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Peroxisomal Proliferator-Activated Receptor-α Protects Renal Tubular Cells from Doxorubicin-Induced Apoptosis

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ABSTRACT

Peroxisome proliferator-activated receptor-α (PPAR-α) is a transcription factor and has been reported to inhibit cisplatin-mediated proximal tubule cell death. In addition, doxorubicin (Adriamycin)-induced nephrosis in rats is a commonly used experimental model for pharmacological studies of human chronic renal diseases. In this study, we investigated the protective effect of PPAR-α on doxorubicin-induced apoptosis and its detailed mechanism in NRK-52E cells and animal models. The mRNA level of PPAR-α was found to be reduced by doxorubicin treatment in NRK-52E cells. PPAR-α overexpression in NRK-52E cells significantly inhibited doxorubicin-induced apoptosis and the quantity of cleaved caspase-3. Endogenous prostacyclin (PGI_2) augmentation, which has been reported to protect NRK-52E cells from doxorubicin-induced apoptosis, induced the translocation and activation of PPAR-α. The transformation of PPAR-α short interfering RNA was applied to silence the PPAR-α gene, which abolished the protective effect of PGI_2 augmentation in doxorubicin-treated cells. To confirm the protective role of PPAR-α in vivo, PPAR-α activator docosahexaenoic acid (DHA) was administered to doxorubicin-treated mice, and it has been shown to significantly reduce the doxorubicin-induced apoptotic cells in renal cortex. However, this protective effect of DHA did not exist in PPAR-α-deficient mice. In NRK-52E cells, the overexpression of PPAR-α elevated the activity of catalase and superoxide dismutase and inhibited doxorubicin-induced reactive oxygen species (ROS). PPAR-α overexpression also inhibited the doxorubicin-induced activity of nuclear factor-κB (NF-κB), which was associated with the interaction between PPAR-α and NF-κB p65 subunit as revealed in immunoprecipitation assays. Therefore, PPAR-α is capable of inhibiting doxorubicin-induced ROS and NF-κB activity and protecting NRK-52E cells from doxorubicin-induced apoptosis.

Doxorubicin (Adriamycin) is the antitumor anthracycline antibiotic of choice for the treatment of many solid malignancies and lymphomas. Rats treated with doxorubicin develop heart failure and a self-perpetuating glomerular nephropathy. Even in the absence of continued doxorubicin exposure, the glomerular damage progresses, and tubular lesions are observed (Bertani et al., 1982; Scholey et al., 1989). A long-term study of this pathological change in rats demonstrated severe renal damage with characteristic features of chronic progressive renal diseases in humans (Okuda et al., 1986). Doxorubicin-induced nephrosis in rats, therefore, is a common experimental model used for pharmacological studies of human chronic renal diseases. Induction of apoptosis is an important cytotoxic mechanism of doxorubicin (Müller et al., 1998). The apoptosis of renal tubular cells has been reported in doxorubicin-treated rats (Zhang et al., 1996) and is believed to be a key feature of tubular atrophy, which is a hallmark of chronic renal diseases (Schelling et al., 1998; Khan et al., 1999).

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the superfamily of ligand-activated nuclear receptors (Issemann and Green, 1990).

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ABBREVIATIONS: PPAR, peroxisome proliferator-activated receptor; PGI_2, prostacyclin; DHA, docosahexaenoic acid; ROS, reactive oxygen species; NF-κB, nuclear factor-κB; siRNA, short interfering RNA; PG, prostaglandin; COX, cyclooxygenase; PGIS, prostacyclin synthase; DMEM, Dulbecco's modified Eagle's medium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PCR, polymerase chain reaction; HPGK, human phosphoglycerate kinase; MCD, malonyl-CoA decarboxylase; β-gal, β-galactosidase; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; DAPI, 4′-6-diamidino-2-phenyindole; TNF-α, tumor necrosis factor-α; ELISA, enzyme-linked immunosorbent assay; DCF, 2′7′-dichlorofluorescein; EMSA, electrophoretic mobility shift assay; m.o.i., multiplicity of infection.

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PPAR-α is a target for various long-chain fatty acids (Lin et al., 1999) and is found predominantly in tissues that exhibit high catabolic rates of fatty acids, such as liver, heart, kidney, and muscle (Kliewer et al., 1994). In the renal proximal tubule, PPAR-α heterodimerizes with the retinoic X receptor and regulates gene transcription by binding to PPAR response elements located in the regulatory regions of target genes involved in lipid metabolism (Kliewer et al., 1994; Yang et al., 1999; Smirnov, 2002). Besides lipid metabolism, PPAR-α also exerts anti-inflammatory activities by transrepressing inflammatory signaling pathways (Duval et al., 2002). Recently, the activation of PPAR-α by fibrate treatment has been found to inhibit cisplatin-mediated renal tubular injury by preventing the activation of various cellular mechanisms that lead to proximal tubule cell death (Nagothu et al., 2005). These data imply the therapeutic potential of PPAR-α for renal tubule damage.

PPAR-α is also a potential nuclear receptor for prostacyclin (PGI_2). PGI_2, one of the major prostaglandins (PGs), is originated from arachidonic acid by the cyclooxygenase (COX) system coupled to the action of PGI_2 synthase (PGIS) (Vane and Botting, 1995). The classic signaling pathway of PGI_2 uses a G protein-coupled cell surface receptor termed IP (Lim and Dey, 2002). Recent reports show that PPARs are potential nuclear receptors for PGs (Lim and Dey, 2002). For example, cPGI and iloprost, the agonists of PGI_2, can effectively induce the activity of PPAR-α and PPAR-δ (Forman et al., 1997). It is interesting that treatment of cells with extracellular PGI_2 did not activate both PPARs, possibly because the chemical instability of this PG precluded it to reach the nuclear target. We have found that selectively augmented PGI_2 production protects rat renal tubular cells from the apoptosis induced by doxorubicin (Chen et al., 2006). In our study system, selective intracellular PGI_2 augmentation is able to be obtained with adenovirus-mediated transfer of genes for COX-1 and PGIS (Chen et al., 2006). There is still a chance for endogenous PGI_2 to interact with and activate PPAR-α. The activation of PPAR-α may be important for PGI_2 protection function on doxorubicin-induced apoptosis in renal tubular cells.

In this study, besides the protective effect of PPAR-α on doxorubicin-induced apoptosis and its detailed mechanism in rat renal tubular cells and animal models, we also intended to evaluate the essential role of PPAR-α in the protective mechanism of PGI_2 using the adenovirus-mediated bicistronic COX-1/PGIS transfection.

Materials and Methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum, and tissue culture reagents were from Invitrogen Corporation (Carlsbad, CA). All other chemicals of reagent grade were obtained from Sigma (St. Louis, MO). Antibodies used in this research were purchased from LabFrontier Co. Ltd. (Seoul, Korea; anti-GAPDH), Cell Signaling Technology, Inc. (Danvers, MA; anti-Caspase-3, anti-NF-κB p65), and Santa Cruz Biotechnology (Santa Cruz, CA; anti-PPAR-α, anti-PPAR-δ, and IP receptor neutralizing antibody).

Cell Culture. We purchased rat proximal renal tubular cells (NRK-52E) from Bioresource Collection and Research Center (Hsinchu, Taiwan), and cultured in DMEM culture medium supplemented with antibiotic/antifungal solution and 10% fetal calf serum. They were grown until the monolayer became confluent. The medium for the cultured cells was then changed to the serum-free medium, and the cells were incubated overnight before the experiment.

