Mice Lacking the 110-kD Isoform of Poly(ADP-Ribose) Glycohydrolase Are Protected against Renal Ischemia/Reperfusion Injury

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The role of poly(ADP-ribose) (PAR) glycohydrolase (PARG) in the pathophysiology of renal ischemia/reperfusion (I/R) injury is not known. Poly(ADP-ribosyl)ation is rapidly stimulated in cells after DNA damage caused by the generation of reactive oxygen and nitrogen species during I/R. Continuous or excessive activation of poly(ADP-ribose) polymerase-1 produces extended chains of ADP-ribose on nuclear proteins and results in a substantial depletion of intracellular NAD⁺ and subsequently, ATP, leading to cellular dysfunction and, ultimately, cell death. The key enzyme involved in polymer turnover is PARG, which possesses mainly exoglycosidase activity but can remove olig(ADP-ribose) fragments via endoglycosidic cleavage. Thus, the aim of this study was to investigate whether the absence of PARG₁₁₀ reduced the renal dysfunction, injury, and inflammation caused by I/R of the mouse kidney. Here, the renal dysfunction and injury caused by I/R (bilateral renal artery occlusion [30 min] followed by reperfusion [24 h]) in mice lacking PARG₁₁₀ the major nuclear isoform of PARG, was investigated. The following markers of renal dysfunction and injury were measured: Plasma urea, creatinine, aspartate aminotransferase, and histology. The following markers of inflammation were also measured: Myeloperoxidase activity, malondialdehyde levels, and plasma nitrite/nitrate. The degree of renal injury and dysfunction caused by I/R was significantly reduced in PARG₁₁₀-deficient mice when compared with their wild-type littermates, and there were no differences in any of the biochemical parameters measured between sham-operated PARG₁₁₀⁻/⁻ mice and sham-operated wild-type littermates. Thus, it is proposed that endogenous PARG₁₁₀ plays a pivotal role in the pathophysiology of I/R injury of the kidney.


Poly(ADP-ribosylation) of proteins in eukaryotic cells is catalyzed by poly(ADP-ribose) polymers (PARP), regulating many cellular processes, such as DNA replication, repair, recombination, cell proliferation, death, gene transcription, and inflammation. Ischemia/reperfusion (I/R) leads to the generation of reactive oxygen species (ROS) and nitrogen species, which cause single- and double-strand breaks in DNA and, hence, activation of the DNA repair enzyme PARP-1 (E.C. 2.4.2.30). PARP-1 binds rapidly to strand breaks in DNA and catalyzes the formation of long and branched poly(ADP-ribose) (PAR) polymers on nuclear target proteins using NAD⁺ as a substrate (1,2). Continuous activation of PARP-1 produces extended chains of ADP-ribose on nuclear proteins (including PARP-1 itself), which results in a rapid and substantial depletion of intracellular NAD⁺ and, subsequently, ATP, leading to cellular dysfunction and, ultimately, cell death (3,4). PAR polymers, however, are extremely short-lived in vivo (t₁/₂, approximately 1 min), being rapidly degraded (2 to 3 min after PAR formation) by the constitutively active poly(ADP-ribose) glycohydrolase (PARG; E.C. 3.2.1.143). Two isoforms of PARG are ubiquitously expressed, a 110-kD isoform (PARG₁₁₀), localized mainly in the nucleus, and a 60-kD isoform (PARG₆₀), localized in the cytoplasm (5). Although both isoforms exhibit exo- and endoglycosidase activity, PARG₁₁₀ is the major form of PARG in the nucleus (6,7).

Inhibitors of PARP activity reduce the tissue injury caused by I/R of the heart (8,9), brain (10), gut (11), liver (12), and kidney (13). Most notable, the degree of tissue injury caused by I/R in the heart (14), brain (15), gut (16), and most recently kidney (17) is reduced in mice in which the gene encoding for PARP-1 has been disrupted (PARP-1⁻/⁻ mice). These studies support the view that the excessive activation of PARP-1 plays a key role in the pathophysiology of I/R injury. The levels of PARP-1 and PARG₁₁₀ proteins seem to be regulated individually (18) but act

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sequentially and are closely coordinated (19). This may suggest that PARG110 could be a possible therapeutic target in I/R injury. Two recent studies have demonstrated that amide-based, DMSO PARG inhibitors attenuate I/R injury of the brain (GPI 16552) in the rat (20) and the organ injury and inflammation caused by zymosan (GPI 18214) in the mouse (21).

