PERP, a p53 proapoptotic target, mediates apoptotic cell death in renal ischemia

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Submitted 19 September 2008; accepted in final form 21 January 2009


The p53 tumor suppressor gene plays a crucial role in mediating apoptotic cell death in renal ischemia-reperfusion injury (IRI). To further elucidate the p53-dependent pathway, we investigated the role of the p53 apoptosis effector related to PMP-22 (PERP), an apoptosis-associated p53 transcriptional target. PERP mRNA and protein are highly induced in the outer medullary proximal tubular cells (PTC) of ischemic kidneys postreperfusion at 3, 12, and 24 h in a p53-dependent manner. In PTC, overexpression of PERP augmented the rate of apoptosis following hypoxia by inducing mitochondrial permeability and subsequent release of cytochrome c, apoptosis-inducing factor (AIF), and caspase 9 activation. In addition, silencing of the PERP gene with short hairpin RNA prevented apoptosis in hypoxia-mediated injury by precluding mitochondrial dysfunction and consequent cytochrome c and AIF translocation. These data suggest that PERP is a key effector of p53-mediated apoptotic pathways and is a potential therapeutic target for renal IRI.

Acute renal failure; mitochondria; caspase; AIF

APOPTOTIC CELL DEATH HAS BEEN documented in humans and in experimental animal models of postischemic acute kidney injury (AKI), and inhibition of apoptotic cell death is shown to ameliorate the injury and inflammation (7, 8). Increased expression of lethal cytokines and receptors (FasL, Fas) (14, 24), aberrant expression of apoptotic regulators (Bcl2, Bcl-xL, Bax) (4, 38), and caspase activation (21) have been implicated in apoptotic cell death in AKI. However, a correlation between the effect of these gene deficiencies and renal functional alterations in the setting of ischemic AKI has not been established (32). Recent studies have demonstrated that activation of the transcription factor p53 can promote apoptosis in renal ischemia (9, 22). p53 and its transcriptional targets regulate diverse cellular functions that include cell cycle, senescence, differentiation, DNA repair, and apoptosis (1, 3, 29, 43). p53 plays a critical role in the biochemical cascade that leads to apoptotic cell death following genotoxic insult and hypoxia (28). p53 can upregulate the expression of several proapoptotic genes including Bax, Fas/Apo-1, PERP, PUMA, and Noxa under adverse cell conditions (44). Although these p53 targets play cell type-specific roles in p53-mediated apoptosis, an obligatory role for any of these targets in p53-dependent apoptosis in all tissues is yet to be defined (17, 18, 42, 49). It is proposed that the ability of p53 to initiate cell death in a particular tissue is related to the subset of p53 target genes that are up- or downregulated in response to apoptotic stimuli (1, 29). The novel finding that p53 inhibitors protect against apoptosis (23) and renal dysfunction following ischemia-reperfusion (23) and cisplatin-induced injury (45) underscores the necessity to uncover the p53 target genes and the mechanisms by which they integrate their signals to execute p53-mediated apoptosis in renal ischemia (9).

The subset of p53 target genes that are up- or downregulated in response to renal ischemic injury has not been defined. Even though the antiapoptotic effects of p53 inhibition can partially be ascribed to a significant reduction in Bax expression, Bax protein was highly expressed in kidneys of sham-operated mice and its increase after ischemia was modest (9, 23). Thus Bax expression may not in its entirety account for apoptotic responses in ischemic renal tissues. To identify the regulatory targets by which p53 induces renal cell death, we screened for p53 transcriptional target genes that are differentially expressed in the setting of renal ischemia. Our data indicate that the level of one of the p53 apoptotic effector genes, PERP (2), is enhanced in a p53-dependent manner in renal ischemia.

PERP induction is shown to be p53 dependent and to function as a novel effector of p53-dependent apoptosis. Reporter assays demonstrated that p53 can interact with the PERP gene through at least three p53 response elements and can direct a p53 response (36). Thus PERP is a direct transcriptional target of p53.

PERP belongs to the peripheral myelin protein 22/growth arrest specific 3 (PMP-22/gas 3) tetraspan membrane protein family, which includes PMP-22 and epithelial membrane proteins-1, -2, and -3 (2). The mechanism by which tetraspan membrane proteins, including PERP that lacks the BH3 domain, induce apoptosis remains to be defined. PERP may stimulate apoptosis through a different mechanism from the BH3-containing proteins such as Noxa, Puma, and Bax (15), molecules whose functions are nearly universally assumed to be modulation of mitochondrial permeability to induce the release of apoptogenic factors (5, 15, 27). Based on its immunolocalization on the plasma membrane, Golgi apparatus, and mitochondria of transfected mouse embryonic fibroblasts, it was suggested that PERP could serve as a cell death receptor or could have channel or pore activity, to allow the transport of some crucial molecules important for activating apoptosis (2).

Expression of PERP in ischemia-reperfusion injury has not been reported in any tissues, and the regulation of its expres-
PERP-mediated apoptosis in renal ischemia

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Animal and surgical procedures. Mice were cared for before and during the experimental procedures in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC), University of Nebraska Medical Center (UNMC) and the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. All protocols had received prior approval from the UNMC IACUC.

