Prostacyclin-induced peroxisome proliferator-activated receptor-α translocation attenuates NF-κB and TNF-α activation after renal ischemia-reperfusion injury

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Chen HH, Chen TW, Lin H. Prostacyclin-induced peroxisome proliferator-activated receptor-α translocation attenuates NF-κB and TNF-α activation after renal ischemia-reperfusion injury. Am J Physiol Renal Physiol 297:F1109–F1118, 2009. First published July 29, 2009; doi:10.1152/ajprenal.00057.2009.—Prostacyclin and peroxisome proliferator-activated receptors (PPAR) protect against ischemia-reperfusion (I/R) injury by the induction of an anti-inflammatory pathway. In this study, we examined the prostacyclin-enhanced protective effect of PPARα in I/R-induced kidney injury. PPAR-α reduced the NF-κB-induced overexpression of TNF-α and apoptosis in cultured kidney cells. In a murine model, pretreating wild-type (WT) mice with a PPAR-α activator, docosahexaenoic acid (DHA), significantly reduced I/R-induced renal dysfunction (lowered serum creatinine and urea nitrogen levels), apoptotic responses (decreased apoptotic cell number and caspase-3, -8 activation), and NF-κB activation. By comparison, I/R-induced injury was exacerbated in PPAR-α knockout mice. This indicated that PPAR-α attenuated renal I/R injury via NF-κB-induced TNF-α overexpression. Overexpression of prostacyclin using an adenovirus could also induce PPAR-α translocation from the cytosol into the nucleus to inhibit caspase-3 activation. This prostacyclin/PPAR-α pathway attenuated TNF-α promoter activity by binding to NF-κB. Using a cAMP inhibitor (CAY10441) and a prostacyclin receptor antibody, we also found that there was another prostacyclin/IP receptor/cAMP pathway that could inhibit TNF-α production. Taken together, our results demonstrate for the first time that prostacyclin induces the translocation of PPAR-α from the cytosol into the nucleus and attenuates NF-κB-induced TNF-α activation following renal I/R injury. Treatments that can augment prostacyclin, PPAR-α, or the associated signaling pathways may ameliorate conditions associated with renal I/R injury.

arachidonic acid; IP receptor; cAMP; docosahexaenoic acid; caspase

RENAL ISCHEMIA-REPERFUSION (I/R) injury is a clinically significant problem that can lead to acute renal failure (32). Recent studies of renal I/R injury have focused on the roles of neutrophils, inflammatory cytokines, the tissue factor thromboplastin, intercellular adhesion molecule-1, oxygen free radicals, vascular plugging, edema, and other mechanisms (3). Tumor necrosis factor-α (TNF-α) has been implicated in the pathogenesis of many inflammatory diseases of the kidney, including glomerulonephritis, septic acute renal failure, and renal I/R injury (10). TNF-α binds to its membrane-bound receptor TNFR1, which activates the Fas-associated death domain (FADD), caspase-8, caspase-3, and ultimately causes cell death (10).

Prostacyclin (PGI2), a prostaglandin-derived product, is synthesized by vascular endothelial and smooth muscle cells. It is a potent vasodilator and anticoagulant that acts on vascular tissues and platelets (25). When PGI2 binds to the PGI2 receptor (IP receptor), it has antipapoptotic effects on renal tubular cells following treatment with radio-contrast medium (43). At the molecular level, activation of the IP receptor induces adenyl cyclase to produce cAMP, which activates numerous intracellular signaling pathways (28, 42). In addition to the IP receptor, endogenous peroxisome proliferator-activated receptor (PPAR)-δ is a PGI2 receptor that is a key signaling protein in PGI2-mediated apoptosis (13).

PPAR-α is a ligand-activated transcription factor that is highly expressed in the kidney (5). Recent studies have shown that PPAR-α contributes to the resolution of inflammation after renal I/R injury using extant ligands (5, 31). PPAR-mediated modulation of gene transcription by PGI2 may indicate a novel role for PPARs in the regulation of gene expression (14). A stable analog of PGI2 has been shown to activate PPARs (13, 14), but little is known about the relationship between PGI2 and PPAR-α in the kidney. Thus the aims of this study were to characterize the effects of PGI2 and PPAR-α in a renal I/R injury model and to investigate the influence of PGI2 on PPAR-α induction and TNF-α-induced cell apoptosis following I/R injury.

