and PARP-deficient mice at 24 h post-ischemic injury.

RESULTS

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Quantitation of the data (Fig. 2) showed that in sham-operated mice, the GFR in wild-type (0.83 ± 0.14 ml·min⁻¹·mg kidney
wt⁻¹; n = 4) and PARP-deficient mice (0.88 ± 0.05 ml·min⁻¹·mg kidney wt⁻¹; n = 4) did not differ significantly. At 24 h
postischemia, GFR was significantly augmented in PARP-defi-
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ny wt⁻¹; n = 5; P < 0.05).

Histological analysis of kidney sections obtained from wild-
type and PARP-deficient mice at 1 and 5 days post-ischemia
injury showed that both groups suffered ischemic damage. Shown in Fig. 3A is a low-magnification photograph of a kidney section originating from a wild-type mice 1 day postinjury, stained with hematoxylin and eosin. A kidney section from PARP knockout mice 1 day postinjury is shown in Fig. 3B. Ischemic kidneys from wild-type mice showed widespread necrosis, tubular obstruction, and sloughed cells in the proximal straight tubule, whereas these features were dramatically reduced in PARP-deficient mice. Erythrocyte trapping was considerable reduced in the corticalmedullary segment of the

cells in the various tubular segments. Images from 10–15 nonover-
lapping fields were randomly selected and counted in a blinded manner as we previously described (30).

Statistics. The paired Student’s t-test was used to compare mean
values within one experimental group. One-way ANOVA with post-
test was used to compare mean values from two groups. A P value of
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PARP-deficient mice compared with that in wild-type mice. High-magnification photographs of kidneys derived from wild-type and PARP-deficient mice, respectively, that were rendered ischemic 5 days before their isolation. The kidney sections were stained with hematoxylin and eosin. All sections are derived from the corticomedullary junction of the kidney. The straight segments of the proximal tubules are visible in each of the sections. The top portion of the sections in each panel is the outer cortex and the bottom portion is the inner medulla. Magnification in A and B is ×40; C, D, E, and F is ×200.

Fig. 3. Comparison of typical histology in 24-h postischemic kidneys derived from wild-type (A and C) or PARP-deficient mice (B and D). The sections in E and F were derived from wild-type and PARP-deficient mice, respectively, that were rendered ischemic 5 days before their isolation. The kidney sections were stained with hematoxylin and eosin. All sections are derived from the corticomedullary junction of the kidney. The straight segments of the proximal tubules are visible in each of the sections. The top portion of the sections in each panel is the outer cortex and the bottom portion is the inner medulla. Magnification in A and B is ×40; C, D, E, and F is ×200.

The changes in histology at 1 day postinjury were quantified by counting the number of necrotic cells and trapped red cells and are shown in Fig. 4, A and B, respectively. Both values were attenuated, demonstrating that gene ablation of PARP-1 reduced cellular necrosis and red cell trapping.

The number of dilated tubules in 5-day postischemic kidneys derived from PARP knockout mice and wild-type mice was counted and quantified as described in MATERIALS AND METHODS. Kidneys derived from the PARP knockout mice had significantly reduced dilated tubules compared with that from wild-type mice (Fig. 4C), indicating that they underwent lesser initial injury.

To elucidate the mechanisms by which PARP deficiency would lead to the functional and morphological protection in the setting of renal ischemia, we examined whether there is any
association between PARP gene ablation and the generation of ROS in ischemic renal tissues. At 12 h postischemia, enhanced levels of ROS were detected in both wild-type and PARP knockout mice. However, as shown in Fig. 5, no variation in the levels of ROS was detected between wild-type and PARP knockout mice that was rendered ischemic. These data suggest that ROS production is induced in renal tissues derived from both wild-type and PARP knockout mice postischemic injury.

To determine whether the induced ROS production is associated with increased DNA damage, we examined ischemic renal tissues for DNA strand breaks using the in situ nick translation method. Light microscopic examination of kidneys derived from both wild-type and PARP knockout mice demonstrated positive signaling for DNA strand breaks (Fig. 6A) localized mostly to the proximal tubules of the corticomedullary junction. The number of positively stained cells in 30–40 high-magnification fields was counted in a blinded manner. As shown in Fig. 6, B and C, the number of cells that were positive for DNA strand breaks was not varied between wild-type and PARP knockout mice. DNA strand breaks were not detected in sham-operated wild-type or PARP knockout mice kidneys.

