Par-4 is a novel mediator of renal tubule cell death in models of ischemia-reperfusion injury

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Xie J, Guo Q. Par-4 is a novel mediator of renal tubule cell death in models of ischemia-reperfusion injury. Am J Physiol Renal Physiol 292: F107–F115, 2007. First published August 8, 2006; doi:10.1152/ajprenal.00083.2006.—Prostate apoptosis response-4 (Par-4) is a leucine zipper protein linked to apoptotic cell death in prostate cancer and neuronal tissues. The leucine zipper domain of Par-4 (Leu.zip) mediates protein-protein interactions that are essential for sensitization of cells to apoptosis, and overexpression of Leu.zip blocks Par-4 activity in a dominant negative fashion. Ischemia-reperfusion-induced renal injury (IRI) is clinically important because it typically damages renal tubular epithelial cells and glomerular cells, and it is the most common cause of acute renal failure (ARF). We now report that Par-4 is expressed in renal tubule cells and that aberrant expression of Par-4 activity plays a crucial role in activating apoptotic pathways in well-characterized models of renal IRI. Increased levels of Par-4 were observed following chemical ischemia-reperfusion in HK-2 cells in vitro and in mouse renal tubular cells following bilateral clamping of renal pedicles in vivo. Inhibition of Par-4 expression by specific par-4 antisense oligonucleotides largely prevented HK-2 cell apoptosis induced by IRI. Overexpression of Par-4 in these cells exacerbated mitochondrial dysfunction and caspase activation and conferred increased sensitivity to IRI-induced apoptosis. Expression of Leu.zip, a dominant negative regulator of Par-4, largely prevented mitochondrial dysfunction and caspase activation and significantly inhibited IRI-induced apoptosis in HK-2 cells. In addition, transfection of Par-4 increased while transfection of Leu.zip decreased necrosis in HK-2 cells following prolonged IRI. These results identify Par-4 as a novel and early mediator of renal tubule cell injury following IRI and provide a potential target for developing new therapeutic strategies for renal IRI and ARF.

prostate apoptosis response-4; apoptosis; necrosis; mitochondria; caspase
renal tubule cells was confirmed by the observation that inhibition of Par-4 expression by par-4 antisense oligonucleotides significantly reduced the death of HK-2 cells induced by IRI. Transfection of Par-4 increased while transfection of Leu.zip decreased necrosis in HK-2 cells following prolonged IRI. These results indicate that Par-4 regulates both apoptosis and necrosis and identify Par-4 as a novel and early mediator of tubular cell death in ischemia-reperfusion-induced renal injury.

MATERIALS AND METHODS

Culture and transfection of human kidney proximal tubular cells. The human kidney proximal tubular cell line HK-2 cells (ATCC) were maintained at 37°C in an atmosphere of 95% air-5% CO₂ in keratinocyte serum-free medium (GIBCO-BRL) with 5 ng/ml recombinant epidermal growth factor and 0.05 mg/ml bovine pituitary extract (complete growth medium), according to the manufacturer’s instructions. A full-length Par-4 cDNA was subcloned into the expression vector pHIP (Invitrogen), yielding a recombinant construct pREP4-Par4 that encodes a 1.2-kb RNA species and a full-length 38-kDa Par-4 protein. A cDNA encoding a deletion mutant of Par-4, Leu.zip (in which only the leucine zipper domain of Par-4 was expressed), was separately subcloned into the expression vector pREP4, yielding a recombinant construct pREP4-Leu.zip (39). Human HK-2 cell lines stably expressing Par-4 or Leu.zip were established by transfection using Lipofectamine 2000 Reagent (Invitrogen) with pREP4-Par4 or pREP4-Leu.zip, respectively. Transfected cells were selected with hygromycin (400 μg/ml) for 4 wk, and surviving clones were selected. Previous studies in our lab established that overexpression of Leu.zip functions as a dominant negative regulator of Par-4 in transfected cells (12, 39). For control purposes, parallel cultures of HK-2 cells were stably transfected with pREP4 vector alone.