RNA Extraction and the Real-Time PCR Analysis. Total RNA was extracted from NRK-52E cells using the TRIzol method according to the protocol recommended by the manufacturer (Invitrogen). After treatment at 37°C for 30 min with 20 to 50 U of RNase-free DNase I (Invitrogen), oligo(dT) primers (Invitrogen) were used to synthesize single-stranded complementary DNA. PPAR-α mRNAs were quantified using SYBR Green Master Mix (QIAGEN GmbH, Hilden, Germany) with specific primers in a GeneAmp ABI prism 7000 (Applied Biosystems, Foster City, CA). The primers of PPAR-α were as follows: anti-sense, 5′-CCA CCA TCG CGA CCA GAT-3′; sense, 5′-GAC GTG CTT CCT GCT TCA TAG A-3′. Calibrated and nontemplate controls were included in each assay. Each sample was run in triplicate. SYBR Green dye intensity was analyzed using the ABI prism 7000 SDS software (Applied Biosystems). All results were normalized to GAPDH. All quantifications were performed in triplicate samples for three separate experiments.

Preparation of Replication-Defective Recombinant Adenoviral Vectors. We constructed the replication-defective recombinant adenoviral vector with two separate human phosphoglycerate kinase (HPGK) promoters to drive COX-1 and PGIS (Ad-COX-1/PGIS), and an HPGK alone to serve as control (Ad-HPGK) as described previously (Lin et al., 2002; Chen et al., 2006).

Western Blot Analysis. A total of 30 μg of NRK-52E lysate proteins were applied to each lane and analyzed by Western blotting. Peroxidase-conjugated anti-rabbit or anti-goat IgG (1:5000 dilution) was used as the second antibody to detect PPAR-α, PPAR-δ, caspase-3, NF-κB p65, and GAPDH bands by enhanced chemiluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The preparation of nuclear protein extracts has been described previously (Chen et al., 2006). The data of cleaved caspase-3 were also analyzed with Image-Pro software (Media Cybernetics Inc., Bethesda, MD).

PPAR-α Activity Assay. The rat malonyl-CoA decarboxylase (MCD)/Luc reporter genes were constructed according to the procedure described by Lee et al. (2004). 5′-Flanking region of rat MCD spanning −2240/+158 bp was cloned into pGL3 Basic vector. PPAR-α transcriptional activity was measured using the pGL3-MCD-Luc reporter construct. Ad-COX-1/PGIS or Ad-HPGK transfected NRK-52E cells grown in six-well plates were transiently transfected with 4 μg of reporter construct and 0.5 μg of RSV-β-galactosidase (β-gal) plasmid as an internal control using the Lipofectamine reagent (Invitrogen) for 6 h under serum-free conditions. After 6 h, medium was replaced with DMEM supplemented with 5% fetal calf serum. Luciferase activity of cell lysates was then determined using a luciferase assay system (Promega, Madison, WI) according to the protocol recommended by the manufacturer. Transfection efficiency was normalized to β-gal activity and expressed as fold induction of vehicle-treated control cells.

PPAR-α cDNA and Short Interfering RNA Transfection. The PPAR-α cDNA in pcDNA3.1 was kindly gifted from Dr. S. K. Shyue (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan), PPAR-α short interfering RNA (siRNA) and PPAR-δ siRNA were purchased from Santa Cruz Biotechnology. Cells with or without Ad-COX-1/PGIS transfection were grown to 70% confluence, and PPAR-α cDNA construct, PPAR siRNA, and their respective control oligonucleotides were transfected using the Lipofectamine reagent according to the manufacturer's instructions. The final concentration of PPAR-α cDNA construct or PPAR siRNA for transfection was 5 and 100 nM, respectively. Transfected cells were washed and resuspended in new culture media for an additional 24 h for doxorubicin treatment and Western blot assays.

Terminal Deoxynucleotidyl Transferase dUTP Nick-End Labeling Stain. Doxorubicin-mediated apoptosis in NRK-52E cells was detected by enzymatic labeling of DNA strand breaks using terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) stain. As described previously (Chen et al., 2006), TUNEL stain with a Cell Death Detection kit (Roche, Mannheim, Germany) was used. To reveal total nuclei, we stained the same slides with 4′-6-diamidino-2-phenyin-

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dole (DAPI) (1 μg/ml) in phosphate-buffered saline plus 0.5% 1,4-diazabicyclo[2,2,2]octane. In addition, we processed kidney slides with an ApopTag Fluorescein in situ apoptosis detection kit (Chemicon International, Temecula, CA) according to the manufacturer's instructions. TUNEL-stained kidney tissue slides were mounted with mounting solution containing DAPI and observed with fluorescent microscope.

Determination of Tumor Necrosis Factor-α. We cultured NRK-52E cells with or without transfection on 10-cm plates with 2 ml of medium in each plate and treated them with doxorubicin at 3 μM for 8 h. The cultured medium was collected and analyzed using the mouse tumor necrosis factor-α (TNF-α) ELISA kit according to instructions provided by the manufacturer (RayBiotech, Inc., Norcross, GA).

Animals and Treatments. The mouse strain 129S4/SvJae was kindly gifted from Dr. C.C. Chen (Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan). The PPAR-α-deficient mice (129S4/SvJae-*Ppara*^{tm1Gonz}/J) were purchased from JAX Mice and Services (The Jackson Laboratory, Bar Harbor, ME). Mice weighing 20 to 25 g and aged 8 weeks were used in this study. All animal experimentation complied with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experimental group animals ($n = 6$) for doxorubicin treatment received intraperitoneal injection with doxorubicin (30 μg/kg/day) for 7 days. The group animals ($n = 6$) for doxorubicin and docosahexaenoic acid (DHA) treatment received i.p. injection with DHA (200 mg/kg/day) 30 min before the doxorubicin treatment each time. Control group mice received the i.p. injection with 0.9% (w/v) saline for 7 days. Treated and control mice were killed 24 h after the last drug injection. Both kidneys were harvested by laparotomy, and the renal cortex tissue was snap-frozen in dry ice and stored at −80°C until in situ TUNEL assays.

The Detection of Intracellular Reactive Oxygen Species. The intracellular reactive oxygen species (ROS) in NRK-52E cells was determined by using 2′7′-dichlorofluorescein (DCF) staining as described in the previous study (Cheng et al., 2006). We also detected the activity of catalase and superoxide dismutase in NRK-52E cells by using Catalase Assay kit and Superoxide Dismutase Assay kit according to instructions provided by the manufacturer (Cayman Chemical Company, Ann Arbor, MI).

Electrophoretic Mobility Shift Assay. The activity of NF-κB in NRK-52E cells was determined using electrophoretic mobility shift assay (EMSA) as described in the previous study (Chen et al., 2006). In brief, cultured NRK-52E cells were suspended in ice-cold buffer containing 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride for 15 min, lysed by adding 10% Nonidet P-40, and then centrifuged at 5000*g* to obtain pellets of nuclei. The nuclear pellets were resuspended in cold buffer containing 20 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 0.4 mM NaCl, vigorously agitated, and then centrifuged. A double-stranded DNA containing a high-affinity sequence for NF-κB from the mouse κ-light chain enhancer was end-labeled with [^{32}P]ATP (Chen et al., 2006). Extracted nuclear proteins were incubated with ^{32}P-labeled DNA for 15 min at room temperature in binding buffer containing 1 μg of poly(dI-dC). The mixtures were electrophoresed on 5% nondenaturing polyacrylamide gels. Gels were dried and imaged by autoradiography.

Immunoprecipitation. Control and transformed cells were starved overnight in serum-free culture medium and treated with or without 3 μM doxorubicin for 24 h. The 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). PPAR-α was collected by using immunoprecipitation kit (Roche) with anti-PPAR-α antibodies (Santa Cruz Biotechnology) according to the manufacturer's instructions.

