Cyclophilin D gene ablation protects mice from ischemic renal injury

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Devalaraja-Narashimha K, Diener AM, Padanilam BJ. Cyclophilin D gene ablation protects mice from ischemic renal injury. Am J Physiol Renal Physiol 297: F749–F759, 2009. First published June 24, 2009; doi:10.1152/ajprenal.00239.2009.—Increased oxidative stress and intracellular calcium levels and mitochondrial overloading of calcium during ischemic renal injury (IRI) favor mitochondrial membrane permeability transition (MPT) opening and subsequent necrotic cell death. Cyclophilin D (CypD) is an essential component of MPT, and recent findings implicate its role in necrotic, but not apoptotic, cell death. To evaluate the role of CypD following IRI, we tested the hypothesis that CypD gene ablation protects mice from IRI. Renal function as assessed by plasma levels of both creatinine and blood urea nitrogen was significantly reduced in CypD−/− mice compared with wild-type mice during the 5-day post-ischemia period. Erythrocyte trapping, tubular cell necrosis, tubular dilatation, and neutrophil infiltration were significantly decreased in CypD−/− mice. To define the mechanisms by which CypD deficiency protect the kidneys, an in vitro model of IRI was employed. Inhibition of CypD using Cyclosporin A in oxidant-injured cultured proximal tubular cells (PTC) prevented mitochondrial membrane depolarization, reduced LDH release, ATP depletion and necrotic cell death. Similarly, oxidant-injured CypD−/− PTC primary cultures were protected from cytotoxicity and necrosis. To conclude, CypD gene ablation offers both functional and morphological protection in mice following IRI by decreasing necrotic cell death possibly via inhibition of MPT and ATP depletion.

Acute renal failure; mitochondria; ATP depletion

APOPTOSIS AND NECROSIS COEXIST in ischemic renal tissues, and inhibition of either form of cell death in the setting of renal ischemia is shown to protect against renal dysfunction (13–16). The mechanisms by which renal cells undergo apoptosis or necrosis post-ischemic renal injury (IRI) are complex and incompletely defined. Necrotic cell death is widely considered to be an unregulated process that cannot be modulated by pharmacological means as opposed to apoptosis. However, recent reports challenge this tenet and indicate that necrosis can be regulated or “programmed” and can be prevented by targeting the molecular components of its signaling pathways (25).

Permeability transition (PT) pores open in the mitochondrial inner membrane in response to stimuli such as increased intracellular Ca2+, inorganic phosphate, alkaline pH, and reactive oxygen species. IRI is associated with ATP depletion, increased reactive oxygen species, and intracellular calcium accumulation, conditions that favor mitochondrial membrane transition pore (MPTP) opening (17, 28, 35, 56). Cyclophilin D (CypD) can associate with inner mitochondrial membrane proteins and regulate the mitochondrial permeability transition (MPT). MPT is a process in which a sudden increase in the inner mitochondrial membrane permeability allows solutes with a molecular mass <1,500 Da to enter the mitochondrial matrix, resulting in the loss of inner mitochondrial membrane potential and matrix swelling. Uncontrolled expansion of the matrix may eventually lead to rupture of the outer mitochondrial membrane, release of various proteins in the intermitochondrial membrane space including the apoptotic effectors such as cytochrome c and AIF (4, 6, 18, 24, 26, 27, 31, 36, 37). The loss of inner mitochondrial membrane potential and the inability to maintain a pH gradient due to proton influx not only disrupt the mitochondrial ATP synthesis but also degrade glycolytic ATP due to reversal of proton-translocating ATPase, thus leading to energy depletion and necrotic cell death (7, 26).

MPT is carried out by the opening of a voltage-dependent, high-conductance channel located in the inner membrane known as the MPTP. Although several proteins such as VDAC, ANT, and CypD are implicated to be components of MPT, gene ablation studies suggest that VDAC and ANT play only a limited role, if any, in MPT, and thus the molecular composition and nature of the channel still remains to be defined (2, 30). However, a role for CypD in MPT is unequivocally established through the development of CypD-deficient mice whose mitochondria do not undergo cyclosporin A (CsA)-sensitive MPT. CypD is considered to regulate MPT by facilitating a calcium-triggered conformational change in the MPT pore by binding to other components of MPT and converting it to an open state (53). Furthermore, CypD-deficient mice are protected from ischemia-reperfusion injury of the heart and focal cerebral ischemia, implicating a compelling role for CypD-dependent MPT opening and subsequent necrotic cell death following IRI (1, 44).

