Knockdown of Cyclophilin D Gene by RNAi Protects Rat from Ischemia/Reperfusion-Induced Renal Injury

Wei Hu^a Zhiqiang Chen^a Zhangqun Ye^a Ding Xia^a Zhongyu Xia^a
Jiefeng Ma^a Min Zhu^b Gang Chen^b

^aDepartment of Urology, and ^bInstitute of Organ Transplantation, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, PR China

Key Words

Cyclophilin D • RNA interference • RNAi • Ischemia/reperfusion • Renal injury

Abstract

Background/Aims: Mitochondrial permeability transition has a critical role in ischemia/reperfusion (I/R)-induced kidney injury. It is thought that mitochondrial permeability transition occurs after the opening of the permeability transition pore, a channel which putatively consists of a voltage-dependent anion channel, adenine nucleotide translocator and cyclophilin D (CypD). Much evidence shows that CypD plays an important role in I/R-induced injury. **Methods:** To evaluate the role of CypD following I/R renal injury, we tested the hypothesis that knockdown of CypD gene by RNA interference (RNAi) protects rat from I/R-induced renal injury. **Results:** Our data show that knockdown of CypD by RNAi protects normal rat kidney cell line from hypoxia-induced necrotic death. Infection of lentivirus expressing CypD RNAi sequence produces a significant reduction of CypD at both mRNA and protein levels. Both pathologic and biochemical analyses show that knockdown of CypD by RNAi protects rat kidney from I/R-induced renal injury. **Conclusion:** Our study provides the evidence that CypD may be a potential target for protecting I/R-induced renal injury.

Copyright © 2010 S. Karger AG, Basel

Introduction

Arterial occlusion, shock and transplantation lead to renal ischemia, which is a common cause of renal cell death, delayed graft function and acute renal failure. Although reperfusion is essential for the survival of ischemic renal tissue, it causes additional damage and contributes to the renal dysfunction and injury associated with ischemia/reperfusion (I/R) injury of the kidney [1, 2].

Mitochondria are critical in I/R-induced injury by their pivotal role in energy production, the generation of reactive oxygen species and the initiation of apoptosis [3–5]. Mitochondrial permeability transition (mPT), which is Ca²⁺-dependent and increases the permeability of the mitochondrial membrane, leads to a loss in mitochondrial membrane potential, mitochondrial swelling and rupture of the outer membrane [6]. The mPT is also implicated in the remodeling of mitochondrial structure with mobilization of cytochrome c stores in cristae during apoptosis [7]. It is thought that the mPT occurs after the opening of the permeability transition pore, a chan-

This project was supported by the National Natural Science Foundation of China (No. 30670820) and the Provincial Natural Science Foundation of Hubei Province, PR China (No. 2006AA301B52-7).

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2010 S. Karger AG, Basel
1420–4096/10/0333–0193\$26.00/0

Accessible online at:
www.karger.com/kbr

Prof. Zhiqiang Chen
Department of Urology, Tongji Hospital, Tongji Medical College
Huazhong University of Science and Technology
Wuhan, Hubei 430030 (PR China)
Tel./Fax +86 27 8366 3406, E-Mail shakurase@163.com

nel which putatively consists of a voltage-dependent anion channel, adenine nucleotide translocator (ANT) and cyclophilin D (CypD) [3, 8].

CypD, a member of the cyclophilin family, is located in mitochondria. It is a peptidylprolyl *cis-trans* isomerase and has a crucial role in protein folding [9]. It is reported that CypD is involved in regulating the mPT, on the basis of the observation that cyclosporine blocks the mPT [10]. Ppif (the gene of CypD) null mice are resistant to mitochondrial swelling and permeability transition in vitro [11]. Moreover, primary hepatocytes and fibroblasts isolated from Ppif null mice are largely protected from Ca^{2+} overload and oxidative stress-induced cell death, whereas CypD overexpression mice show mitochondrial swelling and spontaneous cell death [11].

Ppif null mice are protected from I/R-induced cell death in vivo. The defect of Ppif showed a high level of resistance to I/R-induced cardiac injury and displayed a dramatic reduction in brain infarct size after acute middle cerebral artery occlusion and reperfusion [11–13]. CypD gene ablation offers both functional and morphological protection in mice following ischemic renal injury by decreasing necrotic cell death possibly via the inhibition of mitochondria permeability transition pore and ATP depletion [14]. Genetic ablation of CypD rescues mitochondrial defects and prevents muscle apoptosis in collagen VI myopathic mice [15]. Downregulation of CypD by RNA interference (RNAi) provides the evidence that therapeutic interventions designed to inactivate CypD may be a promising strategy for reducing cardiac injury against myocardial I/R [16].