To determine if the increased DNA damage was associated with an increase in PARP activity, PARP activity assay was performed. At 6 and 12 h and 1 and 5 days postischemia, PARP activity was significantly increased \( (P < 0.05) \) in wild-type mice compared with those in kidneys of sham-operated mice (Fig. 7).

To determine whether PARP activation promoted ATP consumption and PARP deficiency prevented the decline of ATP postischemia, ischemic kidneys were isolated from various time points and the ATP contents were determined. For our studies, kidneys derived from sham-operated mice served as controls. Sham surgery was done by making an incision on the abdominal skin and muscle layers but leaving the kidney untouched. At 6, 12, and 24 h postischemia in wild-type mice, ATP levels were significantly reduced \( (P < 0.05) \) to 28, 26, and 43.64%, respectively, compared with those in kidneys of sham-operated mice (Fig. 8). Levels of ATP in PARP knockout mice that were rendered ischemic remained at 80, 73, and 77% \( (P < 0.05) \) of the levels in sham-operated PARP-deficient mice, consistent with preservation of ATP content postischemia by the absence of PARP.

To determine whether PARP deficiency leads to a variation in the inflammatory response, the infiltration of neutrophils in wild-type and PARP−/− mice at 6 and 12 h and 1, 2, and 5 days postischemic tissues was assessed by immunostaining for PARP AND RENAL ISCHEMIA-REPERFUSION INJURY

Fig. 4. Renal histopathology was scored using quantitative measures as described in MATERIALS AND METHODS. Comparison of the number of necrotic cells or trapped erythrocytes in 24 h postischemic kidneys derived from wild-type and PARP-deficient mice is shown in A and B, respectively. Both values are significantly reduced in PARP-deficient mice compared with that in wild-type animals \( (\ast P < 0.001) \). Comparison of the tubular dilatation in wild-type and PARP-deficient mice kidneys 5-day postischemia is shown in C. The number of dilated tubules is significantly reduced \( (\ast P < 0.001) \) in PARP-deficient mice kidneys compared with that in wild-type animals. The values represent means ± SE.

Fig. 5. Levels of reactive oxygen species (ROS) in wild-type and PARP knockout mice were quantified as described in MATERIALS AND METHODS. The level of ROS was not different in ischemic PARP-deficient mice kidneys compared with that in wild-type at 12 and 24 h postinjury. The values at each time point represent means ± SD.
neutrophils. Representative high-magnification photographs of wild-type and PARP−/− mice kidney sections stained for neutrophils infiltration in the corticomedullary junction at different time points are shown in Fig. 9. The number of positively stained cells in 30–40 high-magnification fields was counted in a blinded manner. A minimum of three animals were used from wild-type and PARP−/− mice for each time point. The data are quantitated (Fig. 10) and demonstrate that the neutrophilic infiltration is attenuated in PARP−/− mice compared with wild-type mice at both time points. At 6, 12, and 24 h postinjury, the number of neutrophils infiltrated in wild-type kidneys were 20.9 ± 2.0, 50.2 ± 8.49, and 15.9 ± 1.25, respectively, compared with 2.83 ± 0.31, 3.1 ± 0.35, and 2.1 ± 0.21 in PARP knockout kidneys at the same time points. At 2 and 5 days postinjury, only a negligible number of neutrophils were observed in both wild-type and PARP knockout mice kidneys (data not shown).

The expression of mRNA for the proinflammatory molecules (TNF-α, ICAM-1, and IL-1β) in sham-operated and ischemic wild-type and PARP−/− mice was determined by...
The expression of all three proinflammatory molecules was significantly \( (P < 0.05) \) reduced post-ischemic injury in PARP\(^{-/-}\) mice compared with wild-type mice at 12 h (Fig. 11). The expression levels of the different molecules varied in sham-operated animals (Fig. 11). No change in the expression of these molecules was observed between sham-operated wild-type or PARP\(^{-/-}\) animals (data not shown).

To determine whether there is a variation in the number of cells undergoing apoptosis in wild-type mice compared with PARP\(^{-/-}\) mice, TUNEL assay was performed in conjunction with nuclear morphology analysis (Fig. 12). Renal tissues derived from both wild-type and PARP\(^{-/-}\) mice at 12 and 24 h post-ischemic injury were assayed and the number of cells positive for TUNEL staining was counted in 10–20 high-magnification fields under a fluorescent microscope. Data from at least three wild-type and PARP\(^{-/-}\) mice were used to quantitate the extent of apoptosis. At 12 h postinjury, the number of TUNEL-positive cells in wild-type kidneys was 20.96 ± 7.99 compared with 6.66 ± 3.19 in PARP-deficient kidneys. At 24 h, however, the number of apoptotic cells in

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**Fig. 9.** Typical neutrophil accumulation in wild-type (wt) and PARP knockout (ko) kidneys at 6, 12, and 24 h postinjury. Neutrophils were identified using a neutrophil-specific antibody as described in MATERIALS AND METHODS.

