Inhibition of PARP prevents oxidant-induced necrosis but not apoptosis in LLC-PK₁ cells

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Filipovic, Dragana M., Xianmin Meng, and W. Brian Reeves. Inhibition of PARP prevents oxidant-induced necrosis but not apoptosis in LLC-PK₁ cells. Am. J. Physiol. 277 (Renal Physiol. 46): F428–F436, 1999.—Oxidant-induced cell injury has been implicated in the pathogenesis of several forms of acute renal failure. The present studies examined whether activation of poly(ADP-ribose)polymerase (PARP) by oxidant-induced DNA damage contributes to oxidant injury of renal epithelial cells. H₂O₂ exposure resulted in an increase in PARP activity and decreases in cell ATP and NAD content. These changes were significantly inhibited by 10 mM 3-aminobenzamide (3-ABA), a PARP inhibitor. In contrast, H₂O₂-induced DNA damage was not prevented by 3-ABA. Exposure of LLC-PK₁ cells to 1 mM H₂O₂ for 2 h induced necrotic cell death as measured by increased lactate dehydrogenase (LDH) release. 3-ABA completely prevented the H₂O₂-induced LDH release. Live/dead fluorescent staining confirmed the protection by 3-ABA. These results are consistent with the view that oxidant-induced DNA damage activates PARP and that the subsequent ATP and NAD depletion contribute to necrotic cell death. Of note, although protected from necrosis, cells treated with H₂O₂ and 3-ABA underwent apoptosis as evidenced by DNA fragmentation and bis-benzimide staining. In conclusion, activation of PARP contributes to oxidant-induced ATP depletion and necrosis in LLC-PK₁ cells. However, PARP inhibition may target cells toward an apoptotic form of cell death.

Oxidant-induced necrosis has been implicated in the pathogenesis of several forms of acute renal failure, such as renal ischemia, rhabdomyolysis, and toxic nephropathies (4, 9). The mechanisms of oxidant injury have been studied extensively in LLC-PK₁ cells, a model of proximal tubular epithelial cells. In these cells, oxidant stress causes ATP depletion, a rise in intracellular calcium, DNA damage, and lipid peroxidation (2, 3, 13, 37, 38). Several mechanisms appear to account for oxidant-induced ATP depletion, with the relative importance of each mechanism varying in different tissues. Thus impaired mitochondrial function, inhibition of glycolysis, and enhanced ATP utilization have been shown to contribute to oxidant-induced ATP depletion (1, 20, 31).

In some cells, oxidant-induced DNA damage leads to increased ATP consumption and subsequent ATP depletion (19, 31, 32). In these cells, the enzyme poly(ADP-ribose)polymerase (PARP) is activated by nicks and breaks in DNA strands. PARP catalyzes the ADP ribosylation of proteins using NAD as the substrate (24) and can produce cellular NAD depletion (32). Subsequently, ATP depletion can result as ATP is consumed in the regeneration of NAD from nicotinamide. In this regard, it has been shown that PARP inhibitors, such as 3-aminobenzamide (3-ABA) and nicotinamide, can prevent energy depletion and improve the viability of certain cells exposed to oxidative stress (17, 32, 43, 46). However, the protective effect of PARP inhibition against oxidant-induced injury is not universal (10, 45), and the role of PARP in oxidant-induced ATP depletion in renal epithelial cells is uncertain. Schnellmann et al. (30) found no protection by 3-ABA against t-butyl hydroperoxide-induced cell death in rabbit proximal tubules; however, PARP activity and nucleotide levels were not measured in that study. Moreover, although PARP inhibitors may improve short-term viability after oxidant injury, their effects on long-term survival have not been widely examined. The present study examined the role of PARP activity in oxidant-induced intracellular ATP depletion in LLC-PK₁ cells and the effects of PARP inhibitors on cell viability after oxidant-induced injury. The results indicate that, in response to oxidant stress: 1) PARP is activated and is a major determinant of ATP depletion; 2) inhibition of PARP prevents ATP depletion and early (<24 h) cell death; and 3) inhibition of PARP leads to late (24–48 h) cell death via apoptosis. Thus oxidant-induced ATP depletion in LLC-PK₁ cells is due largely to increased utilization of ATP. Moreover, maintenance of ATP and NAD stores prevents acute cell necrosis but does not prevent apoptosis in response to oxidant stress.