Statistical Analysis. Data were presented as the mean ± S.D., and groups were compared using the analysis of variance. The differences were considered significant if the p values were smaller than 0.05.

Results

The mRNA Level of PPAR-α in Doxorubicin-Treated NRK-52E Cells. To investigate the role played by PPAR-α in doxorubicin-induced apoptosis, we first monitored the mRNA level of PPAR-α in NRK-52E cells treated with or without doxorubicin. Using quantitative real-time PCR, we found that the PPAR-α mRNA was significantly reduced by more than 50% in doxorubicin-treated cells compared with that in control cells (Fig. 1). This result implies the inverse relationship between PPAR-α and doxorubicin-induced apoptosis. Although PGI_2 augmentation via adenovirus-mediated bicistronic COX-1/PGIS transfection has been found protecting NRK-52E cells from doxorubicin-apoptosis (Chen et al., 2006), it did not influence the PPAR-α mRNA level as shown in Fig. 1.

The Protective Function of PPAR-α on doxorubicin-Induced Apoptosis in NRK-52E Cells. To probe into the function of PPAR-α on the doxorubicin-induced apoptosis, PPAR-α cDNA construct was transfected into NRK-52E cells to overexpress PPAR-α protein. Compared with controls or vector transformants, PPAR-α protein level was significantly increased in PPAR-α construct-transfected cells (Fig. 2A). During doxorubicin treatment, the quantity of cleaved caspase-3, an important apoptosis signal, was significantly reduced by PPAR-α overexpression (Fig. 2B). In TUNEL assays, PPAR-α overexpression significantly reduced doxorubicin-induced apoptosis by over 50% compared with doxorubicin-treated controls or vector transformants (Fig. 2C).

The Induction of PPAR-α Activation by Ad-COX-1/PGIS Transfection. Because PGI_2 is supposed to be a potential ligand of PPAR-α, it is possible that Ad-COX-1/PGIS-mediated endogenous PGI_2 augmentation causes PPAR-α translocation and activation. To evaluate this possibility, the PPAR-α protein in cytosol and nuclei was monitored using Western blot assays. As shown in Fig. 3A, the quantity of PPAR-α protein in the cytosol of Ad-COX-1/PGIS-transfected cells was similar to that in Ad-HPGK-transfected cells. However, the nuclear PPAR-α protein in NRK-52E cells was significantly increased by the 12-h transfection of Ad-COX-

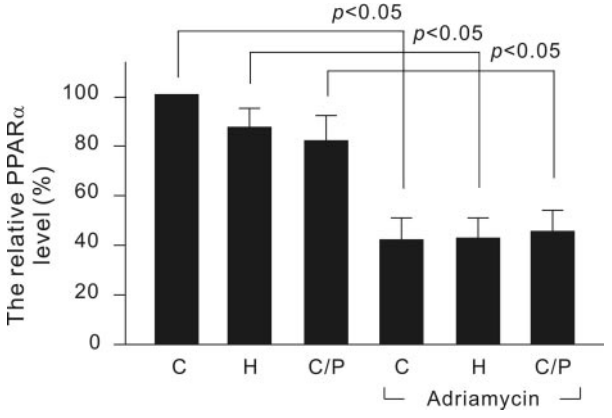

Fig. 1. The relative mRNA levels of PPAR-α in NRK-52E cells. NRK-52E cells were treated with or without 3 μM doxorubicin for 8 h. Doxorubicin decreased PPAR-α mRNA levels more than 50% compared with controls by real-time reverse transcriptase-PCR. NRK-52E cells were also transfected with 40 m.o.i. of Ad-COX-1/PGIS or Ad-HPGK for 2 days and then treated with or without doxorubicin. The transfection did not influence the doxorubicin-induced decrease of PPAR-α mRNA levels. All results were normalized to GAPDH mRNA. A bar is mean ± S.D. of three experiments.

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1/PGIS and ascended along the transfection time. This result reveals that PGI_2 generated by Ad-COX-1/PGIS transfection induces the translocation of PPAR-α. PPAR-α activity was also measured as luciferase activity of acetyl-CoA and was increased by more than 7 times when NRK-52E cells were transfected with 40 m.o.i. of Ad-COX-1/PGIS for 2 days compared with Ad-HPGK-transfected cells, as shown in Fig. 3B.

The Major Role of PPAR-α in the Protective Function of PGI_2. Gene knockdown with siRNA was next applied to analyze the protective function of PPAR-α on doxorubicin-induced apoptosis. NRK-52E cells were transfected with the PPAR-α siRNA, and the PPAR-α protein level was obviously reduced (Fig. 4A). The doxorubicin-induced cleaved caspase-3 generation was not affected by PPAR-α siRNA transfection (Fig. 4B). We further transfected Ad-COX-1/PGIS into the cells and evaluated the influence of siRNA transfection on the protective effect of Ad-COX-1/PGIS. As shown in Fig. 4B, the inhibition effect of Ad-COX-2/PGIS transfection on the cleaved caspase-3 generation was blocked by PPAR-α siRNA transfection. In addition, the excretion of TNF-α, an important mediator for death receptor-mediated apoptosis, was elevated by doxorubicin (Fig. 4C). This doxorubicin-induced elevation was enhanced by PPAR-α siRNA transfection but significantly suppressed in PPAR-α-overexpressed cells.

The signaling pathway of PGI_2 was also involved with IP receptor and PPAR-δ (Lim and Dey, 2002). To identify the crucial role of PPAR-α in the protective function of PGI_2, the neutralizing antibody for IP receptor and PPAR-δ siRNA were applied in Ad-COX-1/PGIS-transfected cells. The PPAR-δ protein level was obviously reduced as shown in Fig. 5A. The doxorubicin-induced apoptotic cells were increased approximately 6% by PPAR-α siRNA transfection and was not affected by IP receptor neutralizing and PPAR-δ siRNA transfection (Fig. 5B). The inhibition effect of Ad-COX-1/PGIS transfection on the doxorubicin-induced apoptosis was reduced approximately 80% by PPAR-α siRNA transfection and approximately 40% by IP receptor neutralizing and not affected significantly by PPAR-δ siRNA transfection. This result reveals the major role of PPAR-α in PGI_2 protection function on doxorubicin-induced apoptosis in NRK-52E cells.

