PARP-1 Inhibits Glycolysis in Ischemic Kidneys

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ABSTRACT

After ischemic renal injury (IRI), selective damage occurs in the S_3 segments of the proximal tubules as a result of inhibition of glycolysis, but the mechanism of this inhibition is unknown. We previously reported that inhibition of poly(ADP-ribose) polymerase-1 (PARP-1) activity protects against ischemia-induced necrosis in proximal tubules by preserving ATP levels. Here, we tested whether PARP-1 activation in proximal tubules after IRI leads to poly(ADP-ribosyl)ation of the key glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a modification that inhibits its activity. Using *in vitro* and *in vivo* models, under hypoxic conditions, we detected poly(ADP-ribosyl)ation and reduced activity of GAPDH; inhibition of PARP-1 activity restored GAPDH activity and ATP levels. Inhibition of GAPDH with iodoacetate exacerbated ATP depletion, cytotoxicity, and necrotic cell death of LLCPK$_1$ cells subjected to hypoxic conditions, whereas inhibition of PARP-1 activity was cytoprotective. In conclusion, these data indicate that poly(ADP-ribosyl)ation of GAPDH and the subsequent inhibition of anaerobic respiration exacerbate ATP depletion selectively in the proximal tubule after IRI.

Compromised perfusion of renal tissues leading to ischemic renal injury (IRI), generally accepted as the major cause of acute kidney injury (AKI), usually results from hypoxia-induced renal vascular and tubular dysfunction. The outer medullary region of the kidney receives <10% of the blood delivered to the kidney *via* the renal artery. After IRI, a persistent perfusion deficit exists even at 24 h after reperfusion, and the outer medullary partial pressure of oxygen is restored to only 10% of its normal levels, rendering this region susceptible to injury at both the tubular and vascular levels.[1–3] Thus, the prolonged perfusion deficit shuts down oxidative phosphorylation in the cells of the outer medullary segments of the nephron and reverts to anaerobic metabolism for ATP synthesis. Nevertheless, ischemia causes selective injury to the outer medullary proximal straight tubules (PST), causing the PST cells to undergo cell death and/or sublethal injury to instigate renal dysfunction.[4,5] The medullary thick ascending limb, although situated in the same region, does not undergo injury to the same level.[6–8] Despite ongoing debate for more than two decades,

the molecular mechanisms by which PST cells undergo selective injury are not known.

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) and reactive nitrogen species.[9,10] The superoxides induce DNA strand breaks in ischemic kidneys as early as 1 h after IRI.[11] The severe DNA damage that ensues results in excessive activation of the DNA repair enzyme poly(ADP-ribose) polymerase-1 (PARP-1), which exacerbates ATP depletion and triggers signaling cascades, leading to cellular suicide.[12] Recent data from our laboratory showed selective
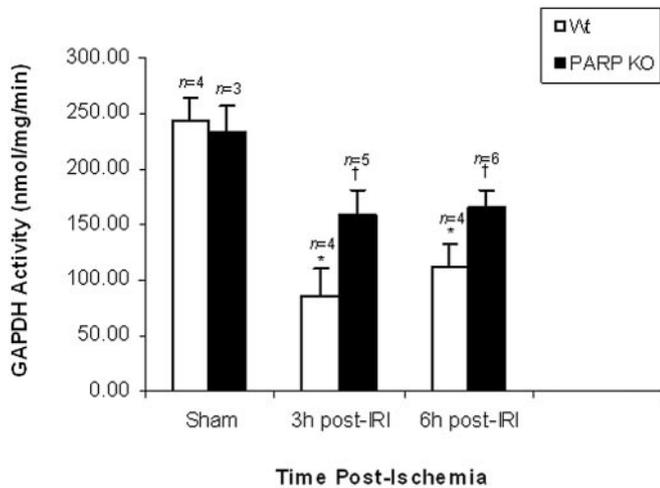

Figure 1. Effect of IRI on GAPDH activity in WT and PARP$^{-/-}$ renal tissues. Each bar represents mean ± SEM. *$P < 0.0005$ *versus* sham-operated WT mice; †$P < 0.005$ *versus* WT renal ischemic mice.

upregulation of PARP-1 expression and its activity in PST cells after renal ischemia.[13,14] Gene ablation or pharmacologic inhibition of PARP-1 activity offers both functional and histopathologic protection from IRI.[13,15] The ATP levels are significantly preserved in both *in vivo* and *in vitro* models of IRI after PARP gene ablation or inhibition, respectively[13,15,16]; however, the exact mechanisms by which PARP activation leads to ATP depletion and whether these mechanisms are linked to selective damage to PST after renal ischemia are not defined.

