Original Paper

CYP2J2 and EETs Protect against Oxidative Stress and Apoptosis *in Vivo* and *in Vitro* Following Lung Ischemia/Reperfusion

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Abstract
Background: Cytochrome P450 epoxygenase 2J2 (CYP2J2) metabolizes arachidonic acids to epoxyeicosatrienoic acids (EETs). EETs exert various biological effects, including anti-inflammatory, anti-apoptotic, pro-proliferation, pro-angiogenesis, anti-oxidation, and anti-fibrosis effects. However, little is known about the role of CYP2J2 and EETs in lung ischemia/reperfusion injury. In this study, we examined the effects of exogenous EETs or CYP2J2 overexpression on lung ischemia/reperfusion injury *in vivo* and *in vitro*. ***Methods and Results:*** CYP2J2 gene was stably transfected into rat lungs via pcDNA3.1-CYP2J2 plasmid delivery, resulting in increased EETs levels in the serum and lung. A rat model of lung ischemia/reperfusion injury was developed by clamping the left lung hilum for 1 hour, followed by reperfusion for 2 hours. We found that CYP2J2 overexpression markedly decreased the levels of oxidative stress and cell apoptosis in lung tissues induced by ischemia/reperfusion. Moreover, we observed that exogenous EETs, or CYP2J2 overexpression, enhanced cell viability, decreased intracellular reactive oxygen species (ROS) generation, inhibited mitochondrial dysfunction, and attenuated several apoptotic signaling events in a human pulmonary artery endothelial cells (HPAECs)-based anoxia/reoxygenation model. These apoptotic events included activation of NADPH oxidase, collapse of mitochondrial transmembrane potential, and activation of

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pro-apoptotic proteins and caspase-3. These effects were mediated, at least partially, by the PI3K/Akt signaling pathway. ***Conclusion:*** These results reveal that CYP2J2 overexpression and exogenous EETs can protect against oxidative stress and apoptosis following lung ischemia/reperfusion *in vivo* and *in vitro*, suggesting that increasing the level of EETs may be a novel promising strategy to prevent and treat lung ischemia/reperfusion injury.

Introduction

Lung ischemia/reperfusion injury (LIRI) occurs in various conditions such as lung transplantation, cardiopulmonary bypass, cardiac bypass surgery, sleeve lobectomy, pulmonary embolism, resuscitation from circulatory arrest, and trauma [1-3]. Clinical studies have indicated that an increase of LIRI-associated mortality and morbidity results in prolonged ventilation, postoperative pulmonary hypertension, longer stay in the intensive care unit, and increased hospital costs [4]. LIRI is a complex pathophysiological process, accompanied by oxidative stress, intracellular calcium overload, release of pro-inflammatory mediators, leukocyte activation, and upregulation of molecules on cell surface membrane [5, 6]. Due to the important role in the pulmonary air-blood barrier, pulmonary artery endothelial cells are recognized as important mediators of LIRI [7]. However, there is no effective method currently available to prevent LIRI, and the precise mechanism of LIRI remains elusive.

Arachidonic acid (AA), the most widely distributed polyunsaturated fatty acid in humans, is located in cell membranes in an inactive state. It can be released by phospholipases A2 in response to various stimuli, such as ischemia [8]. Free AA can then be activated by three enzymatic pathways, including the cyclooxygenase, the lipoxygenase, and the cytochrome P450 (CYP) monooxygenase pathways, to generate numerous active metabolites [9]. CYP epoxygenases, a superfamily of enzymes including the CYP2C and CYP2J subfamilies, metabolize AA to four regioisomeric epoxyeicosatrienoic acids (5,6-, 8,9-, 11,12-, and 14,15-EETs) [10, 11]. Among these, CYP2J2 is expressed in humans, mainly in the cardiovascular system [12]. In previous studies, EETs have been reported to exert diverse biological effects in the cardiovascular and renal systems, such as anti-inflammatory, anti-apoptotic, pro-proliferation, pro-angiogenesis, anti-oxidation, anti-fibrosis, and anti-hypertension effects [13-18].

Either exogenous EETs or CYP2J2 overexpression has been reported to attenuate ischemia/reperfusion injury or hypoxia-reoxygenation injury in myocardium and cerebrum by decreasing infarct size and apoptosis [15, 19], suggesting that EETs and/or CYP2J2 overexpression might also exert a protective role in LIRI. We therefore hypothesized that an increase in EETs may protect against lung ischemia/reperfusion injury *in vivo* and *in vitro*.

In this study, we examined the beneficial effects of CYP2J2 overexpression and exogenous EETs on ischemia/reperfusion or anoxia-reoxygenation induced oxidative stress and apoptosis *in vivo* and *in vitro*. Our data demonstrate that EETs alleviate the levels of oxidative stress and apoptosis induced by LIRI and these effects are mediated, at least in part, by activation of the PI3K/Akt signaling pathway.

Materials and Methods

Construction of the gene delivery vector

The coding DNA fragment of CYP2J2 or green fluorescent protein (GFP) was subcloned into the pcDNA3.1 plasmid as previously described [13, 20]. The pcDNA3.1-CYP2J2 and pcDNA3.1-GFP plasmids were purified using the QIAGEN Plasmid Maxi Kit (QIAGEN, Inc., Chatsworth, CA) according to the manufacturer's instructions.

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Gene delivery and animal model of lung ischemia/reperfusion injury

Animal experimental protocols were approved by the Institutional Animal Research Committee of Tongji Medical College and complied with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Fifty healthy male Wistar rats (280-350 g) were obtained from the Experimental Animal Center of Hubei Province. After 1-week adaptation, rats were randomly divided into five groups (ten in each group): blank control group (Blank), sham group (Sham), lung ischemia/reperfusion group (IR), pcDNA3.1-GFP gene transfection and lung ischemia/reperfusion group (IR+GFP), and pcDNA3.1-CYP2J2 gene transfection and lung ischemia/reperfusion group (IR+2J2). Animals received a tail vein injection of 0.9% NaCl (Blank, Sham, IR group) or an equal volume of plasmid (IR+GFP, IR+2J2 group) at a dose of 3mg/kg body weight, once a week for two consecutive weeks.

One week after the second vein injection, rats in the IR groups were subjected to left thoracotomy via the fifth intercostal space. The left pulmonary hilar, including the left main bronchus, pulmonary artery, and pulmonary vein, were dissected and then occluded for 1 hour with a non-invasive arterial clamp, resulting in complete left lung ischemia and anoxia. At the end of the ischemic time, the clamp was removed. The left lung was then ventilated and reperfused for an additional 2 hours [21, 22]. Time-matched sham rats underwent the identical thoracotomy and hilar dissection, but without hilar occlusion. All operations were carried out under intraperitoneal anesthesia and mechanical ventilation. Animals in the blank group did not receive any other treatment. Animals were sacrificed at the end of experiment. Blood samples were acquired from the left atria prior to sacrifice and the left lungs were then removed for further analysis.

Immunohistochemistry

Immunohistochemical (IHC) staining was performed to detect the expression of CYP2J2 in lung tissues using a Streptavidin/Peroxidase immunohistochemical kit (Fuzhou Maixin Biotechnology Co. Ltd, Fujian, China) according to the manufacturer's instruction [23]. The results were counted by two investigators in a double-blind fashion. At least five randomly-selected fields (×400) were examined.

Detection of 14,15-DHET

EETs are unstable, and can be enzymatically hydrated to the corresponding stable metabolite dihydroxyeicosatrienoic acids (DHETs) by soluble epoxide hydrolases (sEH) [10]. To evaluate the *in vivo* EETs level, an ELISA kit (Detroit R&D, Detroit, MI) was used to determine the concentrations of 14,15-DHET in the rat serum and lung homogenates according to the manufacturer's instructions, as previously described[14, 19, 24].

TUNEL staining for assessment of apoptosis

Apoptotic cell death in the lung was detected in situ by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) staining of fragmented DNA using an In Situ Cell Death Detection Kit, POD (Roche, Mannheim, Germany). The procedure was performed according to the manufacturer's instructions. Cells were identified as TUNEL positive if they showed dark brown staining under a light microscope. More than five randomly-selected high-power fields (×400) were examined for TUNEL-positive cells. The number of TUNEL positive cells was scored and the percentage of apoptotic cells was calculated from the total number of cells.