Induction of ischemia-reperfusion injury in cell culture. These methods combine a widely used and extensively characterized cell culture model of ischemia injury with an in vitro reperfusion protocol described previously for renal tubule cells (15, 20). The in vitro ischemia model takes advantage of the hallmark features of renal ischemic injury: profound intracellular ATP depletion and a fall in tissue oxygen content with a concomitant rise in intracellular calcium. In brief, to induce IRI in cell culture, HK-2 cells (~80% confluent) were washed in glucose-free buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl₂, 1.0 mM MgCl₂, 3.6 mM NaHCO₃, and 5 mM HEPES, pH 7.2) and then incubated with 10 mM antimycin A plus 10 mM 2-deoxyglucose plus 1 μM calcium ionophore (A23187) for 60 min (to induce moderate ischemic injury in vitro) or 120 min (to induce more prolonged ischemic injury in vitro). The in vitro reperfusion was achieved by incubating cells in glucose-replete complete growth medium. Previous studies demonstrated that, without the calcium ionophore, the combination of ATP and glucose depletion with antimycin A and 2-deoxyglucose, respectively, produces ~90% ATP depletion but fails to kill HK-2 cells effectively (16, 20). The addition of 1 μM calcium ionophore (A23187) mimics the rise in intracellular calcium load seen in ischemic renal tubule cells in vivo and facilitates apoptosis of HK-2 cells following in vitro reperfusion (see RESULTS and DISCUSSION).

IRI in animals in vivo. The animal protocols used in this study are consistent with regulations for experimental animal care and use, and have been approved by the Institutional Animal Care and Use Committee at the University of Oklahoma Health Sciences Center. We used the mouse bilateral clamping model of renal ischemia which has been described previously (45). For this experiment, male wild-type C57BL/6 mice aged 8–10 wk were anesthetized with an intraperitoneal injection of an appropriate amount of sterilized 2.5% Avertin (0.583 g/kg body wt) and placed on a homeothermic blanket to maintain body temperature. The skin area in the abdomen was clipped off, and the surgical area was aseptically prepped: the skin was cleaned three times at the intended incision area by first scrubbing the area with betadine followed up by a rinse with 70% alcohol and subsequent application of surgical iodine. A midventral laparotomy was performed under sterile conditions using autoclaved instruments. After the intestine was covered and protected with warm saline-soaked gauze, the renal pedicles were exposed. Renal ischemia was induced by bilateral clamping of renal pedicles with arterial microclamps. For ischemia-reperfusion, clamps were released after 30 min of clamping, and the skin incisions were closed with a continuous subcuticular suture. Sham control animals were subjected to the identical surgery except for renal pedicle clamping. Mice recovering from anesthesia were kept warm on a homeothermic blanket and monitored every 15 min after the surgery until they recovered to a point where they were moving around.

Kidney harvesting and immunohistochemistry. For this procedure, the mice were anesthetized with an intraperitoneal injection of an appropriate amount of sterilized 2.5% Avertin (0.583 g/kg body wt) and exsanguinated by cardiac perfusion with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Both kidneys were removed by midventral laparotomy, cryoprotected in 30% sucrose, frozen, and sectioned (15- to 20-μm thick) on a cryostat. Sections were processed on slides for immunohistochemistry to localize Par-4 expression, according to protocols routinely used in our lab. Briefly, tissue sections were incubated for 16–24 h at room temperature with 1:100 mouse monoclonal anti-Par-4 antibody (49). Sections were washed in phosphate-buffered saline (PBS), incubated in goat anti-mouse Alexa 488 (1:100 dilution, Molecular Probes) secondary antibody for 1 h, and mounted in Vectashield (Vector Labs). Images of Par-4 immunofluorescence were acquired using a Nikon TS 100 microscope equipped for epifluorescence and bright-field microscopy. Images were captured with a Nikon DXM 1200F Digital Camera, and the average pixel intensity of Par-4 immunoreactivity per cell was determined using LSM 510 software (Carl Zeiss MicroImaging, Thornwood, NY). Throughout the experiments, the specificity of the immunoreactivity was confirmed by subjecting additional samples to the immunostaining procedures without primary or the secondary antibody.

