Cytokine 60 (2012) 360–368

Contents lists available at SciVerse ScienceDirect

Cytokine

journal homepage: www.journals.elsevier.com/cytokine

Upregulation of cytochrome P450 2J3/11,12-epoxyeicosatrienoic acid inhibits apoptosis in neonatal rat cardiomyocytes by a caspase-dependent pathway

Hong-Xia Wang [a,1], Dong-Mei Zhang [b,1], Xiang-Jun Zeng [a], Jing Mu [c], Hui Yang [a], Ling-Qiao Lu [a], Li-Ke Zhang [a,*]

[a] Department of Pathophysiology, Capital Medical University, Beijing 100069, China
[b] Department of Pathology, Beijing Xuwu TCM Hospital, Beijing 100050, China
[c] Department of Pathology, Beijing Tuberculosis and Thoracic Tumor Research Institute, Beijing Chest Hospital, Beijing 101149, China

ARTICLE INFO

Article history:
Received 11 August 2011
Received in revised form 10 February 2012
Accepted 18 April 2012
Available online 18 June 2012

Keywords:
Cytochrome P450 2J3
11,12-EET
Postconditioning
Apoptosis
Ischemia

ABSTRACT

Short, nonlethal ischemic episodes administered to hearts directly after ischemic events (ischemic postconditioning, IPost) have an advantage over ischemic preconditioning (IPC). The endogenous cytochrome P450 2J3/11,12-epoxyeicosatrienoic acid (CYP2J3/11,12-EET) is upregulated by IPost, but not IPC, in the rat heart. The CYP epoxygenase inhibitor N-methylsulphonyl-6-(2-propargyloxyphenyl) hexanamide (MS-PPOH) reduces the cardioprotective effects of IPost, but not IPC. We proposed that upregulation of CYP2J3/11,12-EET during IPost induces cardioprotection by inhibiting cardiomyocyte apoptosis and that multiple apoptotic signals, including changes in mitochondrial membrane potential (MMP) and mitochondrial permeability transition pore (mPTP) opening, mitochondrial cytochrome c leakage, caspase-3 levels, and levels of protective kinases such as Bcl-2 and Bax, are involved in the process. Neonatal rat cardiomyocytes underwent 3-h hypoxia followed by 2-, 5-, or 6-h reoxygenation (H/R) or three cycles of 5-min reoxygenation followed by 5-min hypoxia before 90-min reoxygenation (HPost); or were transfected with pcDNA3.1-CYP2J3 for 48 h before H/R; or were treated with MS-PPOH for 10 min before HPost. For HPost alone, pcDNA3.1-CYP2J3 transfection attenuated cardiomyocyte apoptosis to 68.4% ($p < 0.05$) of that with H/R. pcDNA3.1-CYP2J3 transfection significantly decreased MMP and inhibited mPTP opening induced by H/R, reduced mitochondrial cytochrome c leakage, cleaved caspase-3 protein expression, and increased the ratio of Bcl-2 to Bax expression. MS-PPOH abolished this effect. Therefore, upregulation of CYP2J3/11,12-EET during HPost is involved in cardioprotection by inhibiting apoptosis via a caspase-dependent pathway, and the apoptosis-suppressive effect may have important clinical implications during HPost.

1. Introduction

Cycles of brief reperfusion and ischemia performed immediately at the onset of reperfusion following prolonged ischemia markedly limit reperfusion injury [1]. Zhao et al. named this cardioprotective phenomenon "ischemia postconditioning" (IPost), which is as powerful as ischemic preconditioning (IPC) [2,3]. IPost has been demonstrated in isolated perfused hearts [4] and in many *in vivo* models and species [5] such as dogs [2], rabbits [6], rats [4], pigs [7], and, recently, humans [8]. IPost has great advantages over IPC after myocardial infarction. For promising clinical application of IPost, further investigations are necessary to characterize its signal transduction pathway and identify the final end effector.

Our previous work showed that the endogenous cytochrome P450 2J3/11,12-epoxyeicosatrienoic (CYP2J3/11,12-EET) is upregulated by IPost, but not by IPC, in the rat heart. The CYP epoxygenase inhibitor MS-PPOH reduces the cardioprotective effects of IPost, but not IPC [9]. Therefore, upregulation of the CYP2J3/EET system during IPost may be a novel signaling mechanism protecting against ischemia/reperfusion (I/R) injury and may be distinct from IPC. However, the precise mechanisms mediating the protective effects of IPost by CYP2J3/EET remain unclear.

Recently, IPost was suggested to attenuate lethal reperfusion injury by opening mitochondrial K_{ATP} channels and reactive oxygen species signaling, which may lead to reduced mitochondrial calcium accumulation and inhibition of mitochondrial permeability transition pore (mPTP) opening [10–12]. Inhibition of mPTP opening is a critical end effector of the cardioprotective effects of IPost [11,13]. mPTP opening occurs during reperfusion and appears to be

* Corresponding author. Address: Department of Pathophysiology, Capital Medical University, 10 You An Men Wai Xi Tou Tiao, Beijing 100069, China. Tel.: +86 10 83911434; fax: +86 10 83911488.
E-mail address: zlk30@tom.com (L.-K. Zhang).
[1] Hong-Xia Wang and Dong-Mei Zhang contributed equally to this work.

1043-4666/$ - see front matter © 2012 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.cyto.2012.04.029

a key event in the death of cardiomyocytes after a sequence of I/R. Opening of the nonspecific mPTP in the inner mitochondrial membrane results in collapse of the membrane potential ($\Delta\Psi m$), uncoupling of the respiratory chain, and efflux of cytochrome c and other pro-apoptotic factors, which may lead to apoptosis or necrosis [14,15].

In this study, we investigated changes in endogenous CYP2J3/11,12-EET levels in rat neonatal cardiomyocytes treated with hypoxia preconditioning (HPC) and postconditioning (HPost) *in vitro* and observed the effect of upregulation of CYP2J3 or MS-PPOH inhibition on cardiomyocyte apoptosis. We also systematically investigated multiple downstream apoptotic signals, including changes in mitochondrial membrane potential (MMP) and mPTP opening, mitochondrial cytochrome c leakage, levels of cleaved caspase-3 expression, and levels of protective kinases such as Bcl-2 and Bax. Our data suggest that upregulation of CYP2J3/11,12-EET during HPost in neonatal rat cardiomyocytes involves cardioprotection by inhibition of apoptosis via a caspase-dependent pathway.

2. Materials and methods

2.1. Materials

Neonatal Wistar rats (1–3 days old) were provided by the Animal Department of Capital Medical University. Fetal calf serum (FCS), Dulbecco's modified Eagle's medium (DMEM), and streptomycin/penicillin were from Gibco BRL (Life Technologies, Paisley, UK). MS-PPOH was from Cayman Chemical (Ann Arbor, MI, USA), and solid-phase extraction (SPE) cartridges (Oasis HLB) were from Waters (Milford, MA, USA). Lipofectamine™ 2000 Transfection Reagent, JC-1, and calcein-AM were from Invitrogen (Carlsbad, CA, USA), and $CoCl_2$ was from Sigma (St. Louis, MO, USA). Trizol reagent and the reverse transcription system were from Promega (Madison, WI, USA). Sequences of oligonucleotide primers for RT-PCR analysis were synthesized by Sai Bai Sheng (Beijing). CYP2J3 polyclonal antibody was from Beijing Biosynthesis Biotechnology. Anti-GADPH antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-cytochrome c, -Bax, -BCL-2, and -cleaved caspase-3 antibodies were from Cell Signaling Technology (Danvers, MA, USA). Other chemicals and reagents were of analytical grade.

2.2. Isolation and culture of neonatal rat cardiomyocytes

The hearts of rats were excised, and the ventricular myocardium was cut into small pieces (~2 mm^3) in phosphate-buffered saline (PBS) buffer with trypsin (1.125 mg/ml), collagenase I (1 mg/ml), and collagenase II (0.5 mg/ml) and incubated on a shaker for 20 min at 37 °C and 100 rpm. Tissue pieces were allowed to settle, and the supernatant (containing myocytes) was collected and suspended, then centrifuged at 1000 rpm for 10 min. The cell pellet was resuspended and stored at 37 °C. The cells were then resuspended in DMEM with 20% FCS and 0.5% penicillin/streptomycin for 30 min to facilitate separation of ventricular myocytes from the faster-attaching non-myocytes. The ventricular myocytes were collected and plated on gelatin-coated dishes. Cells were used for experiments after demonstrating rhythmic contractions (48–72 h).