129SV (wild-type) male mice (~20 g, Jackson Laboratories) or p53-deficient (KO) mice were anesthetized by intraperitoneal administration of a cocktail containing ketamine (200 mg) and xylazine (16 mg) per kilogram of body weight. Ischemic injury was induced by bilateral renal pedicle clamping using microaneurysm clamps as we previously described (30). After 37 min of occlusion, the clamps were removed and reflow was verified visually. Sham-operated control animals underwent the same surgical procedure, except for renal artery occlusion. At different time points postinjury, mice were euthanized using pentobarbital sodium for blood collection. At the time of death, kidneys of the animals were perfused with sterile phosphate-buffered saline to remove blood. Kidney tissue sections were snap frozen in liquid nitrogen for RNA or protein isolation and were fixed in Bouins or formalin solution for immunohistochemistry using fluorescent-tagged second antibodies.

Western blot analysis for PERP expression. Immunoblotting was performed with 50 μg of protein extracted from whole kidneys and using a PERP-specific antibody (1:1,000 dilution, Novus Biologicals) as we previously described (31). Protein quantitation was performed by densitometric image analysis using LabWorks software (UVP Bioimaging Systems, Upland, CA) as we previously described (33).

Immunohistochemistry. Localization of PERP was performed by immunofluorescent staining of 5-μm-thick Bouins-fixed, paraffin-embedded kidney tissue sections as described previously (31). The sections were incubated overnight at 4°C with the PERP primary antibody and subsequently with corresponding species-specific secondary antibody. Sections were counterstained with the nuclear dye Hoechst 33342 followed by mounting. The tissue sections were assessed for the spatiotemporal expression of PERP. For blocking experiments, 2 μg of PERP antibody was resuspended in 900 μl of PBS and incubated overnight at 4°C with 10 μg of blocking peptide (SC1743p) or a scrambled peptide (SC1744p, Santa Cruz Biotechnology). Formaldehyde-fixed, paraffin-embedded skin tissue sections were used as a positive control.

Cell culture. The porcine renal proximal tubular cell line (PTC) LLC-PK1 was cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Experiments were performed on semiconfluent monolayers of cells as we previously described (33).

Hypoxic injury. Hypoxic culture conditions were achieved with a pouch system providing a CO2-enriched anaerobic environment (BBL GasPak Pouch System, Becton-Dickinson) as previously reported (48). Hypoxic injury was induced by incubating 80–90% confluent monolayers of LLC-PK1 for 4 h in Krebs-Ringer bicarbonate buffer (in mM: 115 NaCl, 1 KH2PO4, 4 KCl, 1 MgSO4, 1.25 CaCl2, and 27 NaHCO3) at 37°C. After hypoxia, cells were allowed to recover for 1–3 h by reoxygenation in normal growth media.

Stable transfection of PERP in renal epithelial cells. LLC-PK1 cells were transiently transfected using Lipofectamine LTX (Invitrogen) with 4 μg of HA-PERP cloned into the pCI-neo Mammalian Expression Vector (a generous gift from Dr. Laura Attardi, Stanford University) or 4 μg of the empty vector pCI-neo (Promega). For selection, transfected cells were cultured in the presence of 1 mg/ml of G-418 antibiotic for 2 wk with a change in the medium every 3 days. Stable colonies were isolated and verified for the stable expression of HA-PERP by immunocytochemistry and Western blot analysis using an antibody against HA-Tag (Cell Signaling Technology) and/or PERP (Orbigen).

Treatment with pifithrin-α. To determine the effect of p53 inhibition, cells were treated with 20 μM pifithrin-α (PFT-α) and cultured for an additional 24 h before experiments as previously described (20).

Immunocytochemistry. LLC-PK1 cells were fixed with methanolacetone (1:1) for 10 min at −20°C and then were air-dried. Permeabilization and blocking were carried out with TBST (20 mM Tris·HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 10% calf serum for 10 min at room temperature and followed by incubation with primary antibodies for 1 h at 37°C. Cells were incubated with species-specific secondary antibodies for 1 h at room temperature and mounted for detection under fluorescent microscopy. Samples were viewed and photographed under either a Leica DMR fluorescent microscope or a Zeiss LSM 510 Meta confocal laser-scanning confocal microscope (UNMC Core Facility). The digital images were processed using Adobe Photoshop software for final layout.

Assessment of mitochondrial transmembrane potential by flow cytometry. Mitochondrial transmembrane potential was measured using the JC-1 dye method (Molecular Probes) as previously described (12). Briefly, cells were incubated for 30 min in the presence of 2 μM of JC-1 in the normal growth culture medium at 37°C, 5% CO2, washed twice, and resuspended in Locke’s solution (in mM: 154 NaCl, 5.6 KCl, 2.3 CaCl2, 1.0 MgCl2, 5.0 NaHCO3, 3.6 glucose, and 5 HEPES, pH 7.2) at a concentration of 1 × 106 cells/ml. Carbonylcyanide m-chlorophenylhydrazine (CCCP) at 50 μM was used to disrupt mitochondrial transmembrane potential as a positive control. Labeled cells were analyzed and quantitated by flow cytometry, with excitation at 488 nm and emission at 530 or 590 nm as recommended by the manufacturer.

