

Inhibition of poly(ADP-ribose) polymerase attenuates ischemic renal injury in rats

DANIEL R. MARTIN, ANDREW J. P. LEWINGTON,
MARC R. HAMMERMAN, AND BABU J. PADANILAM
*George M. O'Brien Center, Renal Division, Department of Medicine,
Washington University School of Medicine, St. Louis, Missouri 63110*

Received 28 February 2000; accepted in final form 10 July 2000

Martin, Daniel R., Andrew J. P. Lewington, Marc R. Hammerman, and Babu J. Padanilam. Inhibition of poly(ADP-ribose) polymerase attenuates ischemic renal injury in rats. *Am J Physiol Regulatory Integrative Comp Physiol* 279: R1834–R1840, 2000.—The enzyme, poly(ADP-ribose) polymerase (PARP), effects repair of DNA after ischemia-reperfusion (I/R) injury to cells in nerve and muscle tissue. However, its activation in severely damaged cells can lead to ATP depletion and death. We show that PARP expression is enhanced in damaged renal proximal tubules beginning at 6–12 h after I/R injury. Intraperitoneal administration of PARP inhibitors, benzamide or 3-amino benzamide, after I/R injury accelerates the recovery of normal renal function, as assessed by monitoring the levels of plasma creatinine and blood urea nitrogen during 6 days postischemia. PARP inhibition leads to increased cell proliferation at 1 day postinjury as assessed by proliferating cell nuclear antigen and improves the histopathological appearance of kidneys examined at 7 days postinjury. Furthermore, inhibition of PARP increases levels of ATP measured at 24 h postischemia compared with those in vehicle-treated animals. Our data indicate that PARP activation is a part of the cascade of molecular events that occurs after I/R injury in the kidney. Although caution is advised, transient inhibition of PARP postischemia may constitute a novel therapy for acute renal failure.

acute renal failure; apoptosis; benzamide; DNA repair

ACUTE RENAL FAILURE (ARF) is the most costly kidney disease in hospitalized patients. At present, there is no pharmacological intervention proven to attenuate cellular injury or augment tissue regeneration after ARF (4, 6, 25). To develop such a treatment, a fundamental understanding of the mechanisms of cellular injury and regeneration after the insult is essential. One means to acquire such an understanding is through the use of animal models of ischemia-reperfusion injury (5, 16).

In models of ischemic tissue injury, cellular DNA damage results from the generation of reactive radicals, such as superoxide anions, hydrogen peroxide, nitric oxide, or peroxynitrite, during tissue reperfusion. In the presence of single- or double-strand breaks in DNA, a chromatin-bound enzyme, poly(ADP-ribose) polymerase (PARP),

transfers the ADP-ribose moiety of nicotinamide adenine dinucleotide (NAD) to nuclear proteins and to itself (3, 8, 14, 23). Proposed functions of PARP include stabilization of V-shaped DNA conformations and facilitation of DNA access for various repair enzymes (14).

The activation of PARP results in a depletion of intracellular NAD, which can only be replenished via a reaction that consumes ATP. Ischemia-reperfusion injury that results in substantial DNA degradation requires that cells consume large amounts of ATP to support poly(ADP-ribosylation). For this reason, whereas a moderate activity of PARP protects cellular genome integrity, its excessive activation can lead to cell death secondary to ATP depletion (28, 35).

Activation of PARP is a consequence of ischemic injury in the brain, retina, heart, and skeletal muscle (10, 15, 27, 28). To characterize the effect of reducing PARP activity in vivo, several inhibitors of PARP, including benzamide and 3-amino benzamide (3-AB) (1), have been administered to animals after ischemic injury to these organs. Administration of PARP inhibitors leads to a significant reduction of brain infarct volume in a model of focal cerebral ischemia in rats (15, 27), an amelioration of the ischemia-reperfusion damage to the retina in rats (10), and a reduction in the infarct size caused by ischemia-reperfusion of the heart or skeletal muscle in rabbits (28).