Thus, it has been suggested that PARG110 contributes to I/R injury of many organs, including the kidney. To test this hypothesis, we performed I/R of the kidney in PARG110-deficient mice (PARG110-/-). We then compared the effects of ischemia and reperfusion on renal function, injury, and inflammation in PARG110-/- mice and in their wild-type littermates. Our data demonstrate that PARG110 plays a pivotal role in I/R of the kidney.

Materials and Methods

Animals

Eighteen male, wild-type mice from a pure genetic background (129/Sv) and 20 male PARG110-/- mice (25 to 30 g) were used in this study. PARG110-/- mice were generated as described previously (6). Genotyping of mice was performed by PCR using two primers located in intron 1 (5’-TCTTITATTGAGCTGGCT-3’ and 5’-GGTTAAACGAGGTTTAAAT-3’) and one primer located in exon 4 (5’-CAACAATCCACGAGACCC-3’) of the PARG gene (6). PARG110-/- mice exhibited no signs of gross or microscopic abnormality (6). Mice were allowed access to food and water ad libitum and were cared for in compliance with French regulations on protection of animals used for experimental and other scientific purposes, as well as with the European Economic Community regulations (O.J. of E.C. L358/1 12/18/1986).

Renal I/R

Mice were anesthetized using chloral hydrate (125 mg/kg, intraperitoneally), and core body temperature was maintained at 37°C using a homeothermic blanket. Renal I/R injury was assessed in wild-type and PARG110-/- mice. After a midline laparotomy was performed, mice were then divided into the following four groups:

1. I/R wild-type group: wild-type mice that underwent renal ischemia for 30 min followed by reperfusion for 24 h (n = 8)
2. I/R PARG110-/- group: PARG110-/- knockout mice that underwent renal ischemia for 30 min followed by reperfusion for 24 h (n = 10)
3. Sham wild-type group: wild-type mice that were subjected to the surgical procedures described above but were not subjected to renal I/R (n = 10)
4. Sham PARG110-/- group: PARG110-/- knockout mice that were subjected to the surgical procedures described above but were not subjected to renal I/R (n = 10)

Mice were maintained under anesthesia for the duration of ischemia (30 min). After a midline laparotomy was performed, mice from the I/R groups were subjected to bilateral renal ischemia for 30 min, during which the renal arteries and veins were occluded using microaneurysm clamps (22). The time of ischemia chosen was based on that found to maximize reproducibility of renal functional impairment while minimizing mortality in these animals (22). After the renal clamps were removed, the kidneys were observed for an additional 5 min to ensure reflow, after which 1 ml of saline at 37°C was injected into the abdomen and the incision was sutured in two layers. Mice then were returned to their cages, where they were allowed to recover from anesthesia and observed for 24 h. Sham-operated mice underwent identical surgical procedures to I/R mice, with the exception that microaneurysm clamps were not applied.

Measurement of Biochemical Parameters

At the end of the 24-h reperfusion period, mice were anesthetized using chloral hydrate (125 mg/kg, intraperitoneally) and 1-ml blood samples were collected from mice via cardiac puncture. The samples were centrifuged (6000 x g for 3 min) to separate plasma. All plasma samples were frozen and stored at –80°C and analyzed for biochemical parameters. Plasma urea and creatinine concentrations were used as indicators of renal (glomerular) dysfunction (22). The rise in the plasma levels of aspartate aminotransferase (AST), an enzyme located in the proximal tubule, was used as an indicator of reperfusion injury (22).

Histologic Evaluation

Kidneys were removed from mice at the end of the experimental period, after the renal pedicle was tied, and cut in a sagittal section into two halves. These tissue samples were fixed in 10% (wt/vol) formaldehyde in PBS (0.01 M; pH 7.4) at room temperature for 1 d. After dehydration using graded ethanol, the tissue was embedded in Paraplast (Sherwood Medical, Mahwah, NJ) and cut in fine (8 μm) sections and mounted on glass slides. Sections then were deparafinized with xylene, counterstained with hematoxylin and eosin, and viewed under a light microscope (Diaphor 22; Leitz, Milan, Italy).