According to the "cell suicide" hypothesis, PARP-1 activation induces energy failure by depleting NAD$^+$, and the cell consumes ATP to replete the NAD$^+$ level, ultimately leading to energy failure and cell death[12]; however, the role of NAD$^+$ in energy depletion is controversial, and its depletion alone may not be lethal to cells.[17,18] Moreover, PST cells can carry out anaerobic respiration under hypoxic conditions and be protected from injury. Nevertheless, under anoxic conditions, significant amounts of ATP are generated by anaerobic glycolysis by thick ascending limbs but not by the proximal tubular cells.[19] These findings prompted us to investigate whether PARP activation interferes with glycolytic ATP synthesis and thus exacerbates ATP depletion and PST cell injury.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a key enzyme in the glycolytic pathway and is susceptible to several modifications that alter its activity, including oxidative modification of thiols and mono-ADP-ribosylation.[20–22] Recently, PARP-1 was reported to inhibit GAPDH activity by poly(ADP-ribosyl)ation after hyperglycemia-induced aortic endothelial cell injury.[23] The temporal and spatial expression pattern of PARP-1 in PST during the time of ischemic injury prompted us to hypothesize that poly(ADP-ribosyl)ation and inhibition of the GAPDH leads to inhibition of glycolysis, re-
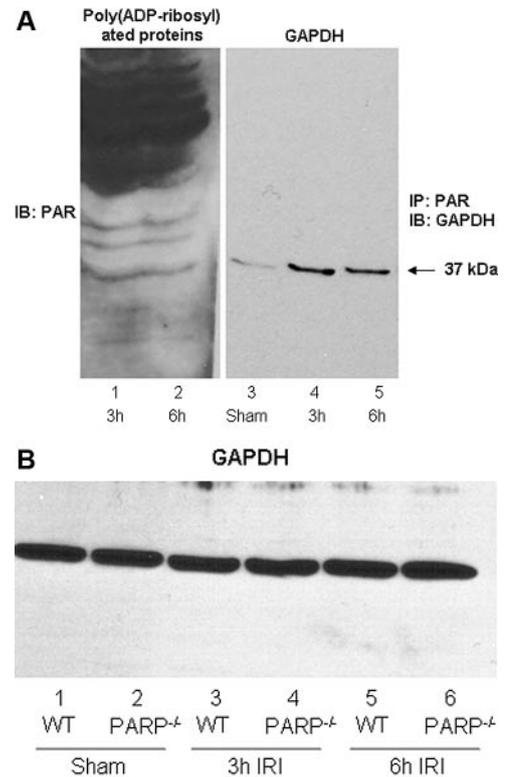
Figure 2. (A) Poly(ADP-ribosyl)ation of GAPDH in renal ischemic tissues. Lanes 1 and 2 represent Western blotting analysis showing poly(ADP-ribosyl)ated proteins in whole-cell lysates of 3 and 6 h postischemic WT outer medullary renal tissues, respectively (100 µg of protein was loaded). Lanes 3, 4, and 5 represent Western blotting analysis using GAPDH antibody on PAR-immunoprecipitated proteins from sham and 3- and 6-h postischemic WT kidney lysates, respectively. (B) Western blotting analysis showing the expression of GAPDH in whole-cell lysates of outer medullary region. Lane 1, WT sham; lane 2, PARP$^{-/-}$ sham; lane 3, WT 3 h after IRI; lane 4, PARP$^{-/-}$ 3 h after IRI; lane 5, WT 6 h after IRI; and lane 6, PARP$^{-/-}$ 6 h after IRI (50 µg of protein was loaded).

ducing ATP synthesis and exacerbating energy depletion and cell injury.

In this study, we evaluated the role of GAPDH-poly(ADP-ribosyl)ation as a mechanism to inhibit GAPDH activity in PST after IRI using *in vitro* and *in vivo* models. We explored the role of GAPDH-poly(ADP-ribosyl)ation in inhibiting glycolysis, exacerbating ATP depletion, and inducing cell death. Our findings suggest that PARP-1–mediated anaerobic glycolytic inhibition is a key mechanism of selective PST injury after IRI.

RESULTS

GAPDH Activity Assay in Wild-Type and PARP$^{-/-}$ Mouse Kidneys after IRI

To test our hypothesis that GAPDH activity is inhibited by PARP, we first evaluated GAPDH activity in wild-type (WT)
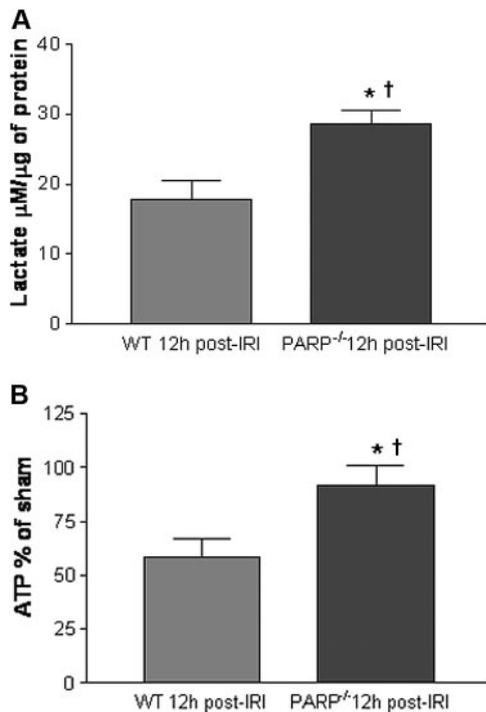
Figure 3. (A) Effect of IRI on lactate production in WT and PARP$^{-/-}$ mice. Each bar represents the mean ± SEM of four separate experiments. *†$P < 0.05$ *versus* WT mice. (B) Effect of IRI on ATP levels in WT and PARP$^{-/-}$ mice. Each bar represents the mean ± SEM of four separate experiments. *†$P < 0.05$ *versus* WT mice.

and PARP$^{-/-}$ renal outer medullary proteins after IRI. As shown in Figure 1, GAPDH activity was significantly inhibited at 3 and 6 h after IRI in WT mouse kidneys compared with that of sham-operated kidneys, whereas its activity was significantly improved at both 3 and 6 h after IRI in PARP$^{-/-}$ mouse kidneys compared with their WT counterparts. These data suggest a role for PARP-1 in the inhibition of GAPDH activity after IRI. No significant change in GAPDH activity was observed between WT and PARP$^{-/-}$ sham-operated kidneys.