2.3. Construction and identification of pcDNA3.1-CYP2J3

The total RNA was extracted from rat livers. The cDNA of CYP2J3 was amplified by RT-PCR and OE-PCR and inserted into a pBS-T vector. The recombinants were checked by PCR and digested by a restriction endonuclease. The CYP2J3 gene was confirmed by DNA sequencing and cloned into a eukaryotic expression vector of the pcDNA3.1, and pcDNA3.1-CYP2J3 was produced. Cultured myocardial cells of rats were transfected with pcDNA3.1-CYP2J3 by a FuGENE HD transfection agent.

2.4. Study groups and experimental protocol

Neonatal rat cardiomyocytes were divided into seven groups for treatment (Fig. 1): Control, no treatment; H/R, 3-h hypoxia followed by 2-, 5-, or 6-h reoxygenation; HPost, three cycles of 5-min reoxygenation followed by 5-min hypoxia before 90-min reoxygenation; HPC, three cycles of 5-min hypoxia followed by 5-min reoxygenation before 2.5-h hypoxia; pcDNA3.1-CYP2J3 + H/R, transfection for 48 h, then H/R; MS-PPOH + HPost, MS-PPOH administered 10 min before HPost; pcDAN3.1-CYP2J3 or MS-PPOH, pcDAN3.1-CYP2J3 transfection or MS-PPOH administration alone.

2.5. Cell viability assay

Cell viability was determined by 3-(4,5)-dimethylthiahiazo (-z-yl)-3,5-di-phenytetrazoliumromide (MTT) assay [16]. Cells were cultured in 96-well plates, and MTT was added to each well under sterile conditions (with a final concentration of 5 mg/ml) immediately after reoxygenation. Plates were then incubated for 4 h at 37 °C. The supernatant was removed, and dimethylsulfoxide was added. Plates were then agitated on a plate shaker. The absorbance of each well was measured at 490 nm with a Wellscan MK 3 automated EIA Analyzer (Labsystems Dragon, Taiwan). The viability of

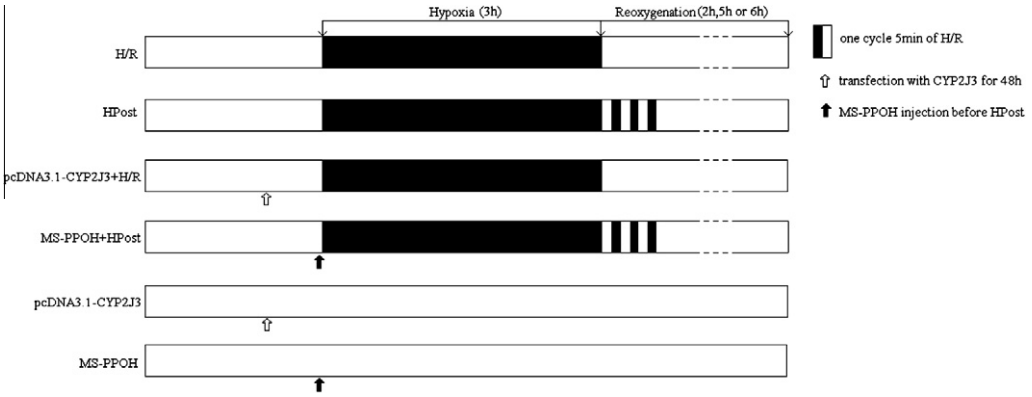

Fig. 1. Study groups and experimental protocol. Protocols for pcDNA3.1-CYP2J3 and MS-PPOH administration and hypoxia–reoxygenation (H/R) in the present study. R/H, reoxygenation–hypoxia; HPost, hypoxia post-conditioning; MS-PPOH, N-methylsulphonyl-6-(2-propargyloxyphenyl)hexanamide, a selective inhibitor of EET synthesis.

control cells was considered to be 100%, and that of other cells was expressed as a percentage of control.

2.6. Quantification of 11,12-EET in cultured medium of neonatal rat cardiomyocytes

11,12-EET was detected by high-performance liquid chromatography (HPLC) (Agilent 1100 System; Agilent Technologies, Palo Alto, CA, USA), as previously described [17]. Briefly, up to 200 μl cultured medium was added to a 1.5-ml microcentrifuge tube with 200 μl methanol and 0.4 μl of 96% formic acid on ice before centrifugation at 14,000*g* for 10 min at 4 °C. The supernatant was transferred to a fresh tube and diluted to 10% methanol by the addition of 1.8 ml water. The diluted supernatant was loaded onto a 1-ml Oasis HLB SPE cartridge (Waters) on a vacuum manifold. The SPE cartridge had been preconditioned by low flow in the sequence 1 ml methanol, 1 ml acetone, 2 ml hexane, 1 ml acetone, 1 ml methanol, and 2 ml water. The cartridge was washed with 3 ml water, then 1 ml of 10% methanol before being allowed to dry under argon for 10 min. The eicosanoid metabolites were collected by elution with 2 ml anhydrous acetonitrile and then dried under a stream of argon. Standards or samples were dissolved in 136 μl anhydrous acetonitrile, 4 μL N,N-diisopropylethylamine, and 10 μl 2-(2,3-naphthalimino)ethyl-trifluoromethanesulfonate and kept at 4 °C for 30 min. The reactions were terminated with argon to evaporate the solution to dryness.

Samples were resuspended in 40 μl methanol and analyzed by HPLC. Mobile phase A comprised 0.5% formic acid in water, and mobile phase B comprised 0.5% formic acid in acetonitrile. A flow rate of 1 ml/min was used to deliver mobile phase A and B gradients as follows: 50–65% mobile phase B for 4 min; 65–100% mobile phase B for 80 min; and 100% mobile phase B for 20 min. The fluorescence detector was set at an excitation wavelength of 260 nm and emission wavelength of 396 nm. Concentrations of 11,12-EET were calculated by comparison against a standard curve.

2.7. Apoptosis assessment

We used two methods to determine apoptosis of cardiomyocytes: nuclear staining with the chromatin dye Hochest 33258 and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL), widely used to detect DNA fragmentation *in situ* and performed according to the manufacturer's protocol. Briefly, cells were fixed with 10% paraformaldehyde for 15 min at 4 °C. After three washes in PBS, cells were permeabilized with 0.2% Triton X-100 for 10 min at room temperature, then incubated with 50 μl TUNEL reaction mixture for 30 min at 37 °C. Diaminobenzidine was used to generate an insoluble colored substrate at the site of DNA fragmentation. Finally, the cells were exposed to Hoechst 33258 (10 μg/ml) for 5 min and examined under a microscope. For TUNEL analysis, the percentage of dead cells was calculated as the ratio of TUNEL-positive cells to total number of cells, counted in three different random fields.

2.8. Measurement of MMP by florescent JC1

MMP was estimated by fluorescence of aggregates of JC-1 (tetrachloro-1,1′,3,3′-tetraethyl-6′,6,5′,5-benzamidazolocarbocyanin iodide) formed as a function of inner MMP. The formation of JC-1 aggregates and their fluorescence linearly corresponds to increases in membrane potential [18]. After treatment, cells plated on 24-well plates were incubated with 10 μg/mL JC-1 for 20 min at 37 °C in a humidified incubator, then washed twice with PBS for detection of the fluorescence ratio (for red fluorescence: excitation, 490 nm and emission, 590 nm; for green fluorescence: excitation, 490 nm and emission, 527 nm). We randomly selected the region of interest, zoomed in the same frames. Data are presented as the relative ratio of red to green fluorescence intensity.

2.9. Determination of mPTP opening

The opening of the transient mPTP was directly assessed by co-loading with calcein-AM and $CoCl_2$ as previously described [19]. Calcein-AM is permeable to intact membranes, but not to intact mitochondrial membranes, and mPTP opening results in exit of calcein. Thus, the condition allows for monitoring of calcein fluorescence in mitochondria of intact cells. In brief, cardiomyocytes were loaded for 15 min with 2 μM calcein-AM in working solution at room temperature and then washed free of calcein-AM and $CoCl_2$. The rates of calcein-AM loading and exit were measured by recording the fluorescence signal every 5 min with the Turner Quantech Digital Filter Fluorometer (Barnstead/Thermolyne, Dubuque, IA, USA; excitation filter NB488 and emission filter SC515) and calculated as the percentage change to maximal fluorescence signal.