A role for MPT has been implicated in hypoxia-reperfusion injury in renal cells (22). Furthermore, Yang et al. (54) have reported that low-dose CsA, a cyclophilin inhibitor, treatment in rats attenuated ischemic renal injury. However, the participation of CypD in MPT and the consequent necrotic cell death in either in vitro or in vivo models of renal ischemia has not been defined. In this study, we tested the hypothesis that CypD gene ablation protects mice from IRI via inhibition of MPT. Our results demonstrate that CypD deficiency offered both functional and morphological protection from IRI by reducing necrotic cell death possibly via inhibition of MPT and ATP depletion.

MATERIALS AND METHODS

Animal care and sampling. CypD−/− mouse breeding pairs were purchased from Jackson Laboratories. CypD−/− mice are viable, fertile, normal in size, and do not display any gross physical or behavioral abnormalities. Their brain architecture and cerebrovasculature are normal (44). The respective wild-type control mice for

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CytoD<sup>−/−</sup> mice (B6129F1) were (~20 g) purchased from Taconic (Hudson, NY) and were used as a control as previously reported (33). The CytoD<sup>−/−</sup> animals were bred, and all the colonies were maintained in the pathogen-free facility of the University of Nebraska Medical Center (UNMC). Mice were cared for before and during the experimental procedures in accordance with the policies of the Institutional Animal Care and Use Committee (IACUC), UNMC, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All animal procedures were approved by the UNMC-IACUC.

**Simulation of renal ischemia in mice.** IRI was induced in mice as described previously (56). The 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 (Roboz Surgical Instrument, Gaithersburg, MD). After 37 min of occlusion, the clamps were removed, and the reflow (reperfusion) was verified visually. Sham-operated control animals underwent the same surgical procedure, except for the occlusion of the renal arteries. All animals were gavaged free access to food and water. Blood samples were collected at 1–5 days post-IRI for the measurement of serum creatinine and blood urea nitrogen (BUN). At the end of each experiment, tissue from the outer medullary region (rich in S3 segments) was obtained (16, 43), snap frozen with liquid nitrogen, and stored at −80°C for future experiments.

**In vitro cell culture models.** LLC-PK<sub>1</sub> cells (a porcine proximal tubular cell line) were purchased from ATCC (Rockville, MD). MCT cells (a murine proximal tubular cell line) were a kind gift from Dr. Eric G. Neilson (Vanderbilt University). Both cell lines were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml. Experiments were performed on 80−90% confluent monolayer cultures.

**Preparation of primary cultures of CytoD<sup>−/−</sup> and wild-type (+/+) proximal tubule cells.** Primary cultures of proximal tubule cells (PTC) were prepared under sterile conditions as described (5). Briefly, renal cortices were microdissected, homogenized, and incubated with 0.5 mg/ml collagenase and 0.5 mg/ml soybean trypsin inhibitor for 30 min. After the removal of large, undigested fragments by centrifugation, the pellets were suspended in growth medium containing a serum-free mixture of DMEM, Ham’s F-12 (1:1), 2 mM L-glutamine, 5 mM HEPES, 15 mM HEPES, 5 mM insulin, 50 μg/ml transferrin, 50 μg/ml streptomycin, and cultured in tissue culture flasks. After 24 h, the monolayers were washed several times to remove unattached tubules and cells, and fresh growth medium was added. The cultures became confluent in 7–9 days. The majority (>90%) of the cells were positive when immunostained for the tubular epithelial cell marker Ksp-Cadherin and the PTC marker aquaporin-1.

**Induction of oxidant injury in cell culture models.** Oxidant injury was used to induce injury in vitro experiments using both LLC-PK<sub>1</sub> and MCT cells. Oxidant injury to confluent monolayer cultures of MCT cells was achieved by exposing them to Krebs-Ringer bicarbonate containing 500 μM H<sub>2</sub>O<sub>2</sub> and incubation at 37°C in a 5% CO<sub>2</sub>-95% O<sub>2</sub> atmosphere for 30 min (42). After the injury, the cultures were rinsed free of the injury medium by washing them three times with PBS. Supplemental medium (DMEM containing 5% FBS) was then added to the cultures and incubated for various recovery time periods. Oxidant injury to confluent monolayer cultures of LLC-PK<sub>1</sub> cells was achieved by exposing them to Krebs-Ringer bicarbonate containing 1 mM H<sub>2</sub>O<sub>2</sub> for indicated time periods by incubating at 37°C in a 5% CO<sub>2</sub>-95% O<sub>2</sub> atmosphere (23).