The role that PARP plays in the process of recovery from the acute renal failure that follows ischemia-reperfusion injury to the kidney is undefined. Accordingly, using a rat model, we determined whether PARP is expressed in kidney postischemia, and we examined the effect of the administration of PARP inhibitors on renal function, cellular regeneration, ATP content, and histopathology after injury. Our data indicate that a transient inhibition of PARP may be a novel approach for the therapy of acute renal failure.

METHODS

Rat model of acute renal failure. Male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing ~225–249 g were

Address for reprint requests and other correspondence: B. J. Padanilam, Renal Division, Campus Box 8126, 660 South Euclid Ave, Washington Univ. School of Medicine, St. Louis, MO 63110 (E-mail: bpadanil@imgate.wustl.edu).

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used in the experiments. Ischemia-reperfusion injury resulting in ARF was induced by 60 min of bilateral renal artery clamping as described previously (2, 17, 20). Blood was collected through a tail vein every 24 h postinjury for 7 days for measurement of creatinine and blood urea nitrogen (BUN) performed as in prior studies (17). The animals were killed at 1 or 7 days postinjury, and both kidneys were processed for histological analysis as before (17).

We have shown in our model (2) and others have shown (32) that it is the cells in the S3 segment of the proximal tubule exclusively that undergo progressive cell injury and death and that are exfoliated into the tubular lumen post-ischemia. It is the S3 segment exclusively that is repopulated via mitotic division of surviving cells beginning 1–2 days after the insult. We have shown in our model (2) that papillary proliferations arise in the S3 segment 5–7 days postischemia as part of the regenerative process. Others have shown (34) that proliferating cell nuclear antigen (PCNA), a marker for the G₁-S transition in the cell cycle and hence mitogenesis, is detected primarily in the S3 segment, peaking at 2 days postischemia.

Administration of PARP inhibitors to rats. The PARP inhibitors benzamide and 3-AB were purchased from Sigma Chemicals (St. Louis, MO). The inhibitors were dissolved in saline at a concentration of 16 mg/ml for benzamide and 5 mg/ml for 3-AB.

Benzamide (40 mg/kg) or 3-AB (10 mg/kg) was administered intraperitoneally every 8 h for 24 h beginning immediately after ischemic injury (4 doses). Vehicle-treated rats received four saline injections instead. In control experiments, we determined that the administration of benzamide or 3-AB to sham-operated rats in this manner had no effect on levels of creatinine or BUN measured daily for 6 days postinjury. Statistical analysis was performed using the ANOVA-Bonferroni multiple comparison test.

Histology and immunohistochemistry. Histology and immunohistochemistry were performed using 5- μ m sections of paraffin-embedded Bouin's-fixed kidneys as before (21). Monoclonal antibody (C2.10) for PARP (Enzyme Systems Products, Livermore, CA) was applied in one-tenth concentration of the blocking buffer for 1 h at room temperature. Detection was performed using the Histostain SP kit (Zymed, San Francisco, CA) as we previously described (19, 21). The specificity of staining was verified by substituting nonimmune antibodies of identical immunoglobulin subtypes for anti-PARP.

PCNA was detected using a PCNA staining kit (Zymed). The kit uses a biotinylated PCNA monoclonal antibody. Color development was with the aminoethyl carbazol staining kit (Zymed). Eight kidney sections originating from four different rats were viewed under $\times 10$ power lens, and the images from nonoverlapping outer medullary (S3) segments were captured on a computer. Levels of creatinine in neither the vehicle-treated group (3.1 ± 0.42 mg/dl) nor the benzamide-treated group (2.7 ± 1.0 mg/dl) of four rats, the tissues of which were used for histological analysis, differed significantly from levels of creatinine at 1 day postinjury in the corresponding groups of 16 rats that were used to generate the data shown in Fig. 3. The purple-colored nuclei were counted using the Image-Pro plus software (Media Cybernetics, Silver Spring, MD). Data were expressed as the number of regenerating cells per square millimeter. To avoid potential error in statistical sampling 20–25 nonoverlapping, 1 square-millimeter fields were randomly selected and counted in a blinded manner. Statistical analysis was done using Student's *t*-test.