Myeloperoxidase Activity

Myeloperoxidase (MPO) activity in kidney samples was determined as an index of polymorphonuclear cell (PMN) accumulation, as described previously (22). There is some evidence that both the MPO and naphthol-AS-D-chloracetate esterase assays can cross-react with monocytes and macrophages (23). We recently demonstrated, however, that PMN counts from histologic sections and MPO activitycorrelate positively (24). Kidneys were homogenized in a solution that contained 0.5% hexa-decyl-trimethyl-ammonium bromide and 10 mM 3-(N-morpholino)-propane-sulfonic acid dissolved in 80 mM sodium phosphate buffer (pH 7.4) and centrifuged for 30 min at 20,000 x g at 4°C. An aliquot of the supernatant then was allowed to react with a solution of tetramethyl-benzenide (16 mM) and 1 mM hydrogen peroxide. The rate of change in absorbance was measured by a spectrophotometer at 650 nm. MPO activity was defined as the quantity of enzyme that degraded 1 μmol of peroxide/min at 37°C.

Malondialdehyde Measurement

Malondialdehyde (MDA) levels in kidney samples were determined as an indicator of lipid peroxidation, as described previously (22). Tissues were homogenized in a 1.15% KCl solution. An aliquot of the homogenate was added to a reaction mixture that contained 200 μl of 8.1% SDS, 1500 μl of 20% acetic acid (pH 3.5), 1500 μl of 0.8% thiobarbituric acid, and 700 μl of distilled water. The mixture then was boiled for 1 h at 95°C and centrifuged at 3000 × g for 10 min. The absorbency of the supernatant was measured by spectrophotometry at 550 nm.

Nitrite/Nitrate Measurement

Nitrate in the plasma was enzymatically converted to nitrite using a protocol similar to previously described (25). Briefly, nitrate was stoichiometrically reduced to nitrite by incubation of the sample aliquot (50 μl) for 15 min at 37°C in the presence of nitrate reductase (E.C. 1.6.6.2, 0.1 iu/ml), β-NADPH (50 μM), and flavin adenine dinucleotide (FAD; 50 μM) in a final volume of 80 μl. When nitrate reduction was complete, unused β-NADPH, which interferes with the subsequent nitrite determination, was oxidized by l-lactate dehydrogenase (LDH; 100 IU/ml)
and sodium pyruvate (100 mM) in a final reduction volume of 100 µl and incubated for 5 min at 37°C. Subsequently, total nitrite in the medium was assayed by adding 100 µl of Griess reagent (0.1% [wt/vol] naphthalhlethylenediamine dihydrochloride in water and 1% [wt/vol] sulfanilamide in 5% [vol/vol] phosphoric acid) to each sample. Optical density at 590 nm (OD590) was measured using a spectrophotometer (Anthos Labtec Microplate Reader HT3, v1.21). Total nitrite/nitrate concentration for each sample was calculated by comparison of the OD590 of a standard solution of sodium nitrate (also stoichiometrically converted to nitrite) prepared in ultradistilled water.

Materials
Unless otherwise stated, all compounds used in this study were purchased from Sigma-Aldrich Company Ltd. (Milan, Italy). All solutions used in vivo were prepared using nonpyrogenic saline (0.9% [wt/vol] NaCl).

Statistical Analyses
All values described in the text and the figures are expressed as mean ± SEM for n observations. Each data point represents biochemical measurements obtained from eight to 10 separate animals. One-way ANOVA with Bonferroni posttest was performed using GraphPad Prism version 4.02 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com), and P < 0.05 was considered to be significant.

Results
Renal Dysfunction (Plasma Urea and Creatinine) in PARG110 Mice
When compared with sham-operated mice, I/R caused a significant increase in the plasma levels of urea and creatinine in wild-type mice (Figure 1), suggesting a significant degree of renal dysfunction. When compared with wild-type mice that were subjected to I/R, plasma levels of urea and creatinine and, therefore, renal dysfunction were significantly lower in PARG110 mice that were subjected to I/R (Figure 1).