Caspase activity assay. Caspase activation was assayed by measuring enzyme activity using luminogenic caspase-specific substrates from Promega according to the manufacturer’s instructions (Caspase-Glo Assay, Promega). Briefly, PERP-overexpressing cells or control cells were cultured to semiconfluency and exposed to hypoxic injury. Three hours following hypoxic injury, cell suspensions were made in normal medium and mixed with either Caspase-Glo 8 Reagent or Caspase-Glo 9 Reagent. Following 30 min of incubation at room temperature, peak luminescent signals were read in a luminometer. Uninjured cells were used as negative controls. Enzyme activity was expressed in relative light units per second and plotted in a graphical form. Results were statistically analyzed using Student’s t-test for two groups and ANOVA with a post hoc test across all groups.

Apoptosis evaluation. Quantification of cells undergoing apoptotic cell death by flow cytometric analysis was performed using the Telford method (12, 41). Briefly, cells in suspension were fixed in ice-cold 70% ethanol, followed by incubation in DNA staining solution containing 20 μg/ml propidium iodide, and 0.2 mg of DNase-free RNase in PBS for at least 30 min at room temperature in the dark.
Gene silencing of PERP in renal epithelial cells using PERP-specific short hairpin RNA. Four different short hairpin (sh) RNA constructs against PERP with the following sequences were employed in the study: 5'-TCCTACGACGATGGCTGCCAGAGCCTCAT-3' (P1), 5'-CTGTCAGCCGCCTCCTACCATC-3' (P2), 5'-CACCATCATCTTTAGGTGTGTCCTCTCT-3' (P3), and 5'-CACTGCGCCATCATCAGATCATCCTC-3' (P4) (Origene). These constructs were transiently transfected in PERP-overexpressing cells, and the shRNA construct (P2) that most effectively downregulated the expression of PERP was selected. Efficacy of shRNA constructs was assessed by Western blot analysis. In experiments using shRNA-mediated gene silencing, LLC-PK1 cells were transiently transfected with 4 μg of the P2 shRNA construct using Lipofectamine LTX (Invitrogen) according to the manufacturer's protocol. One day following the transfection, the cells were exposed to hypoxic injury for 4 h, followed by 1–3 h of incubation under normoxic conditions and assessed for the mitochondrial release of cytochrome c, AIF, and apoptosis.

Statistics. Student’s t-test was used to compare the means of two experimental groups. One-way ANOVA with a post hoc test was used to compare the means of more than two groups. A P value of <0.05 was considered statistically significant.

RESULTS

Differential expression and characterization of PERP mRNA post-ischemic renal injury. To identify genes that are differentially regulated in ischemic renal tissues, we performed a differential screening strategy using a rat cDNA library generated from 1 day posts ischemic renal tissue as we previously described (31). One of the highly upregulated clones that was sequenced and compared across species was found to be the rat homolog of human PERP, a regulator of p53-mediated apoptosis (2, 17).

PERP expression is induced in a p53-dependent manner post-renal ischemia. To confirm whether PERP was induced in a p53-dependent manner after renal injury, kidneys were subjected to 37 min of ischemia followed by reperfusion for 3, 12, and 24 h in wild-type and p53-KO mice. Immunoblot analysis was performed with protein isolated from posts ischemic kidneys of wild-type and p53-KO mice, and data obtained from the 24-h time point are presented in Fig. 1A. Immunoreactivity for PERP was substantially increased in wild-type ischemic kidneys compared with that of sham-operated animals at all of the time points examined. Nonetheless, no expression of PERP was detected in p53-KO ischemic kidney samples at any time point, suggesting that the expression of PERP is p53 dependent in renal ischemia-reperfusion injury. Quantitation of the Western blot data from 3, 12, and 24 h postinjury (Fig. 1B) indicates that the level of PERP expression is significantly elevated at all time points compared with that from sham-operated wild-type mice kidneys. No significant differences in PERP expression were observed among 3, 12, and 24 h.

Subcellular localization of PERP following renal ischemia. To elucidate the spatiotemporal expression pattern of PERP, a p53 transcriptional target, kidney tissue sections obtained up to the 24-h post-ischemic time point from wild-type and p53-KO mice were examined by immunohistochemistry with a PERP-specific antibody.

The data in Fig. 2, D–F, demonstrate that PERP expression was markedly upregulated in wild-type animal kidneys at 3 and 24 h postinjury; however, no expression was observed at the 24-h post-ischemic interval in kidneys of p53-KO mice (Fig. 2B). Similarly, no expression was observed in kidneys of sham-operated p53-KO mice (Fig. 2A). Conversely, a basal cytoplasmic expression of PERP was observed in kidneys of sham-operated wild-type mice (Fig. 2C). Regionally, at 3 h postinjury, PERP localization was restricted to the outer medullary PTC (Fig. 2, D and E). The subcellular localization of PERP at 24-h expression analyzed by confocal and fluorescent high power microscopy demonstrates that in the majority of cells PERP expression was localized to the cytoplasmic region (Fig. 2I, red arrow); however, translocation of PERP to the nuclei occurred in some cells, as shown by white arrows in Fig. 2, H and I. Furthermore, the specificity of PERP antibody was verified by using negative and positive controls. Posts ischemic renal tissue sections were processed with IgG (Fig. 2J), and nonimmune serum (Fig. 2K), antigen-saturated antibody (Fig. 2L), and scrambled antigen peptide (Fig. 2M) were used as different negative controls. Skin tissue of the wild-type mouse
was used as a positive control (Fig. 2N), and PERP was localized on the plasma membrane of keratinocytes as previously described (26). Taken together, these data suggest that PERP was induced in a p53-dependent manner after ischemia-reperfusion injury to the kidney, and it may play a key role in executing downstream p53 apoptotic pathways.