Western blot analysis. Levels of expression of Par-4 were determined by Western blot analysis as described (47). The antibody that specifically recognizes Par-4 was a mouse monoclonal antibody raised against full-length rat Par-4 (Santa Cruz Biotechnology, Santa Cruz, CA). The antibody reacts with Par-4 of mouse, rat, and human origin. Fifty micrograms of solubilized proteins were separated by electrophoresis on a 4–12% gradient SDS-polyacrylamide gel and then transferred to a nitrocellulose sheet. The nitrocellulose sheet was blocked with 5% milk followed by 1-h incubation in the presence of primary anti-Par4 antibody. The membrane was further processed using horseradish peroxidase-conjugated secondary antibody and immunoblotted proteins were detected by chemiluminescence using the ECL system (Amersham). Equal loading was verified by probing the blots with the monoclonal anti-β-actin antibody (Sigma, St. Louis, MO). Western blot images were acquired and quantified using Kodak Image Station 2000R and Kodak Digital Science 1D 3.6. software.

Par-4 antisense oligonucleotides and detection of apoptosis. Par-4 antisense (5′-ATAGCCCGGGTCGGCATTGT-3′) and nonsense (5′-CGGTGCTGATTCGTCGCTG-3′) oligodeoxynucleotides (12) were prepared as 20 μM stocks in the serum-free medium, and 200 nM oligonucleotides were transfected into the cultured HK-2 cells using Oligofectamine Reagent (Invitrogen), according to the manufacturer’s instructions. The effectiveness and specificity of the antisense par-4 oligodeoxynucleotide have been extensively documented in previous studies (12, 39). Quantification of apoptosis using fluorescent microscopy was described previously (12) and involves the staining of cells with the fluorescent DNA-binding dye Hoechst 33342. In brief, cultures of HK-2 cells were stained with 2 μg/ml Hoechst 33342 at 37°C for 1 h. After being washed with 0.2 M HEPES (pH 7.4), cells were visualized under a Nikon TS-100 fluorescent microscope. Images were captured using a
Nikon digital DXM 1200F camera and acquired, processed, and analyzed with Nikon ACT-1 software. At least 200 cells per culture from six random microscopic fields of view at ×200 magnification were counted, and counts were made in at least six separate cultures per treatment condition. Cells showing nuclear chromatin condensation and fragmentation are counted as apoptotic cells. Counts were made without knowledge of cell genotype or experimental treatment history.

**Assessments of mitochondrial transmembrane potential.** The dye rhodamine 123 (Molecular Probes) was employed as a measure of mitochondrial transmembrane potential using methods described previously (12). Briefly, cells were incubated for 30 min in the presence of 10 μM dye and washed twice in Locke’s solution (154 mM NaCl, 5.6 mM KCl, 2.3 mM CaCl2, 1.0 mM MgCl2, 5.0 mM NaHCO3, 3.6 mM glucose, and 5 mM HEPES, pH 7.2). Cellular fluorescence was imaged using a Zeiss LSM 510 laser-scanning confocal microscope with excitation at 488 nm and emission at 510 nm. All images were acquired using the same laser intensity and photodetector gain, to allow quantitative comparisons of relative levels of fluorescence in the cells. The average pixel intensity of fluorescence per cell was determined using the LSM 510 software (Carl Zeiss Micro-Imaging).

**Measurement of caspase activity.** Levels of caspase-3 activity were assessed using a previously described protocol that employs DEVD, a pseudo substrate and inhibitor of caspase-3 (12). Briefly, cultured cells were exposed for 10 min to Locke’s solution containing 0.1% digitonin and were then incubated for 20 min in the presence of 10 μg/ml biotinylated DEVD-CHO (Calbiochem, La Jolla, CA). Cells were washed three times with PBS and fixed for 30 min in a cold solution of 4% paraformaldehyde in PBS. Cells were then incubated for 5 min in PBS containing 0.2% Triton X-100, followed by incubation for 30 min in PBS containing 5 μg/ml streptavidin-Oregon Green conjugate (Molecular Probes, Eugene, OR). Cells were washed twice in PBS and images of cellular fluorescence (corresponding to conjugates of activated caspase-3 with DEVD-biotin) were acquired using a Zeiss LSM 510 confocal laser-scanning microscope (488-nm excitation and 510-nm emission) with a ×60 oil immersion objective. All images were acquired using the same laser intensity and photodetector gain to allow quantitative comparisons of relative levels of fluorescence in the cells. The average pixel intensity per cell was determined using LSM 510 image analysis software.