Cell culture and gene transfection

Human pulmonary artery endothelial cells (HPAECs) (Sciencell Research Laboratories, Carlsbad, CA, USA) were cultured in endothelial cell medium supplemented with 5% fetal bovine serum and penicillin/streptomycin (Sciencell Research Laboratories). Cells were incubated at 37°C in a humidified atmosphere with 5% CO_2/95% air. Cells were grown to ~60% confluency and then transfected with pcDNA3.1/GFP or pcDNA3.1/CYP2J2 plasmids (0.2 ug DNA/cm^2) with the Fugene HD Transfection Reagent (Roche Molecular Biochemicals, Indianapolis, IN). After48-72 hours of transfection, the transfection efficiency was determined by examining pcDNA3.1/GFP-transfected cells for GFP fluorescence under a fluorescence microscope (NIKON) and by flow cytometric analysis. The expression of CYP2J2 was confirmed by Western blotting.

Model of anoxia-reoxygenation and treatment of cultured cells

HPAECs were grown to ~80% confluence in 6-well plates under an atmosphere of 95% air and 5% CO_2. For the experimental groups, the medium on the culture plates was replaced with fresh medium without serum, and then subjected to anoxia-reoxygenation (AR). Cells were maintained at 37°C under an atmosphere of 95% N_2 and 5% CO_2 (anoxia). After 8 h of anoxia, cells were given fresh medium with serum and maintained in normal conditions (reoxygenation) for the next 16 h. The oxygen content (FiO2) during anoxia was continuously monitored to ensure that it remained below 1% (Pro-Ox 110, Biospherix Ltd, Redfield, NY). The cells were pretreated with two inhibitors, LY294002 (PI3K inhibitor, 20 uM) and 14,15-EEZE (EETs inhibitor, 10 uM, Cayman Chemical Co.) for 60 min before addition of vehicle or EETs (1uM), which were applied 60 min prior to AR until the end of the experiment. Normal control cells were treated with the same procedure under normal culture conditions.

Cell proliferation assay

Cell proliferation was monitored using a Cell Counting kit-8 (CCK-8, Beyotime, Haimen, China) according to the manufacture's instruction. Briefly, cells were seeded in 96-well plates at a density of $2×10^3$ per well in 100 ul of complete medium. After being treated with the corresponding drugs and anoxia-reoxygenation as described above, the cells were further incubated with 10 µl of the CCK-8 reagent for 0.5 to 4 hours. The absorbance of 450 nm was measured with a microplate reader (Bio-Tek ELX800, USA).

Detection of reactive oxygen species

2,7-Dichlorodihydrofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA) was used to measure the quantity of reactive oxygen species (ROS) produced by HPAECs following anoxia-reoxygenation. Non-ionized DCFH-DA, which itself is non-fluorescent, can diffuse freely through the cell membrane. Once it is intracellular, DCFH-DA is deacetylated by nonspecific esterases to form DCFH, and then further oxidized by ROS to yield the fluorescent compound DCF. Cells were incubated with 10 µM DCFH-DA at 37°C for 20 min. After staining, cells were harvested immediately to detect DCF fluorescence by flow cytometry.

Measurement of mitochondrial transmembrane potential

5, 5′, 6, 6′-tetrachloro-1, 1′, 3, 3′-tetraethylbenzimidazolyl-carbocyanine iodide (JC-1), a mitochondria-specific membrane potential-sensitive fluorescent probe, was used to determine mitochondrial membrane potential. JC-1 accumulates in the mitochondrial matrix when mitochondrial membrane potential is high, forming J-aggregates which emit a bright yellow-red fluorescence. In contrast, JC-1 presents itself as a green fluorescent monomer with low levels of aggregation when mitochondrial membrane potential is low. During this assay, cells were seeded on 6-well plates. After corresponding treatment in each group, the cells were loaded with 10 µM JC-1 for 20 min at 37°C, and then washed and viewed under a fluorescence microscope with a green excitation filter at 543 nm and a blue excitation filter at 488 nm to visualize yellow-red and green fluorescence, respectively. Subsequently, the cells were harvested to detect fluorescence by flow cytometry.
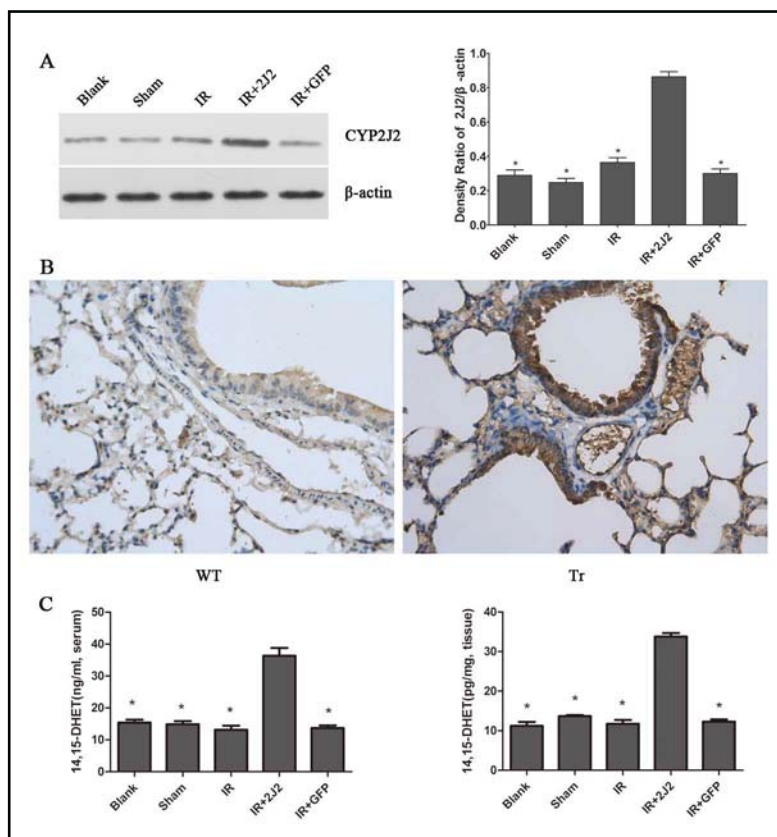
Apoptosis assay using flow cytometry

Cells in 6-well plates were harvested with trypsin/EDTA, resuspended in the binding buffer, and stained with Annexin V-FITC and propidium iodide (PI) using an apoptosis detection kit (KeyGen, Nanjing, China) according to the manufacturer's protocol. Within 1 h, the cells were subjected to flow cytometric analysis with a CycleTEST™ PLUS (Becton Dickinson, San Jose, CA). The annexin V positive (FL1-H) cells were counted for early stages of apoptosis (the lower right quadrant) and late apoptotic or necrotic cells (the upper right quadrant). The PI positive cells were counted for necrotic(the upper left quadrant) and late apoptotic or necrotic cells. The results were then quantitated using CellQuest and ModFit analysis software.

Western blotting analysis

Cellular proteins were extracted with RIPA lysis buffer. Protein concentrations were measured by the Bradford method using BCA Protein Assay Reagent. Western blots were performed according to the methods described previously [25]. The primary antibodies used in the experiment were: PI3-Kinase, phosphor-Thr308-Akt, Akt, Bcl-2, Bcl-xl, Bax, SOD1, SOD2, catalase, p67-phox, gp91-phox, and β-actin (Epitomics, Burlingame, CA, USA); p47-phox (Abgent, San Diego, CA, USA); caspase-3 (Cell Signaling Technology,

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Fig. 1. Effects of pcDNA3.1-CYP2J2 delivery on CYP2J2 expression in the lung and the 14,15-DHET levels in the serum and lung. (A) Western blot showed that CYP2J2 was overexpressed in lung tissues upon gene delivery of pcDNA3.1-CYP2J2. (B) Immunohistochemical staining showed that the CYP2J2 protein expression was significantly increased in lung tissues of transgenic rats, especially in the cytoplasm of vascular endothelial cells and epithelial cells (Tr: transgenic rats, WT: wild type rats). (C) *In vivo* delivery of pcDNA3.1-CYP2J2 increased the 14,15-DHET levels in the serum and the lung. (n=10, *$p < 0.05$ versusIR+2J2).