**Treatment with inhibitors.** CsA was used to inhibit CytoD activity in cell culture studies as described previously (1, 10, 44, 47, 53). CsA stock solution was prepared by dissolving CsA in 100% DMSO. The final concentration of CsA used in the experiments was 1 μM in 0.1% DMSO. A 0.1% DMSO vehicle was used for the control experiments. The cells were preincubated with either vehicle or 1 μM CsA for 45 min before the experiment started, and the drugs were included both during and after the actual treatment.

**Measurement of serum creatinine and BUN.** Serum creatinine and BUN were measured to evaluate renal function using a QuantiChrom Creatinine Assay Kit and QuantiChrom Urea Assay Kit, respectively (BioAssay Systems, Hayward, CA) according to the manufacturer’s protocol.

**Morphological studies.** Wild-type and CytoD<sup>−/−</sup> mice that underwent ischemic injury were killed at various time points, and the kidneys were perfused in vivo with saline warmed to 37°C to remove blood. The kidneys were then fixed in either Bouin’s or formalin fixative, paraffin embedded, and 5-μm sections were cut. The tissue sections were stained with hematoxylin and eosin (H&E).

**Histological changes were evaluated by quantitative measurements of erythrocyte congestion or epithelial necrosis with luminal necrotic debris on S-10 high-power fields/section derived from wild-type and CytoD<sup>−/−</sup> mice. The number of necrotic cells and erythrocytes was counted, and the data were quantified as shown previously (8, 56). The tubular dilatation was analyzed in 5-day-old kidneys derived from wild-type and CytoD<sup>−/−</sup> mice, and the data were quantified as we previously described (35, 56). The average hole area of the dilated tubules was measured using National Institutes of Health Image software (Image J).

**Cell death determination by LDH release assay.** An LDH release assay was performed using the Enlighten ATP assay system (Promega) as we previously described (16, 56). Cellular ATP levels were expressed as nanomoles per milligram protein.

**Cell death determination by trypan blue absorbance assay.** A trypan blue spectrophotometric assay was used to measure cytotoxicity, as previously described with slight modifications (16, 50). Briefly, 0.05% trypan blue was added to each culture well at the end of each treatment and the plate was placed in the incubator at 37°C for 15 min. Dye-containing media was removed by three washes with ice-cold PBS, and the cells were lysed with 1 ml of 1% SDS followed by absorbance measurements at 590 nm. The absorbance value from 2% Triton X-100-treated cells was considered as a high control, whereas the absorbance value from cells growing in normal media without any treatment was considered as low control. The cytotoxicity percentage was measured by the following formula: experimental value-low control/high control-low control × 100.

**Assessment of mitochondrial membrane depolarization by flow cytometry using JC-1.** Mitochondrial membrane depolarization was measured using the JC-1 dye method (Molecular Probes) as previously described (34, 48). Briefly, LLC-PK<sub>1</sub> cells were treated with 1 mM H<sub>2</sub>O<sub>2</sub> for 50 min, and the cells were detached by trypsinization. The cells were then incubated for 15 min in the presence of 2 μM JC-1 in the normal growth culture medium at 37°C, 5% CO<sub>2</sub>. Washed twice, and resuspended in DMEM at a concentration of 1 × 10<sup>6</sup> cells/ml. Carboxyl cyanide 3-chlorophenylhydrazone (CCHP ) at 50 μM was used to disrupt MTP as a positive control. Labeled cells were analyzed and quantitated by flow cytometry with excitation at 488 nm and emission at 530 nm (green) or 590 nm (red) as recommended by the manufacturer.

**Assessment of MTP by JC-1 by scanning of fluorescent intensity.** The JC-1-stained cells referred to above were processed to measure the green/red fluorescent intensity ratio as described previously (55). Briefly, at the end of the JC-1 experiment as mentioned above, cells were seeded in a 96-well plate (flat, clear bottom, Corning) at a density of 50,000 cells/well. The fluorescent intensity was measured with excitation at 488 nm and emission at 530 or 590 nm as...
RESULTS

Evaluation of renal function in wild-type and CypD−/− mice following IRI. To prove that CypD−/− mice are protected from IRI, we first evaluated renal function in wild-type and CypD−/− mice at 1–5 days post-IRI. Renal function was assessed by measuring both BUN and serum creatinine during 1–5 days post-IRI. BUN levels were significantly elevated in wild-type mice at 1–5 days post-IRI compared with wild-type mice following IRI.