The degree of morphological damage was assessed using sections of kidneys stained with hematoxylin and eosin. The number of dilated tubules from 15 to 20 nonoverlapping fields in the outer medullary segment of both kidneys was determined by selecting tubules with a diameter of >20 μ m using light microscopy and the Image-pro plus software. Sections originating from kidneys of four vehicle- or benzamide-treated rats were evaluated. Levels of creatinine in neither the vehicle-treated group (2.9 ± 0.5 mg/dl) nor the benzamide-treated group (2.7 ± 0.5 mg/dl) of four rats, the tissues of which were used for histological analysis, differed significantly from levels of creatinine at 1 day postinjury in the corresponding groups of 16 rats that were used to generate the data shown in Fig. 3. The data were expressed as the number of dilated tubules per square millimeter.

To determine the ATP content, kidneys were snap frozen in liquid nitrogen (<1 s) and homogenized in 2% trichloroacetic acid. A small aliquot of the lysate was neutralized with 0.1 M Tris buffer (pH 9.0), and serial dilutions were made. The diluent was mixed with 100 μ l of luciferase-luciferin reagent (Promega, Madison, WI), and luminescence was detected with a 5-s delay time and a 10-s signal integration time in a Zylux luminometer (Zylux, Maryville, TN). A five-point ATP standard curve was generated using known concentrations of ATP and the corresponding luminescence. The quantity of ATP in the lysate was determined by performing a linear regression on the data from the ATP standard curve. Three animals were used from each group, and ATP assay was performed on samples from both kidneys. Statistical analysis was done using Dunnett's multiple-comparison test.

Western blots were performed exactly as we previously described (20) using 20 μ g of protein extracted from whole kidneys.

RESULTS

To analyze the spatial expression pattern of PARP after renal ischemia, immunohistochemistry was performed using a monoclonal mouse anti-PARP antibody. Before immunohistochemistry, we performed experiments to establish that the antibody recognized a protein in rat kidney with a molecular weight consistent with intact PARP. To this end, we performed Western blots of protein extracted from whole kidneys of sham-operated rats or rats previously rendered ischemic (ARF) at 6 or 12 h or at 5 days. No band is detectable

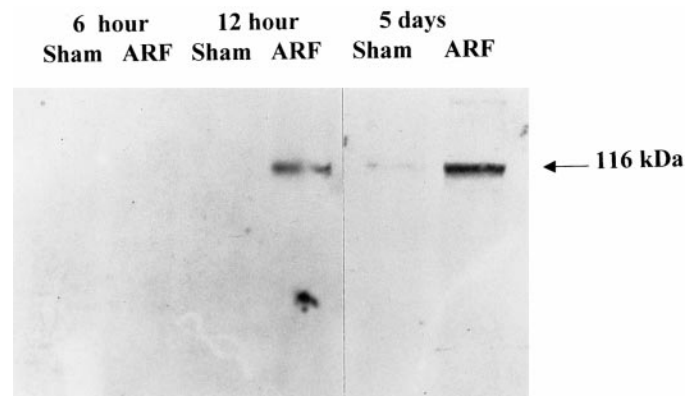


Fig. 1. Western blot of rat kidney extracts. Shown are results using extracts from whole kidneys of sham-operated rats or from kidneys of rats rendered ischemic [acute renal failure (ARF)] 6 or 12 h or 5 days before extraction. The size of the band is shown at *right*.

in extracts of kidneys from sham-operated rats or rats rendered ischemic 6 h before extraction (Fig. 1). However, a 116-kDa band in the extracts of kidneys of rats rendered ischemic 12 h or 5 days before extraction is of a size that would be predicted for intact PARP (35).

Shown in Fig. 2 are sections of the damaged S3 segments of proximal tubules that are observed postischemia in rat and corresponding sections from sham-operated controls. PARP was not detected in sections originating from kidneys of rats 1 day after injury when control antibody was substituted for anti-PARP (Fig. 2A). PARP staining was not observed in sections originating from kidneys of sham-operated rats 1 day post-sham surgery (Fig. 2B). No PARP immunoreactivity was observed in proximal tubular cells at 6 h (Fig. 2C) postischemia. However, positive staining was observed at 12 h (Fig. 2D), 1 day (Fig. 2E), and 5 days

(Fig. 2F) after injury. PARP was localized to nuclei of cells in the S3 segment of the proximal tubule (32), in cells sloughed into the tubule lumen (Figs. 2, D and E), and in papillary proliferations (Fig. 2F).