MATERIALS AND METHODS

Animal model (I/R injury model). We used mouse strain 129 (wild-type PPAR-α+/+/ and the PPAR-α−/− mutant) as a model for the study of renal I/R injury. Our investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85–23, revised 1996). All surgical procedures were performed according to protocols approved by the Institutional Animal Care and Utilization Committee, Academia Sinica. Male mice (aged 8–10 wk; 18–22 g) were subjected to bilateral renal artery occlusion (45 min) and reperfusion (24 h) as previously described (21). Briefly, mice were anesthetized with intraperitoneal pentobarbital (50 mg/kg) and placed on a heating pad to maintain the core body temperature at 37°C. Both kidneys were exposed through the flank. The renal pedicles were clamped with vascular clamps (Roboz Surgical Instrument, Gaithersburg, MD) for 45 min and reperfused for 24 h. Animals received single intraperitoneal doses of vehicle (saline) or docosahexaenoic acid (DHA; 500 mg/kg body wt) 3 days before I/R surgery. The dose of DHA was modified from a previous study (30). Sham control animals underwent the same surgical procedures except for intraperitoneal saline injection.

Terminal deoxynucleotidyl transferase dUTP nick-end labeling. DNA fragments in animal tissues and cells were detected by labeling the terminal ends of nucleic acids using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay. Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Organs were dissected and postfixed with 4% paraformaldehyde overnight, paraffin embedded, and sectioned (5 μm). TUNEL assay followed the manufacturer’s protocol (Roche, Mannheim, Germany).

Nuclear extracts and EMSAs. NF-κB DNA probes containing a consensus NF-κB enhancer element (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). EMSA analysis of nuclear NF-κB was performed as described previously (21). After nuclear extracts were prepared, binding reactions were performed in 20-μl reaction mixtures with 3 μg of nuclear extracts in buffer containing 12 mM HEPES (pH 7.9), 5 mM MgCl2, 60 mM KCl, 4 mM Tris-HCl (pH 7.9), 0.6 mM EDTA, 0.6 mM DTT, 0.5 mg/ml BSA, 1 μg of poly(d-I-C), 12% glycerol, and 20,000 dpm of radio-labeled double-stranded −120 bp ANF GATA probe for 20 min at room temperature. Reactions were loaded on a 5% polyacrylamide gel and run at 200 V at room temperature in 0.25× TBE. The gel was dried and exposed to a PhosphorImager screen (Molecular Dynamics).

Cell culture, infections, and transfections (kidney cell hypoxia-reoxygenation model). Renal tubule cells (NRK-52E) derived from rat kidney were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C with 5% CO2 as previously described (21). NRK-52E cells were either infected or transfected on the following day. Infections were performed using a multiplicity of infection of adenovirus by adding the appropriate recombinant adenovirus, adv-cyclooxygenase (COX)-1/prostacyclin synthase (PGIS) or Ade-HPGK, to the culture media for 2 days, followed by hypoxia for 24 h and reoxygenation for another 24 h. Transfection was performed using lipofectamine with 1.5 μg of luciferase reporter plasmid and varying amounts of PPAR-α, PPAR-α short interference (si) RNA (5′-CCG TTA TCT GAA AA TTC TTA-3′; TRCN 0000025967, National RNAi Core Facility), and Flag PPAR-α expression vectors/6-cm2 culture dish (see legends to Figs. 1, 2, 5, 6, 7). The amount of DNA was kept constant using an empty expression vector. NRK-52E cells were harvested and assayed 16–24 h later.

Immunocytochemistry. NRK-52E cells transfected with Flag-PPAR-α plasmid or infected with Adv-COX-1/PGIS were plated on 35-mm poly-l-lysine-coated glass-bottomed dishes (Matsunami Glass) and fixed for 20 min at room temperature with 4% paraformaldehyde and 0.4% Triton X-100 in PBS. The cells were incubated with an anti-FLAG antibody (rabbit polyclonal, Sigma) in PBST (PBS with 0.05% Tween 20) containing 2% horse serum. After three washes with PBS, the cells were incubated with Alexa 488-conjugated anti-rabbit IgG (Molecular Probes) and Cy3-conjugated anti-mouse IgG (Amersham Biosciences) in PBST containing 2% horse serum for 1 h at room temperature. Fluorescent images were captured and analyzed with a μRadiance Laser Scanning Confocal Microscope System (Bio-Rad).