Statistical analysis. All data are expressed as means ± SE. One-way ANOVA was used to compare the mean values of all groups. An unpaired t-test was used to compare the means of two different groups. A P value < 0.05 was considered statistically significant.

Serum creatinine levels were significantly elevated in wild-type mice at 1 and 3 days post-IRI compared with wild-type sham-operated mice. At 1 and 3 days post-IRI, similar to BUN levels, serum creatinine levels were also significantly blunted in CypD−/− mice compared with wild-type at 1 day post-IRI (Fig. 1B). No significant difference in renal functions existed among three groups preinjury (Fig. 1, A and B). Moreover, no significant difference existed in either BUN or serum creatinine values between wild-type and CypD−/− sham-operated mice (data not shown). These data together suggest that the absence of CypD led to functional protection following IRI.

Morphological evaluation in wild-type and CypD−/− mice following IRI. To examine whether CypD gene ablation leads to histopathological improvement following IRI, we first examined red cell congestion and necrotic cell death in wild-type and CypD−/− mice at 1 day post-IRI. Low-magnification photographs are shown in Fig. 2A (×100, left) of H&E-stained kidney sections originating from wild-type and CypD−/− mice at 1 day post-IRI. Ischemic kidneys from wild-type mice showed widespread necrosis, tubular obstruction, and sloughed cells in the proximal straight tubule, whereas these features were dramatically reduced in CypD−/− mice. Erythrocyte trapping was considerably reduced in the outer medullary region of the CypD−/− mice compared with that in wild-type mice. High-magnification photographs are shown in Fig. 2A (×200, middle) of kidneys derived from wild-type and CypD−/− mice at 1 day post-IRI. Tubular cast formation, cellular necrosis, and red cell trapping are more prominent in the wild-type mouse kidney section compared with that derived from the CypD−/− mice.

The changes in histology at 1 day post-IRI were quantified by counting the number of trapped red blood cells and necrotic cells, and are shown in Fig. 2B, a and b, respectively. Both values were significantly attenuated in CypD−/− mouse kidneys, demonstrating that gene ablation of CypD−/− reduced cellular necrosis and red cell trapping. The number of trapped red blood cells or necrotic cells was negligible in sham-operated animals (data not shown).

The histopathology of the kidneys derived from wild-type and CypD−/− mice at 5 days post-IRI are shown in Fig. 2A (×400 magnification, right). Tubular dilation and number of dilated tubules were prominent in the wild-type mouse kidney section compared with that derived from the CypD−/− mice. No dilated tubules were identified in sham-operated animals (data not shown).

The number of dilated tubules and the average hole area of the dilated tubules in 5-day post-IRI kidneys derived from wild-type and CypD−/− mice were counted and quantified. Ischemic kidneys derived from the CypD−/− mice had a significantly reduced number of dilated tubules and average hole area compared with that in wild-type mice (Fig. 2B, c and d, respectively), indicating that they underwent lesser initial injury. Taken together, the histopathological data derived from wild-type and CypD−/− mice suggest that tissue injury and necrotic cell death were reduced in CypD−/− mice compared with wild-type mice following IRI.

Ca2+-sensitive mitochondrial membrane depolarization in oxidant-injured LLC-PK1 cells. The role for CypD in MPT and subsequent necrotic cell death is unequivocally established through the development of CypD-deficient mice whose mitochondria do not undergo Ca2+-sensitive MPT (1, 44). To further explore