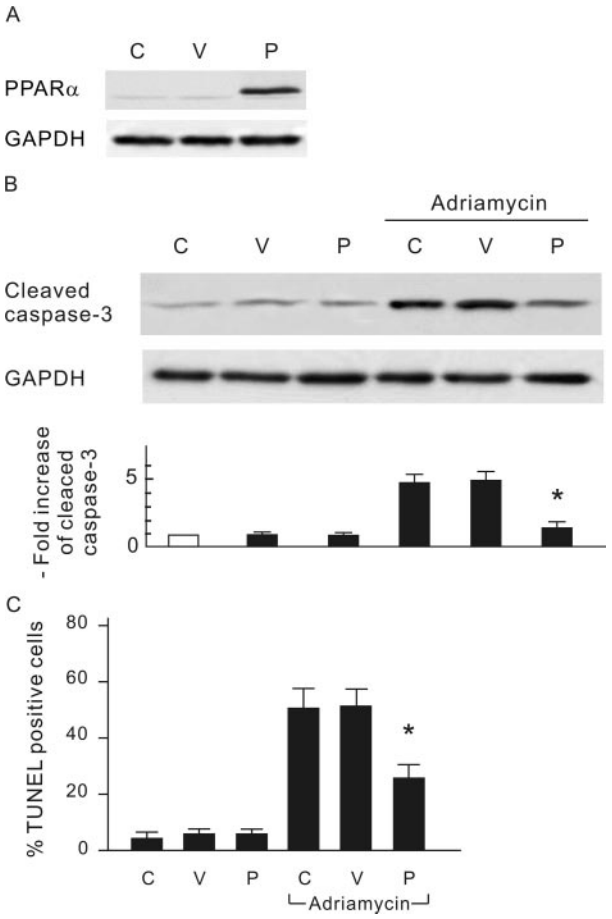

Fig. 2. Effects of PPAR-α overexpression on doxorubicin-induced apoptotic signaling. A, the PPAR-α expression level in NRK-52E cells with PPAR-α cDNA construct transformation. The level of PPAR-α protein in each sample was determined by Western blot analysis with anti-PPAR-α antibodies. B, protein levels of cleaved caspase-3 in transformed NRK-52E cells treated with doxorubicin. Transformed NRK-52E cells were treated with 3 μM doxorubicin for 24 h and then analyzed using Western blotting with the specific antibody against cleaved caspase-3. Data are also presented as the difference relative to the data in untreated control groups. The results are shown as the mean ± S.D. ($n = 5$). *, $p < 0.05$ versus the results in blank vector transformants with doxorubicin treatment. C, the protective effect of PPAR-α overexpression against the doxorubicin-induced apoptosis in NRK-52E cells. Transformed NRK-52E cells were treated with 3 μM doxorubicin for 24 h and then stained with TUNEL assay. Results are the mean ± S.D. ($n = 6$). *, $p < 0.05$ compared with the blank vector transformants with doxorubicin treatment; C, untransformed controls; V, blank vector transformants; P, PPAR-α cDNA construct transformants.

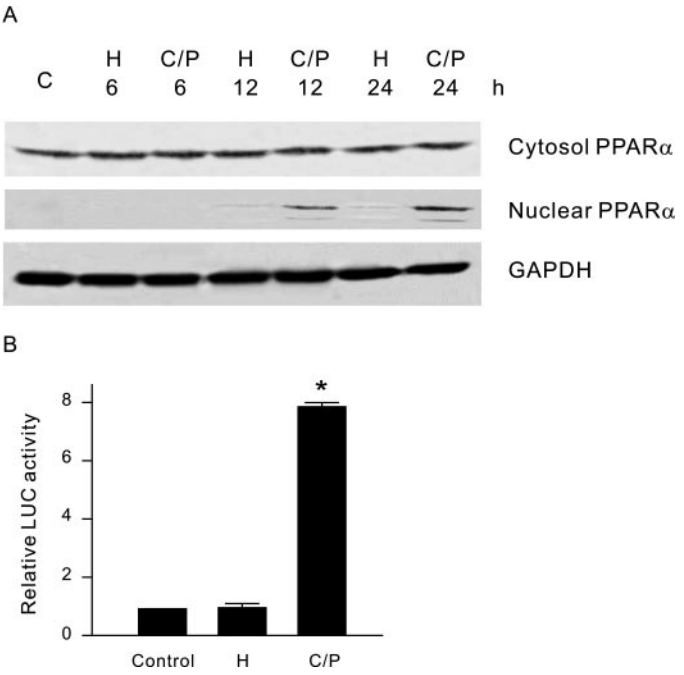

Fig. 3. Effects of Ad-COX-1/PGIS transfection on PPAR-α activation in NRK-52E cells. A, the effect of Ad-COX-1/PGIS transfection on PPAR-α translocation. NRK-52E cells were transfected with Ad-COX-1/PGIS or Ad-HPGK at 40 m.o.i. for different periods. Cell nuclei in each sample were purified. Western blotting was carried out with the specific antibody against PPAR-α. GAPDH was detected as a loading control. C/P, Ad-COX-1/PGIS transfection; H, Ad-HPGK transfection; C, untreated control. B, the luciferase activity assay of NRK-52E cells transfected with the full-length rat MCD luciferase reporter. The cells were cotransfected with β-galactosidase expression vector (as an internal control) and then transfected Ad-HPGK or Ad-COX-1/PGIS as indicated. After transfection, the cells were incubated for 48 h. Transfection efficiency was normalized to β-gal activity and expressed as fold induction of vehicle-treated control cells. Results are the mean ± S.D. ($n = 3$). *, $p < 0.05$ compared with vehicle-treated control cells.

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The Protective Effect of PPAR-α on Doxorubicin-Induced Apoptosis in Vivo. The protective effect of PPAR-α on doxorubicin-induced apoptosis was further proven with docosahexaenoic acid in vivo. DHA, a long-chain fatty acid, is known as one of the PPAR-α activators (Lin et al., 1999). Mice were treated with doxorubicin (30 μg/kg/day) or saline as controls; the experimental groups were treated with DHA (200 mg/kg/day) in addition. At the end of the treatment period (7 days), we examined kidney sections after the detection of DNA fragmentation using in situ TUNEL assay to analyze the doxorubicin-induced apoptosis. As shown in Fig. 6, the scattered and bright nuclei stained by TUNEL staining could easily be detected over the entire cortex from doxorubicin-treated animals, yet they were rarely detected in the specimens of the controls and doxorubicin-DHA-treated animals (Fig. 6A). Most of the TUNEL-labeled nuclei were seen in proximal tubule epithelium. This result reveals that the PPAR-α activator DHA inhibits the doxorubicin-induced cell apoptosis in the renal cortex in mice. In PPAR-α-deficient mice, however, TUNEL-labeled nuclei detected in the cortex from doxorubicin-treated animals were much more than that in normal mice (Fig. 6, B and C). The doxorubicin-induced apoptosis was also rarely reduced by DHA treatment in PPAR-α-deficient mice.