2.10. Isolation of mitochondria

Isolation of mitochondria was performed according to the instructions for the Mitochondria/Cytosol Isolation Kit for Cultured Cells. The cells were harvested and homogenized in 1 ml of ice-cold Mito-Cyto Buffer with a Dounce homogenizer. After centrifugation twice at 800*g* for 5 min at 4 °C, the supernatant was collected, transferred to a fresh microcentrifuge tube, and centrifuged at 12,000*g* for 10 min at 4 °C. The pellet, which contained the mitochondria, was resuspended in 30 μl of Mito-Cyto Buffer for further use.

2.11. Western blot analysis

Total protein (80 or 100 mg) isolated from neonatal rat cardiomyocytes was separated by SDS–PAGE and blotted onto polyvinylidene difluoride (Hybond ECL, Amersham). The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline–Tween (TBST; 10 mM Tris, 150 mM NaCl, and 0.1% Tween-20) for 2 h at room temperature before being incubated overnight at 4 °C with anti-CYP2J3 rabbit polyclonal antibody (1:1000), anti-Bcl-2 mouse polyclonal antibody (1:1000), anti-Bax rabbit polyclonal antibody (1:1000), anti-caspase-3 rabbit polyclonal antibody (1:1000), and anti-GAPDH goat monoclonal antibody (1:1000 in 0.5% milk in TBST). After three washes with TBST, the membranes were incubated with horseradish peroxidase-labeled or fluorescence-labeled secondary antibody or in TBST. After extensive washing in TBST, membranes were developed by use of an enhanced chemiluminescence kit.

2.12. Statistical analysis

All values are expressed as the mean ± SD and were analyzed using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Differences between groups were assessed by one-way ANOVA, and Bonferroni's test was used for multiple comparisons. A p-value of < 0.05 was considered significant.

3. Results

3.1. CYP2J3 epoxygenase overexpression induced 11,12-EET production in neonatal rat cardiomyocytes in vitro

pcDNA3.1-CYP2J3 was introduced into neonatal rat cardiomyocytes and visualized under a fluorescence microscope after 48 h (Fig. 2A). Overexpression of CYP2J3 was associated with a significant increase in the CYP2J3 protein level (by 48.2%) compared with

the control (Fig. 2B). Furthermore, the content of 11,12-EET in the cultured medium was increased by 30.7% with overexpression of CYP2J3 compared with the control (Fig. 2C). Thus, a single administration of pcDNA3.1-CYP2J3 to neonatal rat cardiomyocytes significantly increased both CYP2J3 protein expression and activity *in vitro*.

3.2. Cell viability

Compared with the control, H/R decreased cell viability by 45.5% (0.99 ± 0.03 vs. 0.54 ± 0.03, $n = 8$, $p < 0.01$), which suggests that the H/R model was successful (Fig. 3). Viability was ameliorated with HPost treatment (0.83 ± 0.03, $n = 8$, $p < 0.01$) and overexpression of CYP2J3 with H/R (0.81 ± 0.09, $p < 0.01$), but not inhibition of CYP2J3 (MS-PPOH treatment) with HPost (0.62 ± 0.05, $p < 0.01$). Neither pcDNA3.1-CYP2J3 nor MSPPOH alone had an effect on cell viability.

3.3. CYP2J3/11,12-EET was upregulated during HPost, but not HPC

Compared with the control, CYP2J3 protein expression in H/R group decreased by 79.3% (2.08 ± 0.49 vs. 0.43 ± 0.07, $n = 4$, $p < 0.01$) (Fig. 4A). The content of 11,12-EET with H/R was decreased by 85.7% (0.56 ± 0.07 vs. 0.08 ± 0.02 ng/μl, $n = 8$, $p < 0.01$) (Fig. 4B). HPost, but not HPC, increased CYP2J3 protein expression and 11,12-EET content [(0.43 ± 0.07, H/R and 2.02 ± 0.23, HPost; $n = 4$, $p < 0.01$) (Fig. 4A) and (0.08 ± 0.02, H/R and 0.27 ± 0.02, HPost; $n = 8$, $p < 0.01$) (Fig. 4B), respectively]. Thus, HPost but not HPC upregulated the expression of CYP2J3/11,12-EET.

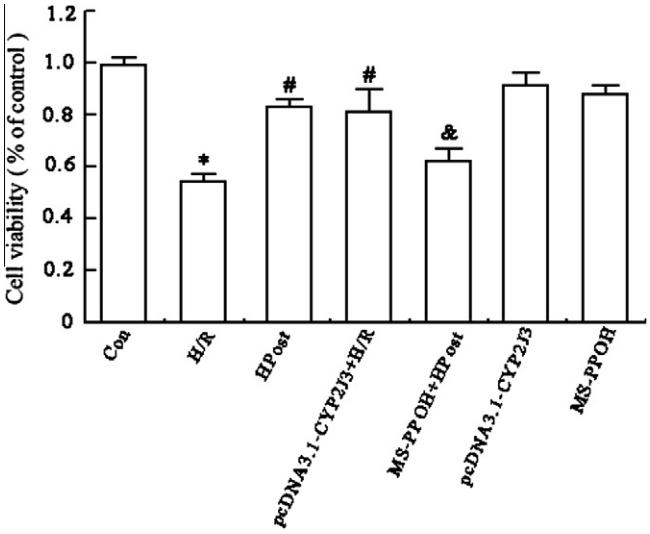

Fig. 3. Cell viability of neonatal rat cardiomyocytes. Cell viability was quantified 48 h after transfection with pcDNA3.1-CYP2J3. Con, no treatment; H/R, 3-h hypoxia followed by 2-h reoxygenation; HPost, three cycles of 5-min hypoxia, then 5-min reoxygenation before 3-h prolonged hypoxia; pcDNA3.1-CYP2J3 + H/R, pcDNA3.1-CYP2J3 for 48 h, then 3-h hypoxia before 2-h reoxygenation; MS-PPOH + HPost, MS-PPOH (20 μM), then three cycles of 5-min hypoxia before 5-min reoxygenation, then 3-h prolonged hypoxia; pcDNA3.1-CYP2J3 or MS-PPOH, pcDNA3.1-CYP2J3 or MS-PPOH alone. Data are mean ± SD of optical density from the MTT assay. Data are mean ± SD ($^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with H/R, $^{\&}p < 0.05$ compared with HPost; $n = 4$).

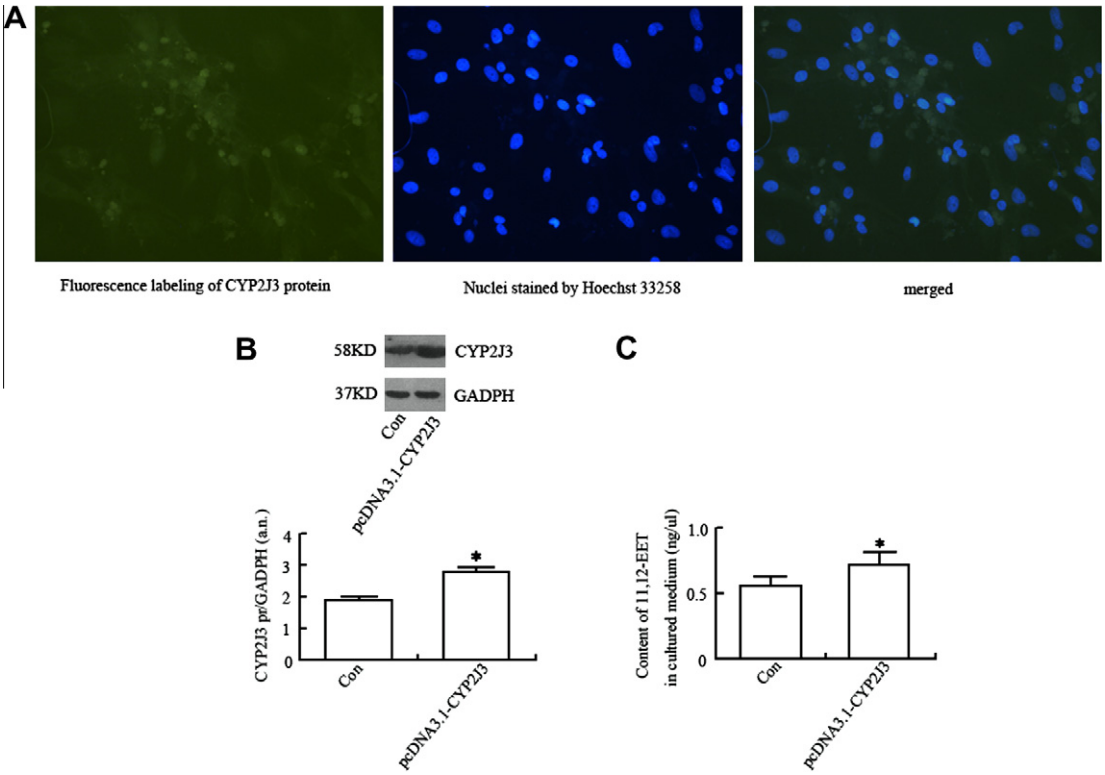

Fig. 2. pcDNA3.1-mediated CYP2J3 epoxygenase overexpression and quantitative analysis of 11,12-EET expression in neonatal rat cardiomyocytes. (A) Fluorescence labeling by Hoechst 33258 staining. Two photographs were taken under the same field and then merged. (B) Western blot analysis of the protein level of CYP2J2 in rat neonatal cardiomyocytes after administration of pcDNA3.1-CYP2J3 for 48 h ($n = 3$ per group). (C) Total 11,12-EET content after administration of pcDNA3.1-CYP2J3 for 48 h ($n = 6$ per group). Data are mean ± SD ($^{*}p < 0.05$ compared with control).