Beverly, MA, USA); and CYP2J2 (Abcam Inc., Cambridge, MA, USA). The horseradish peroxidase-conjugated secondary antibodies (goat anti-rabbit and goat anti-mice) were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Blotting bands were quantified by densitometry using Quantity One software (Bio-Rad, Hercules, CA).

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Comparisons between groups were performed by a one-way analysis of variance (ANOVA). Statistical significance was defined as $p < 0.05$.

Results

Delivery of pcDNA3.1-CYP2J2 induces CYP2J2 overexpression in the lung and increases 14,15-DHET levels in the serum and lung

Rats were injected with either pcDNA3.1-GFP or pcDNA3.1-CYP2J2 once a week for two consecutive weeks. One week after the second gene delivery, CYP2J2 protein levels were increased in the lung of pcDNA3.1-CYP2J2transgenic rats, as evaluated by Western blot (Fig. 1A). Moreover, immunohistochemical staining showed that the expression of CYP2J2 protein was significantly increased in lung tissues of transgenic rats, especially in the cytoplasm of vascular endothelial cells and epithelial cells. CYP2J2+ cells were stained brown and confined to the cytoplasm in transgenic rats (Fig. 1B), as described previously [26]. CYP2J2 metabolizes arachidonic acids to form EETs, which can be quickly hydrolyzed to their corresponding DHETs that exhibit much more stable biological activity. Therefore, we measured the levels of 14,15-DHET in the serum and lung. Interestingly, pcDNA3.1-CYP2J2 injection remarkably increased 14,15-DHET levels in both the serum and lung (Fig. 1C).
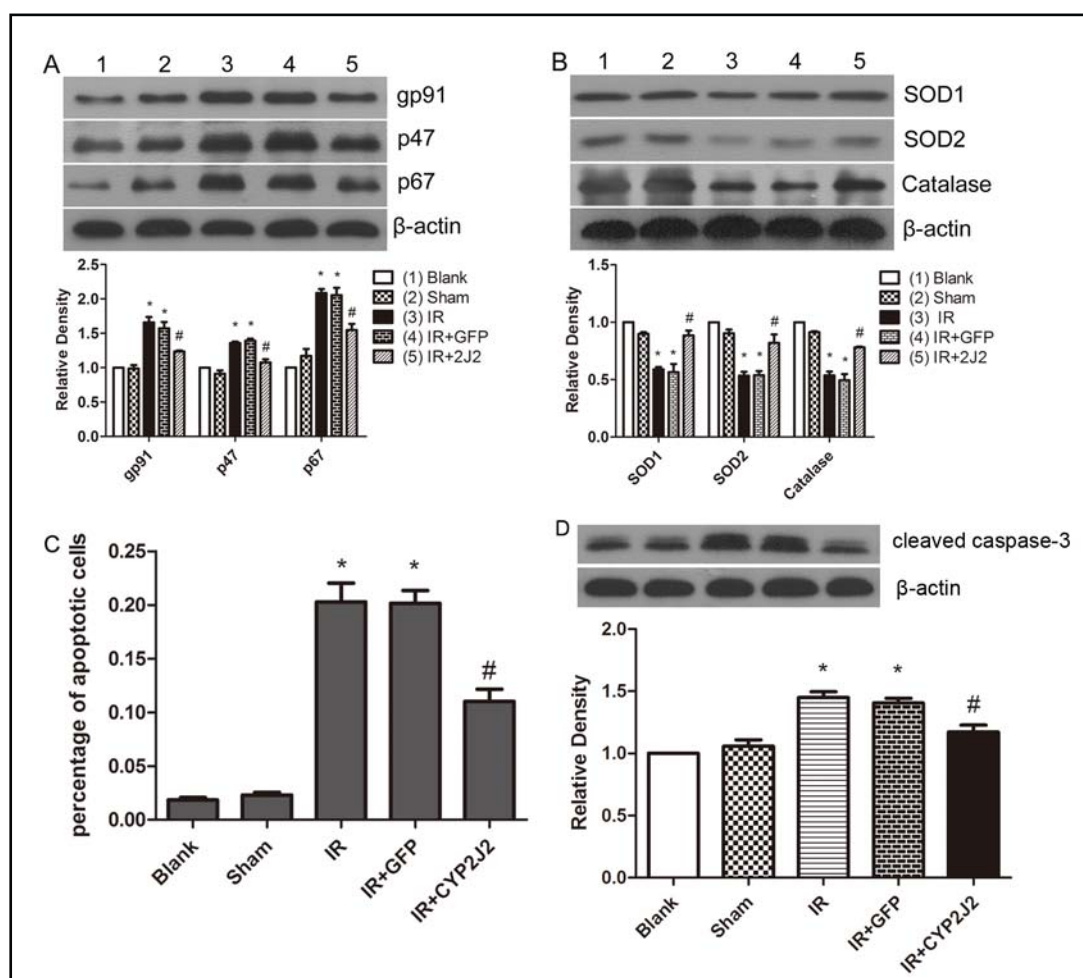
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Fig. 2. CYP2J2 overexpression reduced the level of oxidative stress and apoptosis *in vivo* following ischemia/reperfusion. (A) Ischemia/reperfusion increased the expression of the pro-oxidative proteins gp91, p47, and p67 in lung tissues, an effect that was reversed by CYP2J2 overexpression. (B) CYP2J2 delivery inhibited the reduction of the anti-oxidative proteins SOD1, SOD2, and catalase following ischemia/reperfusion *in vivo*. (n=3 for each experiment, *$p<0.05$ vs. blank or sham group, #$p<0.05$ vs. IR or IR+GFP) (C) Graph representing the mean percentage of TUNEL-positive cells. Cells were identified as TUNEL positive if they displayed dark brown staining. The mean percentage of TUNEL-positive cells significantly increased after ischemia/reperfusion, which was reversed by pcDNA3.1-CYP2J2 transfection. More than 5 randomly-selected high-power fields (×400) were examined. (n=5 for each group, *$p<0.05$ vs. blank or sham group, #$p<0.05$ vs. IR or IR+GFP) (D) The expression of cleaved caspase-3 was remarkably increased in lung tissues after ischemia/reperfusion, an effect that was blocked by CYP2J2 delivery. (n=3 for each experiment, *$p<0.05$ vs. blank or sham group, #$p<0.05$ vs. IR or IR+GFP).

Effects of CYP2J2 overexpression on oxidative stress and apoptosis in vivo

To evaluate the effects of the ischemia/reperfusion model on oxidative stress in this study, we measured the expression of pro-oxidative and anti-oxidative enzymes in lung tissues. As depicted in Fig. 2A, the expression of the pro-oxidative enzymes gp91, p47, and p67 was significantly increased in the lung tissues following ischemia/reperfusion, a phenomenon which was reversed by CYP2J2 overexpression. In contrast, CYP2J2 delivery remarkably inhibited the ischemia/reperfusion-induced reduction in SOD1, SOD2 and catalase protein levels (Fig. 2B).