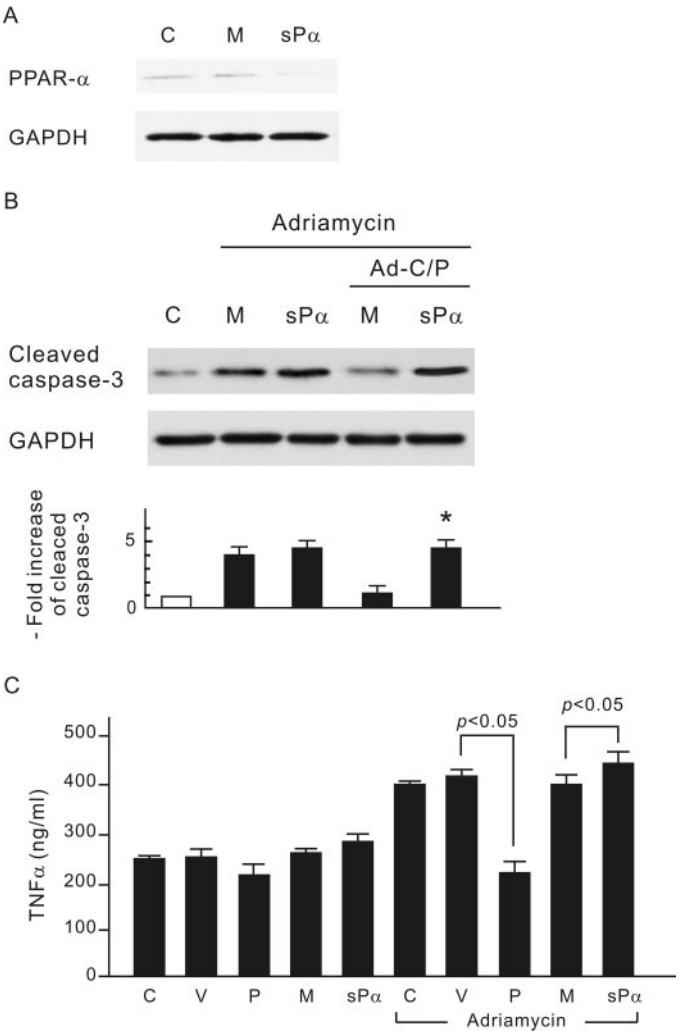

Fig. 4. Effects of PPAR-α knockdown on the protective effect of Ad-COX-1/PGIS transfection in NRK-52E cells. A, the effect of PPAR-α siRNA transfection on PPAR-α protein expression level. NRK-52E cells were either transfected with control siRNA as mock controls (M) or transfected with PPAR-α siRNA (sPα) to obtain PPAR-α knockdown cells. Western blotting was carried out with the specific antibody against PPAR-α. GAPDH was detected as a loading control. B, protein levels of cleaved caspase-3 in siRNA-transfected NRK-52E cells treated with doxorubicin. NRK-52E cells transfected with PPAR-α siRNA or mock control siRNA were transfected with or without Ad-COX-1/PGIS (Ad-C/P) at 40 m.o.i. for 2 days and treated with 3 μM doxorubicin for 24 h. Each sample was analyzed using Western blotting with the specific antibody against cleaved caspase-3. Data are also presented as the difference relative to the data in untreated control groups. The results are shown as the mean ± S.D. ($n = 5$). *, $p < 0.05$ versus the results in mock controls with Ad-COX-1/PGIS transfection and doxorubicin treatment. C, the influence of PPAR-α on doxorubicin-induced TNF-α excretion in NRK-52E cells. Transfected and control cells had been cultured on 10-cm plates with 2 ml of medium in each plate and treated with 3 μM doxorubicin for 8 h. The cultured medium was collected and analyzed using the ELISA kit for TNF-α. Data are expressed as the mean ± S.D. ($n = 3$). The TNF-α excretion was significantly reduced ($p < 0.05$) in PPARα-transformed cells. C, untransfected control; P, PPAR-α cDNA transformants; V, blank vector transformants.

The Influence of PPAR-α on the Doxorubicin-Induced Reactive Oxygen Species Concentration. ROS derived from redox activation of doxorubicin have been proposed to be responsible for doxorubicin-induced cytotoxicity (Singal et al., 2000). To examine the influence of PPAR-α overexpression on doxorubicin-induced ROS, NRK-52E cells were transfected with PPAR-α construct or the blank vector in the absence or presence of doxorubicin. Doxorubicin-induced increases of intracellular ROS were revealed by fluorescent intensities of DCF (Fig. 7A). PPAR-α overexpression

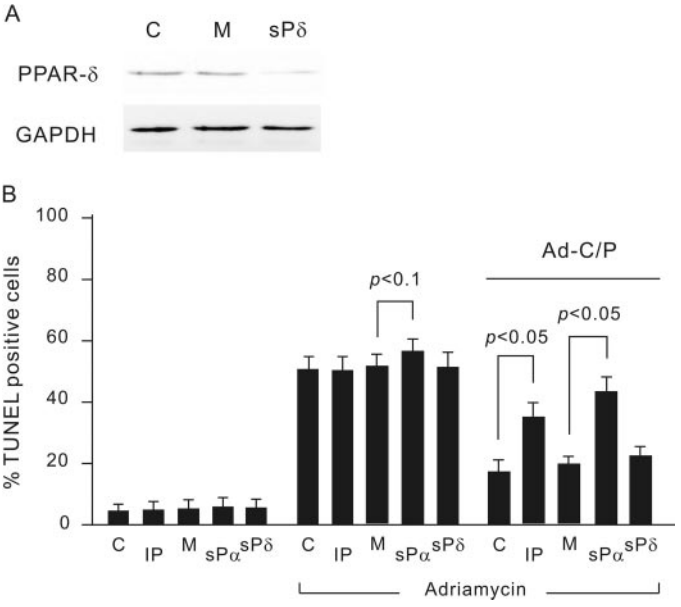

Fig. 5. Effects of PPAR-α and PPAR-δ knockdown and IP receptor neutralizing on the antiapoptotic effect of Ad-COX-1/PGIS transfection in NRK-52E cells. A, the effect of PPAR-δ siRNA transfection on PPAR-δ protein expression level. NRK-52E cells were either transfected with control siRNA as mock controls (M) or transfected with PPAR-δ siRNA (sPδ) to obtain PPAR-δ knockdown cells. Western blotting was carried out with the specific antibody against PPAR-δ. GAPDH was detected as a loading control. B, the influence of PPAR-α and PPAR-δ siRNA and IP receptor neutralizing antibody on the protective effect of Ad-COX-1/PGIS in NRK-52E cells. NRK-52E cells transfected with PPAR-α siRNA (sPα), PPAR-δ (sPδ) siRNA or mock control siRNA (M) were transfected with or without Ad-COX-1/PGIS (Ad-C/P) at 40 m.o.i. for 2 days and treated with 3 μM doxorubicin for 24 h. For blocking IP receptor function, cells were pretreated with IP receptor-neutralizing antibody (1 μg/ml) for 30 min before incubation with doxorubicin. The apoptosis level of each sample was revealed with TUNEL assays. Results are the mean ± S.D. ($n = 6$). C, untransfected control; IP, IP receptor-neutralizing cells.

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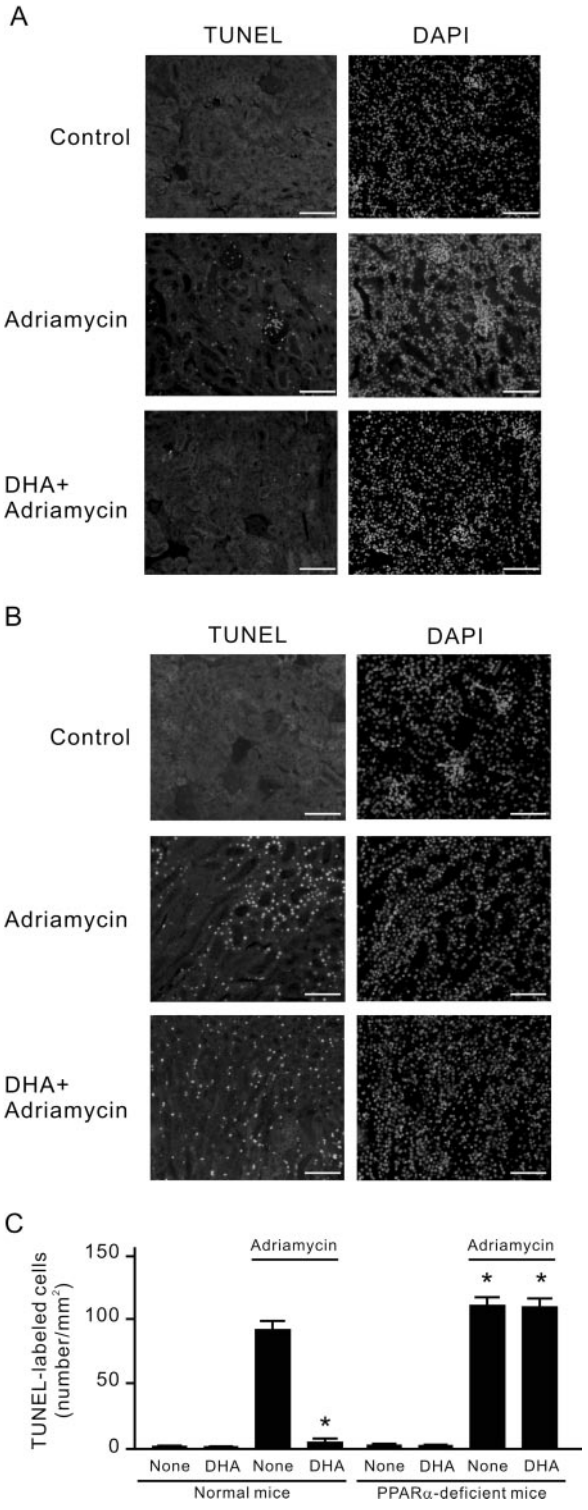