To evaluate the role of CypD following I/R-induced renal injury, we tested the hypothesis that knockdown of CypD gene by RNAi protects rats from I/R-induced renal injury.

Material and Method

Animal Care

Rats about 8–9 weeks old were purchased from the Laboratory Animal Center of Tongji Medical College, Huazhong University of Science and Technology. They were cared for before and during the experimental procedures in accordance with the policies of the China Hubei Provincial Science and Technology Department. All animal procedures were approved by the same department.

Cell Culture

A normal rat kidney (NRK) cell line was purchased from the China Center for Type Culture Collection. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Gibco-BRL, Gaithersburg, Md., USA). Cells were incubated at 37°C in humidified air with 5% CO_2 and subcultured every 2 or 3 days.

RNAi

We used lentivirus-generated siRNA to interfere with the expression of endogenous genes. Four targeted sequences for Ppif were chosen. For lentivirus-mediated RNAi, FG12, a lentivirus vector which has an independent open reading frame of green fluorescence protein (GFP), was used to produce small, double-stranded siRNA to silence target gene expression. The information about this vector system has been described in detail by Qin et al. [17]. After transfection for 72 h, the viruses were harvested from the culture medium, concentrated by centrifugation, and used to infect cells. The infection efficiency was measured by counting GFP-positive cells with a >70% infection efficiency. The negative control sequence is 5'-TTCTCCGAACGTGTCACGT-3'.

RNA Isolation and Real-Time PCR

RNA isolation, reverse transcription and real-time PCR were carried out as described [18]. The sequence of primers are as follows: 5'-GGAGTTAAAGGCAGATGTCGTG-3' and 5'-GAAG-TTCTCGTCAGGAAAGCG-3' for rat Ppif gene; 5'-TTCAAC-GGCACAGTCAAGG-3' and 5'-CTCAGCACCAGCATCACC-3' for rat GAPDH gene.

Western Blot

NRK cells were infected with lentivirus (MOI = 20) for 120 h, washed 3 times in cold phosphate-buffered saline, and then lysed in buffer comprising 100 mM Tris-HCl (pH 6.8), 2% β -mercaptoethanol, 20% glycerol, 4% SDS with a mixture of protease inhibitors and phosphatase inhibitors (Sigma-Aldrich). Samples from cell or tissue lysates were subjected to 95°C for 5 min, followed by Western blotting analysis with primary antibodies anti-CypD (Abcam Inc., Cambridge, Mass., USA) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and a horseradish peroxidase-conjugated secondary antibody (Amersham, UK) and enhanced chemiluminescence from Pierce (Rockford, Ill., USA).

Model of Rat NRK Cell Line Hypoxia Injury and FACS Analysis

NRK cells were determined by flow cytometry using the Annexin V-FITC Apoptosis Detection kit following the manufacturer's instructions. Briefly, 2×10^5 cells were treated with 95% $\text{N}_2/5\%$ CO_2 for 60 min and then incubated in 95% air and 5% CO_2 for another 48 h. The cells were then harvested, washed in PBS, and incubated with Annexin V and propidium iodide for staining in binding buffer at room temperature for 10 min in the dark. The stained cells were analyzed using the FACS Aria instrument.

Model of Rat Kidney I/R Injury

Sixty male Sprague-Dawley rats with initial mean body weights between 200 and 220 g underwent sham operation or kidney I/R injury, which was induced as described previously [19]. The rats 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 Co., Gaithersburg, Md., USA). After 37 min of occlusion, the clamps were removed, and the reflow (reperfusion) was verified visually. Sham-operated control animals underwent the same surgical procedures, except for the occlusion of the renal arteries. All animals were given free access to food and water. Blood samples were collected at 1–5 days post-ischemic renal injury for