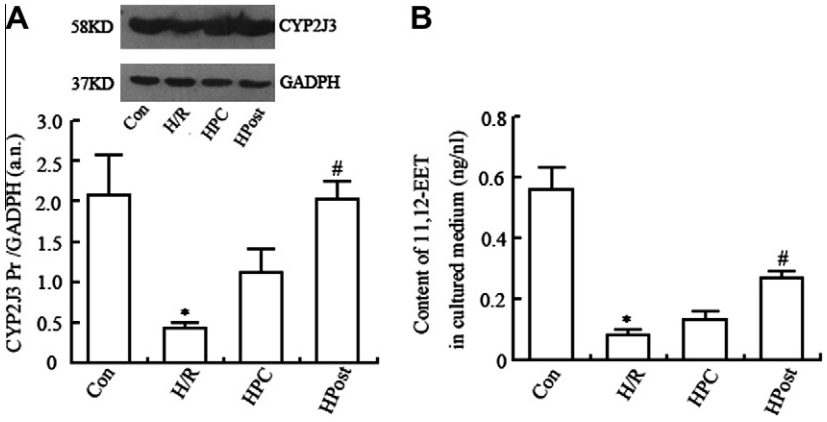

Fig. 4. Changes in CYP2J3 protein expression and 11,12-EET content. Western blot analysis and quantitation of CYP2J3 protein (A) normalized against GAPDH. Concentration of 11,12-EET was determined by reverse-phase high-performance liquid chromatography (B). Treatments as in Fig. 3. Data are mean ± SD ($^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with H/R; $n = 4$).

3.4. Upregulation of CYP2J3/11,12-EET inhibited cardiomyocyte apoptosis

Hoechst 33258 staining revealed control cell nuclei had regular contours and round or elliptical shapes (Fig. 5A). In contrast, H/R cells showed smaller nuclei and condensed chromatin. HPost or overexpression of CYP2J3 improved the morphological features and decreased the number of apoptotic cells induced by H/R; however, MS-PPOH before HPost impaired the morphological features and increased the number of apoptotic cells induced by H/R. The apoptotic results were further confirmed by TUNEL staining (Fig. 5B). Almost no TUNEL-positive cells could be found with control treatment, but many TUNEL-positive cells were found with H/R; the apoptosis ratio was 58.3% higher for H/R than for control treatment (0.38 ± 0.03 vs. 0.24 ± 0.02, $n = 4$, $p < 0.01$). Compared with H/R, HPost or overexpression of CYP2J3 decreased the H/R-induced apoptosis by 31.6% (HPost, 0.26 ± 0.02 and pcDNA3.1-CYP2J3 + H/R, 0.26 ± 0.02, $n = 4$, $p < 0.05$). Inhibition of CYP2J3 with MS-PPOH increased the apoptotic rate of HPost (0.33 ± 0.02, $n = 4$, $p < 0.05$). Neither pcDNA3.1-CYP2J3 nor MS-PPOH alone had an effect on cell apoptosis. Therefore, upregulation of CYP2J3/11,12-EET during HPost inhibited cardiomyocyte apoptosis.

3.5. Upregulation of CYP2J3/11,12-EET during HPost decreased MMP and inhibited mPTP opening induced by H/R

MMP is an important mediator and monitor of key cellular processes. It is a highly sensitive indicator of the energetic state of mitochondria and the health of cells [20]. Detection of mitochondrial permeability provides an early indication of the initiation of cellular apoptosis. By JC-1 analysis, compared with the control, H/R increased MMP by 54.2% (1.0 ± 0.17 vs. 1.54 ± 0.41, $n = 4$, $p < 0.01$) (Fig. 6A). Compared with H/R, HPost and overexpression of CYP2J3 decreased MMP by 47% and 43%, respectively (HPost, 0.81 ± 0.17 and pcDNA3.1-CYP2J3 + H/R, 0.88 ± 0.09, $n = 4$, $p < 0.01$). Inhibition of CYP2J3 with MS-PPOH increased MMP of HPost by 153.1% (1.24 ± 0.32, $n = 4$, $p < 0.01$). Neither pcDNA3.1-CYP2J3 nor MS-PPOH alone had an effect on MMP. Therefore, upregulation of the CYP2J3/11,12-EET system changed HPost-induced MMP.

We investigated the occurrence and mode of mPTP opening in intact cells by monitoring the fluorescence of mitochondrial-entrapped calcein. We used the calcein-AM and $CoCl_2$ co-loaded method to measure mPTP opening (Fig. 6B). Compared with the control, H/R decreased fluorescence intensity by 33.6% (1.52 ± 0.08 vs. 1.01 ± 0.07, $n = 4$, $p < 0.01$), which indicates increased mPTP opening; compared with H/R, HPost or overexpression of CYP2J3 decreased mPTP opening, with fluorescence increased by 28.7% and 25.7%, respectively (HPost, 1.30 ± 0.11 and pcDNA3.1-CYP2J3 + H/R, 1.27 ± 0.04, $n = 4$, $p < 0.01$). Inhibition of CYP2J3 with MS-PPOH decreased the fluorescence intensity of HPost by 37.7% (0.81 ± 0.02, $n = 4$, $p < 0.01$). Neither pcDNA3.1-CYP2J3 nor MSPPOH alone had an effect on mPTP opening. Therefore, the CYP2J3/11,12-EET system decreased mPTP opening induced by H/R.

3.6. Upregulation of CYP2J3/11,12-EET during HPost inhibited mitochondrial cytochrome c leakage and cleaved caspase-3 protein expression

In the mitochondria-mediated apoptotic pathway, disruption of $\Delta\psi_m$ and release of cytochrome c activate a cascade of caspases, including caspase-3, a key and irreversible point in the development of apoptosis. Compared with the control, H/R induced serious damage to the mitochondrial membrane, resulting in the leakage of cytochrome c from the mitochondria. However, the cytochrome c content was significantly preserved in the HPost and pcDNA3.1-CYP2J3 + H/R groups. Inhibition of CYP2J3 with MS-PPOH increased the leakage of cytochrome c in the MS-PPOH + HPost group. Neither pcDNA3.1-CYP2J3 nor MSPPOH alone had an effect on the leakage of cytochrome c (Fig. 7).

Compared with the control, H/R significantly increased cleaved caspase-3 expression by 122.2% (1 vs. 2.22 ± 0.39, $n = 4$, $p < 0.01$). HPost and overexpression of CYP2J3 significantly attenuated the H/R-induced increase in cleaved caspase-3 expression by 41.6% and 46.3%, respectively (HPost, 1.30 ± 0.13 and pcDNA3.1-CYP2J3 + H/R, 1.19 ± 0.23, $n = 4$, $p < 0.05$).

Inhibition of CYP2J3 with MS-PPOH increased cleaved caspase-3 expression of HPost by 59.6.0% (2.07 ± 0.26, $n = 4$, $p < 0.05$). Neither pcDNA3.1-CYP2J3 nor MSPPOH alone had an effect on cleaved caspase-3 expression (Fig. 8). Therefore, the CYP2J3/11,12-EET system improves apoptosis of HPost by decreasing caspase-3 expression.