To elucidate whether inhibition of PARP activity ameliorates the course of ischemic renal injury, we investigated the effects of administering each of two PARP inhibitors, benzamide or 3-AB, to rats rendered ischemic. The levels of serum creatinine (Fig. 3) and BUN (Fig. 4) were no different 24 h after injury in animals that received either benzamide or 3-AB compared with levels in vehicle-treated rats. However, creatinine was significantly reduced during *days 2–5* postischemia and the BUN values at *days 2 and 3*. The mortality rate among vehicle-treated and benzamide-treated rats was identical. In both cases, two animals out of eighteen died within 24 h postinjury. There was

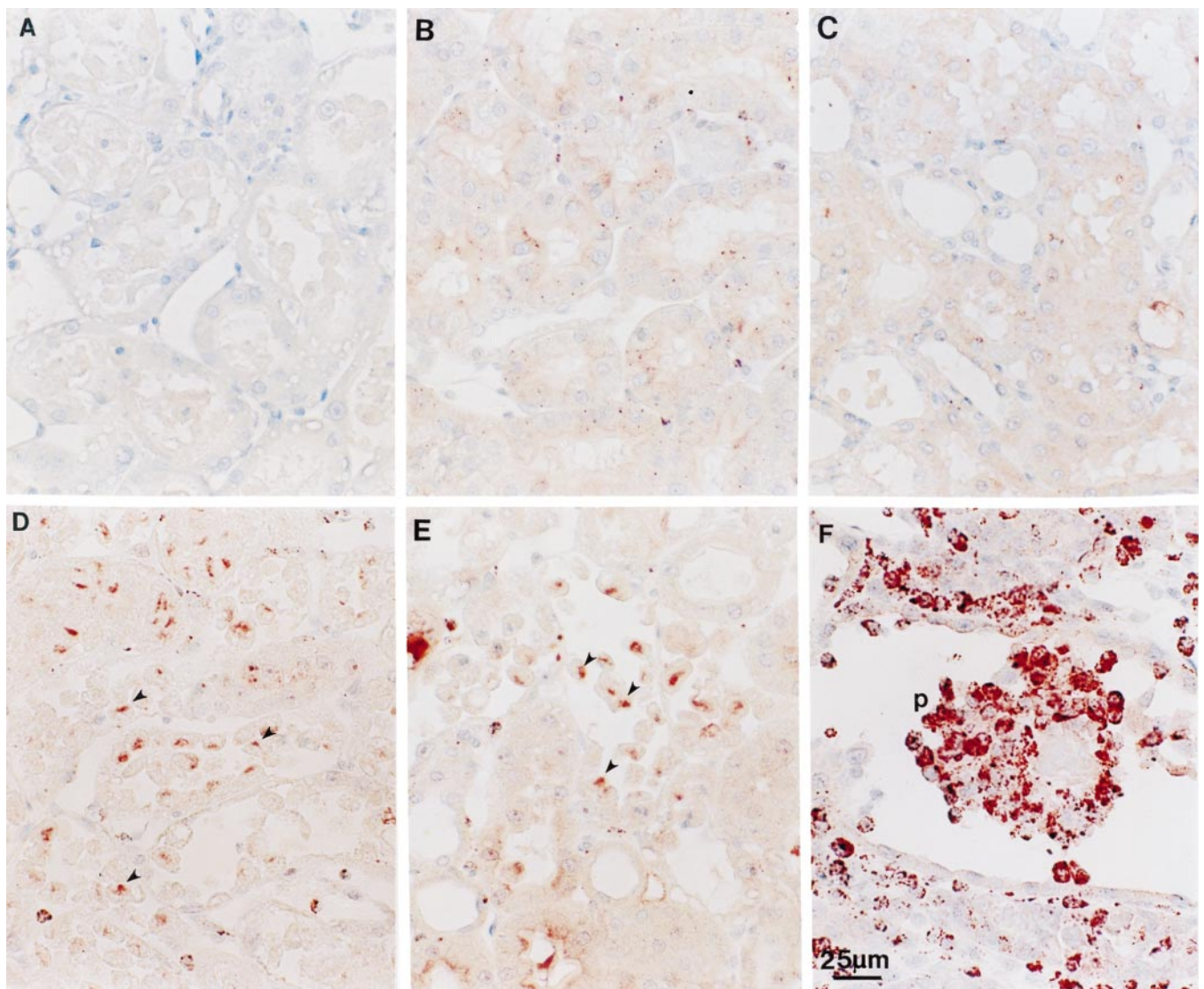


Fig. 2. Immunohistochemical localization of poly(ADP-ribose) polymerase (PARP) in ischemia-injured kidneys. Shown in A is a section from a kidney of a rat rendered ischemic 1 day before death stained using control antibody. Also shown is expression of PARP protein in kidneys from sham-operated rats (B) and rats rendered ischemic 6 h (C), 12 h (D), 1 day (E), or 5 days (F) before death. Arrowheads show positively staining cells. p, Papillary proliferation. Magnification is shown in F.

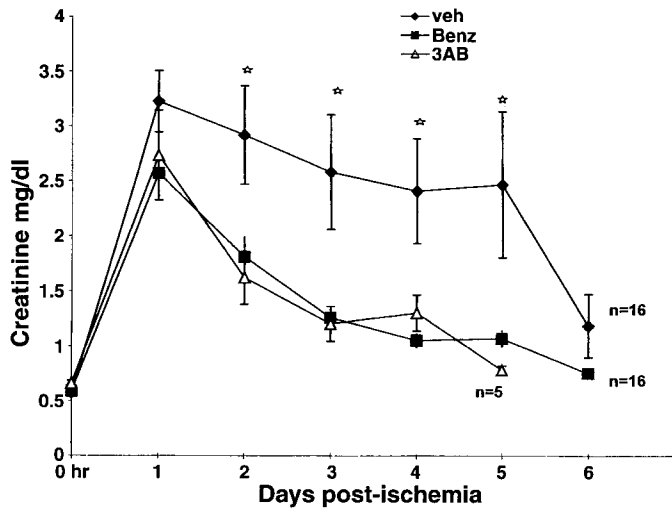


Fig. 3. Effect of administration of PARP inhibitors, benzamide (Benz) or 3-amino benzamide (3AB), on the levels of serum creatinine compared with that from vehicle-treated rats. The values at each time represent means \pm SE. The values at days 2–5 are significantly different ($\star P < 0.05$).