**Assessments of plasma membrane damage in necrotic cells by propidium iodide staining and lactate dehydrogenase release assays.** In contrast to apoptosis, necrosis is characterized morphologically by cellular swelling and lysis, membrane rupture, densities in swollen mitochondria, and pyknotic or irregularly clumped nuclear chromatin. Since the intact plasma membrane of living cells excludes cationic dyes (such as propidium iodide) and prevents extracellular release of the enzyme lactate dehydrogenase (LDH), membrane rupture seen in necrosis can be measured by propidium iodide exclusion and LDH release assays. Propidium iodide is a membrane-impermeant dye that, once inside the cell, stains DNA and RNA by intercalating into nucleic acid molecules. For propidium iodide exclusion experiments, HK-2 cells were subjected to ischemia-reperfusion injury, followed by 10-min incubation with 5 μM propidium iodide. Levels of propidium iodide fluorescence were then visualized by confocal laser-scanning microscopy. Necrotic cells are quickly stained by short incubations with propidium iodide, while apoptotic cells show an uptake of propidium iodide that is much lower than that of necrotic cells (43). LDH release from ischemic HK-2 was determined using a spectrophotometric assay, as described previously (13).

**RESULTS**

Par-4 is expressed in renal tubular epithelial cells: effects of IRI in vitro and in vivo. We first examined whether Par-4 was expressed in a human kidney proximal tubule cell line, HK-2 cells. Fifty micrograms of solubilized proteins from HK-2 cells were separated by electrophoresis on a 4−12% gradient SDS-polyacrylamide gel, transferred to a nitrocellulose sheet, and subjected to Western blotting analysis. As shown in Fig. 1A, Par-4 expression in HK-2 cells was apparent on Western blotting, but at relatively low levels. To examine whether levels of Par-4 are altered following IRI in vitro, HK-2 cells were incubated with 10 mM antimycin A plus 10 mM 2-deoxyglucose plus 1 μM calcium ionophore (A23187) for 60 min. The in vitro reperfusion was achieved by incubating cells in glucose-replete complete growth medium. The addition of 1 μM the calcium ionophore was used to mimic the rise in intracellular calcium induced by ischemia in vivo and to facilitate apoptotic cell death of HK-2 cells following reperfusion. As shown in Fig. 1, A and B, levels of Par-4 in HK-2 cells started to increase quickly within 4 h following ischemia-reperfusion, reaching a maximum level of expression at ~12 h.

The aberrant induction of Par-4 expression in renal tubular cells following ischemia-reperfusion was also confirmed in mice following bilateral clamping of renal pedicles in vivo. Male C57BL/6 mice of 8–10 wk were subjected to sham operation or 30 min of renal ischemia followed by 12 h of reperfusion. Renal tissues were fixed and sectioned on a cryostat and processed for immunofluorescent histochemistry. As shown in Fig. 1, C and D, levels of Par-4 in renal tubule cells were significantly increased following IRI in vivo. The induction of Par-4 expression following IRI was predominantly found in tubular cells in the renal cortex, but not in the glomeruli. These results suggest that Par-4 is likely involved in ischemia-reperfusion-induced renal injury.

**Aberrant expression of Par-4 is essential for apoptosis of kidney proximal tubule cells following ischemia-reperfusion.** We examined whether aberrant expression of Par-4 was involved in apoptotic cell death of HK-2 cells following chemical IRI. We tested whether the chemical ischemia-reperfusion protocol used in the present study would induce apoptosis of HK-2 cells and whether inhibition of aberrant Par-4 expression by antisense oligonucleotides would block apoptotic cell death in these cells. The effectiveness of the par-4 antisense oligonucleotides in blocking Par-4 expression has been documented in our previous studies (12, 39). Cultures of HK-2 cells were transfected with either par-4 antisense (AS par-4) or nonsense DNA (NS par-4) oligonucleotides and were then subjected to chemical ischemia for 60 min followed by reperfusion for 48 h. Apoptotic cell death was then analyzed by counting apoptotic cells that showed nuclear chromatin condensation and fragmentation following staining with the fluorescent DNA-binding dye Hoechst 33342. Significantly amounts of apoptotic HK-2 cells were detected following chemical IRI. Forty-eight hours after reperfusion, over 73% of the HK-2 cells were apoptotic (Fig. 2, A and B). Of importance, apoptosis of HK-2 cells induced by IRI was largely prevented by antisense par-4 treatment (AS Par-4), while nonsense DNA (NS Par-4) was ineffective (Fig. 2, A and B). These results suggest that apoptosis of renal tubule cells plays a significant role in ischemia-reperfusion-induced renal injury and that aberrant induction of Par-4 expression was essential for apoptotic cell death of these cells.