Replication-defective recombinant adenoviral vectors. This procedure was previously described (22). In replication-defective recombinant adenoviral (rAd) vectors, we constructed the following: a human phosphoglycerate kinase (PKG) promoter to drive COX-1 expression (Adv-PGK-COX-1 or Adv-COX-1); two separate PGK promoters (bicistronic) to drive COX-1 and PGIS, respectively (Adv-PGKCOX-1/PGIS or Adv-COX-1/PGIS); and PGK alone to serve as a control (Adv-PGK). Replication-defective rAd vectors were generated by homologous recombination and amplified in 293 cells as described previously (22). rAd stocks were prepared by CsCl gradient centrifugation, aliquoted, and stored at −80°C. Viral titers were determined by a plaque-assay method. The 293 cells were infected with serially diluted viral preparations and then overlaid with low-melting-point agarose. The numbers of plaques that formed were counted within 2 wk.

Eicosanoid measurement. Following the overexpression of PGI2 in NRK-52E cells using an adenovirus that contained the COX-1/PGIS plasmid, we measured eicosanoids in supernatants using immunosassay kits for PGE2, 6-keto-PGF1α, thromboxin X2 (R&D Systems, Minneapolis, MN), and PGD2 (Cayman Chemical, Ann Arbor, MI). All procedures followed manufacturers’ protocols.

RNA extraction and RT-PCR analysis. Total RNA was extracted from NRK-52E cells using the TRIzol method according to the manufacturer’s protocol (Invitrogen, Carlsbad, CA). RT-PCR was performed as previously described (22). Briefly, total RNA extract was treated with 1 U of RQ1 RNase-free DNase (Promega) per microgram of total RNA at 37°C for 1 h. Reverse transcription (RT) was performed at 42°C for 50 min in a total volume of 20 μl containing 5 μg RNA, 0.5 μg of oligo (dT) 12–18, and 200 U of superscript II RNase H (Invitrogen Life Technologies). Subsequently,
RT was inactivated by incubation at 70°C for 15 min, followed by treatment with 1.2 U of RNase H at 37°C for 30 min. PCR was performed with 1/20 of the RT reaction in a total volume of 50 μl using Taq DNA Polymerase (Invitrogen). To control for the generation of PCR products due to residual contamination of genomic DNA, an aliquot of RNA without RT treatment was tested in parallel. PCR was performed for 30 cycles (94°C for 30 s, 57°C for 30 s, and 72°C for 30 s), and the products were visualized on 2% agarose gels by ethidium bromide staining. The PPAR-α primers were anti-sense 5′-CCA CCA TCG CGA CCA GAT-3′ and sense 5′-GAC GTG CTT CCT GCT TCA TAG A-3′. Each sample was run in triplicate and normalized to the level of GAPDH as a “housekeeping” gene.

Cytoplasmic and nuclear protein extraction. Cell and kidney tissue proteins were extracted as nuclear and cytoplasmic fractions according to the manufacturer’s protocol (NE-PER Nuclear and Cytoplasmic Extraction Reagents, Pierce Chemical, Rockford, IL).

Western blotting. Western blotting was performed as previously described (22). Blots were incubated with antibodies against PPAR-α (1:500; Santa Cruz Biotechnology), caspase-3 (1:1,000; Cell Signaling), caspase-8 (1:5,000; Cell Signaling), NF-κB (p65, 1:500; Santa Cruz Biotechnology), IκB (1:500; Santa Cruz Biotechnology), and COX-1 and PGIS (1:1,000; Cayman Chemical).

TNF-α measurement. Culture medium was analyzed for mouse TNF-α by an ELISA kit according to the manufacturer’s protocol (RayBiotech, Norcross, GA).

Immunoprecipitation. Control and transfected cells were lysed at 4°C in lysis buffer [50 mM Tris, pH 7.5, 1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, and protease inhibitors, Complete, Mini protease inhibitor cocktail tablet (Roche)]. PPAR-α-associated proteins were collected with an immunoprecipitation kit (Roche Diagnostics) that included anti-PPAR-α antibodies and protein G-agarose. The precipitates were subjected to Western blotting and hybridization with NF-κB.

Measurement of biochemical parameters. At the end of the reperfusion period, 500-μl blood samples were collected via the tail vein. Samples were centrifuged (6,000 g, 3 min) to separate the serum. Biochemical parameters were measured in the serum within 24 h.

TNF-α promoter assay. A TNF-α reporter gene construct was created by cloning the human TNF-α promoter (−2,000 to +17) into the pGL2-basic promoterless plasmid vector from Promega (Madison, Wisconsin).