METHODS

Cell culture. LLC-PK₁ obtained from the American Type Culture Collection (CRL, Rockville, MD) were maintained in DMEM supplemented with 10% FBS and 2 mM glutamine (GIBCO Laboratories, Grand Island, NY) at 37°C and aerated with 5% CO₂-95% air. Cells were studied 1–2 days after reaching confluence. All experimental maneuvers were performed in a bicarbonate-buffered Ringer’s solution (KRB) containing (in mM) 115 NaCl, 25 NaHCO₃, 3.5 KCl, 1.25 CaCl₂, 1 MgSO₄ (pH 7.4 after bubbling with 95% O₂-5% CO₂). H₂O₂-containing solutions were prepared fresh just before each experiment by dilution of a 30% H₂O₂ stock solution.

Measurement of PARP activity. PARP activity was assayed by measuring the radioactivity incorporated from [³²P]NAD⁺ in TCA-insoluble material (17). LLC-PK₁ cells were grown to confluence in six-well plates and then incubated for 30 min at...
37°C in KRB containing 1 mM H2O2 in the presence or absence of 10 mM 3-ABA. At the end of the incubation, the solution was removed, and the cells were washed briefly with 1 ml PBS containing catalase (13 U/ml) to remove any residual H2O2. Next, 500 µl of PARP assay solution containing 56 mM HEPEs, 28 mM NaCl, 28 mM KCl, 2 mM MgCl2, 0.01% digitonin, and 100 nM [adenylate-32P]NAD were added to each well. The reaction was stopped after 5 min by the addition of 0.2 ml of ice-cold 50% TCA. The contents of the wells were transferred to microcentrifuge tubes and centrifuged at 14,000 g for 10 min. The pellet was washed two times with 500 µl of 10% TCA and then solubilized in 100 µl of 0.2 M NaOH. The 32P content of the solubilized pellet was determined by liquid scintillation counting.

ATP measurements. The ATP content of LLC-PK1 cells was determined using the luciferin-luciferase assay as previously described (16, 29). Briefly, cells were grown to confluence in 12-well tissue culture plates, washed, and incubated at 37°C for 60 min in the indicated test solutions. The solution was then aspirated, and ATP was extracted from the monolayer of cells with 400 µl of 2% perchloric acid (PCA). A 20-µl aliquot of the PCA extract was neutralized with NaOH and diluted 200-fold in 10 mM Tris (pH 7.4). A 20-µl sample of the diluted cell extract was then mixed with 100 µl of luciferin-lucerase (5 mg/ml; Sigma Chemical, St. Louis, MO) in a Turner TD-20e luminometer (Turner Designs, Sunnyvale, CA). The ATP content is expressed relative to the cell protein. Protein content was determined by the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) method with BSA as the standard.

NAD content. NAD content of cells was determined using a bioluminescent assay (21). Cells were grown in six-well tissue culture plates and subjected to the indicated maneuvers. At the end of the test period, the incubation solution was removed, and the cells were extracted in 0.5 ml of 70% ethanol in 10 mM potassium phosphate buffer, pH 7. A 10-µl aliquot of the cell extract was mixed with 390 µl of assay solution containing 25 mM potassium phosphate, 0.1 mM dithiothreitol, 80 U/ml flavin mononucleotide (FMN) reductase (Boehringer Mannheim, Indianapolis, IN), 2.5 µM FMN, 25 mM myristic aldehyde, and 2 µg/ml alcohol dehydrogenase. This mixture was incubated at room temperature for 10 min to allow conversion of NAD to NADH via alcohol dehydrogenase. The sample was then placed in a luminometer and mixed with 100 µl of luciferase solution (25 µg/ml). NAD levels are expressed relative to the protein content of the well.