PERP is induced in hypoxic renal epithelial cells in a p53-dependent manner. Different in vitro models using renal PTC subjected to ATP depletion, oxidant injury, or hypoxic injury have been used to study renal ischemia-reperfusion injury, as these injury models mimic in vivo parameters. In this study, we adapted the in vitro hypoxic model to induce apoptotic stimuli and examine the upregulation of PERP in renal tubular epithelial cells cultured in vitro. Accordingly, LLC-PK₁ cells were exposed to hypoxia for 4 h followed by 1 or 3 h of recovery under normoxic conditions. The expression of PERP was induced at both time points, 1 (Fig. 3b) and 3 h (Fig. 3c), of recovery as examined by immunofluorescence microscopy. Translocation of PERP to the nuclei was observed in some cells at 1 h and in the majority of cells at 3 h.

To test the effect of pharmacological inhibition of p53 on PERP expression, LLC-PK₁ cells were pretreated with pifithrin-α, and the pretreated cells were then exposed to hypoxia for 4 h followed by 3 h of recovery under normoxic conditions. The expression of PERP was undetectable in cells that were pretreated with the p53 inhibitor pifithrin-α (Fig. 3d), similar to uninjured LLC-PK₁ cells (Fig. 3a), suggesting that the induction of PERP in hypoxic renal epithelial cells is p53 dependent.

The induced expression of PERP post-hypoxic injury is further verified by quantitative RT-PCR using PERP-specific oligonucleotide primers (Fig. 3, e and f). The expression of PERP was upregulated by 3.45-fold at 3 h posthypoxia. Western blot analysis was performed to confirm the upregulation of PERP protein in hypoxia-induced cells. Quantitation of the data demonstrate that PERP protein is induced by 1.64-fold at 3 h posthypoxia (see Fig. 8K).

PERP overexpression exacerabtes apoptosis in LLC-PK₁ cells following hypoxic injury. Mouse PERP cDNA cloned in to the pC1-neo Mammalian Expression Vector was stably transfected in LLC-PK₁ cells, and clonal cell lines were established for further studies. The stable cell lines were passaged more than 10 times in normal growth media, and similar to control cells, no changes in cell viability were observed during these passages in any of the PERP-overexpressing clones, suggesting that overexpression of PERP per se does not induce apoptosis in LLC-PK₁ cells under normal growth conditions (data not shown).

PERP-overexpressing cells or control cells were exposed to hypoxic injury for 4 h followed by 1 or 3 h of incubation under normoxic conditions. The cells were stained with Hoechst dye and examined under a confocal microscope to assess morphological changes. DIC images showed hallmark apoptotic changes such as cellular condensation, nuclear fragmentation, and plasma membrane blebbing widely in PERP-overexpressing cells at 3 h (Fig. 4d and inset) compared with that in control vector-transfected cells (Fig. 4c). These morphological changes were, however, minimal at 1 h postinjury in PERP-overexpressing cells (Fig. 4b), and none were observed in control vector-transfected cells at a corresponding interval (Fig. 4a). These data suggest that PERP overexpression expedites the induction of apoptosis in renal tubular epithelial cells.
after hypoxic injury. Quantitation of the percentage of apoptotic cells using the Telford method showed that at 3 h postinjury, 28.4% cells underwent apoptosis in PERP-overexpressing cells (Fig. 4, f and g) compared with 5.7% in control vector-transfected cells (Fig. 4, e and g) (n = 3; P < 0.001). Taken together, these data suggest that PERP overexpression per se is not sufficient to induce apoptosis in renal tubular epithelial cells, but rather an additional factor or factors that are induced during hypoxic injury may cooperate with PERP to elicit exacerbated apoptosis.