**Increased vulnerability to apoptosis induced by ischemia-reperfusion in renal tubule cells transfected with Par-4: effect of Leu.zip, a dominant negative regulator of Par-4.** To further confirm that aberrant induction of Par-4 expression contributes to apoptosis in renal tubule cells, we further examined whether overexpression of Par-4 in transfected HK-2 cells confers...
increased sensitivity to apoptotic cell death following chemical IRI. HK-2 cells were stably transfected with pREP4-Par4 (which encodes the full-length 38-kDa Par-4 protein) using Lipofectamine 2000 Reagent, and parallel control cultures of HK-2 cells were stably transfected with pREP4 vector alone. As shown in Fig. 3, 24 h following IRI, the average percentage of apoptotic cells in untransfected or vector-transfected control groups was 33.5 and 32.8%, respectively, while apoptosis in cells transfected with Par-4 was significantly increased to an average of 76.5%. On the other hand, overexpression of Leu.zip, which has been shown to block Par-4 activity in a dominant negative fashion (12, 39), drastically decreased apoptotic cell death of renal proximal tubule cells induced by chemical ischemia-reperfusion (Fig. 3). These results provide additional convincing evidence that aberrant induction of Par-4 expression plays a key role in apoptotic cell death induced by ischemia-reperfusion in renal proximal tubule cells.

Par-4 functions in the early stages of apoptosis before mitochondrial alterations and caspase activation in renal tubule cells following ischemia-reperfusion. Next, we examined the underlying cellular mechanisms of the proapoptotic action of Par-4 in HK-2 cells following IRI. Since apoptosis often involves mitochondrial dysfunction (47), we examined changes in mitochondrial membrane depolarization using the fluorescent dye rhodamine 123 and confocal laser-scanning microscopy. Overexpression of Par-4 led to an exacerbated decrease in Rh123 fluorescence in HK-2 cells following IRI, while blocking Par-4 activity by expressing Leu.zip significantly preserved Rh123 fluorescence following IRI (Fig. 4, A and B). Because cysteine proteases of the caspase family have a prominent role in apoptosis, we examined levels of caspase-3 activity in HK-2 cells transfected with Par-4 or Leu.zip, using DEVD, a pseudo substrate of caspase-3 (47). Images of cellular fluorescence corresponding to conjugates of activated caspase-3 with DEVD-biotin were acquired by confocal laser-scanning microscopy. As shown in Fig. 4C, transfection of Par-4 drastically exacerbated, while overexpression Leu.zip significantly alleviated, activation of caspase-3 in HK-2 cells following IRI. Collectively, our data demonstrated that blocking Par-4 (by either the antisense approach or overexpression of the dominant negative regulator Leu.zip) significantly inhibited mitochondrial dysfunction and caspase-3 activation and...
largely prevented apoptotic cell death in HK-2 cells following IRI. These results indicate that aberrant induction of Par-4 is an essential and a relatively early step (upstream of mitochondrial dysfunction, caspase activation, and nuclear chromatin condensation and fragmentation) in the apoptotic cascades initiated by IRI.