GAPDH-Poly(ADP-ribosyl)ation in Ischemic Renal Tissues

Because activated PARP-1 ADP ribosylates several different proteins and modulates their activities,[14] we next examined whether poly(ADP-ribosyl)ation of GAPDH could be a possible underlying mechanism for its reduced activity after IRI. Proteins isolated from the outer medullary region of WT or PARP$^{-/-}$ animals that underwent either sham surgery or IRI were subjected to immunoblot analysis. Several poly(ADP-ribosyl)ated protein bands, including a band at approximately 37 kD, were observed at 3 and 6 h after IRI in WT kidney lysates (Figure 2A, lanes 1 and 2, respectively) with anti-PAR antibody, suggesting possible GAPDH-poly-(ADP-ribosyl)ation after IRI. No PAR bands were observed in either sham-operated or PARP$^{-/-}$-ischemic renal tissues (data not shown).
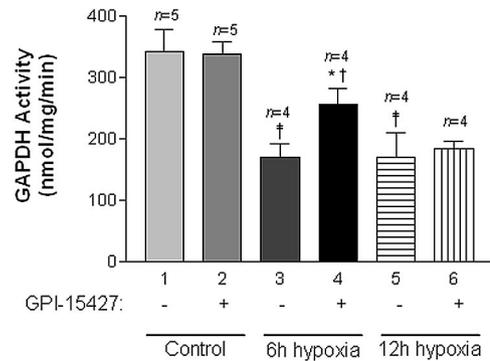
Figure 4. Effect of hypoxia with or without GPI-15427 on GAPDH activity in LLCPK$_1$ cells. Each bar represents the mean ± SEM. ‡$P < 0.02$ *versus* control; *†$P < 0.05$ *versus* 6 h of hypoxia only.

To confirm the possibility that GAPDH is poly(ADP-ribosyl)ated, we performed immunoprecipitation with anti-PAR antibody followed by immunoblotting with anti-GAPDH antibody. The results showed a prominent band at 37 kD at both 3 and 6 h after IRI in WT kidney lysates (Figure 2A, lanes 4 and 5, respectively) but not in sham-operated kidneys (Figure 2A, lane 3) clearly demonstrating GAPDH-poly(ADP-ribosyl)ation after ischemia. No change in the molecular weight of GAPDH was observed as a result of poly(ADP-ribosyl)ation as other PARP-1 targets in the previous reports.[24,25] GAPDH expression *per se* did not change between WT and PARP$^{-/-}$ sham-operated or ischemic renal tissues (Figure 2B).

Lactate and ATP levels in WT and PARP$^{-/-}$ Mouse Kidneys after IRI

To show that the inhibition of GAPDH activity by PARP-1 does indeed inhibit anaerobic glycolysis, we measured lactate production and ATP levels in the outer medulla of WT and PARP$^{-/-}$ mouse kidneys after IRI. As shown in Figure 3A, lactate production was significantly higher in the outer medulla of PARP$^{-/-}$ mice compared with WT mice at 12 h after IRI. As shown in Figure 3B, the ATP levels in the outer medulla of WT mice at 12 h after IRI were decreased to 58.560 ± 8.018% of the sham-operated mice ($P < 0.03$; $n = 4$), whereas the absence of PARP-1 preserved ATP levels to 91.570 ± 9.228% of sham-operated mice. The ATP levels in sham-operated kidneys were measured to be 11.146 nmol/mg protein.

Effect of PARP Inhibition on GAPDH Activity in Hypoxia-Injured LLCPK$_1$ Cells

To confirm our *in vivo* finding that PARP-1 mediates the inhibition of GAPDH activity and to demonstrate that this mechanism occurs in PST cells, we used the porcine-derived proximal tubular cell line LLCPK$_1$. LLCPK$_1$ cells expressed proximal tubular marker aquaporin 1 (AQP1) and the PST marker AQP7 (see Supplementary Figure S1A). Furthermore, 6 h of
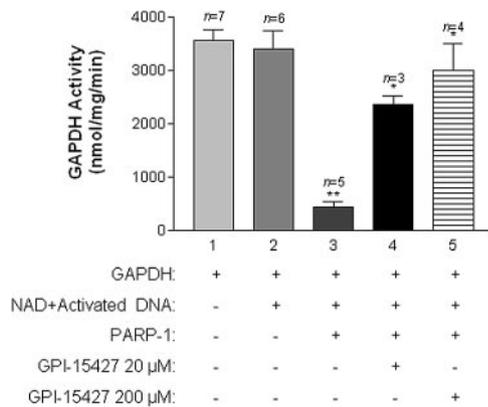
Figure 5. Effect of PARP-1 activity on GAPDH activity in *in vitro* cell-free system. Each bar represents the mean ± SEM. **$P <$ 0.0002 *versus* GAPDH enzyme only; *$P < 0.0005$ *versus* GAPDH enzyme in the presence of NAD^+ and activated PARP-1 enzyme.