Reperfusion Injury (Plasma AST) in PARG110 Mice
When compared with sham-operated mice, I/R caused a significant increase in the plasma level of AST in wild-type mice, suggesting significant reperfusion injury (Figure 2). When compared with wild-type mice that were subjected to I/R, the plasma level of AST and, therefore, reperfusion injury was significantly lower in PARG110 mice that were subjected to I/R (Figure 2), similar to values obtained from sham-operated mice (Figure 2).

Renal Injury (Histologic Evaluation) in PARG110 Mice
When compared with sham-operated mice (Figure 3A), histologic examination of kidneys that were obtained from wild-type mice that were subjected to I/R demonstrated a significant degree of renal injury (Figure 3B). Specifically, kidneys that were obtained from these animals exhibited degeneration of tubular structure, tubular dilation, swelling and necrosis, and luminal congestion. In contrast, renal sections that were obtained from PARG110 mice that underwent I/R (Figure 3C) demonstrated a marked reduction in the severity of these histologic features of renal injury when compared with kidneys that were obtained from wild-type mice that were subjected to I/R only (Figure 3B).

Renal Inflammation (MPO Activity, MDA Levels, and Nitrite/Nitrate Levels) in PARG110 Mice
When compared with sham-operated mice, the kidneys that were obtained from wild-type mice that were subjected to I/R demonstrated a significant increase in MPO activity (Figure 4A).
suggesting increased PMN infiltration into renal tissues. The increase in the tissue level of MPO seen in PARG110 mice that were subjected to I/R (Figure 4A) was significantly smaller than those seen in their wild-type littermates that were subjected to I/R.

When compared with sham-operated mice, the kidneys that were obtained from wild-type mice that were subjected to I/R demonstrated a significant increase in MDA levels (Figure 4B), suggesting increased lipid peroxidation in renal tissues. The increase in the tissue level of MDA seen in PARG110 mice (Figure 4B) was significantly smaller than those seen in their wild-type littermates that were subjected to I/R.

When compared with sham-operated mice, the plasma that was obtained from wild-type mice that were subjected to I/R demonstrated a significant increase in nitrite/nitrate levels (Figure 4C), suggesting increased nitric oxide formation. The increase in the plasma level of nitrite/nitrate seen in PARG110 mice that were subjected to I/R (Figure 4C) was significantly smaller than those seen in their wild-type littermates that were subjected to I/R. There were no differences in any of the above biochemical parameters measured between sham-operated PARG110 mice or sham-operated wild-type littermates (Figures 1, 2, and 4).

**Discussion**

Mice that were subjected to renal I/R demonstrated characteristic signs of renal dysfunction, injury, and inflammation. Specifically, renal I/R caused (1) renal dysfunction (increased plasma creatinine and urea levels), (2) reperfusion injury (increased plasma MPO and MDA levels), and (3) nitric oxide formation (increased plasma nitrite/nitrate levels). The results suggest that PARG110 mice have a reduced response to renal I/R, which may be due to a protective mechanism against oxidative stress and inflammation.

**Figure 2.** Reperfusion injury in PARG110 mice. Plasma aspartate aminotransferase (AST) levels were measured, from PARG110 mice, as biochemical markers of reperfusion injury subsequent to sham operation (WT sham, n = 10; PARG110 sham, n = 10) or renal I/R (WT I/R, n = 8; PARG I/R, n = 10). Data represent mean ± SEM for n observations; *P < 0.05 versus WT I/R group; +P < 0.05 versus WT sham group.

**Figure 3.** Renal injury in PARG110 mice: Histologic examination. A renal section taken from a sham-operated mouse (A) is compared with that of kidney sections prepared from mice that were subjected to ischemia followed by reperfusion for 24 h demonstrated recognized features of renal injury (B), including glomerular degeneration (GD), tubular dilation (TD), and tubular congestion (TC). PARG110 mice that were subjected to I/R (C) displayed reduction in renal injury. Figures are representative of at least three experiments performed on different days. Magnification, ×150 (hematoxylin and eosin).
creased plasma AST levels), (3) characteristic histologic signs of marked tubular injury, (4) PMN infiltration (increased MPO activity), (5) lipid peroxidation (increased MDA levels), and (6) nitric oxide formation (increased plasma nitrite/nitrate levels). All of these data confirmed a well-known pattern of renal dysfunction and injury caused by I/R of the kidney (26–28) and that renal I/R causes both renal and tubular dysfunction (29). We show here for the first time that inhibition of PARG110 via genetic disruption can attenuate the injury, dysfunction, and inflammation caused by renal I/R in the mouse.