Additional evidence suggests Par-4 also exacerbates necrotic cell death induced by prolonged ischemia-reperfusion in renal tubular cells. Ischemia-reperfusion induces both apoptosis and necrosis. Published data indicate that lower intensity, short-duration ischemic insults tend to induce apoptosis, whereas acute, higher intensity and long-lasting ischemic insults often induce necrosis. While data described above provide evidence for a role of Par-4 in apoptosis of renal tubule cells following ischemia-reperfusion, we examined whether overexpression of Par-4 also enhances necrotic cell death induced by ischemia-reperfusion. To effectively induce damage of plasma membrane and necrotic cell death, we subjected HK-2 cells to more intense and prolonged ischemia-reperfusion (120 min of chemical ischemia followed by 48-h reperfusion). Necrotic death of HK-2 proximal tubular cells was then measured by two different approaches: confocal laser-scanning microscopic analysis of propidium iodide fluorescence and LDH release assays. As shown in Fig. 5, transfection of Par-4 significantly increased the amount of propidium iodide-positive necrotic cells induced by ischemia-reperfusion. LDH release induced by intense ischemia-reperfusion was also significantly exacerbated by overexpression of Par-4 in HK-2 cells. Transfection of Leu.zip de-
Fig. 4. Mitochondrial dysfunction and caspase activation are exacerbated in HK-2 cells transfected with Par-4 following chemical IRI: effects of Leu.zip. A: representative confocal laser-scanning microscope images of Rh123 fluorescence, a measure of mitochondrial transmembrane potential, in vector-transfected control cells and Par-4-transfected cells before and after IRI. The cells were subjected to 60 min of chemical ischemia, followed by 12-h reperfusion. Par-4 led to an exacerbated decrease in Rh123 fluorescence following IRI. B: statistical analysis of the average pixel intensity of Rh123 fluorescence/cell in untransfected HK-2 cells (untransfected) and those transfected with vector alone (vector alone), Par-4 or Leu.zip 12 h following IRI. Par-4 and Leu.zip showed contrasting, opposite effects on mitochondrial transmembrane potential in HK-2 cells. ***P < 0.01 compared with the control Rh123 fluorescence levels within their respective groups. ****P < 0.001 compared with corresponding values in untransfected or vector-transfected cell groups. ###P < 0.001 compared with corresponding values in untransfected, vector-, or Par-4-transfected cell groups. C: transfection of Par-4 exacerbates, while overexpression Leu.zip alleviates, activation of caspase-3 in HK-2 cells following IRI. Cultures of indicated lines of HK-2 cells were exposed to 60 min of chemical ischemia, followed by indicated time periods of reperfusion, and levels of DEVD fluorescence, a measure of caspase-3 activation, were quantified. ****P < 0.001 compared with the corresponding values in untransfected, vector-, or Leu.zip-transfected cell groups. ***P < 0.01 compared with the corresponding values in untransfected or vector-transfected cell groups. Similar data were obtained from 3 separate clones of transfected cells (data not shown). Values are means ± SE of determinations made in 6 separate cultures. At least 100 cells per culture were analyzed. ANOVA was used with Scheffe’s post hoc tests.

creased necrotic cell death in HK-2 cells following prolonged IRI. Taken together, our data suggest that Par-4 is involved in both apoptotic and necrotic cell death induced by ischemia-reperfusion in renal tubular epithelial cells.

DISCUSSION

Par-4 is a leucine zipper protein that was initially identified to be associated with apoptosis of prostate cancer cells and neuronal degeneration in Alzheimer’s disease (12, 39). Further studies found that the leucine zipper domain of Par-4 mediates protein-protein interactions that are essential for sensitization of cells to apoptosis (12, 14, 23, 34, 39). Thus, overexpression of a deletion mutant of Par-4 that encodes only the leucine zipper domain of Par-4 (Leu.zip) blocks Par-4 activity in a dominant negative fashion by inhibiting binding of other protein(s) with the full-length Par-4 (12, 14, 23, 34, 39). Cook and
colleagues (5) previously reported that Par-4 protein levels were severely decreased in human renal cell carcinoma specimens relative to normal tubular cells. Replenishment of Par-4 protein levels in renal cell carcinoma cell lines conferred sensitivity to apoptosis (5). These results provide the first hint that Par-4 might be a crucial factor in regulation of renal tubule cell death and survival. Data presented in this study indicate that Par-4 is indeed expressed in human renal proximal tubule cells and that aberrant expression of Par-4 activity plays a crucial role early in activation of cell death pathways in these cells following renal IRI. This notion is supported by several lines of experimental evidence obtained in this study: 1) levels of Par-4 in HK-2 cells started to increase quickly within 4 h following chemical ischemia-reperfusion, reaching a maximum level of expression at ~12 h, indicating that Par-4 functions in the early stages of activation of apoptotic machinery; 2) similar increase in Par-4 expression was also confirmed in mouse renal tubular cells following bilateral clamping of renal pedicles in vivo; 3) inhibition of Par-4 expression by specific par-4 antisense oligonucleotides largely prevented apoptotic cell death induced by IRI, suggesting that Par-4 was essential for apoptosis of HK-2 cells; 4) although overexpression of Par-4 alone was not sufficient to induce spontaneous apoptosis in transfected HK-2 cells under basal culture conditions, it exacerbated mitochondrial dysfunction and caspase activation and conferred increased sensitivity to IRI-induced apoptosis; 5) expression of Leu.zip, a dominant negative regulator of Par-4, largely prevented mitochondrial dysfunction and caspase activation and significantly inhibited IRI-induced apoptosis in HK-2 cells; and 6) transfection of Par-4 increased, while transfection of Leu.zip decreased, necrosis in HK-2 cells following intense prolonged IRI. These results provide convincing evidence that aberrant induction of Par-4 activity is an essential and a relatively early step (upstream of mitochondrial dysfunction, caspase activation, and nuclear chromatin condensation and fragmentation) in the cell death cascades initiated by IRI in renal proximal tubule cells.