Lung tissue apoptosis or necrosis is an important event following ischemia/reperfusion injury, which is associated with the development of post-operative lung dysfunction [6]. In
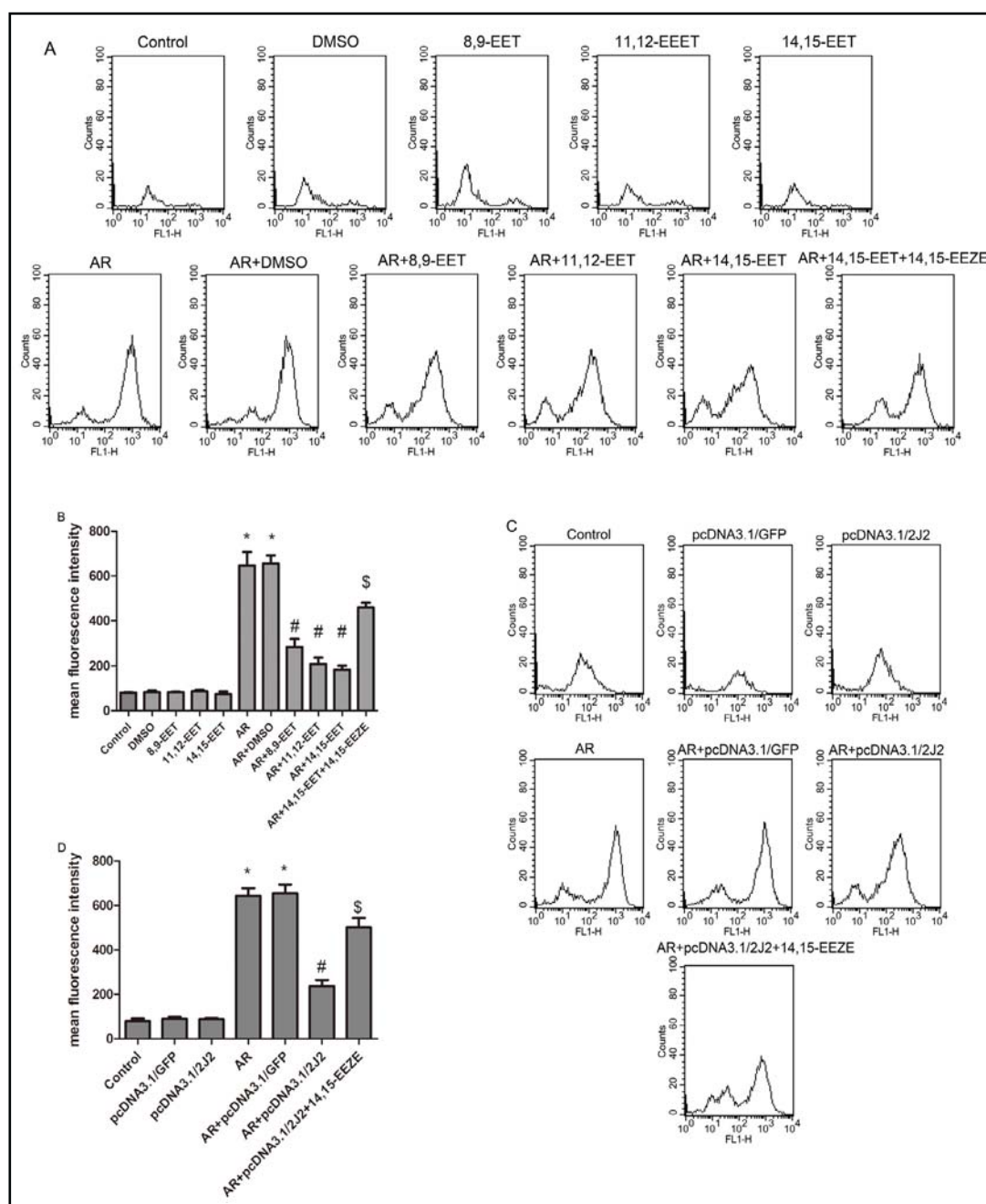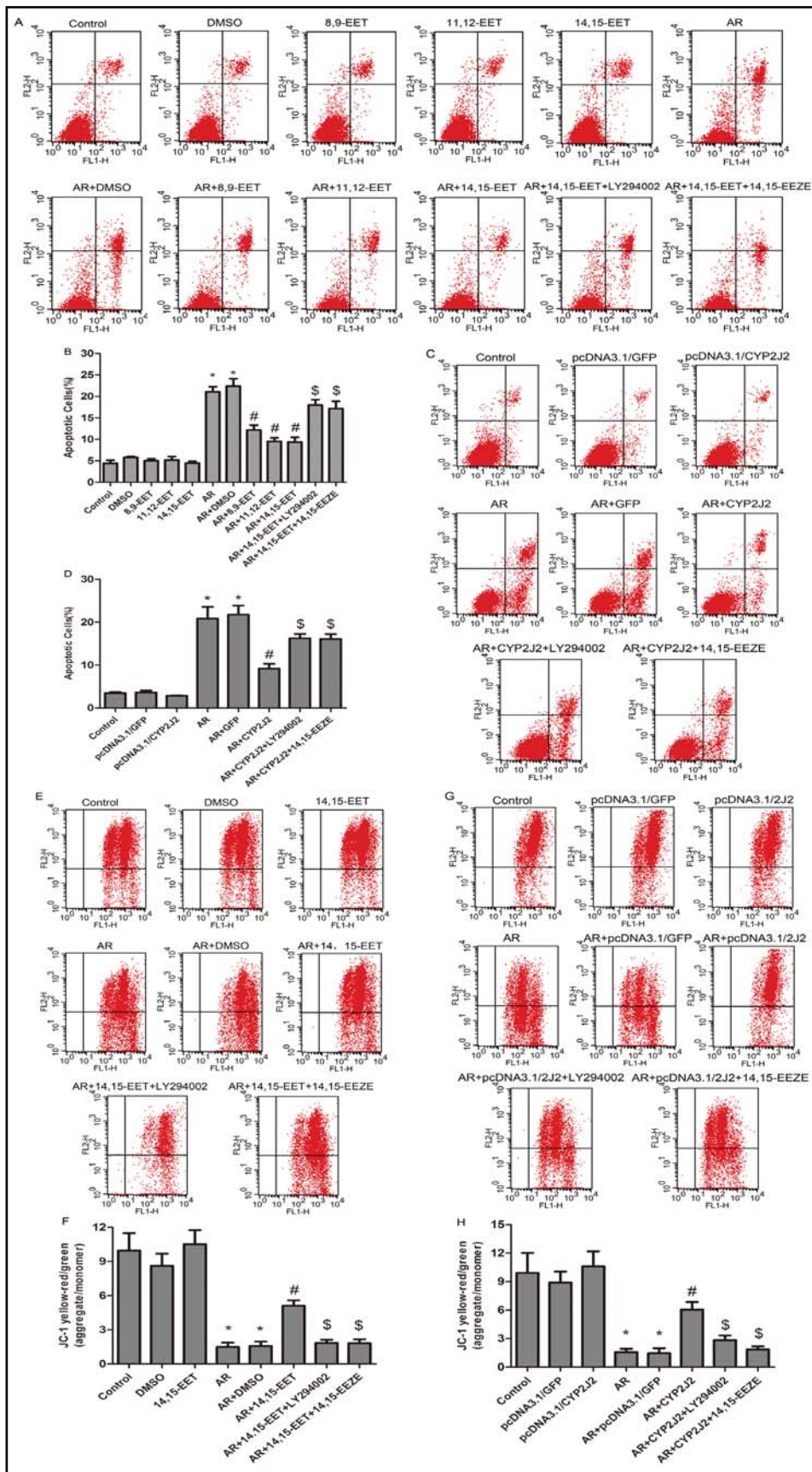
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Fig. 3. EETs and CYP2J2 overexpression suppress ROS production induced by anoxia/reoxygenation in HPAECs. (A) Anoxia/reoxygenation-induced ROS production is suppressed by EETs, which is blocked by 14,15-EEZE (a selective EET antagonist), as detected by flow cytometry. (B) Graphs represent the mean fluorescence intensity of each group. (n=3 for each experiment; *$p < 0.05$ vs. control; #$p < 0.05$ vs. AR or AR+ DMSO; $$p <0.05$ vs. groups of AR+EETs) (C and D) Anoxia/reoxygenation-induced ROS production is inhibited by CYP2J2 overexpression, which is attenuated by 14,15-EEZE, as detected by flow cytometry. (n=3 for each experiment; *$p < 0.05$ vs. control; #$p < 0.05$ vs. AR or AR+pcDNA3.1/GFP; $$p<0.05$ vs. AR+pcDNA3.1/2J2).

this study, apoptotic cell death in lung tissues was examined by TUNEL staining. As depicted in Fig. 2C, treatment with ischemia/reperfusion markedly increased the percentage of