**PERP-induced mitochondrial dysfunction.** Induction of mitochondrial permeability is a common mechanism by which several of the proapoptotic molecules trigger apoptotic signaling. To further explore the cellular mechanisms involved in the PERP-mediated apoptosis after hypoxic injury, we assessed the mitochondrial membrane depolarization using the JC-1 assay (40), and the quantitation was performed by flow cytometry. JC-1 is a cationic dye, which accumulates in mitochondria in a membrane potential-dependent manner and forms red fluorescent J-aggregates, which emit red fluorescence at ~590 nm. Loss of mitochondrial membrane polarization leads to a decrease in red fluorescent J-aggregates and increase in green fluorescent monomers, which emit fluorescence at ~529 nm. Thus the ratio of red to green fluorescence intensity is a measure of mitochondrial membrane potential. PERP-overexpressing cells or controls cells were used to assess mitochondrial dysfunction following injury. Overexpression of PERP per se did not induce membrane depolarization as both PERP-overexpressing cells (Fig. 5B) and control cells (Fig. 5A) equally displayed mitochondrial membrane permeability (MMP) in ~3% of uninjured cells. Conversely, hypoxic injury induced a 10-fold increase in MMP after 1 h in PERP-overexpressing cells (Fig. 5D and F) (29.8%) compared with control cells (Fig. 5, C and F) (3%) [n = 3; P < 0.0001], suggesting that upregulation of PERP following injury mediates mitochondrial dysfunction. CCCP, a potent mitochondrial membrane potential disrupter, was used as a positive control and it induced MMP in 93.79% of uninjured control cells (Fig. 5E).

**PERP overactivation induces mitochondrial release of cytochrome c and AIF.** Apoptotic cell death has been associated with mitochondrial release of cytochrome c, which triggers caspase 9 activation to elicit apoptosis. We examined the distribution of cytochrome c in LLC-PK1 cells as a function of PERP overexpression and hypoxia using a cytochrome c-specific antibody (Calbiochem) and confocal microscopy. In control empty vector-transfected cells, cytochrome c immunoreactivity revealed a punctate mitochondrial pattern in the cytoplasm and colocalized with AIF (Fig. 6, A–C). In addition, overexpression of PERP per se did not alter the expression pattern of cytochrome c (not shown). However, 4 h of hypoxia followed by 1 h of reoxygenation displayed a minimal diffused staining pattern of cytochrome c in the cytoplasm of PERP-overexpressing cells (Fig. 6D; white arrows). However, at 3 h following injury, a more pronounced diffused pattern of cytochrome c staining was observed in the cytoplasm. Conversely, the mitochondrial punctuate pattern of cytochrome c localization was preserved along with AIF colocalization in injured control cells (Fig. 6, A and C) and uninjured cells (Fig. 6, m and n).

AIF is a mitochondrial protein that can translocate to the nuclei to induce caspase-independent apoptosis. It is proposed that AIF binds to chromosomal DNA and elicits peripheral chromatin condensation and large-scale DNA fragmentation. To determine whether PERP overexpression induced AIF translocation after hypoxic injury, we examined the subcellular localization of AIF by immunofluorescence analysis. As shown in Fig. 6B, AIF expression was limited to the mitochondria at 1 h postinjury in control cells, but a diffuse pattern was minimally observed in PERP-overexpressing cells (Fig. 6E, white arrow), suggestive of translocation from mitochondria to the cytoplasm. Nevertheless, the diffuse pattern and nuclear translocation were more pronounced in PERP-overexpressing cells at 3 h following injury (Fig. 6K and inset) than in control cells (Fig. 6H), implying that the upregulation of PERP facilitates the release of AIF from mitochondria following hypoxic injury.
PERP-induced caspase-9 activation. Previous studies have indicated that activation of caspase-9 leads to the initiation of the caspase cascade, resulting in the activation of caspase-3 and the subsequent mitochondrial release of cytochrome c to execute apoptotic cell death. Thus we examined the activation of caspase-9 and caspase-8 by measuring enzyme activity using caspase-specific luminogenic substrates. PERP-overexpressing cells or control cells were subjected to 4 h of hypoxia followed by 3-h postincubation under normoxic conditions. Following injury, cell suspensions were assessed for caspase activity. Compared with control cells (5.7 ± 0.7%; n = 3), PERP-overexpressing cells underwent significant apoptosis (28.4 ± 1.5%; n = 3) (P < 0.001) at 3 h postinjury.

shRNA-mediated knockdown of PERP protected LLC-PK1 cells from hypoxic injury. One day following transient transfection of LLC-PK1 cells with PERP-specific shRNA (Fig. 8, d–f) or control empty shRNA vector (Fig. 8, a–c and g–i), cells were subjected to 4 h of hypoxic injury followed by incubation for 3 h under normoxic conditions. Cells were examined for the expression and translocation of cytochrome c and AIF by immunofluorescence microscopy at ×400 magnification. The number of cells with diffused appearance of cytochrome c and AIF was counted in 10 high-magnification (×400) fields as a function of the expression of PERP shRNA or control vector. Compared with the gene-silenced cells, the number of control vector-transfected cells showed increased release of cytochrome c and AIF, as shown in Fig. 8, b and c, respectively. Higher magnification of the cells with diffused appearance of cytochrome c (Fig. 8h) or AIF (Fig. 8i) and nuclear fragmentation (Fig. 8g, white arrow) are also shown. The number of cells that underwent apoptotic cell death in three separate experiments was assessed by staining the cells with Hoechst dye and examining in 10 high-magnification fields under UV fluorescence for nuclear fragmentation. Quantitation of the data (Fig. 8j) shows that knockdown of PERP significantly reduced the number of cells that underwent mitochondrial release of cytochrome c and AIF and apoptosis (n = 3; P < 0.05) at 3 h postinjury. The efficacy of shRNA was assessed by Western blot analysis with proteins derived from control vector-transfected and sh-PERP-transfected cells at 3 h posthypoxia. In sh-PERP-transfected cells, PERP expression was...
significantly downregulated by 1.62-fold compared with that in control cells following injury \((P < 0.001; n = 3; \text{Fig. 8k})\). Conversely, in injured control vector-transfected cells, PERP expression levels were significantly higher than other groups with or without sh-PERP transfection and/or hypoxic injury \((P < 0.001; n = 3)\).