Fig. 2. Attenuated TNF-α transcription and translation in PPAR-α-transfected NRK-52E rat kidney cells following H/R. A: effects of PPAR-α on TNF-α promoter activity. PPAR-α cDNA and TNF-α promoter plasmid were transfected into NRK-52 cells and subjected to H/R as indicated; cells were harvested and assayed for luciferase activity. *P < 0.05. B: effect of PPAR-α on H/R-induced TNF-α expression in NRK-52E cells. C, no transfection; H, infection with HPGK plasmid; P, infection with PPAR-α plasmid. *P < 0.05. C: effect of PPAR-α on NF-κB nuclear translocation and IκB degradation. EMSA for nuclear extracts of NF-κB from NRK-52E cells after sham operation (C), mock transfection (M), or PPAR-α transfection (PPAR-α) following H/R is shown. D: nuclear and cytosolic extracts from NRK-52E cells were probed with NF-κB and IκB antibodies to quantify protein levels. C, control; M, control plasmid transfection; PPAR-α, PPAR-α plasmid transfection. Results are from 3 independent experiments. Scanning densitometry was used for semiquantitative analysis and compared with β-actin levels. *P < 0.05.
Promoter luciferase activity was measured as previously described (41).

**Statistical analysis.** Results are given as means ± SE. Experimental groups were compared by one-way ANOVA. When there was a significant difference between groups, multiple mean comparisons were made using the Bonferroni procedure with type I error adjustment. All statistical assessments were two-sided and evaluated at the 0.05 level of significance. Statistical analyses used SPSS 15.0 software (SPSS, Chicago, IL).

**RESULTS**

**Effects of PPAR-α on I/R-induced apoptosis.** We first studied the effects of PPAR-α on hypoxia-reoxygenation (H/R) cells by constructing PPAR-α-Flag cDNA and then transfecting NRK-52E cells to induce this protein’s overexpression. Compared with controls or vector transformants, the level of PPAR-α was significantly elevated in cells with the PPAR-α construct (far right lane, Fig. 1A). During H/R treatment, cleaved caspase-3 and -8, which are important signals for TNF-α-induced apoptosis, were both significantly reduced by overexpression of PPAR-α (far right lane; Fig. 1B).

Next, we employed gene knockdown using short interference RNA (siRNA) to evaluate any protective effects of PPAR-α on H/R-induced apoptosis. PPAR-α siRNA specifically and dose-dependently reduced the expression of PPAR-α in transfected NRK-52E cells (Fig. 1, C and D). After H/R, PPAR-α-transfected NRK-52E cells expressed less caspase-3 and -8 (lane 2 vs. lane 4, Fig. 1E). These effects were abolished by cotransfection with PPAR-α siRNA (lane 4 vs. lane 5, Fig. 1E).

**Effects of PPAR-α on TNF-α expression.** Previous studies have shown that following H/R, renal tubule cells are injured by TNF-α and that this causes an overt inflammatory reaction (44). Our results showed that H/R elevated TNF-α promoter activity and the concentration of TNF-α in NRK-52E cells and that these effects were only partially attenuated by infection with PPAR-α (Fig. 2, A and B). As these results were only partially due to the effects of PPAR-α, there may be other I/R-induced TNF-α activation pathways involved (24). Coinfection with PPAR-α siRNA reversed the PPAR-α effect (Fig. 2B).

**Fig. 3.** Docosahexaenoic acid (DHA) reverses renal dysfunction and apoptosis in the mouse kidney following ischemia-reperfusion (I/R) injury. A: effects of DHA on PPAR-α expression, serum urea nitrogen (left), and creatinine (right) levels following I/R after injection with saline (C) or DHA (D) 3 days before I/R injury (45 min/24 h). Kidney extracts were separated and blotted with PPAR-α antibody (Western blotting). Serum urea nitrogen and creatinine levels of mice were measured 24 h after reperfusion or sham surgery. NS, not significant *P < 0.05; n = 6/group. B: detection of apoptotic kidney cells using in vivo terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) staining. Control (A and C) and DHA-treated (B and D) mice underwent a sham operation (A and B) or I/R (45 min/24 h; C and D). *P < 0.05; n = 5/group. C: effect of DHA on expressions of caspase-3 and -8. Kidney extracts from control or DHA-treated mice with or without I/R were probed with specific antibodies against the cleaved, active forms of caspase-3 or caspase-8. Experiments were performed twice with similar results. Results are from 3 independent experiments. Scanning densitometry was used for semiquantitative analysis and compared with β-actin levels. *P < 0.05.
Effects of PPAR-α on the NF-κB-induced TNF-α pathway. TNF-α genes contain functional NF-κB binding sites that are essential for their induction following I/R injury (27, 34). Thus we assessed NF-κB activation using EMSAs. H/R increased the NF-κB DNA binding activity in the nuclear extracts of renal tubular cells, but this was markedly suppressed in PPAR-α-transfected cells (Fig. 2C). We verified the identity of the gel shift band by competition analysis using unlabeled DNA fragments (lanes 25–50, Fig. 2C). In H/R-treated PPAR-α-transfected NRK-52E cells, the level of nucleic NF-κB was reduced and cytosolic IkB, a protein that prevents translocation of NF-κB dimers into the nucleus, was elevated (top, far right lane, Fig. 2D). Taken together, these results suggest that PPAR-α plays a protective role in modulating H/R injury by inhibiting TNF-α expression via inactivation of NF-κB.