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Fig. 4. EETs and CYP2J2 overexpression inhibit anoxia/reoxygenation-induced apoptosis and mitochondrial impairment in HPAECs. (A) Flow cytometric analysis of HPAECs treated with anoxia/reoxygenation and EETs using Annexin V-FITC and PI staining. The annexin V positive cells were counted for early stages of apoptosis (the lower right quadrant) and late apoptotic or necrotic cells (the upper right quadrant). The PI positive cells were counted for necrotic (the upper left quadrant) and late apoptotic or necrotic cells. (B) Graph re-

presents the mean percentage of Annexin V-positive cells in each group. (n=3 for each experiment; *$p < 0.05$ vs. control; #$p < 0.05$ vs. AR or AR+ DMSO; $$p<0.05$ vs. groups of AR+EETs) (C and D) Apoptosis of HPAECs induced by anoxia/reoxygenation is decreased by CYP2J2 overexpression and increased by LY294002 (PI3K inhibitor) and 14,15-EEZE. These data are representative of 3 independent assays. (*$p < 0.05$ vs. control; #$p < 0.05$ vs. AR or AR+GFP; $$p<0.05$ vs. AR+CYP2J2). Mitochondrial transmembrane potential was determined by JC-1 and analyzed by flow cytometry. The upper right quadrant represents cells with normal mitochondrial function, which emit yellow-red fluorescence. The lower right quadrant represents cells with a collapsed mitochondrial transmembrane potential, which emit green fluorescence. (E) Collapse of mitochondrial transmembrane potential induced by anoxia/reoxygenation in HPAECs is mitigated by preincubation with 14,15-EET, an effect which is reversed by LY294002 and 14,15-EEZE. (F) Graph represents the mean ratio of yellow-red/green fluorescence of JC-1 (aggregate/monomer) in each group. (n=3 for each experiment; *p < 0.05 vs. control; # $p < 0.05$ vs. AR or AR+ DMSO; $$P<0.05$ vs. AR+ 14,15-EET) (G and H) CYP2J2 overexpression attenuates mitochondrial impairment induced by anoxia/reoxygenation in HPAECs, an effect that is inhibited by LY294002 and 14,15-EEZE (n=3 for each experiment; *$p < 0.05$ vs. control; #$p < 0.05$ vs. AR or AR+pcDNA3.1/GFP; $$p<0.05$ vs. AR+CYP2J2).

TUNEL-positive cells as compared with the blank and sham groups, an effect which was reversed by pcDNA3.1-CYP2J2 transfection. Moreover, the expression of cleaved caspase-3 was remarkably increased in lung tissues after ischemia/reperfusion, an effect that was blocked by CYP2J2 delivery (Fig. 2D).

Transfection of pcDNA3.1/CYP2J2 leads to CYP2J2 overexpression in HPAECs

After 60 hours of transfection, transfection efficiency of pcDNA3.1-GFP in HPAECs was maintained at 35-60%, as detected by flow cytometry (Fig. 8A) and fluorescent microscopy (Fig. 8B). Expression of CYP2J2 protein was significantly increased in the pcDNA3.1-CYP2J2 group as compared to the control and pcDNA3.1-GFP groups (Fig. 8C).

Effects of exogenous EETs on viability of HPAECs

To assess the impact of anoxia/reoxygenation on cell viability, we developed three different anoxia/reoxygenation models. We found that treatment of HPAECs with anoxia conditions for 8 h, followed by reoxygenation for the next 16 h, led to a significant decrease in cell viability (Fig. 9A). To test effects of exogenous EETs on the viability of HPAECs, we examined proliferation of HPAECs under normoxia and anoxia/reoxygenation conditions, respectively. We found that EETs had no significant effects on cell viability under normal conditions (Fig. 9B). However, the cell viability remarkably decreased following anoxia/reoxygenation, which was reversed by 11,12-EET and 14,15-EET (Fig. 9C).

EETs and CYP2J2 overexpression suppress ROS production induced by anoxia/reoxygenationin HPAECs

ROS play an important role in lung ischemia-reperfusion injury [2, 6]. Therefore, we examined ROS production by flow cytometry in anoxia/reoxygenation-treated HPAECs. The mean fluorescence intensity of DCFH-DA, as detected by flow cytometry, represented the ROS level. The results indicated that pre-incubation with EETs significantly attenuated the anoxia/reoxygenation-induced increase in the ROS level, while EETs alone did not show this effect (Fig. 3A and B). However, the effects of EETs in the anoxia/reoxygenation condition were largely inhibited when cells were pretreated with 14,15-EEZE. Transfection with pcDNA3.1-CYP2J2 displayed similar protective effects on ROS production in anoxia/reoxygenation treated cells (Fig. 3C and D).

Effects of EETs and CYP2J2 overexpression on apoptosis and mitochondrial transmembrane potential in HPAECs following anoxia/reoxygenation

The effects of exogenous EETs and CYP2J2 overexpression on the apoptosis levels of HPAECs were assessed by flow cytometry. Our results revealed that pre-incubation with
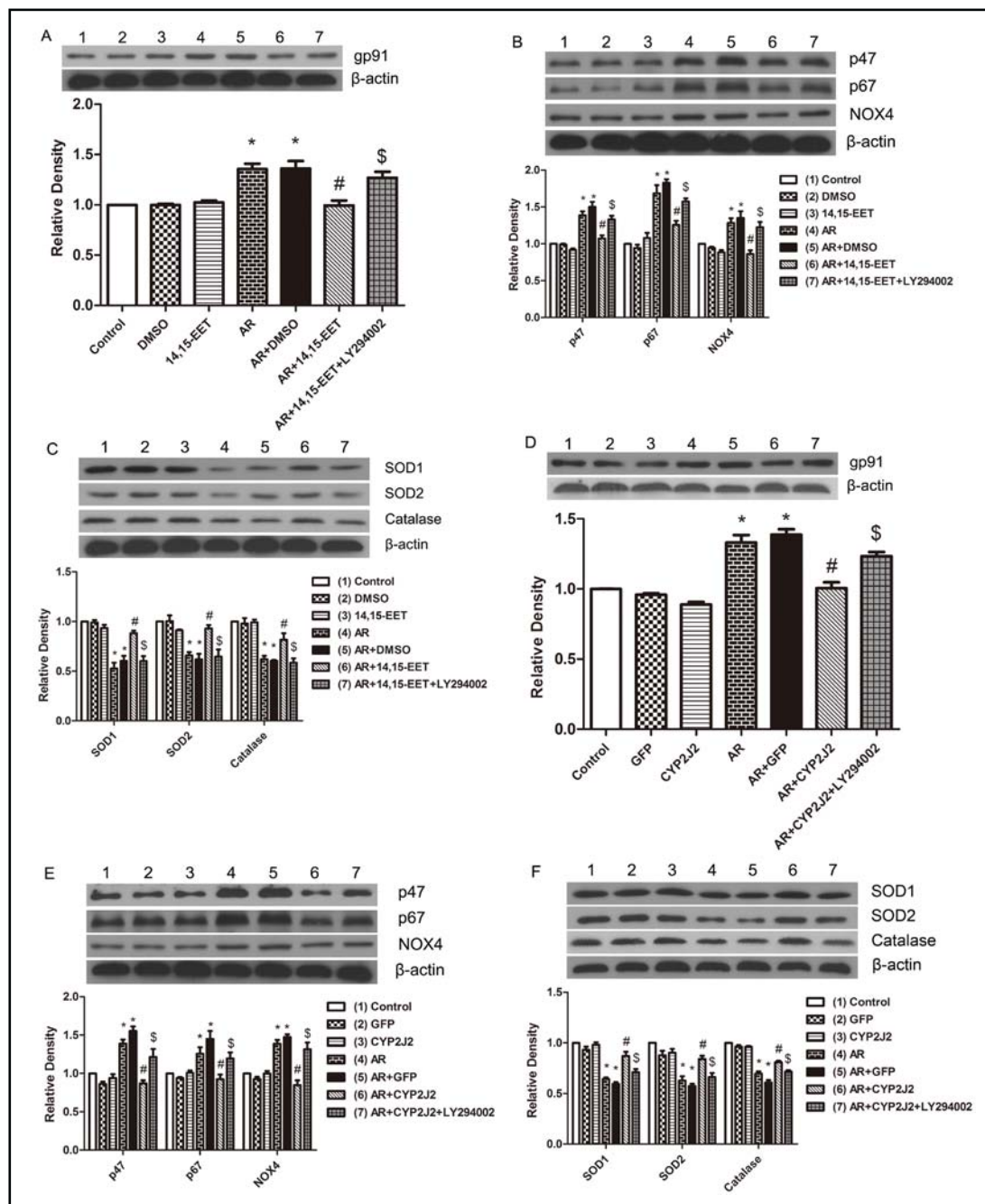
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Fig. 5. 14,15-EET and CYP2J2 overexpression increase the expression of anti-oxidative proteins and decrease the expression of pro-oxidative proteins after anoxia/reoxygenation in HPAECs. (A) The expression of the pro-oxidative protein gp91(a transmembrane protein) is decreased by 14,15-EET after treatment with anoxia/reoxygenation and increased by LY294002. (B) 14,15-EET incubation reduced the expression of the pro-oxidative proteins p47, p67, and NOX4, which were induced by anoxia/reoxygenation. This EET effect was reversed by LY294002. (C) The expression of the anti-oxidative proteins SOD1, SOD2, and catalase in the anoxia/reoxygenation condition is promoted by 14,15-EET but inhibited by LY294002 (n=3 for each experiment; *$p < 0.05$ vs. control; # $p < 0.05$ vs. AR or AR+ DMSO; $^{\$}P<0.05$ vs. AR+ 14,15-EET). (D and E) CYP2J2 overexpression markedly inhibited the expression of gp91, p47, p67 and NOX4, which were induced by anoxia/reoxygenation. This CYP2J2 effect was reversed by LY294002. (F) CYP2J2 overexpression remarkably increased the expression of SOD1, SOD2 and catalase, an effect that is blocked by LY294002. (n=3 for each experiment; *$p < 0.05$ vs. control; # $p < 0.05$ vs. AR or AR+pcDNA3.1/GFP; $^{\$}p<0.05$ vs. AR+CYP2J2).