**DISCUSSION**

Acute ischemia-reperfusion injury to the kidney damages both the renal tubular epithelial cells and the vascular endothelial cells, resulting in cell death by necrosis, apoptosis, or both. Although several molecules are implicated in the mediation of apoptotic cell death to the renal parenchyma, the precise mechanisms remain undefined. Recent studies indicate that the activation of the transcription factor p53 plays a crucial role in apoptotic death of PTC in both in vivo and in vitro models of AKI, and inhibition of p53 results in improved renal functions after ischemia-reperfusion injury \((9, 23)\) and cisplatin-induced nephrotoxicity \((20)\). A role for p53 in cisplatin-induced nephrotoxicity is also implicated in studies demonstrating its induced expression in cultured PTC derived from rabbits \((6)\), pigs \((46)\), and mice \((35)\). A definitive role for p53 in cisplatin-induced nephrotoxicity was shown in studies by Cummings and Schnellmann \((6)\) demonstrating that pharmacological inhibition of p53 partially suppressed caspase-3 activation and protected the cells from cisplatin-induced apoptosis. Recent studies from Dong and his group \((19, 20, 45)\) further confirmed and extended the role of p53 in cisplatin nephrotoxicity using in vitro and in vivo models. These studies prompted us to investigate the mechanisms by which p53 executes apoptosis through its transcriptional targets. Our screening for p53 transcriptional targets that are induced in ischemia-reperfusion injury to the kidney led to the identification of PERP. The p53-dependent increased expression of PERP in ischemic renal tissue suggests that it plays a critical role in integrating p53-mediated apoptosis. Moreover, our in vitro results demonstrate that PERP activation may induce mitochondrial permeability to exacerbate hypoxic injury.

The experimental evidence presented in this study is consistent with the above notion: 1) PERP expression is highly induced in the outer medullary renal PTC following renal ischemia; 2) PERP expression is completely abrogated in p53-KO; 3) PERP expression is induced in renal PTC in vitro in the hypoxic model of simulated ischemic injury; 4) pharmacological inhibition of p53 abrogated PERP induction in simulated ischemia; 5) although overexpression of PERP per se did not augment apoptosis, it exacerbated apoptotic cell death following hypoxic injury by inducing mitochondrial permeability, releasing cytochrome c and AIF and activating caspase-9; and 6) shRNA-mediated knockdown of PERP prevents apoptosis in hypoxia-mediated injury by precluding the mitochondrial dysfunction and the release of cytochrome c and AIF. These results strongly support the hypothesis that PERP expression is downstream of p53 activation and its activation...
plays a major role in the execution of p53 integrated apoptotic signaling pathways in the setting of renal ischemia.

In this study, we found for the first time that PERP is constitutively expressed at a basal level in the cytoplasm of outer medullary PTC. However, the expression of PERP was upregulated in the outer medullary region by 3 h following ischemia-reperfusion injury. Furthermore, renal cells cultured in vitro showed similar upregulation of PERP when subjected to hypoxic injury. Previous studies with PERP-deficient mice have shown reduced apoptotic responses in thymocytes and neurons to external apoptotic stimuli, emphasizing the crucial role played by PERP in apoptosis. Conversely, the involvement of PERP was less pronounced in adenovirus early region-1A (E1A)-expressing mouse embryonic fibroblasts, indicating that a PERP requirement in apoptosis is dictated by the cellular context (17). These findings underscore the need for a complete understanding of the functions of PERP to intervene in apoptotic cell death.

In addition, PERP deficiency significantly attenuates apoptosis induced by either irradiation or hyperproliferative signals in the developing central nervous system, suggesting a crucial role played by PERP in these cell types modulating through multiple target genes such as PUMA and Bax. Nevertheless, studying the role of PERP during development and in adult tissues is limited by the lethality of PERP-KO mice postnatally [10–14 days] (16, 25). The embryonic lethality of PERP-KO mice is partly attributed to a lack of stable assembly of desmosomal adhesive complexes in the skin, resulting in blistering of stratified epithelia, thus compromising epithelial integrity. Furthermore, studies in zebrafish have also demonstrated that overexpression of PERP induces apoptosis and contributes to the p53-dependent cell death after UV induction. On the contrary, during normal development in zebrafish, PERP displays a p53-independent expression, suggesting that the epistatic relationship between p53 and PERP is complex (30). PERP expression during embryonic development is tran-