Fig. 6. The protective effect of DHA against doxorubicin-induced apoptosis in kidneys in vivo. Mice were injected with saline, doxorubicin, or doxorubicin and DHA. Apoptotic cells in kidneys of experimental animals were detected using in vivo TUNEL staining. A, apoptotic cells in the renal cortex of the doxorubicin-treated normal mice. B, apoptotic cells in the renal cortex of the PPAR-α-deficient mice with doxorubicin treatment. TUNEL-labeled nuclei were revealed as bright spots in cortex sections from untreated and treated mice. The identical fields stained for TUNEL were also stained using DAPI to reveal the positions of cell nuclei as bright spots. Scale bar, 100 μm. C, the numbers of TUNEL-labeled cells per millimeter-squared cortex area in each sample were compiled and demonstrated. Results are expressed as the mean ± S.D. ($n = 6$). *, $p < 0.05$ compared with the normal mice treated with doxorubicin alone.

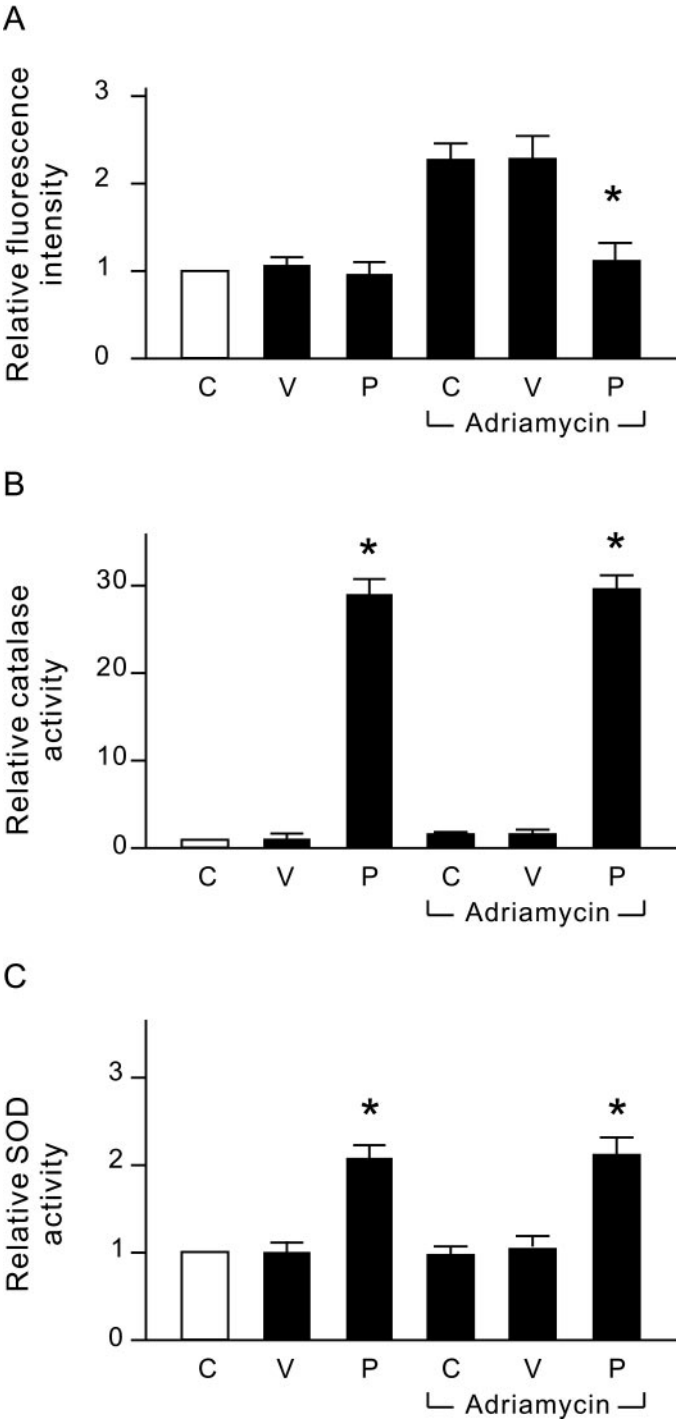

Fig. 7. The influence of PPAR-α overexpression on doxorubicin-induced ROS in NRK-52E cells. A, the inhibition effect of PPAR-α overexpression on doxorubicin-induced ROS. NRK-52E cells were transformed with PPAR-α construct or the blank vector and then treated with 3 μM doxorubicin for 6 h. Doxorubicin-induced increases of intracellular ROS were revealed by fluorescent intensities of DCF. Fluorescence intensities of cells are shown as the relative intensity of experimental groups is compared with untreated control cells. Results are the mean ± S.D. ($n = 6$). *, $p < 0.05$ compared with the blank vector transformants with doxorubicin treatment. B and C, the influence of PPAR-α overexpression on the activity of SOD and catalase in NRK-52E cells. After doxorubicin treatment for 24 h, the transformed cells were harvested, and the activity of SOD and catalase was analyzed using ELISA assay. Results are the mean ± S.D. ($n = 6$). *, $p < 0.05$ compared with the blank vector transformants group with doxorubicin treatment; C, untransfected controls; V, blank vector transformants; P, PPAR-α cDNA transformants.

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significantly inhibited doxorubicin-induced ROS concentration under the treatment of 3 μM doxorubicin for 6 h (Fig. 7A). In addition, the activity of catalase and superoxide dismutase was also increased in PPAR-α-overexpressed NRK-52E cells as revealed by ELISA assays (Fig. 7, B and C).

The Inhibition Effect of PPAR-α on NF-κB Activation Caused by Doxorubicin in NRK-52E Cells. NF-κB is known as an important transcriptional factor and activated by doxorubicin treatment in NRK-52E cells (Chen et al., 2006). The influence of PPAR-α on doxorubicin-induced NF-κB activation was further investigated by using EMSA with nuclear extracts. NRK-52E cells were transfected with PPAR-α construct or the blank vector and then incubated with 3 μM doxorubicin for 12 h. As shown in Fig. 8A, the DNA binding activity of NF-κB was increased by doxorubicin treatment in control cells or vector transformants, and this increase was reduced markedly by PPAR-α overexpression. The reduction of doxorubicin-induced NF-κB activity caused by PPAR-α may result from the inhibition of PPAR-α on doxorubicin-induced ROS because the ROS serves as a stimulus for NF-κB activation (Bonizzi et al., 2000). However, it has been reported that a physical interaction exists between PPAR-α and the p65 subunit of NF-κB and interferes with the activity of both transcription factors (Delerive et al., 1999, 2000). Nuclear extracts isolated from NRK-52E cells were immunoprecipitated using anti-PPAR-α antibody coupled to protein G-agarose beads. Immunoprecipitates were then subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with anti-p65 antibody. Data shown in Fig. 8B demonstrate that the quantity of coimmunoprecipitated NF-κB p65 with doxorubicin treatment was similar to that without doxorubicin in both control cells and vector transformants but was significantly increased by doxorubicin treatment in PPAR-α-overexpressed cells. We also found that p65 translocated into nuclei was reduced in PPAR-α-overexpressed cells with doxorubicin treatment (Fig. 8C).