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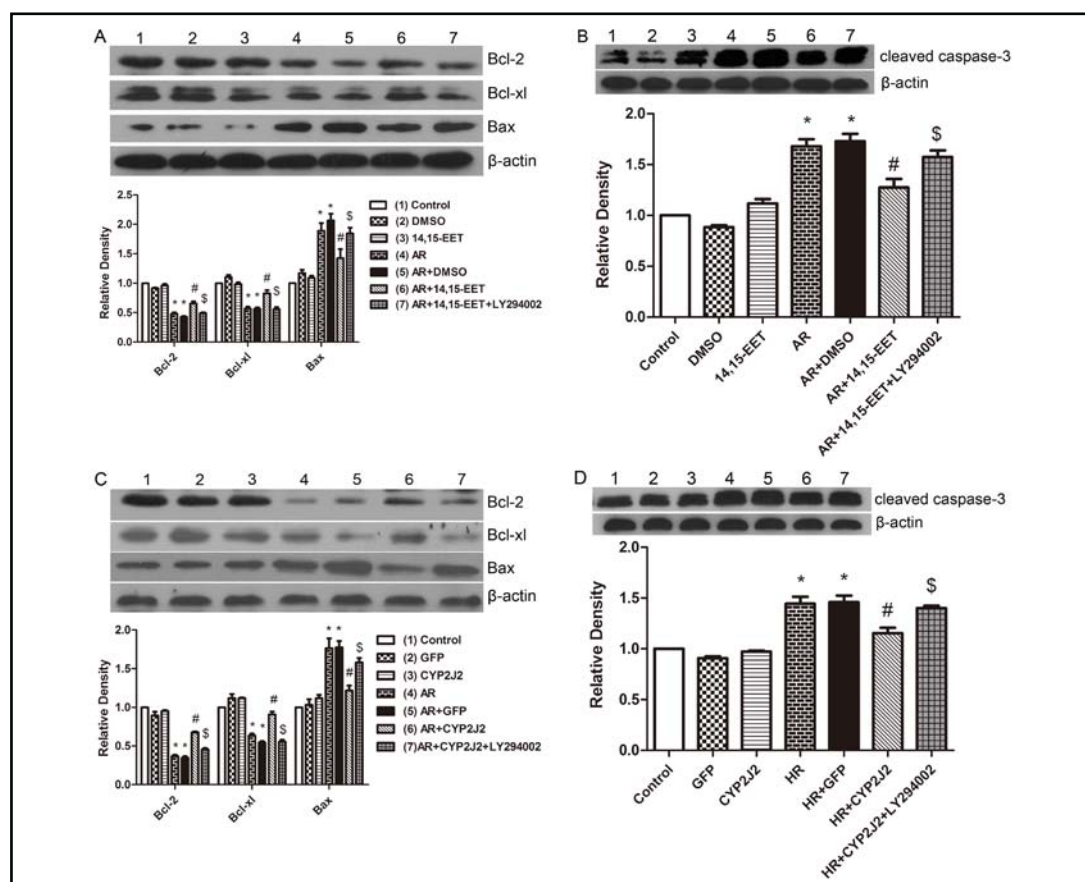Chen et al.: EETs and Lung Ischemia/Reperfusion Injury

Fig. 6. Effects of 14,15-EET and CYP2J2 overexpression on the expression of apoptosis-regulatory proteins in HPAECs. (A and B) Representative Western blots and densitometry results show altered levels of Bcl-2, Bcl-xl, Bax, and cleaved caspase-3 following exogenous administration of 14,15-EET, and the effects of LY294002. (n=3 for each experiment; *$p < 0.05$ vs. control; # $p < 0.05$ vs. AR or AR+ DMSO; $^{\$}p<0.05$ vs. AR+ 14,15-EET) (C and D) Representative Western blots and densitometry results show altered levels of Bcl-2, Bcl-xl, Bax, and cleaved caspase-3 following CYP2J2 overexpression, and effects of LY294002 (n=3 for each experiment; *$p < 0.05$ vs. control; #$p < 0.05$ vs. AR or AR+pcDNA3.1/GFP; $^{\$}p<0.05$ vs. AR+CYP2J2).

EETs significantly decreased apoptosis induced by anoxia/reoxygenation. This protective effect was notably inhibited by LY294002 or 14,15-EEZE (Fig. 4A and B). However, EETs did not show this anti-apoptotic effect in cells under normal conditions. Likewise, CYP2J2 overexpression demonstrated similar effects on apoptosis induced by anoxia/reoxygenation (Fig. 4C and D).

Previous studies have shown that collapse of mitochondrial transmembrane potential is a sign of early programmed cell death [27, 28]. Therefore, we determined mitochondrial transmembrane potential by JC-1 and analyzed it by flow cytometry. We found that mitochondrial transmembrane potential remarkably decreased in the anoxia/reoxygenation treated group as compared with the groups incubated under normal conditions, an effect which was reversed by 14,15-EET (Fig. 4E and F). The protective effect of 14,15-EET, however, was markedly suppressed by LY294002 or 14,15-EEZE. In addition, similar effects were found in the cells transfected with pcDNA3.1/CYP2J2 (Fig. 4G and H).