Fig. 5. Par-4 exacerbates membrane damage in necrotic death of HK-2 proximal tubular cells after prolonged ischemia-reperfusion (I/R) in cell culture. A: representative confocal laser-scanning microscopic images showing specific propidium iodide fluorescence following 10-min incubation with 5 μM propidium iodide. Strong propidium iodide fluorescence represents disintegration of plasma membrane in necrotic cell death of HK-2 cells subjected to 120 min of chemical ischemia followed by 48-h reperfusion. Transfection of Par-4 significantly increased the amount of necrotic cells induced by ischemia-reperfusion. B: LDH release from HK-2 cells subjected to 120 min of chemical ischemia followed by 48-h reperfusion. Membrane rupture seen in necrosis was measured by LDH release assays. Transfection of Par-4 significantly increased, while transfection of Leu.zip significantly decreased, LDH release from HK-2 cells induced by ischemia-reperfusion. Values are means ± SE of determinations made in 6 separate experiments. ***P < 0.01 compared with corresponding before ischemia-reperfusion values within untransfected and vector-transfected control groups. ****P < 0.001 compared with corresponding values in untransfected and vector alone groups. ###P < 0.001 compared with corresponding values in untransfected, vector alone, and Par-4-transfected cell groups. ANOVA was used with Scheffé’s post hoc tests.

The in vitro model of IRI in HK-2 cells used in the present study is a very well-characterized and widely used cell culture model of renal IRI. It reproduces several key hallmark features of IRI, including a profound intracellular ATP depletion, a fall in tissue oxygen and glucose content with a concomitant rise in intracellular calcium. Previous studies demonstrated that, without the calcium ionophore, the combination of ATP and glucose depletion with antimycin A and 2-deoxyglucose, respectively, produces ~90% ATP depletion but fails to kill HK-2 cells effectively (16, 20). The addition of the calcium ionophore (A23187) mimics the rise in intracellular calcium load seen in ischemic renal tubule cells in vivo and facilitates HK-2 cell death in vitro. It was previously shown that addition of high concentrations of the calcium ionophore (over 2 μM), combined with ATP and glucose depletion with antimycin A and 2-deoxyglucose, induced rapid necrotic cell death in HK-2 cells (20), and we found that lower than 1 μM concentrations of the calcium ionophore failed to significantly facilitate HK-2 cells death associated with ATP and glucose depletion (data not shown). The 1 μM calcium ionophore (A23187) concentration was chosen for our studies on apoptosis because, at this concentration, a significant amount of apoptosis of HK-2 cells
was induced over a period of 48 h following in vitro reperfusion (Figs. 2, 3, and 4).

Mounting evidence demonstrates that both apoptosis and necrosis of renal tubule cells play significant roles in the pathogenesis of renal IRI. For example, it has been shown in myocardium that, depending on the ischemic insult, apoptosis may be the predominant form of cell death during the first 8 h of reoxygenation, so that apoptosis is more important than necrosis during the early phase of the ischemic period but the reverse is true after prolonged, intense ischemia (44). We therefore examined whether Par-4 also regulates necrotic cell death in renal tubule cells. To most effectively induce damage of plasma membrane and necrotic cell death, we subjected HK-2 cells to more intense and prolonged ischemia-reperfusion (120 min of chemical ischemia followed by 48-h reperfusion). Necrotic death of HK-2 proximal tubular cells was then measured by two different approaches: confocal laser-scanning microscopic analysis of propidium iodide exclusion and LDH release assays. We found that transfection of Par-4 significantly increased the amount of LDH release and the number of propidium iodide-positive necrotic cells induced by ischemia-reperfusion. Transfection of Leu.zip, a dominant negative regulator of Par-4, significantly ameliorated the necrotic cell death of HK-2 cells induced by ischemia-reperfusion.