DNA damage. DNA damage was determined using the alkaline unwinding assay (7) as reported previously from this laboratory (29). In this assay, the rate of DNA unwinding under mild alkaline conditions is increased by the presence of either single- or double-strand DNA breaks. The amount of residual double-stranded DNA after alkaline treatment is expressed as a percentage of the total DNA in the sample.

Lactate dehydrogenase release. At the indicated times, the incubation medium was removed, and the cells were lysed in 0.2% Triton X-100. The activity of lactate dehydrogenase (LDH) in the incubation solution (supernatant) and in the detergent extract of cells was determined spectrophotometrically from the oxidation of NADH (6). The results are expressed as the percentage of the total LDH content of the well that appeared in the supernatant: %release=LDHsuper/(LDH supernatant + LDH cell)×100, where LDH supernatant is the amount of LDH in the supernatant and LDH cell is the amount of LDH in the cell. None of the test agents or vehicles interfered with the assay for LDH.

Live/dead assay. Plasma membrane integrity was assessed using the fluorescent probes calcein AM and ethidium homodimer (LIVE/DEAD Viability kit; Molecular Probes, Eugene, OR). Viable cells take up and retain the green calcein dye while excluding the orange ethidium dye. Cells with impaired plasma membrane integrity stain orange due to the entry of ethidium and the failure to retain calcein. LLC-PK1 cells were grown to confluence on plastic chamber slides (Nunc, Naperville, IL). Cells were then incubated in KRB containing 1 mM H2O2 in the presence or absence of 10 mM 3-ABA for 2 h at 37°C. At the end of the incubation, the cells were washed with PBS and then incubated with PBS containing 4 µM ethidium homodimer and 2 µM calcein AM for 30 min at room temperature. The cells were then rinsed with PBS and viewed under fluorescence microscopy.

MTS assay. Cell survival was quantified using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfo- phenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay; Promega, Madison, WI) following the manufacturer’s instructions. For this assay, LLC-PK1 cells were grown in 96-well tissue culture plates until confluence. Cells were then treated for 2 h at 37°C in KRB or KRB containing 1 mM H2O2 in the presence or absence of 10 mM 3-ABA. After the 2-h treatment, the KRB solution was replaced with culture medium, and the plates were returned to the incubator. Cell survival was measured at 6, 12, 24, and 48 h after the initial treatment using the MTS assay. Briefly, 20 µl of the MTS solution were added to the appropriate wells, and the plates were incubated for 2 h at 37°C. The absorbance at 490 nm was measured with a microplate reader (Vmax Kinetic Microplate Reader; Molecular Devices, Sunnyvale, CA) and corrected for background absorbance. Preliminary experiments showed a linear relationship between the absorbance at 490 nm and cell number between 103 and 105 cells/well.

Bis-benzimide staining. Nuclear morphological changes were assessed by staining with the nuclear stain bis-benzimide (H33258; Molecular Probes). At the end of the treatment period, cells were washed with PBS and fixed in 4% paraformaldehyde in PBS for 10 min. The cells were then washed with PBS and stained with 5 µM bis-benzimide in PBS for 10 min. Cells were photographed with epifluorescence microscopy.

Electron microscopy. LLC-PK1 cells were grown to confluence on permeable tissue culture inserts (Millipore, Bedford, MA). Cells were then treated with 1 mM H2O2 in the presence or absence of 10 mM 3-ABA for 2 h at 37°C. The incubation solution was replaced with culture medium, and the cells were incubated at 37°C for an additional 24 h. At that time, the culture medium was removed, and the cells were fixed in 4% glutaraldehyde solution at room temperature for both the preparation of unlysed cells. SDS and RNase A were added to the supernatant and incubated at 56°C for 2 h followed by digestion with proteinase K for 2 h at 37°C. The DNA fragments were precipitated with ammonium acetate and ethanol and then separated by electrophoresis in 1% agarose gels.
RESULTS