Effects of 14,15-EET and CYP2J2 overexpression on anti-oxidative and pro-oxidative enzymes in HPAECs

Oxidative stress plays a central role throughout the process of lung ischemia/reperfusion injury. We investigated the regulation of anti-oxidative and pro-oxidative enzymes by 14,15-
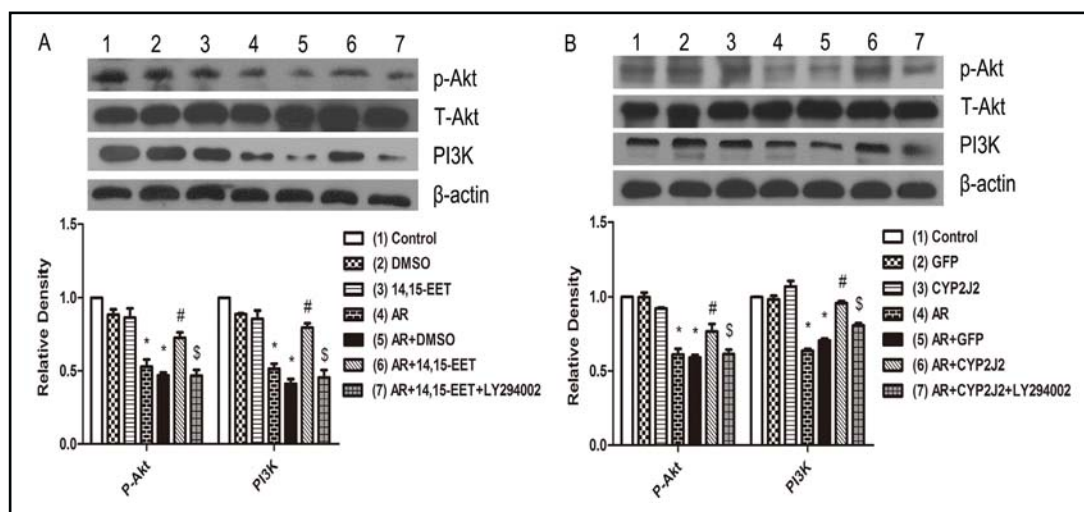
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Fig. 7. 14,15-EET and CYP2J2 overexpression notably promote activation of the PI3K/Akt signaling pathway. (A) Representative Western blots and densitometry results of phospho-Akt/Akt and PI3K/β–actin levels upon incubation with 14,15-EET, with or without LY294002 (n=3 for each experiment; *$p < 0.05$ vs. control; #$p < 0.05$ vs. AR or AR+ DMSO; $$p<0.05$ vs. AR+ 14,15-EET). (B) Representative Western blots and densitometry results of phospho-Akt/Akt and PI3K/β-actin levels upon CYP2J2 overexpression with or without LY294002 (*$p < 0.05$ vs. control; #$p < 0.05$ vs. AR or AR+GFP; $$p<0.05$ vs. AR+CYP2J2).
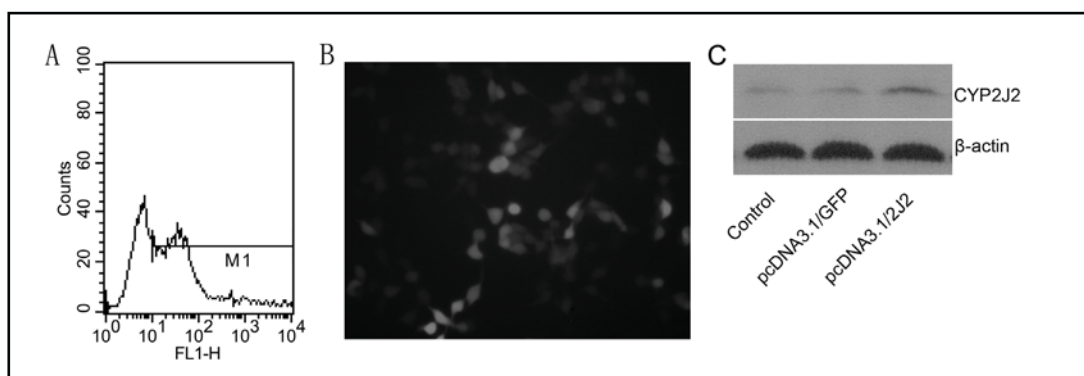

Fig. 8. Transfection of pcDNA3.1/CYP2J2 results in CYP2J2 overexpression in HPAECs. Transfection efficiency, detected by flow cytometry (A) and fluorescent microscopy (B), was maintained at 35-60% within 60 hours after transfection. (C) CYP2J2 protein was overexpressed after pcDNA3.1/CYP2J2 transfection, which was measured by Western blot analysis. The data are representative of 3 independent assays.

EET and CYP2J2 overexpression in HPAECs. The results indicated that 14,15-EET and CYP2J2 overexpression significantly attenuated the anoxia/reoxygenation-induced increase in gp91 (a transmembrane protein), p47, p67 and NOX4 protein levels (Fig. 5A, B, D and E). Those effects, however, were reversed by LY294002. In contrast, 14,15-EET and CYP2J2 overexpression remarkably increased the expression of the anti-oxidative proteins SOD1, SOD2, and catalase, which were obviously decreased in the anoxia/reoxygenation condition (Fig. 5C and F). However, these protective effects were markedly decreased by LY294002. Collectively, these results suggest that the anti-oxidative effect of 14,15-EET and CYP2J2 overexpression might be mediated by PI3K activation.

Effects of 14,15-EET and CYP2J2 overexpression on apoptosis-related protein expression in HPAECs treated with anoxia/reoxygenation

The expression of the anti-apoptotic proteins Bcl-2 and Bcl-xl was decreased after an-oxia/reoxygenation treatment in HPAECs, an effect which was reversed by 14,15-EET and
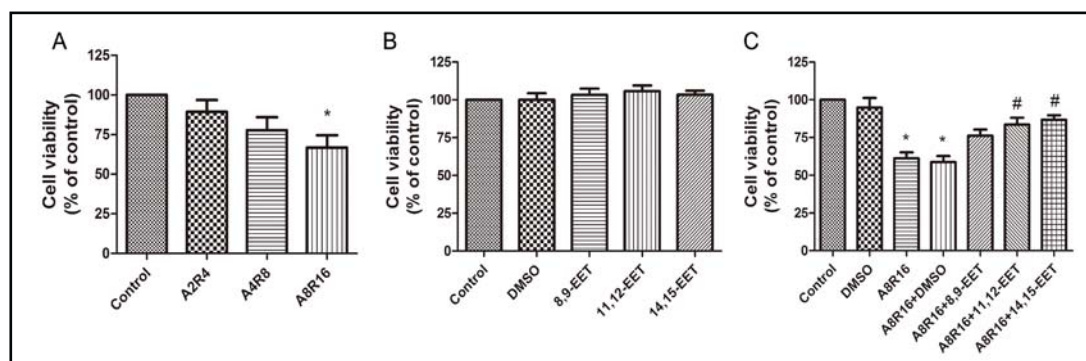
Fig. 9. Effects of exogenous EETs on viability of HPAECs. Cell viability was measured by cell counting kit-8 (CCK-8) assay. (A) Effects of anoxia/reoxygenation at different times on cell viability. Anoxia for 8 hours and reoxygenation for next 16 hours (A8R16) leads to significant decrease in cell viability. (B) Effects of EETs on cell viability in normal condition. (C) Effects of the combination of EETs and anoxia/reoxygenation on cell viability. (n=3 for each experiment; *p < 0.05 versus control; # p < 0.05 versus A8R16).

CYP2J2 overexpression (Fig. 6A and C). In contrast, the expression of Bax was increased after the anoxia/reoxygenation treatment, while this increase was attenuated by 14,15-EET and CYP2J2 overexpression (Fig. 6A and C). Moreover, the expression of cleaved caspase-3 was also increased after anoxia/reoxygenation treatment, while this increase was inhibited by 14,15-EET and CYP2J2 overexpression (Fig. 6B and D). Remarkably, the effects of 14,15-EET and CYP2J2 overexpression were blocked by LY294002, supporting a role of the PI3K/Akt signaling pathway in EET-mediated regulation of apoptosis.

Effects of 14,15-EET and CYP2J2 overexpression on the PI3K/Akt pathway in HPAECs treated with anoxia/reoxygenation

Anoxia/reoxygenation treatment significantly inhibited the expression of PI3K and the PI3K-dependant Akt phosphorylation in HPAECs, which were reversed by 14,15-EET and CYP2J2 overexpression (Fig. 7A and B). Interestingly, these effects of 14,15-EET and CYP2J2 overexpression were notably blocked by LY294002.

Taken together, these data indicate that exogenous 14,15-EET and CYP2J2 overexpression activate the PI3K/Akt signaling pathway, which may contribute to the anti-oxidative and anti-apoptotic effects observed *in vivo* and *in vitro* following lung ischemia/reperfusion.