How does Par-4 modulate both apoptotic and necrotic cell death in the kidney following ischemia-reperfusion? The usual circumstances initiating apoptotic and necrotic cell death, as well as the characteristics of each, differ so greatly that these processes were once thought to be fundamentally separate and unique. However, apoptosis and necrosis have been shown to be more similar in their regulation than previously believed, sharing several common signaling pathways (24). Many pro-apoptotic proteins also exacerbate necrotic cell death. For example, mutations in presenilin-1 are causally linked to the pathogenesis of Alzheimer’s disease and have been shown to exacerbate apoptotic neuronal death in vitro in response to a variety of apoptotic insults (13, 46, 48). The same mutant protein was, however, also found to increase neuronal vulnerability to kainic acid-induced necrotic cell death in the hippocampus (11). Another example of cell death-related proteins that promote both apoptotic and necrotic cell death is Bid, a member of the bcl-2 family of proteins. Bid is a well-characterized proapoptotic protein that promotes cytochrome c release from mitochondria. However, in Bid knockout mice, both apoptotic and necrotic tubular cell death were ameliorated. A role for Bid in necrosis was also suggested by the results from a hypoxia injury model (42, 45). Secondary necrosis may occur if an apoptotic cell is not phagocytosed after its period of surface blebbing and becomes highly rigid, blisters, and finally ruptures, releasing its contents into the surroundings (24). This, as a consequence, may promote a proinflammatory response. Production and availability of ATP change drastically under ischemic conditions. Ischemic ATP depletion may lead to necrotic cell death following apoptotic initiation, while apoptotic cell death may increase following reperfusion (24). Necrosis, originally believed to be a random and chaotic means of cell death, is being found to possess a higher degree of order than expected. For example, PS externalization, an event previously considered unique for apoptosis, may also occur in cells undergoing necrosis. As apoptotic DNA displays laddering on gel electrophoresis, necrotic DNA displays a “smear,” presumably representing DNA fragments of variable length. However, in early necrosis, DNA degradation also occurs in an orderly manner, with DNA fragments showing surprisingly little variation (24). Recent research shows that, following induction of necrosis by various stimuli, the orderly destruction of chromatin is carried out by serum DNAse I along with plasmin, which together penetrate the cell to induce nuclear breakdown. The variable length of DNA fragments likely results from nonspecific degradation caused by lysosomal rupture and the release of their contents, as well as macrophage involvement and other enzymatic degradations (24). Thus, the apoptotic and necrotic modes of death may share more common pathways than previously believed, and, in many diseased conditions, there often exists a delicate balance between them (2, 22, 30, 31, 37, 41, 42). It is therefore highly possible that Par-4 modulates both apoptotic and necrotic cell deaths induced by renal ischemia-reperfusion by initiating similar or interlinked cell death pathways in the early phases of cell death. The increased necrosis induced by Par-4 might be secondary to an early apoptotic initiation, or might have occurred in an apoptosis-independent manner, or both. The cells that survive the insult may undergo a process resembling development, which includes dedifferentiation, reentry into the cell cycle, and proliferation to replace the dead cells (21). We also observed that, when cell death was prevented by knocking down Par-4, HK-2 cells reenter cell cycle and grow well in subcultures (data not shown).

Renal IRI is clinically important because it typically damages renal tubular epithelial cells and glomerular cells and is the most common cause of ARF, a major clinical problem with exceptionally high morbidity and mortality (2, 7, 10, 27, 29, 42). Effective treatment for ARF has been lacking. Although the precise mechanisms by which Par-4 alters mitochondrial function and various elements of cell death pathways in renal tubule cells still need to be further investigated, this study identifies Par-4 as a potential target for developing novel therapeutic strategies for renal IRI and ARF.

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REFERENCES

PAR-4 AND RENAL ISCHEMIA-REPERFUSION INJURY