no mortality among the five rats that were treated with 3-AB. No change in the levels of creatinine or BUN were detected over a 6-day time period among sham-operated rats that received vehicle, benzamide, or 3-AB (data not shown).

Histological analysis of kidney sections obtained from rats that were treated with vehicle or one of the PARP inhibitors, benzamide, 7 days postischemic injury, showed that both groups suffered ischemic damage. Shown in Fig. 5A is a section from a kidney originating from a vehicle-treated rat stained with hematoxylin and eosin. A section of a kidney from a benzamide-treated rat is shown in Fig. 5B. Kidneys from animals that received benzamide had fewer dilated tubules compared with kidneys from a rat that received vehicle (Fig. 5C).

To assess whether PARP inhibition affects proximal tubular regeneration, we analyzed PCNA expression immunohistochemically in kidneys from vehicle- or benzamide-treated rats obtained 24 h postischemic injury. PCNA-positive cells were found both in the cortex and in the outer medullary segments in kidneys from the vehicle- or benzamide-treated rats. Shown in Fig. 5D is a section from a kidney originating from a vehicle-treated rat stained for PCNA. A section from a benzamide-treated rat shows more PCNA-positive cells in a comparable area (Fig. 5E). Kidneys originating from vehicle-treated rats had a relatively lower number of PCNA-positive cells in the cortex and outer medullary segments (Fig. 5F).