Fig. 1. Ppif is effectively downregulated by RNAi. **a** Four target sequences are shown in the table. **b** NRK cells were highly infected with lentivirus expressing both GFP and Ppif RNAi as evidenced by representative bright-field or fluorescence photos. **c** The mRNA levels of Ppif were downregulated to about 50%. NRK cells were infected with lentivirus expressing target se-

quence 1–4, respectively. After infection for 72 h, total RNA isolation and real-time PCR were carried out. CON = Control; NC = negative control. Fold changes of Ppif mRNA compared with that of GAPDH are shown as mean $\pm$ SD. **d** Lentivirus-mediated knockdown of Ppif in NRK cells was confirmed by Western blotting.

the measurement of serum creatinine (CR) and blood urea nitrogen (BUN). At the end of each experiment, tissue from the outer medullary region (rich in S3 segments) was obtained [20, 21] for real-time PCR, Western blot and histological analyses.

Histological Analysis and Histopathological Evaluation

The kidneys were removed from the rats at the end of the experimental period and were cut in a sagittal section into two halves. Renal tissue was fixed in 4% polyformaldehyde solution at 4°C overnight, and then embedded in paraffin. Paraffin kidney sections (4 μ m) were prepared and stained with hematoxylin and eosin. Evaluation of renal injury was performed in a blinded manner by a pathologist of the Tongji Medical College, Huazhong University of Science and Technology, and renal sections were scored with a semiquantitative scale designed to evaluate the degree of tubular necrosis. Injury was graded on a 5-point scale: 0 = normal kidney; 1 = minimal damage (<5% involvement of the

cortex or outer medulla); 2 = mild damage (5–25% involvement of the cortex or outer medulla); 3 = moderate damage (25–75% involvement of the cortex or outer medulla), and 4 = severe damage (>75% involvement of the cortex or outer medulla).

Measurement of CR and BUN

According to the manufacturer's instructions, CR and BUN were measured to evaluate renal function using a Quantichrom Creatinine Assay Kit and Quantichrom Urea Assay Kit (BioAssay Systems, Hayward, Calif., USA), respectively.

Statistical Analysis

Values are represented as means $\pm$ SD. A sufficient number of animals was studied in each group to obtain valid results. One-way analysis of variance (ANOVA) was used for comparison of animals with different operation, and statistical significance was established at $p < 0.05$.

Fig. 2. Knockdown of Ppif protects NRK cells from hypoxia-induced necrosis. **a** FACS analysis of NRK cells infected with indicated lentiviruses after I/R treatment. CON = Control; NC = negative control. **b** Statistical analysis of FACS results. Values are mean percentages from three independent experiments. * $p < 0.05$ compared with control.

Results

Ppif Is Effectively Downregulated by RNAi

To knock down the endogenous expression of Ppif, four target sequences have been chosen (fig. 1a). Lentiviruses that express negative control or Ppif RNAi sequence were packaged, concentrated and titrated. NRK cells were highly infected as evidenced by the expression of GFP (fig. 1b). The knockdown effect was determined by real-time PCR. As shown in figure 1c, the mRNA levels of Ppif were downregulated to 50–60%. Among the four sequences, the third one (KD3) is the most effective. To further confirm the knockdown effect of KD3 target sequence, the endogenous protein level of Ppif was checked by Western blotting. As shown in figure 1d, the expression of Ppif was effectively reduced by RNAi. Therefore, the KD3 target sequence was used in our following analyses.

Knockdown of Ppif Protects NRK Cells from Hypoxia-Induced Necrosis

It was reported that CypD-dependent mPT regulated several forms of necrotic death, but not apoptotic death [11] and Ppif null mice were protected from I/R-induced cell death in vivo [12]. To investigate whether knockdown of Ppif could protect NRK cells from hypoxia-induced cell death, we treated NRK cells with 95% N_2 /5% CO_2 for 60 min and then incubated in 95% air/5% CO_2 for another 48 h. Thereafter, we analyzed the cells with FACS. As shown in figure 2, in the control and negative control group, about 30% of the cells were necrotic. In contrast, about 17% of the cells were necrotic in the Ppif RNAi group ($p < 0.05$). Therefore, knockdown of Ppif protects NRK cells from hypoxia-induced necrosis.

Fig. 3. Evaluation of the expression of Ppif in the kidney tissue after I/R injury. **a** The mRNA levels of Ppif in rat kidney tissue were downregulated to about 40%. CON = Control; NC = negative control. Fold changes of Ppif mRNA compared with that of GAPDH are shown as mean $\pm$ SD. **b** Lentivirus-mediated knockdown of Ppif in rat kidney tissue was confirmed by Western blotting.