**Fig. 10.** Number of neutrophils in high-magnification fields were counted and quantitated as described in MATERIALS AND METHODS. Neutrophil infiltration is significantly increased \( (*P < 0.001) \) in wild-type compared with that in PARP-deficient mice kidneys at 6, 12, and 24 h postinjury. The values at each time point represent means ± SE.
wild-type (14.85 ± 6.1) and in PARP-deficient kidneys (10.8 ± 3.23) was comparable. The data presented in Fig. 13 demonstrate that the number of cells undergoing apoptosis at 12 h postinjury was enhanced in wild-type mice compared with PARP−/− mice. By 24 h, however, an equal number of cells were found to undergo apoptosis in both wild-type and PARP−/− mice. Only negligible numbers of apoptotic cells were detected in kidneys of both wild-type and PARP−/− mice at 2 and 3 days postinjury (data not shown).

**DISCUSSION**

This study focuses on the role of PARP in acute ischemia-reperfusion injury and the subsequent inflammatory response in the kidney. The major findings of our study are that 1) mice deficient for PARP-1 gene demonstrate accelerated recovery of normal renal function after ischemia-reperfusion injury compared with wild-type mice as determined by the levels of plasma creatinine and GFR and 2) the improved renal function is associated with decreased inflammation and ATP depletion and improved histopathological appearance but with no variation in the number of cells undergoing apoptosis at 24 h postreperfusion. These findings suggest that PARP plays an important role in the functional and morphological consequences of ischemic acute renal failure.

**Fig. 11.** Levels of expression of TNF-α, IL-1β, ICAM-1, and GAPDH were semiquantitatively determined as described in MATERIALS AND METHODS. The level of intensity of each of the bands was normalized relative to the level of the intensity of the respective GAPDH band to standardize for variations in probe-specific activity. RNA derived from at least 3 animals was used in hybridization experiments. The values at each time point represent means ± SE. Expression levels of TNF-α, IL-1β, and ICAM-1 were significantly reduced (*P < 0.05) in ischemic PARP-deficient mice kidneys compared with that from wild-type at 1 day postinjury.

**Fig. 12.** The number of cells that underwent apoptosis was assayed by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling method (A-D) in kidneys derived from wild-type and PARP-deficient mice that underwent 37 min of ischemia followed by 12- or 24-h reperfusion. The same tissue sections were also stained with nuclear Hoechst dye to confirm changes in nuclear morphology (E-H).

**Fig. 13.** The number of apoptotic cells in 10–15 high-power fields was counted in tissues derived from wild-type and PARP-deficient mice and the data were compared. Apoptosis was significantly (*P < 0.005) decreased in ischemic PARP-deficient mouse kidneys compared with that from wild-type at 12 h postinjury. Kidney sections derived from at least 3 animals were used in the apoptosis assay. The values at each time point represent means ± SD.
PARP-deficient mice and wild-type mice were subjected to ischemia-reperfusion injury for 37 min followed by reperfusion for various periods of time. Our data show that the absence of PARP activity following renal ischemia-reperfusion injury accelerates recovery as reflected by lower concentrations of creatinine during days 1-3 postischemia (Fig. 1) and improved GFR at 24 h postinjury (Fig. 2). The accelerated recovery in renal function is coupled with improved renal histology at 1 and 5 days following injury and reduced erythrocyte congestion (Figs. 3 and 4). Quantitation of tubular dilation in kidneys that underwent ischemic injury followed by 5 days of reperfusion showed that there are fewer dilated tubules in PARP-deficient kidneys (Fig. 4C). This data suggest that there is enhanced recovery in PARP-deficient kidneys that could be attributed to both attenuated injury following the insult and an accelerated regeneration process. The mechanism by which PARP deficiency leads to improved renal functions or improved histopathology is not elucidated.