To determine whether PARP inhibition increased levels of renal ATP postischemia, kidneys from vehicle- or benzamide-treated rats that underwent 60 min of ischemia followed by reperfusion for 24 h were isolated, and the ATP contents were determined. At 24 h postischemia, ATP levels in kidneys rendered ischemic would be expected to be $\sim 60\%$ of normal (9).

For our studies, kidneys derived from sham-operated rats served as controls. At 24 h postischemia in vehicle-treated rats, ATP levels were significantly reduced ($P < 0.05$) to 54% (0.683 ± 0.064 nmol/mg protein) of those in kidneys of sham-operated rats (1.256 ± 0.157 nmol/mg protein). Levels of ATP in benzamide-treated rats that were rendered ischemic were significantly ($P < 0.05$) greater (85%) than those in vehicle-treated controls (1.073 ± 0.273 nmol/mg protein), consistent with preservation of ATP content postischemia by benzamide.

DISCUSSION

Ischemia-reperfusion injury of the kidney results in the generation of reactive oxygen species such as the superoxide radical and its reduction products hydrogen peroxide and the hydroxyl radical (29–31). In addition, the inducible form of nitric oxide synthase (iNOS) is activated, and nitric oxide is generated postischemic injury. Nitric oxide reacts with superoxides to form peroxynitrite. A role for the reactive oxygen species and peroxynitrite in mediating renal damage has been substantiated by the observation that pretreatment of rats with free radical scavengers and inhibition of iNOS ameliorates the course of ischemic injury (18, 22).

There is evidence that reactive oxygen species and peroxynitrite induce cellular injury by inducing nicks in DNA. DNA damage is repaired via the activity of several DNA repair enzymes, including PARP (7, 23, 24, 31). PARP activation occurs in the settings of cerebral, cardiac, and skeletal muscle ischemia. The data shown in Figs. 1 and 2 demonstrate that PARP is also induced after ischemia-reperfusion injury of the kidney in the S3 proximal tubule segment (Fig. 2).

PARP maintains genome integrity after cellular exposure to genotoxic agents in the setting of ischemia-

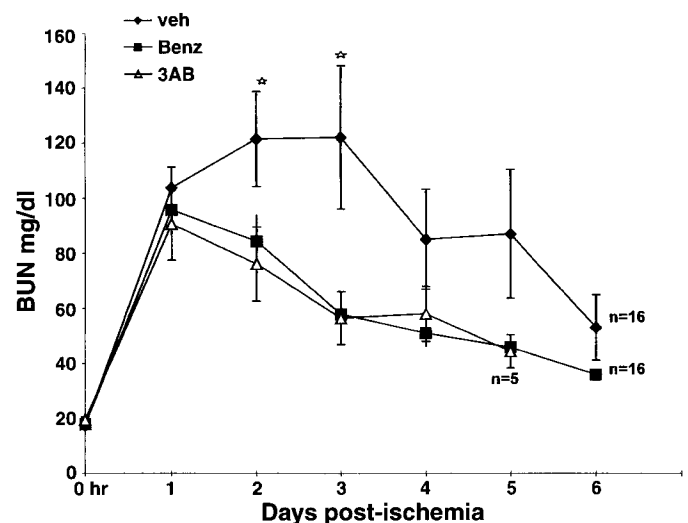


Fig. 4. Effect of administration of PARP inhibitors, benzamide or 3-amino benzamide, on the levels of blood urea nitrogen (BUN) compared with that from vehicle-treated rats. The values at each time point represent means \pm SE. The values at days 2–5 are significantly different ($\star P < 0.05$).