Fig. 1. A: comparison of blood urea nitrogen (BUN) levels in wild-type (wt) and cyclophilin D-deficient (CypD−/−) sham-operated (sh) or renal ischemia (37 min)-induced mice at various time points post-renal ischemia. **P < 0.005, †P < 0.006, and ‡P < 0.03 compared with wt-sh. TP < 0.008 compared with wt-1 day (d) post-ischemic renal injury (IRI). ¶P < 0.05 compared with wt-3d post-IRI; n = 5 for 1d samples and for n = 3 for the rest. B: comparison of serum creatinine levels in wild-type and CypD−/− sham-operated or renal ischemia (37 min) induced mice at various time points post-renal ischemia. **P < 0.005 and †P < 0.006 compared with wt-sh. TP < 0.008 compared with wt-1d post-IRI. ¶P < 0.05 compared with wt-3d post-IRI; n = 8 for 1d samples and n = 3 for the rest.
the cellular mechanisms involved in CypD-mediated necrotic cell death of PTC following IRI, we assessed the mitochondrial membrane depolarization in oxidant-injured cultured LLC-PK1 cells using the JC-1 assay. Shown in Fig. 3A are images from flow cytometric analysis of LLC-PK1 cells by the JC-1 method. The cells that fell in the bottom right quadrant were considered to be the cells undergoing mitochondrial depolarization since they have decreased red fluorescence as a result of exclusion of dye from mitochondria.

Figure 3B is a graph showing the percentage of cells with complete mitochondrial membrane depolarization out of the total population. As shown in Fig. 3, A and B, treating LLC-PK1 cells with 1 mM H2O2 for 50 min significantly increased mitochondrial membrane depolarization compared with uninjured control (by \( \sim 28\% \)). Treatment of cells with CsA, a cyclophilin inhibitor, significantly decreased mitochondrial membrane depolarization compared with H2O2 with vehicle. CCCP was used as a positive control, and it induced MPT in 97.23% of cells. These data clearly suggest that oxidant injury induced LLC-PK1 cells to undergo CsA-sensitive MPT in a CypD-dependent pathway.

CypD-mediated ATP depletion in in vitro and in vivo models of IRI. Next, we examined whether the loss of CsA-sensitive MPT leads to ATP depletion (7, 11, 26) in PTC following IRI. ATP levels were measured in oxidant-injured LLC-PK1 cells in the presence/absence of CsA. ATP levels were significantly decreased following treatment with 1 mM H2O2 for 1 h compared with uninjured control (Fig. 4A).

To determine whether energy depletion occurs in a CypD-dependent pathway, we measured ATP levels in the outer medulla of wild-type and CypD\(^{-/-}\) mice kidneys following IRI. ATP levels were significantly decreased following treatment with 1 mM H2O2 compared with wild-type mice at 1 day post-IRI compared with those in sham-operated mice (Fig. 4B). Levels of ATP were significantly preserved in the kidneys of CypD\(^{-/-}\) mice compared with wild-type mice at 1 day post-IRI. Taken together, these data suggest that ATP depletion occurs in PTC in a CypD-dependent pathway following IRI.
To determine whether the mitochondrial membrane depolarization and changes in energy depletion occur as a result of alterations in the expression of CypD, we compared the expression of CypD by immunoblotting between 1) wild-type sham-operated and wild-type 1-day post-ischemic kidneys and 2) uninjured MCT (Ctl) and oxidant-injured MCT cells (500 μM H2O2 30 min posttreatment with normal DMEM, 45 min). No change in the expression of CypD protein was observed between the treated and untreated groups (data not shown).

CypD-mediated cytotoxicity in oxidant-injured LLC-PK1 cells. Next, we examined whether loss of CsA-sensitive MPT and subsequent ATP depletion result in necrotic cell death in oxidant-injured LLC-PK1 cells. For this, we measured LDH released from cells as an indicator of plasma membrane damage. Figure 5A is a graph depicting LDH released into the media as a percentage of total LDH in oxidant-injured LLC-PK1 cells in the presence or absence of CsA. The percentage of LDH release compared with H2O2 vehicle (Fig. 5A).

Our results from LDH assay experiments in LLC-PK1 cells were further bolstered by a trypan blue spectrophotometric assay for necrotic cell death (16). LLC-PK1 cells were subjected to oxidant injury by 1 mM H2O2 for 2 h in the presence of oxidant-injured LLC-PK1 cells.
or absence of CsA, and trypan blue absorbance was measured. As in the case of LDH assay experiments, the presence of CsA significantly decreased the percentage of CypD-mediated cytotoxicity (Fig. 5B). The data from LDH release experiments and trypan blue spectrophotometric assay suggest that cytotoxicity associated with necrotic cell death was significantly increased in a CypD-dependent manner in oxidant-injured LLC-PK1 cells.