In some cells, the activation of PARP, an enzyme involved in repair of oxidant-induced DNA damage, contributes to oxidant-induced ATP depletion (35). To evaluate this possibility in renal epithelial cells, we first determined the activity of PARP in LLC-PK1 cells treated with 1 mM H2O2 in the presence and absence of 3-ABA, an inhibitor of PARP. As shown in Fig. 1, treatment with H2O2 resulted in a large increase in PARP activity (0.043 ± 0.001 vs. 0.275 ± 0.04 pmol·mg protein⁻¹·5 min⁻¹, P < 0.0002). Moreover, 3-ABA almost completely prevented this increase in PARP activity.

Figure 2 shows the results of experiments that measured the NAD content of LLC-PK1 cells exposed to H2O2 in the presence or absence of 3-ABA. The results mirrored those of PARP activity, that is, H2O2 resulted in a large fall in NAD content (2.31 ± 0.06 vs. 0.84 ± 0.10 nmol/mg protein, P < 0.0001, n = 6). In contrast, NAD levels in cells treated with H2O2 in the presence of 3-ABA (1.95 ± 0.06 nmol/mg protein) were only slightly lower than controls. The regeneration of NAD consumes four molecules of ATP for each NAD molecule restored (46). As a result, PARP activation, with consequent NAD depletion, can lead to ATP depletion (19, 32, 45). Because the results in Figs. 1 and 2 indicate that H2O2 activates PARP in LLC-PK1 cells and leads to NAD depletion, we examined the effects of H2O2 and 3-ABA on the ATP content of LLC-PK1 cells. As shown in Fig. 3, exposure to 1 and 5 mM H2O2 for 1 h resulted in marked, dose-dependent ATP depletion (control = 32.8 ± 1.6 nmol ATP/mg protein vs. 17.1 ± 0.6 and 9.9 ± 0.9 nmol ATP/mg protein with 1 and 5 mM H2O2, respectively, n = 6–8, P < 0.0001 vs. control). The extent of ATP depletion in H2O2-treated cells was significantly reduced in the presence of 10 mM 3-ABA. 3-ABA alone had no effect on the ATP content of cells (31.9 ± 2.3 nmol/mg protein, n = 5, P = not significant vs. control).

The trigger for activation of PARP is DNA damage (24). We confirmed that brief exposure (10 min) of LLC-PK1 cells to H2O2 produced significant DNA damage (82 ± 3 vs. 35 ± 4% residual double-strand DNA, n = 6, P < 0.0001, Fig. 4). However, in contrast to its effects on ATP and NAD content, 3-ABA did not prevent the H2O2-induced DNA damage (37 ± 5% residual double-strand DNA).

The results provided in Figs. 1–4 are consistent with the view that oxidant-induced DNA damage leads to activation of PARP and subsequent depletion of both NAD and ATP stores. The next series of experiments examined the effects of PARP inhibition on H2O2-induced cell death. LDH release into the extracellular fluid was measured as a marker of lethal cell injury (Fig. 5). For these studies, cells were exposed to 1 mM H2O2 for 2 h in the absence or presence of the indicated concentrations of 3-ABA. H2O2 increased LDH release...
Over the range of 1–10 mM, 3-ABA produced a dose-dependent inhibition of H2O2-induced LDH release. In contrast, 10 mM 3-aminobenzoic acid, an analog of 3-ABA that does not inhibit PARP, had no effect on LDH release. Also, as expected for H2O2-induced cell damage, catalase, which converts H2O2 into H2O and molecular oxygen, prevented H2O2-induced LDH release.