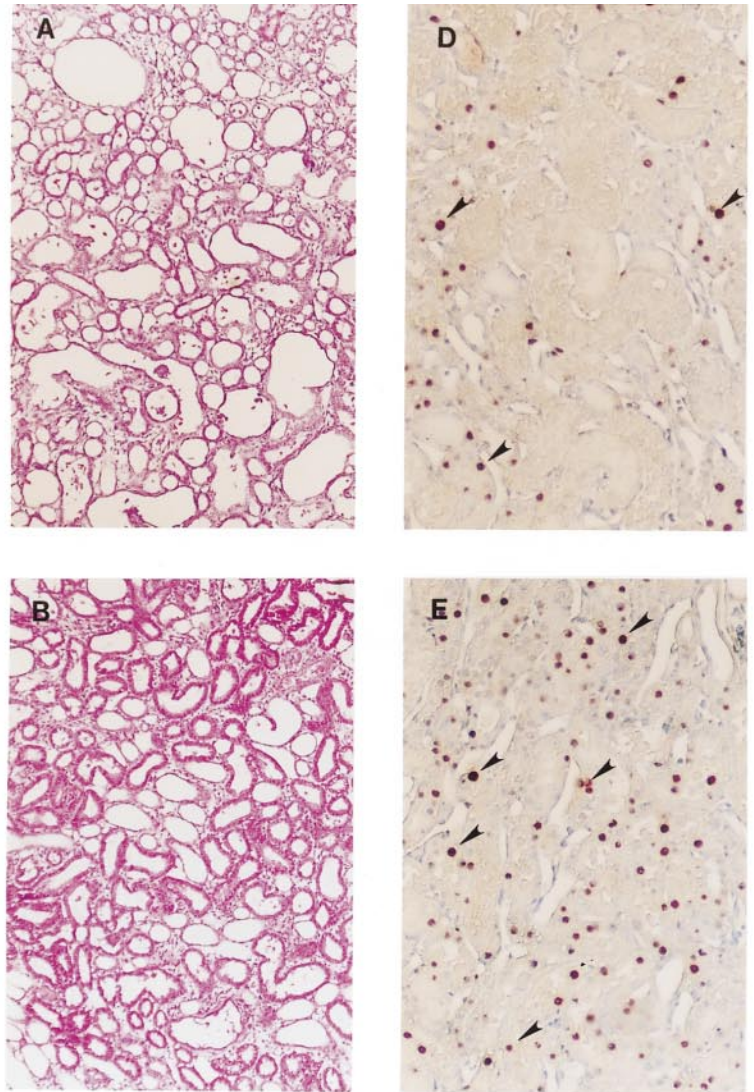
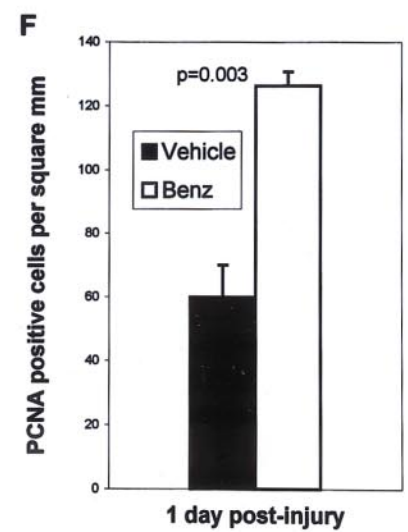
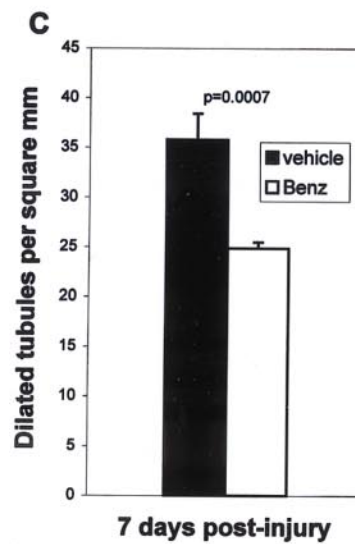


Fig. 5. Effect of administration of vehicle (*A, D*) or PARP inhibitor benzamide (*B, E*) on renal histopathology 7 days postinjury (*A-C*) and proliferation (*D-F*) 1 day postinjury. *A* and *B* show hematoxylin and eosin-stained sections. *D* and *E* show proliferating cell nuclear antigen (PCNA)-stained sections. Arrowheads show PCNA-positive nuclei (*D* and *E*).



reperfusion injury. However, extensive DNA damage after ischemic injury may lead to excessive PARP activation that consumes large quantities of cellular NAD, resulting in ATP depletion and death (35). Therefore, it has been proposed that, although chronic inhibition of the activity of PARP is likely to be harmful to the cell, transient inhibition after ischemia-reperfusion injury may prevent cell death (28).