Fig. 6. Overexpression of PERP exacerbated cytochrome c and apoptosis-inducing factor (AIF) release in hypoxia-induced renal cells. LLC-PK1 cells were stably transfected with PERP (D–F and J–L) or the control vector (A–C and G–I). The cells were grown to semiconfluence and exposed to hypoxic injury for 4 h followed by 1 or 3 h of reoxygenation. The cells were fixed and processed for immunofluorescence to visualize cytochrome c (A, D, G, and J) or AIF (B, E, H, and K). At 3 h postinjury, both cytochrome c (J) and AIF (K) were released from mitochondria in PERP-overexpressing cells while minimal or no release of cytochrome c and AIF was observed in control cells (G and H, respectively). Similarly, at 1 h postinjury both cytochrome c (D, white arrows) and AIF (E, white arrow) were minimally released in PERP-overexpressing cells while no release of cytochrome c (A) and AIF (B) was observed in control cells. Images were obtained using confocal microscopy. The images are representative of results obtained from experiments repeated at least 3 times. Brightness and contrast optimization and merging of images (m and n) were performed using ImageJ 1.38x (NIH) software, and extraneous portions were excluded using Adobe Photoshop 7.0.
sient and is mostly found in epithelium-forming tissues, including the skin, ear vesicles, and the pronephric duct during the differentiation process while it is absent in terminally differentiated cells. However, our studies in adult mouse kidneys had showed a basal level of PERP expression in mostly cytoplasmic and not in the plasma membrane of proximal tubular epithelial cells.

We investigated whether upregulation of PERP plays a role in eliciting apoptosis in the setting of renal ischemia. Our results show that PERP expression is induced in the proximal straight tubule cells of the outer medullary region of ischemic kidneys as early as 3 h postischemia and its expression persisted at 24 h postinjury, suggesting that aberrant PERP activity may play a critical role in apoptotic signaling events in ischemia-reperfusion-injured renal PTC.

p53 can upregulate the expression of several proapoptotic genes including Bax, Fas/Apo-1, PERP, PUMA, and Noxa (44); however, the role of each of these molecules appears to differ in different cell types and the apoptotic stimuli (17, 18, 42, 49). The expression of PERP is regulated in a p53-

Fig. 7. Overexpression of PERP induced activation of caspase-9 after hypoxic injury. Subconfluent LLC-PK1 cells transfected with PERP or control vector were exposed to 4 h of hypoxia followed by 3 h of recovery under normoxia. Cells were harvested and assayed for caspase-8 (left) or caspase-9 (right) activity with enzyme-specific substrates (Promega). Following hypoxic injury, PERP-overexpressing cells showed a significant increase in caspase-9 activity compared with injured and uninjured control cells (right). Although caspase-8 activity was mildly elevated in injured cells, there was no difference between injured PERP-overexpressing and injured control cells (left). The data are presented from 4 independent experiments from normoxic (n = 4) and hypoxic (n = 4) cell preparations. *P < 0.05 and **P < 0.0001.

Fig. 8. Short hairpin (sh) RNA-mediated knockdown of PERP protected LLC-PK1 cells from hypoxic injury. LLC-PK1 cells were transiently transfected with PERP shRNA (d–f) or the control vector (a–c and g–i) and cultured for 24 h. The cells were exposed to hypoxic injury for 4 h followed by 3 h of reoxygenation. The cells were fixed and processed for immunofluorescence to visualize mitochondrial release of cytochrome c (b, e, h) and AIF (c, f, i). Images were obtained using a fluorescent microscope. The images are representative of results obtained from experiments repeated at least 3 times. The number of cells with translocation of cytochrome c, AIF, or nuclear fragmentation (g, white arrow) were counted in 10 high-magnification fields (×400). Quantitation of the data (j) shows that shRNA-mediated knockdown of PERP significantly reduced the mitochondrial release of cytochrome c, AIF, and the induction of apoptosis (P < 0.05; n = 3). To assess the efficacy of shRNA to downregulate PERP expression, Western blot analysis was performed with proteins derived from control vector-transfected and sh-PERP-transfected cells at 3 h posthypoxia and from uninjured cells. Quantitation of the Western blot data (k) demonstrates that PERP expression was downregulated by 1.62-fold in sh-PERP-transfected cells compared with that in control vector-transfected cells following hypoxic injury. Furthermore, PERP expression was significantly elevated in control vector-transfected cells compared with that in other groups with or without shRNA transfection and/or hypoxic injury (P < 0.001; n = 3; ANOVA with post hoc test).
dependent manner in both wild-type and injured renal tissues, suggesting that PERP is a downstream apoptotic effector of p53 in the setting of renal ischemia.

Studying the detailed mechanisms by which PERP augments apoptosis may provide novel insights for devising newer therapeutic targets. The characterization of the protein structure suggests that PERP has four transmembrane domains. Therefore, it is possible that PERP may stimulate apoptosis through a different mechanism from the BH3-containing proteins such as Noxa, Puma, and Bax (15), molecules whose functions are universally assumed to modulate mitochondrial permeability to induce the release of apoptogenic factors (5, 15, 27). PERP belongs to the peripheral myelin protein 22/growth arrest specific 3 (PMP-22/gas 3) tetraspan membrane protein family, which includes PMP-22 and epithelial membrane proteins-1, -2 and -3 (2). The mechanism by which tetraspan membrane proteins, including PERP, that lack the BH3 domain induce apoptosis remains to be defined. It was suggested that PERP could serve as a cell death receptor, albeit a different type with four membrane-spanning domains, to receive either autocrine or paracrine signals. Alternatively, a sequence similarity between PERP and the calcium channel γ-subunit suggests that it could have channel or pore activity, perhaps allowing some crucial molecules important for activating apoptosis to be transported (2).