3.7. Upregulation of CYP2J3/11,12-EET increased the ratio of Bcl-2 to Bax expression induced by H/R

The Bcl-2 family proteins Bax and Bcl-2 play important roles in initiating the mitochondrial death cascade. We used western blot analysis to investigate the expression of pro-apoptotic Bax and anti-apoptotic Bcl-2 underlying apoptosis reduced by the CYP2J3/EET system. Compared with the control, H/R significantly decreased the ratio of Bcl-2 to Bax expression by 31.2% (1 vs. 0.69 ± 0.07, $n = 4$, $p < 0.01$) (Fig. 9). Compared with H/R, HPost and overexpression of CYP2J3 significantly increased the H/R-in-

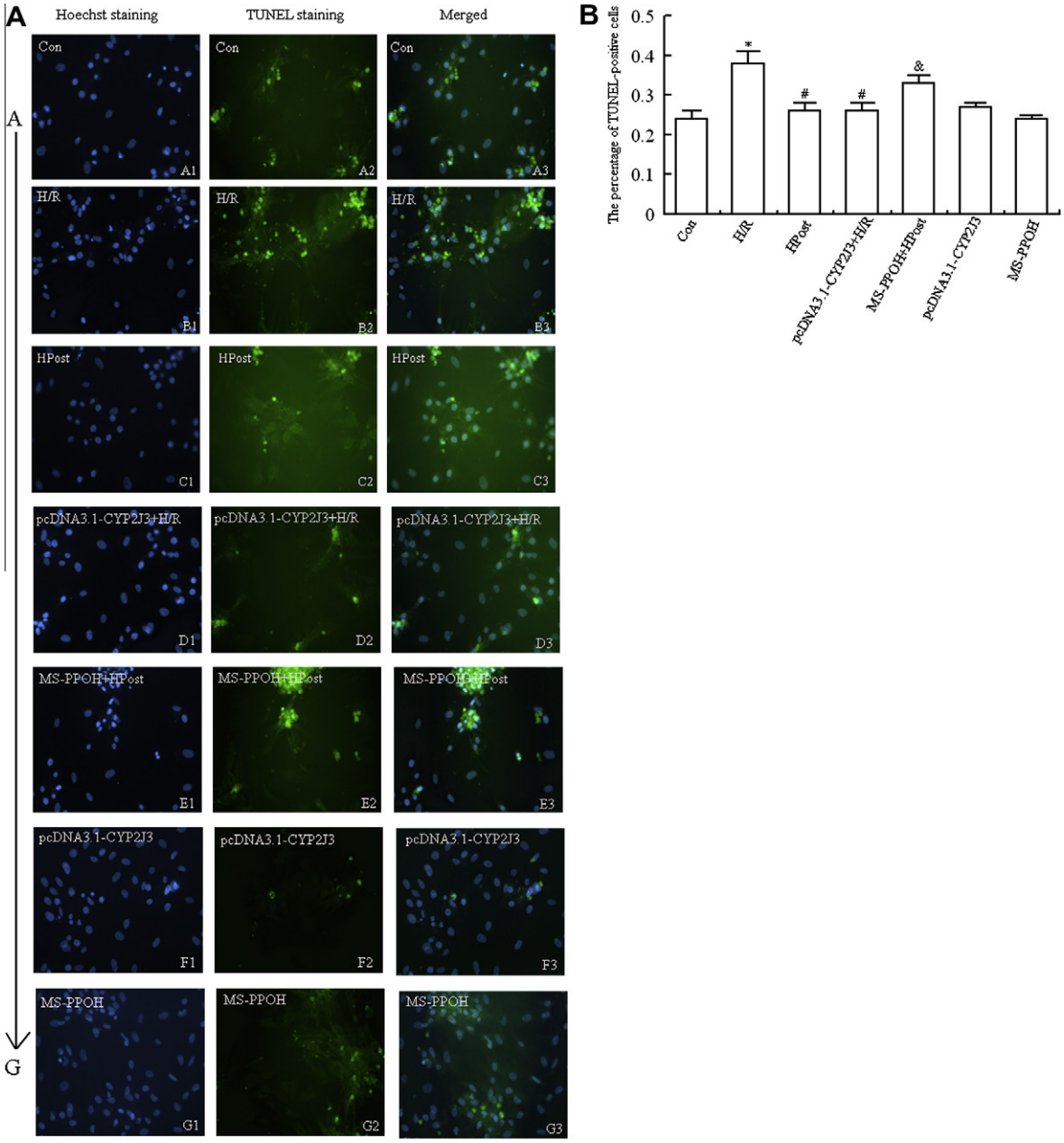

Fig. 5. Effect of pcDNA3.1-CYP2J3 or MS-PPOH on apoptosis. (A) (A1–G1), Effect of upregulation of CYP2J3/11,12-EET during HPost on apoptosis by Hoechst 33258 staining. (A2–G2), Detection of apoptosis by TUNEL staining. (A3–G3), Two photographs taken under the same field and then merged. (A1–A3), Con treatment. (B1–B3), H/R. (C1–C3), HPost. (D1–D3), pcDNA3.1-CYP2J3 + H/R. (E1–E3), MS-PPOH + HPost. (F1–F3), pcDNA3.1-CYP2J3 alone. (G1–G3), MS-PPOH alone. (B) Apoptotic ratio further analyzed by TUNEL staining. Data are mean ± SD ($^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with H/R, $^{\&}p < 0.05$ compared with HPost).

duced decrease in the ratio of Bcl-2 to Bax expression by 26.6% and 25.1%, respectively (HPost, 0.94 ± 0.05 and pcDNA3.1-CYP2J3 + H/R, 0.91 ± 0.13, $n = 4$, $p < 0.05$). Inhibition of CYP2J3 with MS-PPOH decreased the ratio of Bax to Bcl-2 expression of HPost by 24.5% (0.71 ± 0.12, $n = 4$, $p < 0.05$). Neither pcDNA3.1-CYP2J3 nor MS-PPOH alone had an effect on the ratio of Bcl-2 to Bax expression. Thus, the CYP2J3/11,12-EET system improves apoptosis of HPost by decreasing the ratio of Bax to Bcl-2 expression.

4. Discussion

IPost administered to hearts was previously found to have an advantage over IPC; the endogenous CYP2J3/11,12-EET system was found to be upregulated by IPost, but not IPC, in the rat heart. We proposed that upregulation of CYP2J3/11,12-EET during IPost induces cardioprotection by inhibiting cardiomyocyte apoptosis and that multiple apoptotic signals, including changes in MMP and mPTP opening, mitochondrial cytochrome c leakage, caspase-3 levels, and the ratios of expression of protective kinases such as Bcl-2 and Bax, are involved in the process. Neonatal rat cardiomyocytes were exposed to H/R or HPost alone; transfected with pcDNA3.1-CYP2J3 for 48 h, then H/R; or treated with the CYP epoxygenase inhibitor MS-PPOH 10 min before HPost. As with HPost alone, pcDNA3.1-CYP2J3 transfection attenuated cardiomyocyte apoptosis to 68.4% of that with H/R. pcDNA3.1-CYP2J3 transfection significantly decreased MMP levels and inhibited mPTP opening and mitochondrial cytochrome c leakage and cleaved caspase-3