CypD-mediated mitochondrial membrane depolarization and subsequent cytotoxicity in oxidant-injured MCT cells. Another proximal tubular cell line, MCT (9), was used to confirm the role of CypD in MPT and subsequent cytotoxicity. The images from flow cytometric analysis of oxidant-injured MCT cells by the JC-1 method are shown in Fig. 6A. The cells that fell in the bottom right quadrant were considered to be the cells undergoing mitochondrial depolarization since they have decreased red fluorescence as a result of exclusion of dye from mitochondria.

Figure 6B is a graph showing the percentage of cells with mitochondrial membrane depolarization out of the total population. As shown in Fig. 6B, treating MCT cells with 500 μM H2O2 for 30 min and postincubation for 45 min with normal DMEM significantly increased (∼56%) mitochondrial membrane depolarization compared with uninjured control.

Treatment of cells with CsA significantly decreased mitochondrial membrane depolarization compared with H2O2+vehicle. CCCP, a potent mitochondrial membrane potential disrupter, was used as a positive control, and it induced MPT in 91.58% of cells.

Figure 6C is a graph depicting the ratio of green to red fluorescence following the JC-1 method using a 96-well fluorescent intensity reader. An increase in the green/red ratio indicates an increase in mitochondrial membrane depolarization. The green-to-red ratio was significantly elevated by treating MCT cells with 500 μM H2O2 for 30 min followed by a postincubation of 45 min with normal DMEM compared with uninjured control cells, suggesting that mitochondrial membrane depolarization was increased with H2O2 treatment. Treatment of cells with H2O2 along with CsA significantly decreased the green-to-red ratio compared with H2O2 with vehicle.

The percentage of LDH released into the media was significantly elevated following treatment with 500 μM H2O2 for 30 min and postincubation for 60 min with normal DMEM compared with uninjured control. Inclusion of 1 μM CsA in the incubation media significantly decreased the percentage of LDH release compared with H2O2+vehicle (Fig. 6D). Taken together, these data suggest that CypD facilitates MPT and subsequent cytotoxicity in oxidant-injured MCT cells.
Fig. 6. A: mitochondrial membrane potential assay in oxidant-injured MCT cells by flow cytometry following JC-1 probing (a–d). Percentage of cells that fell in the bottom right quadrant of each panel were considered as the percentage of cells that underwent mitochondrial membrane depolarization and are denoted in the top right quadrant of the respective panels. B: graphical representation of percentage of oxidant-injured MCT cells with mitochondrial membrane depolarization; 

- 500 μM H$_2$O$_2$, 30 min + post-treatment with normal DMEM 45 min + vehicle
- 50 μM CCCP:
- 500 μM H$_2$O$_2$, 30 min + post-treatment: +
- Vehicle: -
- 1 μM CsA: -

C: green/red fluorescent intensity ratio in oxidant-injured MCT cells following JC-1 assay; 

- 50 μM CCCP:
- 500 μM H$_2$O$_2$, 30 min + post-treatment: +
- Vehicle: -
- 1 μM CsA: -

D: comparison of LDH released into media in oxidant-injured MCT cells with or without CsA.
- 50 μM mH$_2$O$_2$, 30 min + 45 min post-treatment: +
- Vehicle: -
- 1 μM CsA: -

a: Uninjured MCT cells
b: Positive control (treated with 50 μM CCCP)
c: 500 μM H$_2$O$_2$, 30 min + post-treatment with normal DMEM 45 min + vehicle
d: 500 μM H$_2$O$_2$, 30 min + post-treatment with normal DMEM 45 min + 1 μM CsA

*P < 0.0001 compared with a. \( \frac{\lambda}{\lambda} P < 0.03 \) compared with c. C: green/red fluorescent intensity ratio in oxidant-injured MCT cells following JC-1 assay; n = 4 for all groups. **P < 0.0005 compared with a. ***P < 0.0005 compared with a. **P < 0.0005 compared with b.
CypD−/− PTC are resistant to oxidant-injured cytotoxicity and necrotic cell death. CsA has been shown to inhibit cyclophilins other than CypD. To rule out the possibility that the protective effect demonstrated by CsA treatment is not due to its side effects, we employed primary cultures of CypD-deficient PTC. Primary cultures of PTC derived from kidneys of wild-type and CypD−/− mice were treated with 500 μM H2O2 for 30 min and postincubation for 60 min with normal DMEM. The percentage of LDH released into the media was significantly reduced in CypD−/− PTC compared with that in wild-type PTC (Fig. 7A).