Effects of DHA on renal function and apoptosis. DHA, a long-chain fatty acid, is an activator of PPAR-α (26). I/R-injured mice showed increased serum levels of urea and creatinine (right) levels were measured 24 h after reperfusion or sham surgery. PPARα−/− mice showed more severe renal failure compared with WT mice. Values are means ± SE; n = 7 animals/group. *P < 0.01, WT vs. PPARα−/− mice. B: detection of apoptotic kidney cells in WT and PPAR-α−/− mice using in vivo TUNEL staining. WT (A and C) and PPAR-α−/− mice (B and D) mice underwent a sham operation (A and B) or 45 min of renal ischemia followed by 24 h of reperfusion (C and D). TUNEL staining of representative kidney sections from each experimental group are shown. Bar = 200 μm. Proportions of TUNEL-positive renal epithelial nuclei for total nuclei in WT and PPAR-α−/− mice subjected to sham operation or I/R injury are shown. *P < 0.05; n = 5 animals/group. C: presence of active caspase-3 and -8 in WT and PPAR-α−/− mice with or without IR. Experiments were performed twice with similar results. Scanning densitometry was used for semiquantitative analysis and compared with β-actin levels.*P < 0.05. D: effect of PPAR-α on NF-κB nuclear translocation. EMSA for renal nuclear extracts of NF-κB in WT and PPAR-α−/− mice subjected to sham operation (P), cold probe competitor (50×) following IR is shown.
inulin compared with sham-operated mice (Fig. 3A). Treatment with DHA increased intracellular PPAR-α expression and lowered serum urea and creatinine levels compared with non-DHA-treated I/R mice (Fig. 3A), suggesting a marked reduction of renal I/R injury.

Apoptosis has been implicated in the pathogenesis of renal I/R injury (23, 40). We used a TUNEL assay to evaluate any role for PPAR-α in renal tubular cell apoptosis following renal I/R. TUNEL-positive cells were virtually undetectable in the kidneys of sham-operated mice and DHA-treated mice without I/R (Fig. 3B). Following I/R, we observed large numbers of TUNEL-positive cells in untreated mice (primarily renal tubular cells) but significantly fewer TUNEL-positive cells in DHA-treated mice (Fig. 3B).

Caspases, a family of cysteine proteases involved in apoptosis, necrosis, and inflammation, are induced during renal I/R (22a). TNF-α has been implicated in TNF-α receptor-induced caspase-8 and -3 pathways in I/R-induced apoptosis (33). Thus we examined the activations of caspase-8 and -3 in the kidneys of untreated and DHA-treated mice. Consistent with the results of our TUNEL assay, DHA-treated mice under I/R showed significantly less caspase-8 and -3 activation than untreated mice (Fig. 3C). These results suggest that PPAR-α is involved with renal tubular cell apoptosis during I/R injury.