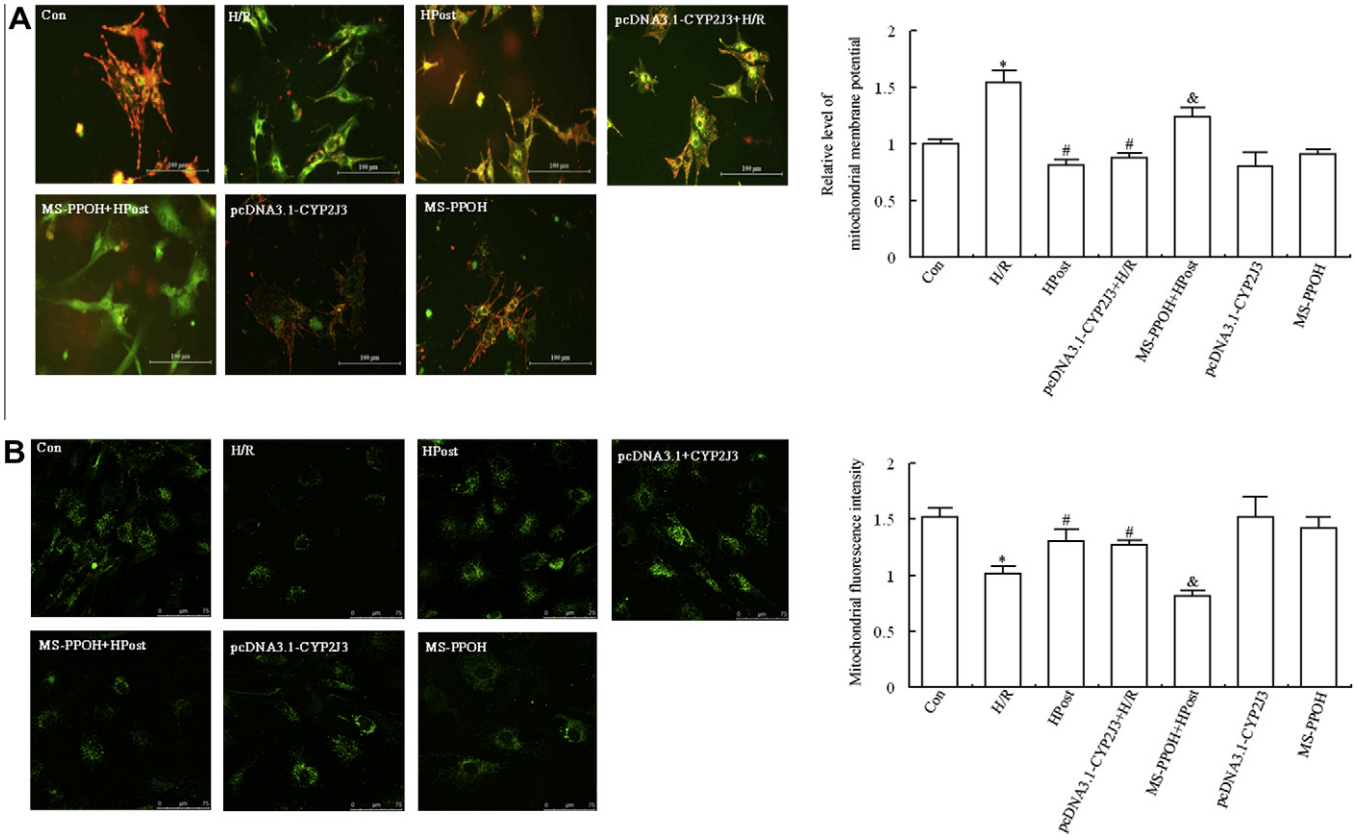

Fig. 6. Effect of pcDNA3.1-CYP2J3 or MS-PPOH on mitochondrial membrane potential (MMP) and mitochondrial permeability transition pore (mPTP) opening. To reveal MMP change (A), cells were incubated with 10 μg/mL JC-1 for 20 min at 37 °C. Treatment as in Fig. 2. To reveal mPTP opening (B), cells were co-loaded for 15 min with 2 μM calcein-AM and 4 mM $CoCl_2$ at 37 °C. The rates of calcein-AM loading and exit were measured by fluorescence signal recording every 5 min by confocal microscopy. The relative level of fluorescence was quantified by the relative ratio of red to green fluorescent intensity per cell. Data are mean ± SD ($^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. H/R, $^{\&}p < 0.05$ vs. HPost; $n = 4$).

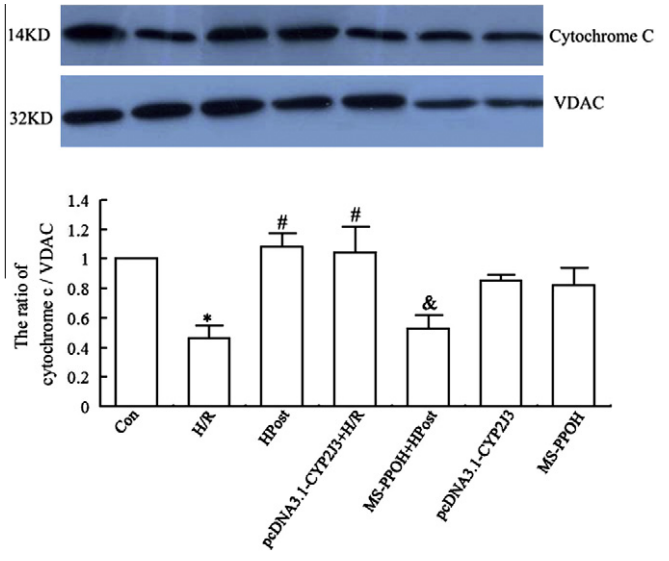

Fig. 7. Effect of pcDNA3.1-CYP2J3 or MS-PPOH on mitochondrial cytochrome c leakage. (A) Western blot analysis of mitochondrial cytochrome c protein expression (upper panel) and VADC (lower panel) as a loading control. (B) Densitometry of cytochrome c level normalized to VADC in each treatment condition in (A). Data are mean ± SD ($^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. H/R, $^{\&}p < 0.05$ vs. HPost; $n = 4$).

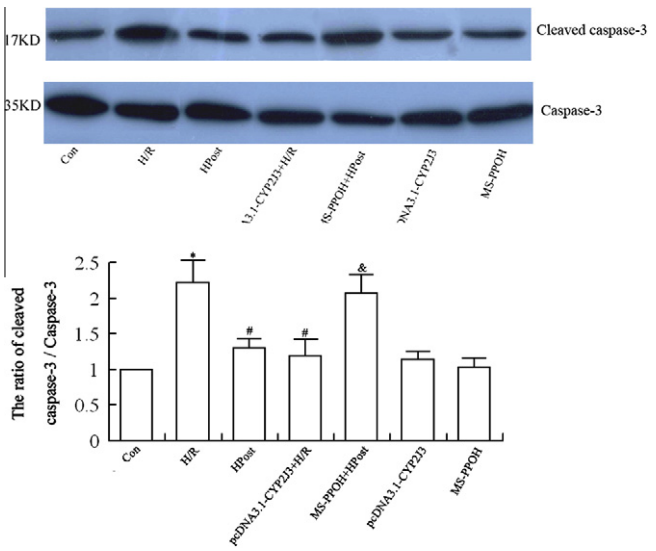

Fig. 8. Effect of pcDNA3.1-CYP2J3 or MS-PPOH on cleaved caspase-3 protein expression. (A) Western blot analysis of cleaved caspase-3 expression (upper panel) and caspase-3 (lower panel). (B) Densitometry of cleaved caspase-3 expression normalized to total caspase-3 in each treatment condition in (A). Data are mean ± SD ($^{*}p < 0.05$ vs. control, $^{\#}p < 0.05$ vs. H/R, $^{\&}p < 0.05$ vs. HPost; $n = 4$).

protein expression induced by H/R, as well as increasing the ratio of Bcl-2 to Bax expression. The CYP epoxygenase inhibitor MS-PPOH abolished this effect of HPost. Therefore, upregulation of CYP2J3/11,12-EET during HPost caused cardioprotection by

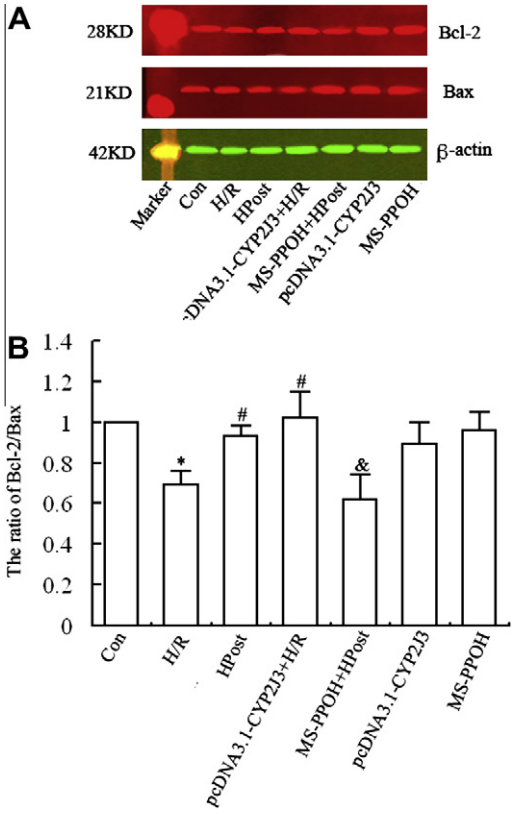

Fig. 9. Effect of pcDNA3.1-CYP2J3 or MS-PPOH on ratio of Bcl-2 to Bax expression. (A) Western blot analysis of protein expression of Bax and Bcl-2 (upper panels) and GADPH (lower panel) as a loading control. (B) Densitometry of Bax and Bcl-2 levels normalized to GADPH in each treatment condition in (A). Data are mean ± SD ($^{*}p < 0.05$ vs. control. $^{\#}p < 0.05$ vs. H/R, $^{\&}p < 0.05$ vs. HPost; $n = 4$).

inhibiting apoptosis via a caspase-dependent pathway. This apoptosis-suppressive effect may have important clinical implications during HPost.