We also examined the effects of H2O2 and 3-ABA on the cell membrane integrity to small probes. Because cell membranes undergo a progressive loss of integrity during injury (11, 15), the permeability to small molecules may be an earlier marker of injury than the release of macromolecules such as LDH. We used three probes with molecular weights less than 1,000 (trypan blue and the fluorescent probes calcein and ethidium homodimer). Figure 6 shows the staining of cells with calcein AM and ethidium homodimer. Under control conditions (A) and with 3-ABA alone (D), viable cells took up, deesterified, and retained the green calcein dye but excluded the ethidium homodimer. Cells treated with H2O2 (Fig. 6B) were unable to retain the intracellular calcein or to exclude the ethidium. However, 3-ABA prevented the increase in membrane permeability to both probes (Fig. 6C). Similar results were obtained using trypan blue (not shown).

The effects of 3-ABA on long-term cell survival were determined using the MTS assay of mitochondrial function. Cells were exposed to 1 mM H2O2 for 2 h in the presence or absence of 10 mM 3-ABA. After the 2-h treatment, the incubation solution was replaced by growth medium, and cells were incubated for up to 48 additional hours. Figure 7 shows the results of the MTS assay at 6, 12, 24, and 48 h after H2O2 treatment. By 12 h, the viability of H2O2-treated cells was markedly reduced (15 ± 3%) compared with untreated cells. At 6 and 12 h, the viability of cells treated with H2O2 in the presence of 3-ABA (78 ± 7 and 46 ± 11%, respectively) was about threefold greater than cells treated with H2O2 alone (P < 0.001, n = 6 in each group). However, this advantage in cell survival was not durable, as the viability of the 3-ABA-treated cells fell to levels indistinguishable from those of H2O2-treated cells after 24 to 48 h. Treatment of cells with 3-ABA in the absence of H2O2 had no effect on cell viability (not shown).

We examined cells 24 h after exposure to H2O2 and 3-ABA to determine the nature of the delayed cell death. Figure 8 shows cells stained with bis-benzimide. Control cells (Fig. 8A) showed faintly stained nuclei and homogeneously dispersed chromatin. H2O2-treated cells (Fig. 8B) showed random clumping and vacuolization of chromatin, typical for necrosis, and rare apoptotic bodies. In contrast, cells treated with 3-ABA and H2O2 for 24 h (Fig. 8C) showed extensive apoptotic changes such as highly condensed chromatin and small dispersed apoptotic bodies. Cells treated with 3-ABA...
alone (Fig. 8D) showed no nuclear alterations. These results suggest that, while cells treated with H2O2 alone die a necrotic death, cells treated with H2O2 and 3-ABA undergo an apoptotic death. Electron micrographs (Fig. 9) also support this view. Specifically, control cells (Fig. 9A) showed a typical morphology of polarized cells organized in monolayers with scattered apical microvilli and tight junctions. H2O2-treated cells (Fig. 9B) showed typical necrotic changes with cell swelling, large cytoplasmic vesicles, and ruptured plasma membranes. Cells treated with H2O2 and 3-ABA for 24 h (Fig. 9C) showed structural characteristics of apoptosis, such as condensation, margination, and fragmentation of chromatin, a relative reduction in cell size, and loss of microvilli with plasma membrane blebbing and budding.

Finally, we examined H2O2- and 3-ABA-treated cells for evidence of endonuclease-mediated DNA fragmentation. DNA was isolated from cells 24 h after exposure to H2O2 in the presence or absence of 3-ABA. The agarose gel electrophoresis (Fig. 10) showed no low molecular weight fragments in either control cells or in H2O2-treated cells. However, a ladder pattern of 200-bp fragments, typical of endonuclease-mediated DNA damage, was readily apparent in the cells treated with H2O2 and 3-ABA.