Fig. 5. Effects of PGI2 on PPAR-α translocation. A and B: changes in COX-1, PGIS, and eicosanoid levels in NRK-52E cells as a function of time. Cells were infected with Adv-COX-1/PGIS for 1–3 days, with Adv-PGK as a control. Cell extracts were assayed by Western blotting using antibodies specific for COX-1 and PGIS. Additionally, the indicated prostaglandins in supernatants were assayed by ELISA. C: effect of PGI2 on translocation of PPAR-α from the cytosol into the nucleus. NRK-52E cells were infected with Adv-COX-1/PGIS for 24 h; cells were harvested and nuclear and cytosolic fractions were isolated and subjected to Western blotting using anti-PPAR-α. D: COX-1-specific inhibitor SC560 attenuates PPAR-α translocation. SC560 (10 nM) was administered 30 min before NRK-52E cells were transfected with a Flag-PPAR-α plasmid. Cell extracts were separated into nuclear and cytosolic fractions and subjected to Western blotting using a Flag antibody. Results are from 3 independent experiments. Scanning densitometry was used for semiquantitative analysis and compared with β-actin levels. *P < 0.05. E: subcellular localization of PPAR-α-FLAG in transiently transfected NRK-52E cells. A and B: in the absence of Adv-COX-1/PGIS infection, the majority of PPAR-α-FLAG localizes to the cytosolic fraction. PPAR-α-FLAG was detected using an anti-FLAG monoclonal antibody and Alexa Fluor anti-mouse 488 secondary antibody. C: Adv-COX-1/PGIS infection results in a significant redistribution of PPAR-α-FLAG selectively concentrated in the nucleus (arrow). D: with SC560 pretreatment, PPAR-α-FLAG is preferentially found in the cytosolic fraction and is significantly decreased in the nucleus. Scale bar = 200 μm.
Effects of IR on renal function in wild-type and PPARα−/− mice. We subjected wild-type (PPARα+/+) and mutant (PPARα−/−) mice to I/R and compared their renal functions and the levels of cleaved caspase-3 and -8. After I/R, PPARα−/− mice had increased levels of serum urea and creatinine, TUNEL-positive cells, and expressions of caspase-3 and -8 (Fig. 4, A–C). We also assessed NF-κB activation by EMSA. I/R increased the NF-κB DNA binding activity in the renal nuclear extracts of WT mice, and this was further increased in the PPARα−/− mice (Fig. 4D). These results suggest that PPARα has a protective role in renal I/R by inhibiting NF-κB binding.

Effects of PGI2 on PPARα translocation. Prostaglandin-derived products, such as PGI2, are ligands for PPARs (2, 18, 21). It is not known whether PGI2 plays any physiological role in mice with I/R injury, although PGI2 can serve as a ligand for the translocation of PPARα. We first showed that 6-keto-PGF1α levels dramatically increased up to 3 h during reperfusion, but returned to basal levels after 24 h (Supplemental Fig. 1; all supplemental material for this article are accessible on the journal web site). Next, we selectively overexpressed PGI2 in NRK-52E cells using an adenovirus that contained COX-1/PGIS (Adv-COX-1/PGIS; Fig. 5, A and B). The levels of cytosolic PPARα were similar in HPV- and COX-1/PGIS-infected cells (lanes 5 and 7, Fig. 5C, bottom), but the nuclear level of PPARα was significantly elevated only in COX-1/PGIS-infected cells (lanes 5 and 7, Fig. 5C, bottom). In addition, neutralization of PGI2 by a COX-1-specific inhibitor, SC560, attenuated the translocation of PPARα from the cytosol into the nucleus (Fig. 5D). Immunostaining also showed that Flag-PPARα was translocated from the cytosol into the nucleus after infection with Adv-COX-1/PGIS (Fig. 5E, bottom left). However, this phenomenon was significantly diminished after treatment with SC560 (Fig. 5E, bottom right). These results indicate that PGI2 may play an important role in the translocation of PPARα into the nucleus.

Effects of PGI2 on PPARα and NF-κB. The physical interaction between PPARα and the p65 subunit of NF-κB interferes with the activity of both transcription factors (6, 8). Thus we investigated whether PGI2 and PPARα could inhibit H/R-induced apoptosis via the NF-κB pathway. PPARα-trans-

Fig. 6. Effects of PGI2 on PPARα-associated NF-κB-induced apoptosis. A: effect of overexpression of PGI2 by Adv-COX-1/PGIS on the association of PPARα with NF-κB in the nucleus. NRK-52E cells were transfected with PPARα or mock control for 48 h and separately infected with Adv-HPGK and Adv-COX-1/PGIS for 24 h before H/R (1 day/1 day). Cells were harvested, and nuclei were immunoprecipitated with PPARα antibody, then subjected to Western blotting using an anti-NF-κB (p65) antibody (see MATERIALS AND METHODS). B and C: effect of cotreatment with Adv-COX-1/PGIS and PPARα on attenuation of TNF-α-induced apoptosis. NRK-52 cells were cotransfected with siRNA, PPARα for 48 h then infected with Adv-COX-1/PGIS following I/R. Cell lysates were subjected to Western blotting of cleaved caspase-3 antibody; β-actin was used as a loading control. Supernatants were assayed for TNF-α concentrations by ELISA (C). Results are from 3 independent experiments. Scanning densitometry was used for semiquantitative analysis and compared with β-actin levels. *P < 0.05.
fected NRK-52E cells were immunoprecipitated with a PPAR-α antibody and then immunoblotted with an anti-p65 antibody. Our results (Fig. 6A) showed that after H/R, the levels of PPAR-α that coimmunoprecipitated with p65 were similar in control (lane 6) and HPKG cells (lane 7). However, either treatment with PPAR-α (lane 9) or Adv-COX-1/PGIS transfection (lane 8) significantly increased the level of the immunoprecipitated protein. Simultaneous treatment with PPAR-α and transfection with Adv-COX/PGIS resulted in the highest level of immunoprecipitated protein (lane 10).