The general idea that PERP is localized in the plasma membrane was conceived based on the structural similarity to PMP-22, its role in the development of stratified epithelia during embryogenesis, and results from in vitro cultured cells (2, 17). It should be emphasized that our data do not support the concept that PERP is exclusively a plasma membrane protein, as the localization is mostly to the cytoplasm in renal PTC. Therefore, PERP acting as a plasma membrane protein in eliciting apoptosis through the above discussed mechanisms appears not to be exclusive. In addition, our data demonstrate that cells overexpressing PERP when exposed to hypoxic injury elicit increased mitochondrial permeability and caspase-9 activation. Thus, given the mitochondrial permeability and caspase-9 activation in our model, it is possible that PERP may localize via the four transmembrane domains to mitochondria, the endoplasmic reticulum, and/or the nucleus to induce pore activity for transporting molecules or ions across the membrane to regulate apoptosis.

We acknowledge that the functional significance of PERP in renal ischemia may not be clearly known until loss-of-function studies are performed in PERP-deficient animal models. Systemic KO of PERP is postnatally lethal (10–14 days), and therefore a kidney- or renal PTC-specific KO mouse needs to be generated to undertake such experiments. An alternative approach that may be used is to design and administer effective antisense oligonucleotides that may downregulate the expression of PERP in the setting of renal ischemia in mice. We are currently pursuing this approach to further define the functional role of PERP in renal ischemia. Nevertheless, our in vitro studies showing a fundamental role of PERP in eliciting apoptotic cell death under simulated ischemic conditions are consistent with results from the renal ischemia-reperfusion injury model.

Under the simulated ischemic conditions to deplete ATP in LLC-PK1 cells in vitro, PERP expression was induced in the cytoplasmic region. Nevertheless, overexpression of PERP per se did not induce apoptosis; rather, the hypoxic injury to the PERP-overexpressing renal cells exacerbated apoptotic cell death, indicating that PERP may require additional stimuli such as the synergetic activation of other proapoptotic molecules. This notion is further supported by the observation that PERP inhibition protected cells from apoptotic cell death, albeit quantitation of apoptotic cell death showed that PERP inhibition resulted in inhibition of apoptosis by ~50%, a value far less than that achieved by p53 inhibition (23). Collectively, the temporal expression of PERP and the functional relevance of its expression demonstrate that PERP is a downstream effector of p53-directed apoptotic pathways. However, this result also suggests that PERP may play only a partial role in eliciting the p53-triggered downstream apoptotic pathways and its function is dependent on the activation of other pathways or apoptotic molecules.

We have previously reported (31) that the TNF receptor CD27 and its death domain-containing ligand Siva are expressed in kidney and may be involved in cell death pathways in pathological conditions such as ischemic renal injury. Siva is a proapoptotic protein that contains a death domain homology region, a box-B-like ring finger, and a zinc finger-like domain (34). Siva binds to the cytoplasmic domain of CD27, a member of the tumor necrosis factor receptor (TNFR) superfamily (13, 34). In addition to its interaction with TNFR superfamily members, Siva has also been shown to interact with antiapoptotic Bcl-XL and sensitize MCF7 breast cancer cells to UV-induced apoptosis (47).

Recognition sequences for p53 binding were found in the first intron of the mouse and human Siva gene. Binding studies and reporter assays showed that p53 binds to the consensus sites on the Siva gene promoter and Siva is a direct transcriptional target (11). Thus we are currently investigating the mechanisms by which PERP and Siva coordinate their functions to integrate p53-dependent apoptosis in renal ischemia in a separate study.

To our knowledge, this is the first study to show that the induction of the p53 proapoptotic transcriptional target, PERP, plays a crucial role in eliciting the p53-dependent apoptotic pathways in pathological conditions such as ischemic renal injury. The basal level of PERP expression is upregulated following ischemia-reperfusion injury in the outer medullary PTC, orchestrating apoptosis through mitochondrial dysfunction and the activation of caspase-9. These results provide new insights into the mechanisms by which one of the p53 proapoptotic transcriptional targets, PERP, may participate in integrating signals to elicit apoptosis in the setting of renal ischemia. To date, there is no specific therapeutic approach to inhibit apoptosis in AKI. Thus understanding the p53-mediated signaling pathways and a thorough evaluation of the role of the p53 transcriptional targets such as PERP is crucial for a complete understanding of the specific apoptotic pathways activated in the course of renal ischemia. Such information will provide the basis for the design of new therapeutic strategies for treating or preventing AKI.

ACKNOWLEDGMENTS

Part of this work was presented at the Experimental Biology 2008 meeting and at the Renal Week 2004 annual meeting of the American Society of Nephrology and published as abstracts (10, 39).
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GRANTS

This work was supported by a Nebraska Kidney Association research grant to B. J. Padanilam and a University of Nebraska Medical Center fellowship to K. Singaravelu.

REFERENCES