DISCUSSION

Oxidant-induced cell injury is believed to contribute to the pathogenesis of some forms of acute renal failure (4, 9). A number of perturbations, such as ATP depletion, DNA damage, lipid peroxidation, and calcium overload, have been documented in renal epithelial cells exposed to oxidant injury (2, 3, 13, 37, 38). However, the relations between these events and subsequent cell death are not completely defined. In this regard, the studies reported here provide several insights into the mechanism of acute oxidant injury to renal tubular epithelial cells. First, our results indicate that the activation of PARP plays an important role in oxidant-induced ATP depletion and early necrotic cell death. Second, while confirming the importance of oxidant-induced DNA damage in the initiation of cell death, our results indicate that cell necrosis is not a direct consequence of DNA damage and can be dissociated from oxidant-induced DNA damage. Third, depletion of NAD and/or ATP stores appears to be a requisite for oxidant-induced necrotic cell death. Finally, oxidant-induced damage to DNA, or the inability to repair such damage, may lead to apoptosis in settings where necrosis is inhibited.
Oxidant stress results in DNA damage via both direct oxidant attack, resulting in single strand breaks and base modifications, and activation of endonucleases (38). DNA damage is an obligatory trigger for the activation of the abundant nuclear enzyme PARP (35). PARP catalyzes the transfer of ADP-ribose from NAD to certain nuclear acceptor proteins, e.g., histones, DNA polymerases, and PARP itself, to form poly-ADP-ribose chains (24). Although the physiological function of this process remains unclear, the pathophysiological consequences of PARP activation may be dramatic. A number of investigators have determined that the activation of PARP can lead to rapid depletion of NAD and, as discussed below, ATP with subsequent cell death (17, 32, 43, 46). Moreover, inhibition of PARP activity using 3-ABA or related analogs can preserve NAD stores and prevent oxidant-induced cell death. However, the role of PARP in oxidant-induced injury to renal epithelial cells has not been established. We demonstrated clearly that PARP is activated in LLC-PK1 cells as a result of oxidant stress (H2O2) and that pretreatment of cells with 3-ABA completely prevented the H2O2-induced increase in PARP activity (Fig. 1). Likewise, H2O2 decreased cell NAD content, and this decrease was prevented by 3-ABA.

Oxidant stress results in a rapid depletion of intracellular ATP in renal epithelial cells (2, 3, 16). As noted above, the activation of PARP by oxidant-induced DNA damage may result in NAD depletion. The regeneration of NAD from nicotinamide consumes four ATP molecules for each molecule of NAD regenerated and can lead to ATP depletion (46). The results in Fig. 3 indicate that, in fact, a large component of the H2O2-induced fall in ATP in LLC-PK1 cells was prevented by 3-ABA. Finally, because DNA damage is the trigger for PARP activation, we confirmed that exposure of LLC-PK1 cells to H2O2 resulted in DNA damage (Fig. 4). Taken together, the results in Figs. 1–4 are consistent with the view that the H2O2-induced DNA damage results in activation of PARP and leads to NAD depletion and, in turn, ATP depletion in LLC-PK1 cells. It should be noted also that 3-ABA did not completely prevent the H2O2-induced fall in ATP. Thus other mechanisms may also account for a component of H2O2-induced ATP depletion, particularly at higher concentrations of H2O2.
Exposure of LLC-PK₁ cells to 1 mM H₂O₂ causes necrotic cell death over the course of 2–6 h (2, 38). In a variety of cells, inhibition of PARP reduces oxidant-induced cytotoxicity (reviewed in Ref. 35). The results in Figs. 5–7 demonstrate that 3-ABA prevented necrotic cell death within the first 24 h of H₂O₂ exposure. The effect of 3-ABA on cell viability appears to be the result of PARP inhibition, since an inactive structural analog did not reduce cell death. Ueda and Shah (38) have shown that DNA damage is an important determinant of oxidant-induced cell death in LLC-PK₁ cells. Our results indicate that DNA damage itself is not sufficient to result in necrotic cell death. Specifically, inhibition of PARP prevented early necrotic cell death (Figs. 5–7) without preventing DNA damage (Fig. 4). Likewise, Andreoli and Mallett (2) found that certain antioxidants could prevent H₂O₂-induced cell death without preventing oxidant-induced DNA damage. Thus it appears that DNA damage is an early consequence of oxidant injury that triggers a pathological cascade, involving activation of PARP, NAD depletion, and ATP depletion, which ultimately results in necrotic cell death.