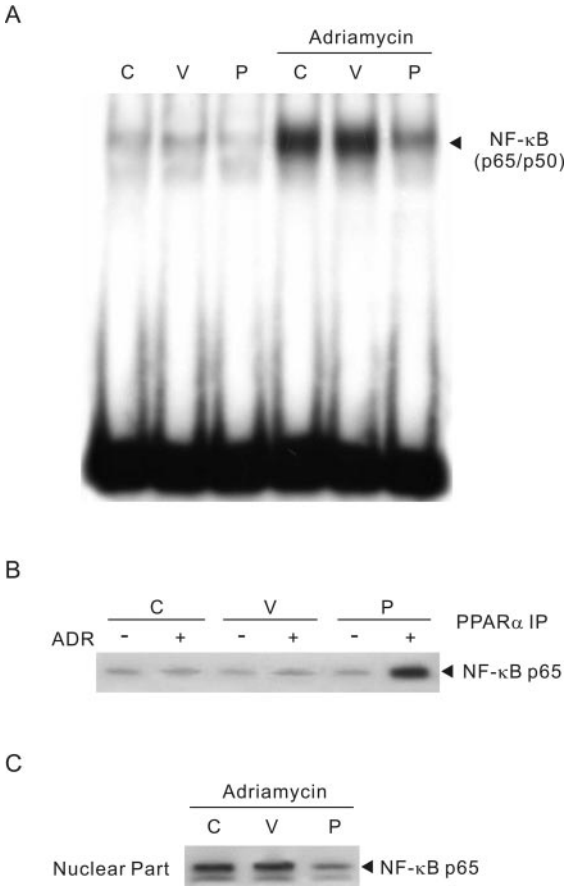

Fig. 8. The influence of PPAR-α on doxorubicin-induced NF-κB signaling pathways in NRK-52E cells. A, the DNA binding activity of NF-κB in doxorubicin-treated NRK-52E cells. The nuclear proteins were extracted and analyzed by EMSA with NF-κB binding nucleotides. B, the interaction between PPAR-α and NF-κB p65. NRK-52E cells were transformed with PPAR-α construct or the blank vector and then treated with 3 μM doxorubicin for 8 h. PPAR-α in each sample was immunoprecipitated with an anti-PPAR-α antibody. Coimmunoprecipitated NF-κB p65 was detected by using Western blotting with an anti-p65 antibody and was significantly elevated in PPAR-α-transfected cells with doxorubicin (ADR) treatment. C, the levels of nuclear NF-κB p65 in doxorubicin-treated cells. After doxorubicin treatment, nuclear protein was also extracted and detected by Western blotting assay with the specific antibody against NF-κB p65. The nuclear NF-κB p65 was significantly reduced in PPAR-α-transfected cells. C, untransfected controls; V, blank vector transformants; P, PPAR-α cDNA transformants.

Discussion

In our results, doxorubicin-induced apoptosis was reduced in PPAR-α-overexpressed NRK-52E cells (Fig. 2) and even more serious in renal tubular cells in PPAR-α-deficient mice compared with that in normal mice (Fig. 6). These data indicate that PPAR-α is associated with the inhibition of doxorubicin-induced apoptosis in renal tubular cells. Because some PPAR-α ligands, such as certain fatty acids, originally are present in cytosol, the overexpression of PPAR-α in NRK-52E cells is supposed to increase the active form of PPAR-α. Therefore, the protective function of PPAR-α on doxorubicin-induced apoptosis may result from the activation of PPAR-α. In our previous study, the selective augmentation of PGI_2 caused by Ad-COX-1/PGIS transfection was found to protect NRK-52E cells from doxorubicin-induced apoptosis (Chen et al., 2006). COXs and PGIS mRNA were present in the whole kidney in vivo, but PGIS was not in any nephron segment. However, PGI_2 synthesis along the nephron is quite significant despite the lack of PGIS detection throughout the nephron (Lim and Dey, 2002). PGI_2 was able to cause PPAR-α translocation and activation and lost the protective effect in PPAR-α siRNA-transfected cells (Figs. 4 and 5). These data reveal that the activation of PPAR-α is essential for the protective function of PGI_2 on doxorubicin-induced apoptosis in renal tubular cells.

The detailed mechanism regarding PPAR-α activation protecting renal tubular cells from doxorubicin-induced apoptosis is not clear yet. We found the overexpression of PPAR-α elevated the activity of catalase and superoxide dismutase and reduced doxorubicin-induced ROS concentration in NRK-52E cells (Fig. 7). This inhibition of ROS concentration is supposed to be a crucial mechanism for the protective function of PPAR-α because ROS are mediators in doxorubicin-induced apoptotic injury (Singal et al., 2000). ROS also serve as a stimulus for NF-κB activation (Bonizzi et al., 2000). In our study system, the activation of NF-κB was always associated with doxorubicin-induced apoptosis and was reduced by the overexpression of PPAR-α (Fig. 8A). This reduction of NF-κB activity may result from the inhibition of PPAR-α on doxorubicin-induced ROS. However, there is another possibility about the reduction of NF-κB activity caused by PPAR-α. A physical interaction between PPAR-α and the

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p65 subunit of NF-κB existed in doxorubicin-treated NRK-52E cells (Fig. 8B). In PPAR-α-expressed cells, a lot of NF-κB p65 bound to PPAR-α under doxorubicin treatment. This interaction has been reported to interfere with the activity of PPAR-α and NF-κB (Delerive et al., 1999, 2000). But the expression level of PPAR-α was significantly reduced by doxorubicin treatment in control cells (Fig. 1). This will cause less PPAR-α to interact and interfere with activated NF-κB in doxorubicin-treated NRK-52E cells. In fact, we also found the quantity of nuclear p65 in doxorubicin-treated control cells was obviously higher than that of PPAR-α-overexpressed cells with doxorubicin treatment (Fig. 8C). Therefore, the interaction between PPAR-α and NF-κB p65 is another possible mechanism for the protective function of PPAR-α in doxorubicin-treated renal tubular cells.

Although PGI_2 is supposed to be the ligand of PPARs, the connection between endogenous PGI_2 and the activation of PPAR-α is still not conclusive (Lim and Dey, 2002). The data in this study prove that Adv-COX-1/PGIS transfection induces the activation of PPAR-α in NRK-52E cells. Previous studies have shown that Adv-COX-1/PGIS transfection is effective in augmenting COX-1 and PGIS expression and increases PGI_2 without overproduction of other prostanoids in endothelial, cerebral, and rat renal tubular cells (Shyue et al., 2001; Lin et al., 2002; Chen et al., 2006). It is possible that this endogenous augmented PGI_2 is able to interact with and activate PPAR-α without the interference of other PGs. With PPAR-α signaling pathway, PGI_2 shows a protective effect on doxorubicin-induced apoptosis. However, several recent independent reports show that endogenously produced PGI_2 indeed activates PPAR-δ in vivo (Lim et al., 1997, 1999; Gupta et al., 2000) and then uses PPAR-δ to modulate apoptotic process as well (Hatae et al., 2001; Lim and Dey, 2002). These data indicate that PGI_2 plays a proapoptotic role, which is opposite to our finding. Actually, in our study, PPAR-δ knockdown did not affect the protective function of PGI_2 on doxorubicin-treated NRK-52E cells (Fig. 5). Besides different kinds of experimental cells, the data on the proapoptotic effect of PGI_2 are often obtained with COX-2/PGIS cotransfection but not Ad-COX-1/PGIS transfection applied in this study. The difference between the physiological effects of COX-1 and COX-2 may cause the experimental diversity of PGI_2. So far, it seems that PGI_2 possesses proapoptotic and antiapoptotic effects via the activation of PPAR-δ and PPAR-α, respectively. The mechanism of how both opposite effects of PGI_2 are regulated is still unknown.