Discussion

The current study tested the effect of the administration of exogenous EETs or CYP2J2 transfection on lung ischemia/reperfusion-induced oxidative stress and apoptosis *in vivo* and *in vitro*. It has been documented that CYP2J2 is predominantly expressed in human cardiomyocytes and endothelial cells [13, 29].Based on previous studies [13, 14], we chose the pcDNA3.1 plasmid as a vector to transfect CYP2J2. Immunohistochemical staining confirmed that CYP2J2 expression was remarkably elevated in lung tissues of transgenic rats, especially in vascular endothelial cells and epithelial cells. Elevated CYP2J2 effectively increased the concentration of circulating EETs. CYP2J2 overexpression in the lung significantly inhibited apoptosis and the levels of oxidative stress via regulating the expression of pro-oxidative (NADPH oxidase) and anti-oxidative enzymes (SOD1, SOD2, catalase). Therefore, we further investigated the effects of lung ischemia/reperfusion and its possible mechanisms *in vitro*. We found that anoxia/reoxygenation treatment attenuated the viability of human pulmonary artery endothelial cells in a time-dependent manner, an effect that was reversed by pre-incubation with exogenous EETs. Exogenous EETs, particularly 14,15-EET, and CYP2J2 overexpression, suppressed ROS production, inhibited apoptosis,

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and attenuated mitochondrial impairment induced by anoxia/reoxygenation in HPAECs. These effects were blocked by 14,15-EEZE and LY294002. Moreover, 14,15-EET and CYP2J2 overexpression significantly attenuated the anoxia/reoxygenation-induced increase in the expression of the pro-oxidative proteins gp91, p47, p67, and NOX4, the apoptosis-related protein Bax, and the activity of caspase-3, and inhibited the anoxia/reoxygenation-induced decrease in the expression of the anti-oxidative proteins SOD1, SOD2, and catalase, and the pro-survival proteins Bcl-2 and Bcl-xl. Furthermore, we demonstrated that these protective effects were mediated, at least in part, via the PI3K/Akt signaling pathway.

Unlike other organs, lungs acquire oxygen from both blood via a dual circulatory system and alveolar gas exchange. Hence, various animal models have been used to explore the mechanism and possible treatment of LIRI [30]. In this study, we developed a stable and reproducible lung ischemia/reperfusion model in rats, by clamping the left pulmonary hilum to create complete ischemia and anoxia. Due to the critical role of endothelial cells in LIRI [2, 31], we further developed a HPAECs-based anoxia/reoxygenation model *in vitro*.

Lung ischemia/reperfusion injury is a complex pathophysiologic process, in which oxidative stress and apoptosis play critical roles. During ischemia, endothelial cells and macrophages generate ROS, activate nicotinamide adenine dinucleotide phosphate (NADPH), nitric oxide synthases (NOS), nuclear factor-κB (NF-κB), and pro-inflammatory cytokines. When reperfusion occurs, ROS activates neutrophils. These reactions result in vascular damage, characterized by increased pulmonary vascular resistance and microvascular permeability [2], eventually leading to pulmonary edema and gas exchange dysfunction with severe ventilation-perfusion imbalance immediately following reperfusion [32]. It has also been reported that ROS cause an inflammatory response and dysfunction in remote organs, which further exacerbate ischemia/reperfusion injury [33]. In addition, apoptosis, which is only found during reperfusion, also plays an important role in the development of lung dysfunction [6, 34]. The intrinsic pathway of apoptosis, known as the mitochondrial pathway, is activated by oxidative stress [6]. Previous studies have shown that EETs can protect heart, brain, and aortic endothelial cells against ischemia/reperfusion injury [19, 20, 35]. However, the effect and mechanism of EETs in lung ischemia/reperfusion injury are still elusive.

ROS, such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen, are products of normal cellular metabolism [36]. It is well recognized that ROS play a dual role as both beneficial and harmful effects. ROS exert beneficial effects at low or moderate concentrations, and involve physiological roles, such as in defence against infectious agents, induction of a mitogenic response, and regulation of several cellular signaling pathways [36, 37]. On the contrary, overproduction of ROS leads to oxidative stress, a harmful process resulting in cellular structural damage through lipid peroxidation, inactivation of various proteins and DNA damage [2, 37]. Therefore, oxidative stress plays an important role in various human diseases.

During LIRI, mitochondria and NADPH oxidase are the most important pathways through which ROS are generated [2]. Mitochondria are not only a major source of ROS generation, but also sensitive targets for oxygen radical [38]. During lung ischemia/reperfusion, mitochondrial damage occurs mainly during ischemia and not reperfusion [39]. During ischemia, the lack of oxygen ends ATP synthesis. The rapid depletion of ATP causes a decrease in mitochondrial membrane potential, triggering apoptotic process and ROS overproduction [2]. Excessive ROS causes mitochondrial outer membrane permeabilization, leading to release of cytochrome c and overexpression of pro-apoptotic proteins, which can further trigger caspase activation and cell apoptotis [38].

NADPH oxidase is present in endothelial cells, macrophages and vascular smooth muscle cells, including gp91[phox], p47[phox], p67[phox], p40[phox], p22[phox], and Rac subunits [40]. Gp91[phox], located in the plasma membrane, is the main functional subunit. NADPH oxidase is also a major source of ROS in endothelial cells during lung ischemia [41, 42]. The rapid depletion of ATP causes inactivation of ATP-sensitive K^+ channels, resulting in endothelial cell membrane

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depolarization, followed by the activation of endothelial cell-localized NADPH oxidase [43], with subsequent increased production of ROS [44]. It has been demonstrated that inactivation of NADPH oxidase can attenuate LIRI in rat and pig models of lung ischemia/reperfusion [45, 46].

It is well known that the PI3K/Akt signaling pathway plays an important role in promoting cell proliferation and anti-apoptosis via regulating the expression of Bcl-2 family proteins, which is located in mitochondrial outer membrane [47, 48]. Although lack of detailed mechanisms, previous studies have confirmed that EETs activate the PI3K/Akt signaling pathway and protect against apoptosis in pulmonary artery endothelial cells [49, 50]. Activation of the PI3K/Akt pathway has emerged as a critical event for survival and proliferation in various ischemia/reperfusion models [19, 51]. PI3K has also been proved to regulate NADPH oxidase assembly and activation via its products PtdIns(3,4)P2 and PtdIns3P binding directly to oxidase subunits [40]. It has been reported that melatonin blocks the translocation p47[phox] and p67[phox] to the membrane via a PI3K/Akt-dependent signaling pathway, inhibits the binding of p47[phox] to gp91[phox], and impairs NADPH oxidase assembly, eventually resulting in inhibition of ROS production [52]. Therefore, PI3K/Akt inhibits apoptosis at a pre-mitochondrial level via regulating the activity of NADPH oxidase.

In the current study, we found that lung ischemia/reperfusion led to mitochondrial dysfunction and activation of NADPH oxidase, resulting in ROS overproduction. Excessive ROS further damaged mitochondria, triggering apoptotic process via regulating the expression of Bcl-2 family proteins and activation of caspase-3. These deleterious effects induced by lung ischemia/reperfusion were attenuated by either CYP2J2 overexpression or exogenous EETs. Moreover, the protective effects of CYP2J2 and EETs were blocked by LY294002. Taken together, these results indicated that the protective effects of EETs might be mediated, at least partially, by the PI3K/Akt pathway. EETs might inhibit the activity of NADPH oxidase and promote the expression of anti-oxidative proteins following lung ischemia/reperfusion via activating the PI3K/Akt pathway, leading to inhibition of ROS production which further alleviated mitochondria dysfunction. This would decrease ROS generation by mitochondria which further blocked apoptotic process [53]. In addition, EETs might promote cell proliferation and inhibit cell apoptosis via PI3K/Akt activation, as previously reported [18].

Since lung IR injury is a complicated process, further studies are needed to clarify whether EETs also affect other events including inflammation, intracellular calcium overload, leukocyte activation, and the corresponding signaling pathways, similar to its involvement with PI3K/Akt following ischemia/reperfusion.

In conclusion, our results demonstrate the protective effects of CYP2J2 expression and its metabolites, EETs, on oxidative stress and apoptosis after lung ischemia/reperfusion. The mechanisms involved may include reduction of the intracellular ROS levels, mitigation of mitochondrial dysfunction, and suppression of cell apoptosis. CYP2J2 overexpression or administration of exogenous EETs could be a novel strategy for the prevention and treatment of lung ischemia/reperfusion injury.

Disclosure Statement

The authors have no conflict of interests to declare.

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