To determine the effect of CypD deficiency on necrotic cell death, PTC primary cultures derived from wild-type and CypD−/− mice were treated with 500 μM H2O2 for 30 min and postincubation of 120 min with normal DMEM. The number of cells undergoing necrotic cell death was analyzed by trypan blue exclusion studies and expressed as a percentage of the total number of cells in the microscopic field. As shown in Fig. 7B, H2O2 induced 63.5% necrotic cell death in wild-type PTC while it was significantly reduced to 24.3% in PTC deficient for CypD.

DISCUSSION

In this study, we analyzed the role of CypD in mediating acute cell injury following IRI. Our in vivo findings suggest that mice deficient for CypD maintained renal function closer to normal levels following IRI compared with wild-type mice as determined by the levels of serum creatinine and BUN. The preservation of renal function in CypD−/− mice following IRI which could be attributed to attenuated cell injury and an accelerated regenerative process. CypD is a member of the cyclosporin A-binding cyclophilin family of proteins, which are peptidyl-prolyl cis-trans isomerases (PPIase). CypD is exclusively expressed in the mitochondrial matrix and is the only molecule that is established as an essential component of the MPT (29). It is hypothesized that the PPIase function of CypD induces a calcium-triggered conformational change in a component of the MPT pore to facilitate pore opening. A physiological role for CypD or MPT during development or normal cellular homeostasis has not been established as mice deficient for CypD show no obvious phenotype and grow normally (4). However, reports from independent studies indicate a role for CypD in regulating MPT in a Ca2+-dependent and CsA-sensitive manner to induce necrotic cell death following ischemia-reperfusion injury in animal models of heart failure, focal cerebral ischemia, and liver injury (1, 10, 38, 44). Loss of CypD resulted in smaller myocardial infarcts and reduced cerebral infarcts. Brain mitochondria, in particular, are more resistant to the Ca2+-induced MPT compared with mitochondria from other tissues, and a recent report attributed this resistance to the lower expression levels of CypD in the adult brain (21). It is also interesting to note that CypD may play opposing roles in the postnatal brain vs. the adult brain as CypD deficiency resulted in significantly increased hypoxia-induced ischemic (HI) brain injury at postnatal day 9 (52). The differences in HI sensitivity is unlikely to be attributable to age-dependent differences in CypD levels (21, 52) as its expression levels were not altered between the neonatal and adult brain. Although we have not analyzed the expression levels of CypD in an age-dependent manner or in various tissues, our studies demonstrated that CypD protein expression is not altered in ischemic kidneys or oxidant-injured MCT cells compared with their respective controls (data not shown) and does not account for the increased resistance to injury.

A characteristic feature of MPTP is its inhibition by CsA, although the inhibition can be relieved by increasing the Ca2+ load (12). The role of CypD in MPTP is confirmed by studies showing that CypD-deficient mitochondria fail to undergo CsA-sensitive MPT (1, 38, 44). CsA is demonstrated to bind CypD and inhibit its PPIase activity; however, the requirement for PPIase function in pore modulation has not been clearly established (45). A role for MPT in the kidney was previously reported by Feldkamp et al. (22), demonstrating that the proximal tubules undergo condensation and MPT following hypoxia-reoxygenation injury. Furthermore, Yang et al. (54) demonstrated that low-dose CsA pretreatment attenuated subsequent IRI in rat kidneys, although the precise mechanism for the ischemic tolerance was not defined. Our in vitro data in two independent cultured PTC demonstrate that MPT was significantly inhibited by CsA treatment following oxidant injury, which suggests that these cells may undergo MPT following hypoxia-reoxygenation injury in a CypD-dependent manner. The use of CsA has some disadvantages as an inhibitor of CypD since CsA when complexed with the cytosolic cyclo-
Cyclophilin A can inhibit the phosphatase activity of calcineurin and function as an immunosuppressant. Thus the data from in vivo studies should be interpreted cautiously as CsA may alter additional cellular functions independent of MPT opening (40). Furthermore, CsA also binds to other nonmitochondrial cyclophilin isoforms that regulate many other cellular activities, and thus the results observed with CsA cannot be conclusively related to MPT inhibition. Although our in vitro studies did not address the effect of CsA on other cyclophilins, the data provide support for the notion that the effect of CsA is independent of its immunosuppressant functions. In addition, our data from primary PTC cultures derived from CypD knockout mice demonstrating that they are protected from oxidant injury, an effect similar to that observed following CsA treatment, further indicate that CsA’s effect is mediated by CypD.