Figure 6. Effect of hypoxia on ATP levels in LLCPK$_1$ cells with or without PARP/GAPDH inhibitors. Each bar represents the mean ± SEM of four separate experiments except 6 h of hypoxia ($n = 5$). **$P < 0.0002$ *versus* control; *†$P < 0.05$ *versus* 6 h of hypoxia only; *$P < 0.0005$ *versus* 6 h of hypoxia.

hypoxia induced poly(ADP-ribose) (Figure S1B) and several poly(ADP-ribosyl)ated protein bands, including a band at approximately 37 kD, (Supplemental Figure S1C, lanes 3 and 4), suggesting potential PARP activation and GAPDH-poly(ADP-ribosyl)ation in LLCPK$_1$ cells after hypoxia. We subjected LLCPK$_1$ cells to hypoxia with or without the PARP inhibitor GPI-15427 and assayed GAPDH activity. GAPDH activity was significantly inhibited in LLCPK$_1$ cells after 6 or 12 h of hypoxic treatment (Figure 4, bars 3 and 5, respectively) compared with untreated control cells (Figure 4, bar 1). Subjecting the cells to hypoxia in the presence of GPI 15427 resulted in significant improvement of GAPDH activity at 6 h (Figure 4, bar 4) but not at 12 h (Figure 4, bar 6), compared with hypoxia treatment only (Figure 4, bars 3 and 5). Inhibition of PARP in untreated control cells (Figure 4, bar 2) did not cause significant changes in the GAPDH activity compared with controls (Figure 4, bar 1).

Inhibition of GAPDH Activity by PARP-1 Is Due to its Poly(ADP-ribosyl)ation

To confirm further the direct role of PARP-1 in the inhibition of GAPDH activity, we performed an *in vitro* cell-free assay involving purified PARP-1 and GAPDH proteins. GAPDH activity was significantly inhibited by activated PARP-1 (PARP-1 + activated DNA) in the presence of NAD^+ compared with GAPDH alone (Figure 5, bar 3 *versus* bar 1). Presence of the PARP-1 inhibitor GPI-15427 reversed the effect of activated PARP-1 on GAPDH activity (Figure 5, bars 4 and 5 *versus* bar 3), indicating a direct role of PARP-1 activity in the inhibition of GAPDH activity. Inclusion of NAD^+ alone in the incubation medium, without activated PARP-1, did not change GAPDH activity significantly (Figure 5, bar 2 *versus* bar 1). The proteins from the *in vitro* cell-free assay, when subjected to immunoblotting with anti-PAR antibody, demonstrated PAR modification of PARP and GAPDH in a PARP-1–dependent manner (see Supplementary Figure S2, B through E).
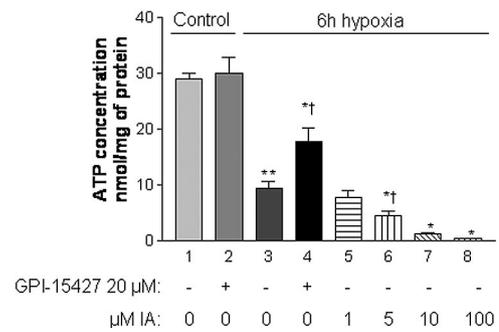
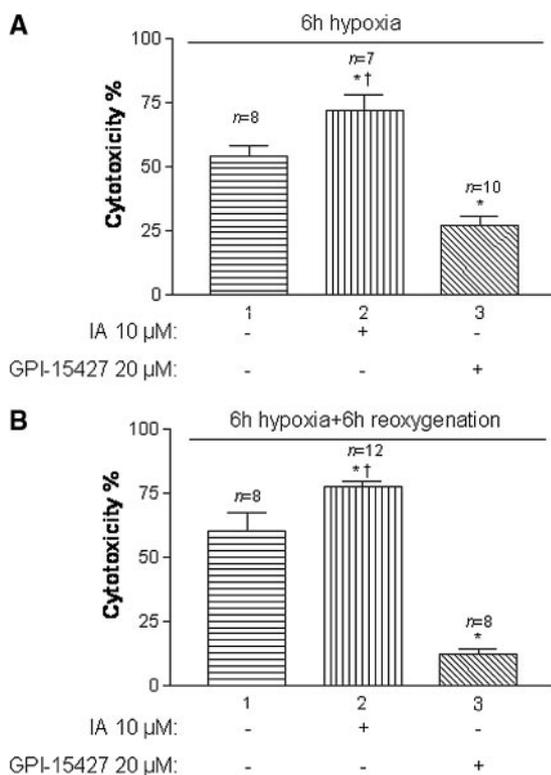### Effect of Hypoxia on ATP Levels in Cultured LLCPK$_1$ Cells