Our data show that transiently inhibiting PARP activity after renal ischemia-reperfusion injury accelerates recovery, as reflected by lower levels of creatinine and BUN during *days 2–5* postischemia (Figs. 3 and 4) and improved renal histology at 7 days after injury (Fig. 5). Inhibiting PARP activity led to an increase in the number of regenerating cells at 24 h postinjury (Fig. 5), consistent with acceleration of the tubular repair process.

We cannot exclude the possibility that the data shown in Figs. 3 and 4 reflect actions of PARP inhibition to reduce BUN generation and creatinine release in the setting of acute ischemic renal injury. However, the data shown in Fig. 5, *A–F*, considered together with those shown in Figs. 3 and 4 and our finding that neither inhibitor affects levels of creatinine or BUN in sham-operated rats over a period of 7 days, render it likely that the reductions in creatinine and BUN (Figs. 3 and 4) reflect an amelioration of ischemic injury. Inhibition of PARP after renal ischemia-reperfusion injury did not affect levels of creatinine or BUN measured at 24 h postischemia (Figs. 3 and 4). This finding could indicate that the extent of the renal damage was not affected by inhibiting PARP. Alternatively, an amelioration of injury effected by PARP inhibition may be inadequately reflected by measurements of creatinine and BUN so soon after injury but better reflected by the increased ATP levels we at this time find postinjury.

The regenerative capacity of the renal proximal tubule immediately after ischemia-reperfusion injury is dependent on the number of noninjured or sublethally injured tubular cells that survive and can initiate the reparative mechanisms that restore the structure and physiological function of the renal tubular epithelium (4, 11, 12). One of the major requirements for initiation of the cellular repair process is the repletion of intracellular ATP. ATP levels fall to undetectable levels after 60 min of renal ischemia. During the first 2 h after 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 (26, 33).

The restoration of ATP levels back to normal takes >48 h (9). Our data showing that PARP inhibition postinjury restores levels of ATP close to normal levels at 24 h are consistent with one of the mechanisms by which PARP inhibition ameliorates the course of injury being preservation of ATP levels.

It is of interest, that at 7 days postischemia, renal cortices from benzamide-treated rats appear more normal than those of vehicle-treated rats, whereas at the same time, there are no significant differences in se-

rum creatinine or BUN (Figs. 3 and 4). Such a dissociation between histology at 7 days after renal ischemia and levels of creatinine and BUN was previously observed in rats treated with insulin-like growth factor-I (IGF-I) or epidermal growth factor compared with vehicle-treated rats (16, 17) and a similar dissociation between disturbances of structure and function is well described in human acute renal failure (13).

Perspectives

We showed that PARP is expressed in the damaged S3 segment of the renal proximal tubule beginning within 12 h of renal injury and that transient inhibition of PARP activity postischemia ameliorates the course of acute renal failure. As is the case for other agents with similar effects on the course of renal injury (16, 17), such as IGF-I, the exact mechanism by which PARP inhibition is salutary remains undefined and may reflect direct or indirect actions on renal tissue. However, whatever the basis for their beneficial action may be, our findings provide a rationale for the development and pharmacological use of suitable inhibitors of PARP to accelerate recovery from acute renal failure in humans.

It is necessary to proceed with caution. In certain circumstances, inhibition of PARP may be harmful because this enzyme facilitates DNA repair that may be required in the recovery phase of acute tubular necrosis. For this reason, determination of the proper timing for PARP inhibition postinjury may be a crucial factor to permit the use of PARP inhibitors.

We thank Dr. Helen Liapis (Washington University) for helpful suggestions on evaluating renal pathology.

B. J. Padanilam is supported by National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) Grant DK-52907. D. R. Martin and M. R. Hammerman are supported by NIDDK Grant DK-45181. A. J. P. Lewington was supported by Barnes-Jewish Hospital, St. Louis, MO.

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