Several reports have indicated that CsA can limit cardiac ischemia-reperfusion injury in experimental models (18, 19). In a recent pilot clinical trial, administration of cyclosporine reduced infarct size during acute myocardial infarction compared with that in placebo-treated patients (41). It is well known that long-term use of CsA can induce renal and hepatic toxicity and increased susceptibility to infections and cancers. It should be noted that in the pilot study, cyclosporine was administered as a single intravenous bolus, which may have prevented toxicity in the patients. Our in vitro study also demonstrated that long-term incubation of the cells with CsA induced cytotoxicity while a low-dose treatment for a short period of time protected the cells. Similar protective effects demonstrated in oxidant injured CypD-deficient PTC further confirm that the cytotoxicity is directly mediated by CypD and may not be due to the effect of CsA on other cyclophilins. Collectively, these data suggest that CypD mediates necrotic cell death following IRI. Following MPT, mitochondrial swelling occurs, leading to the rupture of the outer mitochondrial membrane, causing release of various proapoptotic factors (18, 36). However, CypD is shown to be dispensable for apoptosis (32, 49). On the other hand, studies from Yang et al. (54) show that low-dose CsA treatment attenuates apoptotic cell death following IRI in rats. Nevertheless, we did not evaluate the role of CypD in apoptotic cell death following IRI in vivo in our studies. However, no apoptosis is detected in vitro at the time points we examined for LDH release (data not shown).

Mitochondria play a pivotal role in the generation of ATP. The loss of inner mitochondrial membrane potential not only disrupts the mitochondrial ATP synthesis due to uncoupling of oxidative phosphorylation but also degrades glycolytic ATP due to the reversal of proton-translocating ATPase, thus leading to energy depletion (7, 11, 26). Data from our both in vivo and in vitro studies suggest that ATP depletion occurs in a CypD-dependent manner following ischemia or oxidant injury.

It has been shown that IRI can be ameliorated by inhibiting molecules involved in apoptosis, necrosis, or inflammation, suggesting that multiple injury and death mechanisms may be involved in IRI (reviewed in Ref. 39). Necrotic cell death post-renal ischemia in animal studies has been shown to be attenuated by the inhibition of molecules such as poly(ADP-ribose)polymerase (PARP)-1 (56) and calpain (20). Are these different molecules and their signaling pathways operating in the renal cell in a sequential manner and/or in parallel to elicit necrosis? Numerous studies have demonstrated intracellular accumulation of Ca\(^{2+}\) under ischemic conditions and activation of Ca\(^{2+}\)-dependent enzymes such as calpain. Calpain-mediated cleavage of the Na\(^{+}\)/Ca\(^{2+}\) exchanger in the plasma membrane results in a sustained secondary intracellular Ca\(^{2+}\) overload and subsequent necrotic cell death (3). Ca\(^{2+}\) is a major factor, if not the most important factor, that triggers MPT pore opening. Data from our laboratory demonstrated that PARP activation can lead to energy depletion, glycolytic inhibition, and activation of an inflammatory cascade post-renal ischemia. In the context of neuronal NMDA toxicity, PARP-1 is shown to induce mitochondrial Ca\(^{2+}\) dysregulation by an undefined mechanism and mediates mitochondrial calpain activation (51). Can PARP activation and CypD act in a sequential manner to induce necrosis post-renal ischemia? PARP-1 activation post-renal ischemia can deplete NAD+ and can induce MPT and result in further loss of NAD through the MPTP (46) and its metabolism by cytosolic/nuclear PARP-1. These studies suggest that cross talk between the different signaling pathways may occur to instigate necrosis. In addition, apoptosis may be occurring in parallel or may be induced as a compensatory mechanism during inhibition of necrosis. Thus to prevent cell death in IRI may require combined inhibition of both necrotic and apoptotic pathways. Identification of key molecules that trigger necrotic and apoptotic cascades is essential to develop strategies to completely inhibit cell death in the setting of IRI.

In conclusion, CypD gene ablation in mice led to both functional and histopathological protection following IRI. The recovered renal function/tissue injury in CypD−/− mice can be attributed to the inhibition of MPTP formation, preservation of ATP levels, and subsequent reduction of necrotic cell death. Our data suggest that CypD is a central component that mediates acute cellular injury following IRI and can be targeted to develop novel therapeutic agents to treat IRI.

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