Oxidative injury plays an important role in the inflammatory response post-ischemic renal injury (27, 39). ROS such as the superoxide radical and its reduction products hydrogen peroxide and the hydroxyl radical are generated in the kidney post-ischemia-reperfusion injury (57). In addition, nitric oxide-generated post-ischemic injury reacts with superoxides to form peroxynitrite as early as 40 min postinjury (62). A role for the ROS and peroxynitrite has been implicated in the causation of inflammation post-ischemic injury (34). The data presented here indicate that ROS levels are induced following renal ischemia-reperfusion injury. However, no changes in the level of ROS between wild-type and PARP-deficient mice kidneys are observed, suggesting that PARP activation may not influence the generation of ROS posts ischemia.

Oxidative stress can induce DNA strand breaks in ischemic kidneys as early as 1 h postreperfusion (10). Quantitative analysis of DNA degradation in ischemic kidney cells derived from wild-type or PARP-deficient mice demonstrated no differences in the extent of DNA damage (Fig. 6, A-C). DNA strand breaks can induce excessive activation of PARP (50) leading to rapid ATP depletion. Depending on the severity of DNA damage, genotoxic stimuli can trigger three different pathways (61). In the case of mild DNA damage, PARP facilitates DNA repair and thus survival. More severe DNA damage induces apoptotic or necrotic cell death depending on the severity of the injury. Renal ischemia induces a large number of sublethally injured cells in the proximal tubules, and their survival depends to a great extent on the cellular energy levels (12). Our data demonstrate that PARP activity is enhanced in ischemic kidneys as early as 6 h and its activation persisted at 12 h, 1 and 5 days postinjury (Fig. 7). Inhibition of PARP may preserve the ATP levels in these cells and may aid the cells in recovering from the insult. The most severe DNA damage may cause excessive PARP activation, depleting NAD\(^+\) and ATP stores. It is highly unlikely that cells where massive DNA damage occurred may be able to survive. The reduced number of necrotic cells in the PARP knockout mice may partly be due to the absence of PARP-mediated ATP depletion in the sublethally injured cells.

Restoration of cellular ATP levels is a major requirement for initiation of the cell survival and regeneration process. The levels of ATP fall to undetectable levels in kidney following a major ischemic insult in animal models. During the first 2 h following the ischemic insult, ATP recovery occurs in two phases. There is a rapid initial increase in levels of ATP that occurs immediately on reflow followed by a more gradual elevation to normal levels (53, 63). The restoration of ATP levels back to normal takes more than 48 h (20).

Mice that are lacking PARP-1, although subject to oxidative stress similar to wild-type mice, do not see their intracellular energy sources depleted. Therefore, PARP-deficient mice are tolerant to various conditions where oxidant injury plays a key role in inducing the injury. Inhibition of PARP activity using various pharmacological agents also has been shown to prevent ischemia-reperfusion injury in the kidney and various other organs. Our data (Fig. 8) showing that levels of ATP are restored to close to normal levels in PARP-deficient mice compared with wild-type mice indicate that PARP-mediated energy depletion plays an important role in ischemic renal injury. This suggests that one of the mechanisms by which PARP ablation ameliorates the course of injury is by preserving cellular ATP levels.

Ischemic renal injury is associated with significant necrosis in the renal tissue, which can initiate an inflammatory cascade involving infiltration of neutrophils and subsequent release of inflammatory cytokines. In addition, necrotic tissue itself may activate various cytokines that can exacerbate necrotic cell death. The molecular components that initiate an inflammatory cascade post-ischemic renal injury are not clearly defined. PARP has been implicated in inflammation-induced tissue injury and its activation represents a cytoplasmic pathway in the inflammatory response (54, 59). Several studies have demonstrated that ischemia-reperfusion of the kidney induces leukocyte-endothelial cell interaction within the renal microvasculature (46, 47). A recent study in an ischemia-reperfusion injury of the liver showed that the number of rolling and adherent leukocytes and platelets was significantly lower in PARP−/− mice, suggesting a role for PARP in triggering these cell-cell interactions (23). PARP activation also is shown to be important in leukocyte infiltration in myocardial ischemia-reperfusion injury and in mucosal colitis (66, 67). The data presented in this study (Figs. 9 and 10) demonstrate that the number of neutrophils infiltrated into the renal tissue postischemia-reperfusion injury is dramatically reduced in PARP−/− mice compared with their wild-type counterparts.