To examine whether energy levels are compromised after hypoxic injury in LLCPK$_1$ and whether PARP inhibitor preserves them, we measured ATP levels in LLCPK$_1$ cells immediately after hypoxic treatment so as to isolate ATP production by anaerobic respiration from that of aerobic respiration. As a positive control to indicate the significance of GAPDH activity in anaerobic ATP production, we used a GAPDH inhibitor, sodium iodoacetate (IA), as described previously.[26,27] A 45-min incubation of LLCPK$_1$ cells with IA resulted in significant inhibition of GAPDH activity compared with control. The inhibitory effect of IA was dosage-dependently enhanced with the increased concentration of the inhibitor (see Supplementary Figure S3). The concentration of iodoacetamide that was used in the study is comparable or lesser than the concentrations used in previous reports.[26,28,29] ATP levels were significantly decreased in LLCPK$_1$ cells after 6 h of hypoxic treatment when compared with control treatment (Figure 6, bar 3 *versus* bar 1). The PARP inhibitor GPI-15427 significantly preserved ATP levels in 6-h hypoxic-LLCPK$_1$ cells compared with hypoxia only (Figure 6, bar 4 *versus* bar 3). Interestingly, inhibiting GAPDH enzyme activity by IA along with 6 h of hypoxic treatment further decreased ATP levels significantly compared with hypoxia only, and this decrease was enhanced with the increasing concentration of GAPDH inhibitor (Figure 6, bars 6 through 8 *versus* bar 3), suggesting the significance of preserving GAPDH activity and ATP production from anaerobic glycolytic pathway. A concentration of 1 μM IA did not significantly decrease ATP levels compared with hypoxia only (Figure 6, bar 5 *versus* bar 3). Inhibition of PARP activity in control LLCPK$_1$ cells did not cause significant change in ATP levels (Figure 6, bar 2 *versus* bar 1).

Effect of GAPDH/PARP Inhibition on Cytotoxicity in Hypoxic LLCPK$_1$ Cells
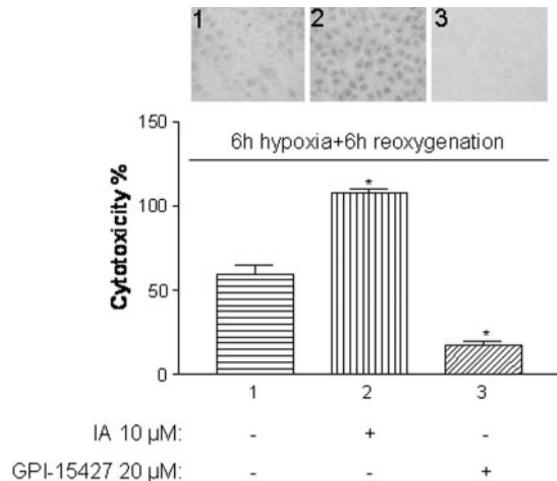
We then examined the percentage of cytotoxicity in hypoxic LLCPK$_1$ cells by measuring the concentration of lactate dehydrogenase (LDH) that was released from injured cells. We sub-

jected the LLCPK$_1$ cells to hypoxia with and without reoxygenation and in the presence/absence of GAPDH or PARP inhibitors and measured the percentage of cytotoxicity. The cytotoxicity percentage was significantly increased when GAPDH inhibitor was used along with 6 h of hypoxia compared with hypoxia only treatment (Figure 7A, bar 2 *versus* bar 1), whereas PARP inhibition significantly protected cells from injury after 6 h of hypoxia (Figure 7A, bar 3 *versus* bar 1). Similar results were found when LLCPK$_1$ cells were subjected to 6 h of hypoxia followed by 6 h of reoxygenation; GAPDH inhibition significantly increased cytotoxicity, whereas PARP inhibition protected cells (Figure 7B).

Our results from LDH assay experiments were further bolstered by Trypan blue spectrophotometric assay for necrotic cell death. We subjected LLCPK$_1$ cells to 6 h of hypoxia followed by 6 h of reoxygenation and measured Trypan blue absorbance, which was directly proportional to necrotic cell death. As in the case of LDH assay experiments, GAPDH inhibition together with hypoxia/reoxygenation significantly increased cell death compared with hypoxia/reoxygenation only (Figure 8, bar 2 *versus* bar 1), whereas PARP inhibition signif-

Figure 8. Trypan blue absorbance cytotoxicity assay. Effect of hypoxia and reoxygenation on cytotoxicity in LLCPK$_1$ cells in the presence/absence of GAPDH or PARP inhibitors. Each bar represents the mean ± SEM of four separate experiments. *$P < 0.0005$ *versus* 6 h of hypoxia with 6 h of reoxygenation. (Top) Trypan blue staining images of the respective treatments.

icantly decreased cell death (Figure 8, bar 3 *versus* bar 1). The images in Figure 8 represent Trypan blue–stained cells corresponding to the respective treatments. Trypan blue staining seemed to be enhanced when GAPDH inhibitor was used along with hypoxia/reoxygenation compared with hypoxia/reoxygenation only. Only trace levels of Trypan blue staining were visible in the hypoxic cells treated with the PARP inhibitor GPI-15427. Collectively, these data clearly indicate that PARP-mediated GAPDH inhibition after simulated ischemia led to exacerbation of cell injury in LLCPK$_1$ cells.