PGI₂ is known to attenuate adriamycin-induced apoptosis (42). Thus we determined the levels of the cleaved form of caspase-3 in NRK52E cells following H/R (Fig. 6B). Following H/R, the level of caspase-3 was lower in cells receiving both treatment with PPAR-α and transfection with COX/PGIS (lane 5) than for cells receiving either of these treatments alone (lanes 3 and 4). Furthermore, gene knockdown with siRNA (lane 6) increased the level of caspase-3.

**Effects of PGI₂ on TNF-α.** A recent study showed that treatment with a PGI₂ analog, beraprost, reduced radio contrast-induced hypoxia injury via a PKA-dependent CREB phosphorylation pathway (43). Thus we examined the signal transduction pathways for PGI₂ inhibition of TNF-α production following H/R. H/R-treated NRK-52E cells released large amounts of TNF-α into cell lysates (lane 2, Fig. 6C). However, Adv-COX-1/PGIS and PPAR-α transfections, both separately and synergistically, reduced the levels of TNF-α (lanes 3–5, Fig. 6C); these effects were reversed by PPAR-α siRNA (lane 6, Fig. 6C).

In addition, TNF-α promoter activity was attenuated in NRK-52E cells that were transfected either separately or synergistically with PPAR-α and/or Adv-COX-1/PGIS (lanes 6 and 7 vs. lanes 8–10, Fig. 7). These Adv-COX-1/PGIS or PPAR-α transfection effects were only partially reversed when cells were given CAY10441, a cAMP inhibitor (lanes 8–10 vs. lanes 13–15, Fig. 7) and an IP receptor antibody (lanes 8–10 vs. lanes 18–20, Fig. 7). Clearly, PPAR-α and cotransfection with Adv-COX-1/PGIS results in greater downregulation of TNF-α promoter activity than either of these alone, and these effects were only partially abolished by a cAMP inhibitor and an IP receptor antibody. Taken together, these results imply that PGI₂ has effects on TNF-α transcription and translation by dual antiapoptotic pathways: the exogenous PGI₂/IP/cAMP pathway and the endogenous PGI₂/PPAR-α pathway.

**DISCUSSION**

All PPARs can regulate gene expression by forming heterodimers with the retinoid X receptor (RXR), which then binds to PPAR response elements (PPREs) in the promoter regions of target genes (4). RXR also forms heterodimers with vitamin D, thyroid hormone, and other receptors (45). PPARs are also known to bind free fatty acids and eicosanoids, including PGI₂ (7).

In addition to regulating transcription, several studies have shown that PPARs interfere with inflammatory signaling pathways by interacting with either the activator protein (AP-1) complex (including c-fos, c-jun, and NF-kB) or with signal transduction and activation of transcription proteins (STATs) (4, 6, 8). A study using the human colorectal carcinoma cell line SW620 showed that PPAR-α ligands inhibited tumor promoter PMA-mediated induction of genes associated with inflammation and tumor growth, including cyclooxygenase 2 (COX2) and vascular endothelial growth factor (VEGF) (12). PPAR-α activators also reduced the transcriptional induction of COX-2 and VEGF by inhibiting AP-1-mediated transcriptional activation induced by PMA or by the overexpression of c-Jun (12). PGI₂ is derived from ω-6 arachidonic acid and is rapidly broken down in the endothelium to 6-keto-PGF₁α, which has weaker vasodilator effects (29). There is evidence that the interaction of PGI₂ with thromboxane is involved in cardiovascular homeostasis that effectively reduces vascular damage (29).

In this study, we demonstrated that increased PPAR-α expression can inactivate the NF-κB-dependent TNF-α gene and protect against renal I/R (H/R) injury both in vivo and in vitro. We also showed that PGI₂ will translocate PPAR-α from the cytosol to the nucleus where it binds with NF-κB to inhibit TNF-α activation. However, this effect could not be completely reversed by either a cAMP inhibitor or an IP receptor antibody. Taken together, our findings support the hypothesis that PGI₂ has potent antiapoptotic effects during renal I/R injury and that the PPAR-α and IP/cAMP receptor pathways both mediate these effects.

Previous studies showed that renal I/R downregulated PPAR-α and that a PPAR-α agonist (clofibrate) was the most important factor for reducing renal I/R injury damage by PPAR-α (31, 38). Our results also indicate that PPAR-α inhibits H/R induced caspase-8-dependent apoptosis (Fig. 1).