ICAM-1 is constitutively expressed on the surface of endothelial cells, and its interaction with CD11/CD18 is important in neutrophil and macrophage recruitment post-ischemia-reperfusion injury of the kidney (46). ICAM-1 expression is induced in ischemic renal tissues, and its inhibition is shown to attenuate renal injury (22). In this study, we semiquantitatively analyzed the gene transcription of TNF-α, IL-1β, and ICAM-1 in renal tissues derived from wild-type or PARP−/− mice that underwent ischemic injury or sham surgery. No difference in the expression of these molecules between sham-operated wild-type and PARP−/− mice was observed (data not shown). However, PARP ablation led to reduced expression of proinflammatory molecules TNF-α, IL-1β, and ICAM-1 compared with that in wild-type mice post-ischemic renal injury (Fig. 11). The mechanism by which PARP activation contributes to the expression of ICAM-1 is not fully understood. ICAM-1 expression is increased by IL-1 and TNF-α in vitro (52). A recent report indicates that PARP inhibition can completely abrogate the release of cytokines such as TNF-α and IL-1β in
postischemic liver (8) as well as suppress TNF-α-induced expression of ICAM-1 in human umbilical vein endothelial cells (66). PARP can interact with transcription factors such as NF-kB and enhance its activity. NF-kB activity is impaired and iNOS, TNF-α, and IFN-γ expression is reduced in PARP−/− mice subjected to endotoxic shock (35). However, transcription of ICAM-1 following ischemic renal injury is shown to be independent of TNF-α and IL-1β functions (6). The role of PARP in regulating TNF-α and IL-1β-mediated inflammatory response is under investigation.

Inhibition of PARP activity in vitro models of ischemic injury is shown to protect epithelial cells from necrotic injury, but it either enhanced or allowed apoptotic cell death to ensue (17, 31, 40, 60). Similarly, when PARP-deficient mice are subjected to ischemic injury, enhanced apoptotic cell death is observed in various tissues (14, 18, 31). Apoptosis is mediated by a variety of intracellular enzymes, among which are Ca2+/ Mg2+-dependent endonucleases (CMEs) that catalyze the inter-nucleosomal fragmentation of DNA. CME activity is inhibited by poly(ADP-ribose)lation (4, 48). Thus cleavage of PARP during apoptosis may lead to activation of CMEs required for DNA fragmentation. DNASIL3 is a unique CME that is localized to the nucleus and regulated by PARP. Poly(ADP-ribose)ylation of DNASIL3 leads to downregulation of its activity (5, 51). Inactivation of PARP by caspase 3-dependent cleavage may interfere with its poly(ADP-ribose)ylation function and thus inhibition of nuclear proteins such as DNASIL3 could be compromised. In this study, we analyzed the apoptotic cell death in kidneys that were derived from wild-type and PARP knockout by the TUNEL method (Fig. 12). Quantitation of the number of cells undergoing apoptotic cell death in kidneys from wild-type or PARP knockout mice shows that in wild-type kidneys, the number of cells of the proximal tubules in the outer medulla that are positive for TUNEL is significantly increased compared with that in PARP knockout mice kidneys at 12 h postinjury. However, at 24 h, the number of apoptotic cells was not different in kidney sections originating from the PARP knockout mice and wild-type mice (Fig. 13).

The data presented demonstrate that the absence of PARP protects the cells by inhibition of necrosis and possibly due to a delay but not inhibition of apoptosis. Previous reports have shown that in mice that underwent 32 min of bilateral clamping or 45 min of unilateral clamping of the renal pedicle, inhibition of apoptosis improves renal functions and histopathology (11, 21). These data suggest that both apoptosis and necrosis contribute to the functional and morphological consequences of ischemic renal injury.

In summary, we showed that the PARP-deficient mouse is protected against ischemic renal injury as demonstrated by improved GFR, reduced plasma creatinine levels, decreased tubular dilatation, and ATP depletion. The exact mechanism by which PARP inhibition attenuates renal injury remains undefined. The data presented in this study demonstrate that PARP gene ablation protects mice from ischemia-reperfusion injury not only by restoring the levels of ATP in kidney but also by decreasing the expression of proinflammatory agents and the adhesion molecule ICAM-1. Our previous demonstration that pharmacological inhibition of PARP prevents ischemia-reperfusion injury combined with the evidence presented in this article supports the view that PARP-1 activation contributes significantly to the pathophysiology of ischemia-reperfusion injury in the kidney. Pharmacological inactivation of PARP-1 might be a therapeutically viable strategy to prevent acute cell injury and improve the outcome in ischemic acute renal failure.

GRANTS

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