Downregulation of Ppif Protects Rat from I/R-Induced Renal Injury

Next, we evaluated the knockdown effect of Ppif in rat kidney tissue. Compared to the control or negative control group, the expression of Ppif in the kidney was significantly reduced at both mRNA and protein levels (fig. 3). To further study the role of Ppif on the renal injury caused by I/R, a rat kidney I/R animal model was set up. As shown in figure 4a, rat after operation showed lesions in the kidney at 24 h after the reperfusion. Specifically, the most severe and pronounced injuries were observed in the cortex and the outer stripe of outer medulla with a typical tubular necrosis pattern, which included widespread degeneration of tubular architecture, detachment of epithelial cells from the basement membrane, tubular cell necrosis, intratubular cast formation and luminal congestion with loss of brush border (fig. 4a). The histological features of renal injury of the control and negative control groups were characterized as score 2 or 3. In contrast, histopathological examination revealed that the Ppif RNAi group had much less injury after 24 h of reperfusion and its histological features of renal injury were characterized as score 0 or 1 (fig. 4b). Furthermore, the measurement of CR and BUN is consistent with the histopathological examination. Compared to the control group, BUN and CR of the Ppif RNAi group were significantly decreased (fig. 4c). Collectively, the above data showed that knockdown of Ppif protected rat from I/R-induced renal injury.

Discussion

I/R-induced renal failure is associated with a high morbidity and mortality rate in hospitalized patients. The mechanisms underlying I/R-induced renal injury are complicated. Here, we evaluated the role of CypD following I/R-induced renal injury. Our current study clearly demonstrates that knockdown of CypD protects the rat kidney in a model of I/R injury. According to our results, infection of lentiviruses that express a Ppif RNAi sequence before the onset of ischemia produces a significant reduction in tubular injury, which was accompanied by a marked amelioration of renal functional impairment as assessed by biochemical parameters (fig. 4). This cytoprotective action seems to be associated with the biological function of CypD, a peptidylprolyl isomerase F that is located in mitochondria [10]. A genetic deficiency in CypD protects mice from Ca^{2+} - and oxidative stress-induced cell death, whereas CypD overexpression mice show mitochondrial swelling and spontaneous cell death [11]. All these findings shed light on the role of CypD, which is required to mediate Ca^{2+} - and oxidative stress-induced necrotic cell death.

In addition, knockdown of CypD resulted in a marked reduction of necrotic cell death, but had no visible effect on apoptotic death (fig. 2). This finding is in agreement with previous studies reporting that CypD and the opening of the PT pore are required to mediate calcium- and oxidative stress-induced necrotic cell death but are not

Fig. 4. Evaluation of renal histopathology and renal function in the control, negative control and Ppif RNAi rats after I/R injury. **a** Comparison of renal histopathology of normal and I/R injured rats. The kidney sections were stained with hematoxylin and eosin. All sections are derived from the outer medullary region of the kidney. Left: sections derived from normal rats. Right: sections derived from I/R-injured rats. **b** Comparison of renal histo-

pathology of the control, negative control and Ppif RNAi rats after I/R injury. CON = Control; NC = negative control. **c** Biochemical analyses of rat renal function in the control, negative control and Ppif RNAi groups. Renal function parameters are BUN (left) and CR (right) at 24 h after reperfusion. Each column and bar represents the mean $\pm$ SD (n = 30). * p < 0.05 compared with (i.v.) vehicle-treated acute kidney injury rats.

essential to Bax-induced apoptotic cell death [11]. In fact, I/R-induced renal injury caused apoptotic cell death and led to an increased expression of Bax protein in distal, and especially in proximal tubular cells at 48 h of reperfusion. Based on the TUNEL method, only a slight decrease in apoptosis has been identified (data not shown). A possible explanation for this is that CypD alone is essential for I/R-induced necrosis rather than apoptotic death.

All these results gave us the hints that targeting CypD by RNAi may protect the kidney from I/R injury and our results indeed proved it. It is worth mentioning that CypD-dependent mPT regulates several forms of necrotic death [12]. Therefore, loss of CypD through gene

knockout may lead to cancer. However, knockdown of CypD gene by lentivirus-mediated RNAi technology avoids this risk. The present study provides the evidence that Ppif may be a potential target for protecting I/R-induced renal injury. Especially therapeutic interventions designed to inactivate CypD may be a promising strategy for reducing I/R-induced injury.