In 1997, we demonstrated for the first time that inhibitors of PARP activity reduce the tissue injury associated with I/R of the heart and skeletal muscle in vivo. We therefore proposed that the activation of PARP contributes to the pathophysiology of I/R injury (30). In 1999, we demonstrated that activation of PARP mediates the cell death caused by hydrogen peroxide in cultured proximal tubule cells. On the basis of this work, we proposed that the excessive activation of PARP plays a role in the pathophysiology of renal disorders associated with oxidant stress (31). In 2000, we (32) and others (33) reported that inhibitors of PARP activity reduce the tissue injury caused by I/R of the kidney. What is the role of PARP-1 in cell injury? Exposure of cells to oxidants such as superoxide, hydrogen peroxide, and nitric oxide leads to the subsequent generation of hydroxyl radicals and peroxynitrite, which causes DNA damage within seconds, leading to excessive activation of PARP-1 (34,35). Excessive activation of PARP-1 results in the subsequent depletion of NAD+ pools within minutes, the main consequence of which is ATP depletion (36). NAD+ is an essential co-factor in glycolysis. It is required for the production of 1,3-diphosphoglycerate from inorganic phosphate and glyceraldehyde-3-phosphate. NAD+ is also required in fatty acid oxidation, which therefore is also affected by PARP-1 activation (37). In addition, ATP is consumed in the resynthesis of NAD+ from nicotinamide-released ADP-ribosylation (38). Treatment with PARP inhibitors, such as 3-aminobenzimide, does not affect the development of DNA strand breaks but does block NAD+ and ATP depletion, improving cell viability (34,39). Studies on PARP-1 mice have also supported the role of PARP-1 in oxidant-mediated cell injury. Pulmonary epithelial cells from PARP-1 mice are more resistant to ROS-mediated injury when compared with wild-type littermates (40), as are pancreatic islet cells against nitric oxide–mediated injury (41). Although cellular suicide may seem drastic, it represents a physiologic mechanism by which cells with severe DNA damage are eliminated. The “suicide hypothesis” is a safety mechanism that prevents cells with severe DNA damage from attempting ineffectually to repair themselves and consequently surviving with high mutation frequency. This reduces the likelihood of cells’ surviving with highly mutant phenotypes and therefore reduces the potential for malignant transformation.

The pharmacologic tools that are available to study the role of PARG in disease models are very limited, but some synthetic and nonsynthetic PARG inhibitors are available. In 2001, Ying et al. (42) used hydrolyzable tannins such as gallotannin (extracted from green tea and pine cones) and nobotanin B (extracted from the plant Tibouchina semidecandra Cogn.) to inves-
tigate their beneficial effects in PARP-mediated neuronal cell death. These compounds are able to inhibit PARP by competing with PAR at binding sites on PARP. They demonstrated that these natural PARP inhibitors are unable to inhibit PARP-1 directly but by preventing PARP-1-mediated cell death by slowing the turnover of PAR and thus limiting NAD+ consumption (42). However, it is possible that (at least some) of the observed beneficial effects of these agents are due to nonspecific effects. For instance, Falsig et al. (43) reported that the cytoprotective properties of gallotannin were not due to the inhibition of PARP but secondary to the ability of gallotannin to scavenge ROS. In fact, gallotannin enhanced the damage in in vivo models, and the maximal inhibition of PARP achieved with GPI 16552 was only 40% (43). It was concluded that neither GPI 16552 nor gallotannin is suitable for the evaluation of PAR in cellular death models and that any previous conclusions drawn from the use of these compounds should be interpreted with caution (43).

The use of genetically modified mice has advanced our knowledge of mechanisms that are involved in conditions such as I/R of the kidney (44). This study uses, for the first time, mice deficient for PARG110 in a model of brain I/R (20). It was found that GPI 16552 had no effect on astrocyte death models, and the maximal inhibition of PARP activity with GPI 16552 was only 40% (43). It was concluded that neither GPI 16552 nor gallotannin is suitable for the evaluation of PAR in cellular death models and that any previous conclusions drawn from the use of these compounds should be interpreted with caution (43).

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