Arachidonic acid (AA), a polyunsaturated fatty acid normally found esterified to cell membrane glycerophospholipids, can be released by phospholipases in response to ischemia [21]. Free AA is then available for metabolism by prostaglandin H2 synthases, lipoxygenases, and CYP450 epoxygenases to generate numerous metabolites, collectively termed eicosanoids [21]. Cardiac CYP epoxygenases metabolize AA to four regioisomeric EETs (5,6-; 8,9-; 11,12-; and 14,15-EET), and CYP ω-hydroxylases metabolize AA to 20-hydroxyeicosatetraenoic acid (20-HETE) [22]. The CYP450 pathway has recently been investigated in several animal models of I/R injury. Administration of EETs and overexpression of mouse CYP2J3 resulted in cardioprotection [23]. Similarly, two selective EET antagonists, 14,15-EEZE and 14,15-EEZE-PEG, and an EET synthesis inhibitor, MS-PPOH, blocked the cardioprotective effects of IPost in the canine heart at doses that had no effect on ischemia when given alone [24]. In a recent study of CYP2J2 transgenic mice, the protective effect was observed in an isolated Langendorff mouse heart [25]. In addition, EET biosynthesis was enhanced in stenosed coronary arteries [26] and during cardiac I/R injury [27]. All of these data strongly suggest that EETs are involved in cardioprotection, but the mechanisms by which EETs initiate their actions are not clear.

EETs prevented apoptosis in rat neonatal cardiac myocytes and in a mouse atrial cardiomyocyte cell line (HL-1) subjected to HR injury [28]. A finding in a renal proximal tubular epithelial cell line demonstrated that EETs inhibited apoptosis induced by serum withdrawal and etoposide [29]. Exogenous EETs increased human endothelial cell survival and attenuated apoptosis [30]. CYP epoxygenases markedly attenuated apoptosis and prevented both TNF-α activation of caspase-3 and TNF-α reduction of Bcl-2 expression in cultured bovine aortic endothelial cells [31]. All of these data suggest that EETs are involved in cardioprotection by inhibition of apoptosis.

We aimed to investigate the effect of upregulating CYP2J3 or administering a specific inhibitor of CYP2J3 (MS-PPOH) on neonatal rat cardiomyocytes to explore whether the CYP2J3/11,12-EET system is involved in the cardioprotection of HPost by inhibiting apoptosis. Endogenous CYP2J3/11,12-EET upregulated by HPost, but not HPC, in rat neonatal cardiomyocytes *in vitro* was consistent with our previous results in rat hearts *in vivo* [9]. Similar to HPost, CYP2J3 overexpression significantly increased neonatal rat cardiomyocyte viability, as previously observed [29]. Moreover, similar to HPost, CYP2J3 overexpression significantly protected neonatal rat cardiomyocytes against H/R-induced apoptosis as evaluated by Hoechst staining and TUNEL analysis. Importantly, inhibition of CYP2J3 with MS-PPOH increased the apoptotic rate of HPost. Therefore, the CYP2J3/11,12-EET pathway is an important regulator of neonatal rat cardiomyocyte apoptosis.

The high energy demands of cardiac function are supplied by ATP, produced mainly by mitochondria through oxidative phosphorylation, glycolysis, and the Krebs cycle [32,33]. Ischemic events can greatly alter mitochondrial function and thereby decrease cardiac efficiency. This damage ultimately contributes to contractile dysfunction, mPTP opening, and cell death. mPTP formation may be the event that leads to irreversible changes in cellular function and cell death [34]. During the early minutes of reperfusion, IPost reduces oxidative stress and inhibits mPTP opening, independent of altered oxidative phosphorylation or $\Delta\Psi m$ [35]. Perfusion with the mPTP-opener atractyloside abolished improved the post-ischemic functional recovery observed in hearts with CYP2J2 overexpression [36]. Moreover, UA-8, a synthetic compound with both EET-mimetic and sEH-inhibitory properties, provided significant cardioprotection against I/R injury by limiting mitochondrial dysfunction [37]. Our results demonstrated that similar to HPost, CYP2J3 overexpression significantly decreased MMP and mPTP opening as evaluated by JC-1 staining and calcein-AM and $CoCl_2$ co-loading analysis. Furthermore, MS-PPOH abolished this effect; thus, CYP2J3/11,12-EET inhibited apoptosis by decreasing MMP and mPTP opening.

A diverse array of intrinsic and extrinsic stimuli regulates endothelial cell apoptosis by modulating the balance between the pro-apoptotic caspases and various anti-apoptotic proteins such as Bcl-2. CYP epoxygenase overexpression significantly inhibited caspase-3 activity, and the time-dependent downregulation of Bcl-2 expression induced by TNF- α [36], which suggests that EETs inhibit the activity of pro-apoptotic proteins and maintain levels of anti-apoptotic proteins. Our results showed that, similar to HPost, CYP2J3 overexpression significantly inhibited mitochondrial cytochrome c leakage, ameliorated increased cleaved caspase-3 expression and the ratio of Bcl-2 to Bax expression. Furthermore, MS-PPOH abolished this effect; thus, mitochondrial cytochrome c release, pro-apoptotic caspase-3 and apoptosis proteins such as Bcl-2 and Bax are involved in the cardioprotection of CYP2J3/11,12-EET upregulation during HPost.

In addition, several papers have suggested that the EETs are potent activators of the sarc KATP channel [38,39]. One paper suggests that they may also act via the mito KATP channel [40]. In a more recent study from this laboratory, Lu et al. [39] demonstrated that the 11(S),12(R)-EET regioisomer activated the rat ventricular myocyte sarc KATP channel. Seubert et al. showed that flavoprotein fluorescence, a marker of mito KATP channel activity [22], was significantly higher in cardiomyocytes obtained from CYP2J2 transgenic animals than in those from wild-type mice. Taken together,

these results suggest that the beneficial effects of activating the CYP epoxygenase pathway in the heart are mediated, at least partly, via opening of the sarc and/or mito KATP channel. Whether KATP is involved in this cardioprotection effect of CYP2J3/11,12-EET during HPost should be further studied.

In conclusion, our study revealed that upregulation of CYP2J3/11,12-EET during HPost induced cardioprotection by inhibiting apoptosis via the mitochondrial pathway. This cardioprotection effect appeared to involve decreased mPTP opening and attenuation of the H/R-mediated mitochondrial cytochrome c leakage and activation of pro-apoptotic caspase-3 and ratio of expression of apoptotic proteins such as Bcl-2 and Bax. Increased 11,12-EET biosynthesis may contribute to the survival of neonatal rat cardiomyocytes. The apoptosis-suppressive effect of EETs may have important clinical implications during HPost, and modulation of CYP2J3-mediated 11,12-EET biosynthesis may represent a novel therapeutic strategy for the clinical establishment of IPost.

Acknowledgments

This work was supported by the National Natural Science Foundation of the People's Republic of China (no. 30770874).