Figure 7. LDH cytotoxicity assay. (A) Effect of hypoxia on cytotoxicity in LLCPK$_1$ cells in the presence/absence of GAPDH or PARP inhibitors. Each bar represents the mean ± SEM. *†$P < 0.05$ *versus* 6 h of hypoxia only; *$P < 0.0005$ *versus* 6 h of hypoxia only. (B) Effect of hypoxia and reoxygenation on cytotoxicity in LLCPK$_1$ cells in the presence/absence of GAPDH or PARP inhibitors. Each bar represents the mean ± SEM. *†$P < 0.05$ *versus* 6 h of hypoxia with 6 h of reoxygenation; *$P < 0.0005$ *versus* 6 h of hypoxia with 6 h of reoxygenation.

DISCUSSION

The S3 segment of the proximal tubule or PST is extremely susceptible to IRI compared with other segments of the nephron[4,5]; however, the S$_3$ segment is less vulnerable to injury compared with S$_1$ and S$_2$ segments after selective inhibition of glycolytic process or mitochondrial respiration as opposed to IRI.[30] Although altered hemodynamic factors and differential glycolytic enzyme concentrations or activities were proposed to be the underlying causes,[1,31–33] the exact mechanisms leading to selective PST injury are largely unknown.

Our data clearly demonstrated that GAPDH activity is improved in the absence of PARP-1 compared with its presence after renal ischemia in the outer medullary nephron segments. We showed that GAPDH is poly(ADP-ribosyl)ated after IRI, suggesting the reduced GAPDH activity is due to its modification by poly(ADP-ribosyl)ation. Our previous finding that PARP-1 expression and activity are selectively upregulated in the S$_3$ segments[13,14] but not in other tubular segments after renal ischemia suggests that GAPDH-poly(ADP-ribosyl)ation and consequent inhibition of its activity exclusively occur in

the S_3 segment. Thus, although significant, the relatively small absolute difference that is observed in GAPDH activity (Figure 1) may be due to the relative changes in its activity in the whole outer medullary nephron segments used in the assay. GAPDH expression level was not altered among sham, WT, or PARP$^{-/-}$ ischemic renal tissues, thereby excluding the possibility of reduced expression as a mechanism of altered activity. Although we did not examine the role of ROS in GAPDH activity inhibition in this study, our previous studies did not observe any changes in the generation of ROS between WT and PARP$^{-/-}$ ischemic renal tissues.[13] Moreover, data from other laboratories suggested that oxidative modification is not responsible for the reduced GAPDH activity.[23,34]

To define further the mechanism and the significance of PARP-1–mediated inhibition of anaerobic ATP synthesis in exacerbation of hypoxic cell injury, we used LLCPK$_1$ cells as an *in vitro* cell culture model of IRI. It has been shown that, under *in vitro* cell culture conditions, LLCPK$_1$ cells tend to depend mainly on glycolysis.[35] Similarly, under control conditions, the freshly isolated proximal straight tubular cells have higher glycolytic rates compared with proximal convoluted tubules.[36] Hence, proximal straight tubular cells can revert to anaerobic glycolysis and be protected under hypoxia. Our data also show that lactate and ATP levels were significantly increased in the outer medulla of PARP$^{-/-}$ mouse kidneys compared with WT. This suggests that, under ischemia/hypoxic conditions, the glycolytic capacity of proximal straight tubular cells can be enhanced in the absence of PARP-1. We acknowledge that, although we cannot compare the glycolytic capacities of PST cells and LLCPK$_1$ cells, our data suggest that the glycolytic capacity is increased in PST cells under hypoxia and thus the use of LLCPK$_1$ cells as a model system can be justified. We conducted most of our *in vitro* experiments immediately after subjecting LLCPK$_1$ cells to hypoxia to isolate anaerobic respiration from aerobic metabolism as the sole source of ATP production. Our data clearly demonstrated that GAPDH activity was significantly blunted in LLCPK$_1$ cells subjected to 6 or 12 h of hypoxia, whereas PARP inhibition restored its activity after 6 h of hypoxic treatment. PARP inhibition could not restore GAPDH activity after 12 h of hypoxia, suggesting that either PARP-independent inhibition of GAPDH activity occurred under severe hypoxia or the injury at 12 h was too severe for recovery of cellular functions. Although GAPDH-poly(ADP-ribosyl)ation has been suggested to be the underlying mechanism for its inhibited activity in hyperglycemic aortic endothelial cells[23] and in both our *in vivo* and *in vitro* models of ischemia, direct evidence is lacking toward this end. To confirm this proposed mechanism, we performed *in vitro* cell-free GAPDH assay using purified GAPDH and PARP-1 enzymes. We showed that GAPDH activity was significantly inhibited in the presence of NAD$^+$ and activated PARP-1. That GAPDH activity was restored to control levels in the presence of PARP inhibitor clearly indicates that GAPDH modification by poly-(ADP-ribosyl)ation leads to inhibition of its activity. Furthermore, it rules out the possibility of direct binding of PARP-1 to

GAPDH as an alternate mechanism of inhibition. Western blot analysis of these proteins at the end of the *in vitro* cell-free GAPDH assay experiment revealed that both PARP-1 and GAPDH were poly(ADP-ribosyl)ated. To rule out the possibility that the reduction in GAPDH activity is due to nonspecific leakage of GAPDH out of the cytosol, possibly as a result of membrane damage, we assessed the levels of GAPDH protein and its activity after 6 h of hypoxia with or without PARP inhibitor in the cultured medium. Unlike the changes in the level of LDH, we observed no changes in the protein levels or activity of GAPDH (data not shown).