In summary, our present study presents the protective effect of PPAR-α on doxorubicin-induced apoptosis in renal tubular cells. With doxorubicin treatment, the expression level of PPAR-α was significantly reduced in NRK-52E cells, which may cause apoptotic signals to overcome the protective effect of PPAR-α. When we overexpressed PPAR-α or transfected Ad-COX-1/PGIS to activate PPAR-α in cultured cells, doxorubicin-induced apoptosis was extensively inhibited. The PPAR-α ligand DHA also protected renal tubular cells from doxorubicin-induced apoptosis in mice but not in PPAR-α-deficient mice. Altogether, these findings suggest that the activation of PPAR-α can ameliorate the doxorubicin-induced injury in kidney.

References

Bertani T, Poggi A, Pozzoni R, Delaini F, Sacchi G, Thoua Y, Mecca G, Remuzzi G, and Donati MB (1982) Adriamycin-induced nephrotic syndrome in rats: sequence of pathologic events. *Lab Invest* **46:**16–23.

Bonizzi G, Piette J, Merville MP, and Bours V (2000) Cell type-specific role for reactive oxygen species in nuclear factor-kappaB activation by interleukin-1. *Biochem Pharmacol* **59:**7–11.

Chen CH, Lin H, Hsu YH, Sue YM, Cheng TH, Chan P, and Chen TH (2006) The protective effect of prostacyclin on Adriamycin-induced apoptosis in rat renal tubular cells. *Eur J Pharmacol* **529:**8–15.

Cheng CY, Sue YM, Chen CH, Hou CC, Chan P, Chu YL, Chen TH, and Hsu YH (2006) Tetramethylpyrazine attenuates Adriamycin-induced apoptotic injury in rat renal tubular cells NRK-52E. *Planta Med* **72:**888–893.

Delerive P, De Bosscher K, Besnard S, Vanden Berghe W, Peters JM, Gonzalez FJ, Fruchart JC, Tedgui A, Haegeman G, and Staels B (1999) Peroxisome proliferator-activated receptor alpha negatively regulates the vascular inflammatory gene response by negative cross-talk with transcription factors NF-κB and AP-1. *J Biol Chem* **274:**32048–32054.

Delerive P, Gervois P, Fruchart JC, and Staels B (2000) Induction of IκBα expression as a mechanism contributing to the anti-inflammatory activities of peroxisome proliferator-activated receptor-α activators. *J Biol Chem* **275:**36703–36707.

Duval C, Chinetti G, Trottein F, Fruchart JC, and Staels B (2002) The role of PPARs in atherosclerosis. *Trends Mol Med* **8:**422–430.

Forman BM, Chen J, and Evans RM (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. *Proc Natl Acad Sci U S A* **94:**4312–4317.

Gupta RA, Tan J, Krause WF, Geraci MW, Willson TM, Dey SK, and DuBois RN (2000) Prostacyclin-mediated activation of peroxisome proliferator-activated receptor δ in colorectal cancer. *Proc Natl Acad Sci U S A* **97:**13275–13280.

Hatae T, Wada M, Yokoyama C, Shimonishi M, and Tanabe T (2001) Prostacyclin-dependent apoptosis mediated by PPAR δ. *J Biol Chem* **276:**46260–46267.

Issemann I and Green S (1990) Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* **347:**645–650.

Khan S, Cleveland RP, Koch CJ, and Schelling JR (1999) Hypoxia induces renal tubular epithelial cell apoptosis in chronic renal disease. *Lab Invest* **79:**1089–1099.

Kliewer SA, Forman BM, Blumberg B, Ong ES, Borgmeyer U, Mangelsdorf DJ, Umesono K, and Evans RM (1994) Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci U S A* **91:**7355–7359.

Lee GY, Kim NH, Zhao ZS, Cha BS, and Kim YS (2004) Peroxisomal-proliferator-activated receptor alpha activates transcription of the rat hepatic malonyl-CoA decarboxylase gene: a key regulation of malonyl-CoA level. *Biochem J* **378:**983–990.

Lim H and Dey SK (2002) A novel pathway of prostacyclin signaling-hanging out with nuclear receptors. *Endocrinology* **143:**3207–3210.

Lim H, Gupta RA, Ma WG, Paria BC, Moller DE, Morrow JD, DuBois RN, Trzaskos JM, and Dey SK (1999) Cyclo-oxygenase-2-derived prostacyclin mediates embryo implantation in the mouse via PPARdelta. *Genes Dev* **13:**1561–1574.

Lim H, Paria BC, Das SK, Dinchuk JE, Langenbach R, Trzaskos JM, and Dey SK (1997) Multiple female reproductive failures in cyclooxygenase 2-deficient mice. *Cell* **91:**197–208.

Lin H, Lin TN, Cheung WM, Nian GM, Tseng PH, Chen SF, Chen JJ, Shyue SK, Liou JY, Wu CW, et al. (2002) Cyclooxygenase-1 and bicistronic cyclooxygenase-1/prostacyclin synthase gene transfer protect against ischemic cerebral infarction. *Circulation* **105:**1962–1969.

Lin Q, Ruuska SE, Shaw NS, Dong D, and Noy N (1999) Ligand selectivity of the peroxisome proliferator-activated receptor alpha. *Biochemistry* **38:**185–190.

Müller I, Niethammer D, and Bruchelt G (1998) Anthracycline-derived chemotherapeutics in apoptosis and free radical cytotoxicity. *Int J Mol Med* **1:**491–494.

Nagothu KK, Bhatt R, Kaushal GP, and Portilla D (2005) Fibrate prevents cisplatin-induced proximal tubule cell death. *Kidney Int* **68:**2680–2693.

Okuda S, Oh Y, Tsuruda H, Onoyama K, Fujimi S, and Fujishima M (1986) Adriamycin-induced nephropathy as a model of chronic progressive glomerular disease. *Kidney Int* **29:**502–510.

Schelling JR, Nkemere N, Kopp JB, and Cleveland RP (1998) Fas-dependent fratricidal apoptosis is a mechanism of tubular epithelial cell deletion in chronic renal failure. *Lab Invest* **78:**813–824.

Scholey JW, Miller PL, Rennke HG, and Meyer TW (1989) Effect of converting enzyme inhibition on the course of Adriamycin-induced nephropathy. *Kidney Int* **36:**816–822.

Shyue SK, Tsai MJ, Liou JY, Willerson JT, and Wu KK (2001) Selective augmentation of prostacyclin production by combined prostacyclin synthase and cyclooxygenase-1 gene transfer. *Circulation* **103:**2090–2095.

Singal PK, Li T, Kumar D, Danelisen I, and Iliskovic N (2000) Adriamycin-induced heart failure: mechanism and modulation. *Mol Cell Biochem* **207:**77–86.

Smirnov AN (2002) Nuclear receptors: nomenclature, ligands, mechanisms of their effects on gene expression. *Biochemistry (Mosc)* **67:**957–977.

Vane JR and Botting RM (1995) Pharmacodynamic profile of prostacyclin. *Am J Cardiol* **75:**3A–10A.

Yang T, Michele DE, Park J, Smart AM, Lin Z, Brosius FC 3rd, Schnermann JB, and Briggs JP (1999) Expression of peroxisomal proliferator-activated receptors and retinoid X receptors in the kidney. *Am J Physiol* **277:**F966–F973.

Zhang J, Clark JR Jr, Herman EH, and Ferrans VJ (1996) Doxorubicin-induced apoptosis in spontaneously hypertensive rats: differential effects in heart, kidney and intestine, and inhibition by ICRF-187. *J Mol Cell Cardiol* **28:**1931–1943.

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