References

- 1 Paller MS: The cell biology of reperfusion injury in the kidney. *J Investig Med* 1994;42: 632–639.
- 2 Lien YH, Lai LW, Silva AL: Pathogenesis of renal ischemia/reperfusion injury: lessons from knockout mice. *Life Sci* 2003;74:543–552.
- 3 Jassem W, Heaton ND: The role of mitochondria in ischemia/reperfusion injury in organ transplantation. *Kidney Int* 2004;66:514–517.
- 4 Tsujimoto Y, Nakagawa T, Shimizu S: Mitochondrial membrane permeability transition and cell death. *Biochim Biophys Acta* 2006;1757:1297–1300.
- 5 Novgorodov SAP, Gudzi TI: Ceramide and mitochondria in ischemia/reperfusion. *J Cardiovasc Pharmacol* 2009;53:198–208.
- 6 Halestrap AP, McStay GP, Clarke SJ: The permeability transition pore complex: another view. *Biochimie* 2002;84:153–166.
- 7 Scorrano L, Ashiya M, Buttke K, Weiler S, Oakes SA, Mannella CA, Korsmeyer SJ: A distinct pathway remodels mitochondrial cristae and mobilizes cytochrome c during apoptosis. *Dev Cell* 2002;2:55–67.
- 8 Crompton M: On the involvement of mitochondrial intermembrane junctional complexes in apoptosis. *Curr Med Chem* 2003; 10:1473–1484.
- 9 Galat A, Metcalfe SM: Peptidylproline *cis/trans* isomerases. *Prog Biophys Mol Biol* 1995;63:67–118.
- 10 Broekemeier KM, Dempsey ME, Pfeiffer DR: Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J Biol Chem* 1989;264: 7826–7830.
- 11 Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD: Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature* 2005; 434:658–662.
- 12 Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y: Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature* 2005;434:652–658.
- 13 Schinzel AC, Takeuchi O, Huang Z, Fisher JK, Zhou Z, Rubens J, Hetz C, Danial NN, Moskowitz MA, Korsmeyer SJ: Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. *Proc Natl Acad Sci USA* 2005;102:12005–12010.
- 14 Devalaraja-Narashimha K, Diener AM, Padanilam BJ: Cyclophilin D gene ablation protects mice from ischemic renal injury. *Am J Physiol Renal Physiol* 2009;297:F749–F759.
- 15 Palma E, Tiepolo T, Angelin A, Sabatelli P, Maraldi NM, Basso E, Forte MA, Bernardi P, Bonaldo P: Genetic ablation of cyclophilin D rescues mitochondrial defects and prevents muscle apoptosis in collagen VI myopathic mice. *Hum Mol Genet* 2009;18:2024–2031.
- 16 Kato M, Akao M, Matsumoto-Ida M, Makiyama T, Iguchi M, Takeda T, Shimizu S, Kita T: The targeting of cyclophilin D by RNAi as a novel cardioprotective therapy: evidence from two-photon imaging. *Cardiovasc Res* 2009;83:335–344.
- 17 Qin XF, An DS, Chen ISY, Baltimore D: Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci USA* 2003;100:183–188.
- 18 Ding Q, Jin T, Wang Z, Chen Y: Catalase potentiates retinoic acid-induced THP-1 monocyte differentiation into macrophage through inhibition of peroxisome proliferator-activated receptor γ . *J Leukoc Biol* 2007; 81:1568–1576.
- 19 Zheng J, Devalaraja-Narashimha K, Singaravelu K, Padanilam BJ: Poly(ADP-ribose) polymerase-1 gene ablation protects mice from ischemic renal injury. *Am J Physiol Renal Physiol* 2005;288:F387–F398.
- 20 Devalaraja-Narashimha K, Padanilam BJ: PARP-1 Inhibits glycolysis in ischemic kidneys. *J Am Soc Nephrol* 2009;20:95–103.
- 21 Ruegg CE, Mandel LJ: Bulk isolation of renal PCT and PST. I. Glucose-dependent metabolic differences. *Am J Physiol Renal Physiol* 1990;259:F164–F175.