Under hypoxic/ischemic environment, cells cannot carry out oxidative phosphorylation because of unavailability of oxygen and hence revert to anaerobic metabolism. Measurement of ATP levels immediately after hypoxia shows the level of activation of anaerobic respiration. Our data demonstrate that ATP levels were depleted in hypoxic-LLCPK$_1$ cells in concordance with reduced GAPDH activity. PARP inhibition preserved ATP levels, suggesting restoration of anaerobic glycolysis by improved GAPDH activity; however, PARP inhibition could not replete ATP levels completely, suggesting that GAPDH may not be the "rate-limiting" enzyme and modulation of other key glycolytic enzymes such as phosphofructokinase or its product, fructose 1,6-biphosphate,[37] may also be involved in the metabolic control of anaerobic ATP synthesis under hypoxic conditions.[34,38] Nevertheless, the significant preservation of energy levels may be sufficient to reduce the necrotic cell death that was revealed by our cytotoxicity experiments.

To confirm that reduced GAPDH activity in fact leads to decreased ATP production by anaerobic glycolysis, we inhibited GAPDH activity using iodoacetamide, a GAPDH inhibitor, which inhibits the activity of GAPDH by reacting with the cysteines in its active site.[26,27] We demonstrated that IA dosage-dependently (1 to 100 μM) inhibited GAPDH activity. Subjecting LLCPK$_1$ cells to hypoxia in the presence of IA resulted in ATP depletion in a dosage-dependent manner, suggesting that inhibiting GAPDH activity prevents anaerobic glycolysis and consequent ATP production.

We and others previously showed that PARP-1 mediates ATP depletion and subsequent necrotic cell death after simulated renal ischemia[13,16]; however, the mechanisms by which PARP-1 elicits ATP depletion and exacerbation of PST injury after simulated ischemia was not elucidated. In this study, we showed that necrotic cell death was significantly enhanced in LLCPK$_1$ cells subjected to hypoxia with or without reoxygenation in a PARP-1–dependent manner; however, we did not observe any apoptotic cells immediately after hypoxia (data not shown). PARP inhibition offered significant protection whereas inhibition of GAPDH enhanced necrotic cell death in hypoxic LLCPK$_1$ in concordance with ATP depletion, suggesting GAPDH is a limiting factor in mediating anaerobic ATP synthesis and subsequent cell survival.

In summary, the study provides novel insights into the mechanisms by which PARP-1 mediates targeted acute PST

cell injury under ischemia/hypoxia. We showed that GAPDH was poly(ADP-ribosyl)ated and its activity was inhibited in both *in vivo* and *in vitro* models of simulated renal ischemia. Inhibition of GAPDH activity by PARP-1 led to decreased ATP production by anaerobic glycolysis. PARP-1–mediated inhibition of anaerobic ATP synthesis resulted in exacerbation of proximal tubular cell injury and consequent necrosis. Taken together, our data indicate that GAPDH-poly(ADP-ribosyl)-ation and subsequent inhibition of anaerobic respiration lead to exacerbation of ATP depletion in PST to induce selective injury after IRI.

CONCISE METHODS

Materials
All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO) unless otherwise noted.

Animal Models, Surgical Procedures, and Tissue Preparation
All animal procedures were performed after previous approval by the Institutional Animal Care and Use Committee at the University of Nebraska Medical Center. A total of 129 SV (WT) or PARP-1$^{-/-}$ mice (approximately 20 g) were purchased from Jackson Laboratories (Bar Harbor, ME). IRI was induced by bilateral renal pedicle clamping, as described previously.[13] Briefly, we clamped both renal pedicles for 37 min by using microaneurysm clamps and observed reflow visually after the clamps were removed. The mice were allowed to recover for variable duration before being killed. Sham-operated mice underwent the same surgical procedure except the renal pedicles were not occluded. All mice were given free access to food and water. At the end of the each experiment, we obtained tissue from the outer medullary region (rich in PST/S3 segments) from slices made using a Stadie-Riggs microtome (Thomas Scientific, Swedesboro, NJ),[36] snap-frozen with liquid nitrogen, and stored at −80°C for future experiments.

Cell Culture Conditions
The porcine-derived proximal tubular cell line LLCPK$_1$ (ATCC, Rockville, MD) was cultured to 80 to 90% confluent monolayer cultures as described previously.[39] We used hypoxia to simulate ischemic injury in *in vitro* experiments using a pouch system providing a CO_2-enriched anaerobic environment (BBL GasPak Pouch System; Becton Dickinson, Sparks, MD) as described previously.[40] Hypoxic injury was induced by incubating 80 to 90% confluent monolayer of LLCPK$_1$ cells for 6 or 12 h in Krebs-Ringer bicarbonate buffer (in mM: 115 NaCl, 1 KH$_2$PO$_4$, 3.5 KCl, 1 MgSO$_4$, 1.25 CaCl$_2$, and 25 NaHCO$_3$) at 37°C.[41,42] In some experiments, cells were allowed to recover after hypoxia for 6 h by reoxygenation in normal growth medium. GAPDH inhibition was achieved by exposure to 1 to 100 μM (10 μM in most of the experiments) sodium IA (a specific GAPDH inhibitor).[26,27] A novel highly potent PARP inhibitor, GPI-15427 (MGI Pharmaceuticals, Andover, MA), was used in some studies.[43,44,45] The final concentration of GPI-15427 used in the experiments was 20 μM unless otherwise stated.