The TNF-α induced inflammatory cascade for renal injury involves endothelial, epithelial, and infiltrating inflammatory cells (37). The infiltrating cells include macrophages and cytotoxic T cells, which increase the levels of E-selectin, TNF-α, and IFN-γ, resulting in renal dysfunction and apoptosis (17, 35). TNF-α production is triggered by locally produced reactive oxygen species (ROS), as well as by H/R injury, which activate transcription factor NF-κB and cause renal dysfunc-
tion (17). Our results are in agreement with recent studies that showed that H/R-induced TNF-α activation in cultured renal epithelial cells is mediated by NF-κB and that inhibition of PPAR-α by siRNA exacerbated H/R injury by enhancing NF-κB-mediated TNF-α induction (Fig. 2). In addition, an omega-3 fatty acid, DHA, which is a PPAR-α activator, can also inhibit leukocyte adhesion to cytokine-stimulated human umbilical vein endothelial cells (36). Accordingly, we showed that renal dysfunction was markedly ameliorated in DHA-treated mice in response to I/R injury (Fig. 3).

However, both TNF-α activation and cell apoptosis were only partially reduced when cells overexpressed PPAR-α or when mice were injected with DHA. The reason for these partial effects may be that there are multiple pathways involved in I/R-induced kidney injury via NF-κB-induced TNF-α activation (11). Thus overexpression of PPAR-α by a plasmid or DHA treatment can block only a part of the NF-κB-induced inflammatory pathway. In addition, the partial protective effects by PPAR-α in renal I/R injury may arise, as most tissues in humans and rodents express three PPAR subtypes: α, β, and γ (39). Each member of this subfamily of nuclear transcription factors has antiatherogenic and anti-inflammatory functions (9). Experiments using PPAR-α, β, γ-null mice as a model showed more severe cortical necrosis and renal dysfunction after I/R injury (9).

Although PPAR-β and PPAR-γ agonists have shown therapeutic effects against I/R injury (1, 16), the effects of endogenous PPAR-α on conditions associated with I/R injury have not been investigated to date. A recent study of NRK-52E cells examined gentamicin-induced apoptosis, in which PGI2 production was increased by infecting cells with an adenovirus carrying COX-1 and prostacyclin synthase (15). These authors concluded that elevated production of endogenous PGI2 protected renal tubule cells from apoptosis by a PPAR-α-associated pathway (15). Our study also provides strong evidence that renal I/R protection provided by PPAR-α is mediated by PGI2 and that PGI2 enhances PPAR-α translocation from the cytosol into the nucleus (Fig. 5).

PGI2 is known to regulate growth, fibrosis, and apoptotic responses via the IP and PPAR pathways (19). Hatae et al. (13) demonstrated that activation of the PGI2/PPAR-δ pathway was associated with apoptosis, whereas the PGI2/IP/cAMP pathway had antiapoptotic effects in human embryonic kidney (HEK-293) cells. However, our study indicates that not only the PGI2/IP/cAMP pathway but also the PGI2/PPAR-α pathway has antiapoptotic effects in the kidney during I/R injury (Fig. 7). In the presence of endogenous COX-1/PGIS (Supplemental Fig. 3) and PPAR-α (Supplemental Fig. 2) in NRK-52E cells, transfection of PPAR-α or Adv COS-1/PGIS individually could partially reduce TNF-α activity (lanes 8 and 9, Fig. 7). The presence of endogenous PPAR-α and the antiapoptotic PGI2/PPAR-α pathway also explains why a cAMP inhibitor or an IP receptor antibody could not completely reverse the protective effects of COX-1/PGIS (lane 8 vs. lanes 13 and 18, Fig. 7).

Furthermore, we demonstrated that the antiapoptotic PGI2/PPAR-α pathway is mediated by PGI2 by enhancing PPAR-α translocation from the cytosol into the nucleus, which then inhibits NF-κB-induced TNF-α activation (Figs. 5D and 6). However, it is not known whether there is a PGI2/PPAR-α complex that binds with a corepressor or another transcription factor that can protect against renal I/R. Also, it is not known whether another PPAR subfamily member might influence these results.

In summary, our results indicate that PGI2-induced PPAR-α translocation plays an important role in protecting the kidney against I/R injury, in addition to its traditional IP/cAMP signaling pathway. Pharmacological modulation of renal PGI2 and PPAR-α induction or its signal transduction may be a viable strategy for improving renal functional outcomes after I/R injury.

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