We also demonstrated that, although inhibition of PARP prevented H₂O₂-induced ATP and NAD depletion and early (<24 h) cell death, cells subsequently underwent an apoptotic phenotype (Figs. 8–10). Similar findings have been reported in intestinal epithelial cells (43), thymocytes (41), and leukemic myeloid cells (27), although PARP inhibitors may also reduce oxidant-induced apoptosis in some tissues (14, 26). Our results do not indicate whether the apoptosis we observed was initiated by the oxidant injury and simply uncovered by the prevention of early necrosis or whether the inhibition of PARP, in the setting of DNA damage, led to apoptosis. Either possibility is plausible. Oxidant stress is a known inducer of apoptosis in a number of cell types (26, 27, 34), and the severity of the stress may determine whether cells manifest necrotic or apoptotic death. Using a model of ATP depletion in mouse proximal tubular cells, Lieberthal et al. (23) demonstrated that mild ATP depletion led to apoptosis, whereas severe ATP depletion led to necrosis. Moreover, ATP is required for apoptosis such that severe ATP depletion inhibits the apoptotic pathway in a number of cells (12, 33), including LLC-PK₁ cells (25). Thus, by preventing severe ATP depletion (Fig. 3), 3-ABA may have allowed oxidant-induced apoptosis to proceed. Alternatively, inhibition of PARP may have played a more direct role in promoting apoptosis. PARP is a substrate for the caspase proteases (36). Upon activation of caspase-3 during apoptosis, PARP is cleaved from its 113,000 molecular weight active form to an inactive 85,000 molecular weight COOH-terminus fragment. Thus inhibition of PARP by 3-ABA might mimic the effects of caspase-3 on PARP during apoptosis. However, the importance of the inactivation of PARP during apoptosis remains uncertain, since PARP-deficient cells show no greater sensitivity to several apoptosis-inducing stimuli (22).

Regardless of the mechanism, the observation that cells exposed to H₂O₂ in the presence of 3-ABA survived for 12–24 h but subsequently developed apoptosis raises a caution regarding in vitro studies of renal epithelial cell injury and cytoprotection. In general, such studies have employed either freshly isolated tubules or cultured cells and have focussed on necrosis (e.g., LDH release, vital dye exclusion) over only a limited time (0.5–6 h) after the insult (28, 40, 42, 44). It is possible that some of the maneuvers reported to prevent early cell death, e.g., antioxidants, glycine, acidosis, etc., may have no long-term benefit and that some of these maneuvers may also result in apoptosis.

The finding that inhibition of PARP prevented necrosis but not apoptosis may also be relevant to the differential sensitivity of various nephron segments to acute renal injury (8). In ischemic acute renal failure, necrosis affects predominantly the proximal tubule (39), whereas evidence of apoptosis is more prevalent in the distal tubule (5). It is possible that different levels of PARP activity, i.e., high activity in the proximal tubule and low activity in the distal tubule, may account for the different patterns of injury in these segments. Measurements of PARP activity in specific nephron segments will be required to address this issue.

In summary, PARP is activated during oxidant injury to LLC-PK₁ renal epithelial cells, and inhibition of PARP attenuates the depletion of ATP and NAD and prevents necrosis without affecting oxidant-induced DNA damage. These results suggest that ATP utilization to regenerate NAD is a major factor leading to ATP depletion during oxidant injury and that DNA damage is not a direct cause of necrotic cell death. In addition, prevention of cell necrosis by the inhibition of PARP may target cells toward an apoptotic death. In vivo

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Fig. 10. Analysis of DNA fragmentation by agarose gel electrophoresis. Cells were treated for 2 h with 1 mM H₂O₂ in the presence or absence of 10 mM 3-ABA and then incubated in culture medium for 24 h. Typical ladder pattern of DNA fragments (which were multiples of ~200 bp) can be recognized readily in the 3-ABA + H₂O₂-treated cells. DNA laddering was not observed in control or H₂O₂-treated cells.
studies will be needed to determine the role of PARP in acute renal injury.

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