References

[1] Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986;74:1124–36.
[2] Zhao ZQ, Corvera JS, Halkos ME, Kerendi F, Wang NP, Guyton RA, et al. Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol Heart Circ Physiol 2003;285:H579–88.
[3] Kin H, Zhao ZQ, Sun HY, Wang NP, Corvera JS, Halkos ME, et al. Postconditioning attenuates myocardial ischemia-reperfusion injury by inhibiting events in the early minutes of reperfusion. Cardiovasc Res 2004;62:74–85.
[4] Tsang A, Hausenloy DJ, Mocanu MM, Yellon DM. Postconditioning: a form of "modified reperfusion" protects the myocardium by activating the phosphatidylinositol 3-kinase-Akt pathway. Circ Res 2004;95:230–2.
[5] Skyschally A, Van CP, Iliodromitis EK, Schulz R, Kremastinos DT, Heusch G. Ischemic postconditioning: experimental models and protocol algorithms. Basic Res Cardiol 2009;104:469–83.
[6] Yang XM, Proctor JB, Cui L, Krieg T, Downey JM, Cohen MV. Multiple, brief coronary occlusions during early reperfusion protect rabbit hearts by targeting cell signaling pathways. J Am Coll Cardiol 2004;44:1103–10.
[7] Iliodromitis EK, Georgiadis M, Cohen MV, Downey JM, Bofilis E, Kremastinos DT. Protection from post-conditioning depends on the number of short ischemic insults in anesthetized pigs. Basic Res Cardiol 2006;101:502–7.
[8] Staat P, Rioufol G, Piot C, Cottin Y, Cung TT, L'Huillier I, et al. Postconditioning the human heart. Circulation 2005;112:2143–8.
[9] Yu GG, Zeng XJ, Wang HX, Lu LQ, Zheng SP, Ma LQ, et al. Cytochrome P450 2J3/epoxyeicosatrienoic acids mediate the cardioprotection induced by ischaemic post-conditioning, but not preconditioning, in the rat. Clin Exp Pharmacol Physiol 2011;38:63–70.
[10] Penna C, Rastaldo R, Mancardi D, Raimondo S, Cappello S, Gattullo D, et al. Post-conditioning induced cardioprotection requires signaling through a redox-sensitive mechanism, mitochondrial ATP-sensitive K(+) channel and protein kinase C activation. Basic Res Cardiol 2006;101:180–9.
[11] Argaud L, Gateau-Roesch O, Augeul L, Couture-Lepetit E, Loufouat J, Gomez L, et al. Increased mitochondrial calcium coexists with decreased reperfusion injury in postconditioned (but not preconditioned) hearts. Am J Physiol Heart Circ Physiol 2008;294:H386–91.
[12] Sun HY, Wang NP, Kerendi F, Halkos M, Kin H, Guyton RA, et al. Hypoxic postconditioning reduces cardiomyocyte loss by inhibiting ROS generation and intracellular Ca2+ overload. Am J Physiol Heart Circ Physiol 2005;288:H1900–8.
[13] Bopassa JC, Ferrera R, Gateau-Roesch O, Couture-Lepetit E, Ovize M. PI3-kinase regulates the mitochondrial transition pore in controlled reperfusion and postconditioning. Cardiovasc Res 2006;69:178–85.
[14] de Moissac D, Gurevich RM, Zheng H, Singal PK, Kirshenbaum LA. Caspase activation and mitochondrial cytochrome C release during hypoxia-mediated apoptosis of adult ventricular myocytes. J Mol Cell Cardiol 2000;32:53–63.
[15] Borutaite V, Budriunaite A, Morkuniene R, Brown GC. Release of mitochondrial cytochrome c and activation of cytosolic caspases induced by myocardial ischaemia. Biochim Biophys Acta 2001;1537:101–9.
[16] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55–63.
[17] Yue H, Strauss KI, Borenstein MR, Barbe MF, Rossi LJ, Jansen SA. Determination of bioactive eicosanoids in brain tissue by a sensitive reversed-phase liquid chromatographic method with fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci 2004;803:267–77.
[18] Smiley ST, Reers M, Mottola-Hartshorn C, Lin M, Chen A, Smith TW, et al. Intracellular heterogeneity in mitochondrial membrane potentials revealed by a J-aggregate forming lipophilic cation JC-1. Proc Natl Acad Sci U S A 1991;88:3671–5.
[19] Boengler K, Hilfiker-Kleiner D, Heusch G, Schulz R. Inhibition of permeability transition pore opening by mitochondrial STAT3 and its role in myocardial ischemia/reperfusion. Basic Res Cardiol 2010;105:771–85.
[20] Feeney CJ, Pennefather PS, Gyulkhandanyan AV. A cuvette-based fluorometric analysis of mitochondrial membrane potential measured in cultured astrocyte monolayers. J Neurosci Methods 2003;125:13–25.
[21] Sinal CJ, Miyata M, Tohkin M, Nagata K, Bend JR, Gonzalez FJ. Targeted disruption of soluble epoxide hydrolase reveals a role in blood pressure regulation. J Biol Chem 2000;275:40504–10.
[22] Seubert J, Yang B, Bradbury JA, Graves J, Degraff LM, Gabel S, et al. Enhanced postischemic functional recovery in CYP2J2 transgenic hearts involves mitochondrial ATP-sensitive K+channels and p42/p44-MAPK pathway. Circ Res 2004;95:506–14.
[23] Gross GJ, Falck JR, Gross ER, Isbell M, Moore J, Nithipatikom K. Cytochrome P450 and arachidonic acid metabolites: role in myocardial ischemia/reperfusion injury revisited. Cardiovasc Res 2005;68:18–25.
[24] Gross GJ, Gauthier KM, Moore J, Campbell WB, Falck JR, Nithipatikom K. Evidence for role of epoxyeicosatrienoic acids in mediating ischemic preconditioning and postconditioning in dog. Am J Physiol Heart Circ Physiol 2009;297:H47–52.
[25] Batchu SN, Law E, Brocks DR, Falck JR, Seubert JM. Epoxyeicosatrienoic acid prevents postischemic electrocardiogram abnormalities in an isolated heart model. J Mol Cell Cardiol 2009;46:67–74.
[26] Rosolowsky M, Falck JR, Willerson JT, Campbell WB. Synthesis of lipoxygenase and epoxygenase products of arachidonic acid by normal and stenosed canine coronary arteries. Circ Res 1990;66:608–21.
[27] Nithipatikom K, DiCamelli RF, Kohler S, Gumina RJ, Falck JR, Campbell WB, et al. Determination of cytochrome P450 metabolites of arachidonic acid in coronary venous plasma during ischemia and reperfusion in dogs. Anal Biochem 2001;292:115–24.
[28] Dhanasekaran A, Gruenloh SK, Buonaccorsi JN, Zhang R, Gross GJ, Falck JR, et al. Multiple antiapoptotic targets of the PI3K/Akt survival pathway are activated by epoxyeicosatrienoic acids to protect cardiomyocytes from hypoxia/anoxia. Am J Physiol Heart Circ Physiol 2008;294:H724–35.
[29] Chen JK, Capdevila J, Harris RC. Cytochrome p450 epoxygenase metabolism of arachidonic acid inhibits apoptosis. Mol Cell Biol 2001;21:6322–31.
[30] Dhanasekaran A, Al-Saghir R, Lopez B, Zhu D, Gutterman DD, Jacobs ER, et al. Protective effects of epoxyeicosatrienoic acids on human endothelial cells from the pulmonary and coronary vasculature. Am J Physiol Heart Circ Physiol 2006;291:H517–31.
[31] Yang S, Lin L, Chen JX, Lee CR, Seubert JM, Wang Y, et al. Cytochrome P450 epoxygenases protect endothelial cells from apoptosis induced by tumor necrosis factor-α via MAPK and PI3K/Akt signaling pathways. Am J Physiol Heart Circ Physiol 2007;293:H142–51.
[32] Ussher JR, Lopaschuk GD. The malonyl CoA axis as a potential target for treating ischaemic heart disease. Cardiovasc Res 2008;79:259–68.
[33] Stanley WC, Morgan EE, Huang H, McElfresh TA, Sterk JP, Okere IC, et al. Malonyl-CoA decarboxylase inhibition suppresses fatty acid oxidation and reduces lactate production during demand-induced ischemia. Am J Physiol Heart Circ Physiol 2005;289:H2304–9.
[34] Cohen MV, Downey JM. Ischemic postconditioning: from receptor to end-effector. Antioxid Redox Signal 2011;4:821–31.
[35] Paillard M, Gomez L, Augeul L, Loufouat J, Lesnefsky EJ, Ovize M. Postconditioning inhibits mPTP opening independent of oxidative phosphorylation and membrane potential. J Mol Cell Cardiol 2009;46:902–9.
[36] Katragadda D, Batchu SN, Cho WJ, Chaudhary KR, Falck JR, Seubert JM. Epoxyeicosatrienoic acids limit damage to mitochondrial function following stress in cardiac cells. J Mol Cell Cardiol 2009;46:867–75.
[37] Batchu SN, Lee SB, Qadhi RS, Chaudhary KR, El-Sikhry H, Kodela R, et al. Cardioprotective effect of a dual acting epoxyeicosatrienoic acid analogue towards ischaemia reperfusion injury. Br J Pharmacol 2011;162:897–907.
[38] Lu T, Hoshi T, Weintraub NL, Spector AA, Lee HC. Activation of ATP-sensitive K+ channels by epoxyeicosatrienoic acids in rat cardiac ventricular myocytes. J Physiol 2001;537:811–27.
[39] Lu T, Vanrollins M, Lee HC. Stereospecific activation of cardiac ATP sensitive K+ channels by epoxyeicosatrienoic acids: a structural determinant study. Mol Pharmacol 2002;62:1076–83.