GAPDH Activity Assay
We centrifuged protein lysates from frozen kidney tissue or cultured LLCPK$_1$ cells at 4°C for 10 min at 13,000 rpm to remove cellular debris. We prepared the cytosolic fraction by centrifuging the tissue or cell lysate at $100,000 \times g$ at 4°C for 30 min. We measured protein concentration with Bio-Rad D$_c$ protein assay reagents using the manufacturer's protocol. We determined the GAPDH enzyme activity by the reduction of NAD$^+$ with GAPDH as a substrate in arsenate buffer, as described previously.[23,45] Briefly, we added 1 to 5 μg of cytosolic protein to 1 ml of assay buffer at 25°C and measured the rate of absorbance by continuous monitoring for 30 min at 340 nm using a spectrophotometer (Biomate 3; Thermo Scientific, Waltham, MA). The enzyme activity was expressed in nmol/min per mg of protein.

Immunoprecipitation
We immunoprecipitated total protein (500 μg) from tissue lysate with 4 μg of indicated antibody and 20 μl of protein A/G plus (Santa Cruz Biotechnology, Santa Cruz, CA) as per the manufacturer's protocol. We resuspended the immunoprecipitated protein in SDS loading dye and performed Western blotting analysis as described previously,[13] using antibodies against PAR (BD Biosciences, San Jose, CA), GAPDH (Novus, Littleton, CO), or PARP (Trevigen, Gaithersburg, MD). The signal was detected with ECL-plus system according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). For biotinylated proteins, we performed blocking with 1% BSA in PBST, followed by biotin detection with 1:50,000 dilutions of streptavidin–horseradish peroxidase.

Immunofluorescence Microscopy
We performed immunofluorescence staining of LLCPK$_1$ cells with primary antibodies against PAR (Trevigen), AQP1 (Alpha Diagnostic, San Antonio, TX), or AQP7 (Santa Cruz Biotechnology) as described previously.[39] Samples were viewed using a Leica DMR fluorescence microscope and the images were captured with an Optronics digital camera.

In Vitro Biotinylation Assay to Detect PARP Activity
We performed *in vitro* biotinylation to detect PARP activity in hypoxia-injured LLCPK$_1$ cells as described previously.[21,46] Briefly, 50 μg of protein was incubated with 60 μM biotinylated NAD in a 50-μl final volume of PARP reaction buffer (50 mM Tris-HCl [pH 8.0] and 25 mM MgCl$_2$) at 37°C for 1 h. We terminated the reaction with SDS loading dye and subjected the samples to Western blot analysis to detect biotin expression, an indirect method to detect PAR expression.

ATP Assay
We performed ATP assay on tissue/cellular extracts using the Enlighten ATP assay system (Promega, Madison, WI) as previously described.[13] Cellular ATP levels were expressed as nmol/μg protein.

Lactate Assay
We used the same tissue extracts used to measure ATP levels to measure lactate levels. We measured the tissue lactate levels using Lactate Assay Kit, EnzyChrom (BioAssay Systems, Hayward, CA) according

to the manufacturer's protocol. The lactate levels were expressed in $\mu M/\mu g$ protein.

We performed *in vitro* cell-free GAPDH assay as described previously,[47,48] using unlabeled NAD^+ or 6-biotin-17-NAD^+ (biotin-NAD^+), purified rabbit muscle GAPDH enzyme, activated DNA, and recombinant human PARP-1 enzyme (Trevigen, MD). The final reaction was performed in a reaction buffer (50 mM Tris-HCl [pH 8.0] and 25 mM $MgCl_2$) containing 5 μg of GAPDH, 5 μg of activated DNA, 10 μM biotin-NAD^+ or 1 mM of NAD^+, 20 or 200 μM GPI-15427, and 1 μl of PARP-1 enzyme (20 U). The sample was mixed well and incubated at 37°C for 1 h. Immediately after the reaction, we used 1 μl of the sample to measure GAPDH activity and mixed the rest of the sample with SDS loading dye. We performed Western blot analysis to detect biotin-labeled proteins, PAR, GAPDH, or PARP-1 with the indicated antibodies.

Cell Death Determination by LDH Release Assay

We performed LDH release assay using the cytotoxicity detection kit (Roche, Indianapolis, IN). We performed cell death determination by Trypan blue absorbance assay as described previously,[49] with slight modifications. Briefly, we added 0.05% Trypan blue to each culture well at the end of each treatment and placed the plate in the incubator at 37°C for 15 min. We removed dye-containing medium by three washes with ice-cold PBS and lysed the cells with 1 ml of 1% SDS followed by absorbance measurements at 590 nm. The absorbance value from 2% Triton-X 100–treated cells was considered as a high control, whereas absorbance value from cells growing in normal medium without any treatment was considered as low control. We measured the cytotoxicity percentage by the formula

$$\frac{\text{Experimental value-low control}}{\text{High control-low control}} \times 100$$

Statistical Analysis

All data are expressed as means $\pm$ SEM. We used one-way ANOVA to compare the mean values of all groups. We used unpaired t test to compare the means of two different groups. $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS

DISCLOSURES
None.

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