EETs and CYP2J2 inhibit TNF-α-induced apoptosis in pulmonary artery endothelial cells and TGF-β1-induced migration in pulmonary artery smooth muscle cells

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Abstract. Cytochrome P450 epoxygenase-derived epoxyeicosatrienoic acids (EETs) have multiple biological functions in cardiovascular homeostasis. The anti-inflammatory, anti-migratory and pro-proliferative effects of EETs suggest a possible beneficial role for EETs in the apoptosis, proliferation and migration of pulmonary vascular cells. In this study, we investigated the effects of exogenous EETs and cytochrome P450 2J2 (CYP2J2) overexpression on tumor necrosis factor-α (TNF-α)-induced pulmonary artery endothelial cell (PAEC) apoptosis, and transforming growth factor-β1 (TGF-β1)-induced pulmonary artery smooth muscle cell (PASMC) proliferation and migration. PAECs and PASMCs were cultured from porcine pulmonary arteries. Our findings indicated that EETs or CYP2J2 overexpression significantly protected the PAECs from TNF-α-induced apoptosis, as evaluated by cell viability and flow cytometry. Two mechanisms were found to be involved in these important protective effects: firstly, EETs and CYP2J2 overexpression inhibited the decrease in the expression of the antiapoptotic proteins, Bcl-2 and Bcl-xL, as well as the increase in the expression of the pro-apoptotic protein, Bax, mediated by TNF-α; secondly, they activated the phosphoinositide 3-kinase (PI3K)/Akt and extracellular signal-regulated kinase (ERK) signaling pathways. We also found that 11,12-EET and 14,15-EET significantly inhibited TGF-β1-stimulated PASMC migration. However, EETs did not suppress TGF-β1-induced PASMC proliferation *in vitro*. These data may represent a novel approach to mitigate pulmonary vascular remodeling in diseases, such as pulmonary arterial hypertension.

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Introduction

Pulmonary vascular remodeling is one of the most important pathological changes in patients with pulmonary arterial hypertension (PAH). Endothelial cells are recognized as major regulators of vascular function. The balance between endothelial cell survival and death is critical in various processes, such as the regulation of vasoconstriction and vasodilation, smooth muscle cell growth and migration, thrombotic formation, intravascular inflammation and vascular remodeling (1,2). Inflammation is one of the main features of PAH, and circulating levels of cytokines, including tumor necrosis factor-α (TNF-α), are elevated in patients with PAH (3,4). During the early stages of vascular remodeling, inflammatory infiltration directly participates in the apoptosis of endothelial cells. Considerable experimental evidence suggests that the apoptosis of endothelial cells induces the release of mediators, in particular, transforming growth factor-β1 (TGF-β1), which induces the proliferation and migration of vascular smooth muscle cells (5,6). Sturrock *et al* (7) demonstrated that TGF-β1 is abundantly expressed in patients with pulmonary hypertension, and promotes pulmonary artery smooth muscle cell (PASMC) proliferation in low serum medium. The inhibition of TGF-β1 signaling has been demonstrated to attenuate pulmonary vascular remodeling and increase right ventricular pressure in animal models (8).

Arachidonic acid (AA) is an essential polyunsaturated fatty acid which is esterified to membrane phospholipids. Four epoxyeicosatrienoic acid (EET) regioisomers (5,6-, 8,9-, 11,12- and 14,15-EET) are the metabolites of AA by the cytochrome 450 (CYP) monooxygenase pathway (9,10). Similar to many eicosanoids, EETs have multiple biological functions, including the regulation of cardiovascular inflammation, the reduction of blood pressure, as well as anti-atherosclerotic functions in multiple model systems. EETs inhibit endothelial activation and leukocyte adhesion by suppressing nuclear factor (NF)-κB activation (11), thus activating peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ (12-14), and increasing heme oxygenase (HO)-1 expression (15). In addition to their potent vasodilatory action and anti-inflammatory effects, EETs have been associated with a number of physiolog-

ical functions, such as vascular protection and cardiovascular homeostasis. EETs upregulate endothelial nitric oxide synthase (eNOS) expression and activity (16), and stimulate angiogenesis and endothelial cell growth (17,18).

Evidence suggests that EETs exert many protective effects on systemic vascular homeostasis (19). However, the precise mechanisms responsible for the effects of EETs on pulmonary vascular homeostasis remain to be elucidated. Our group recently demonstrated the protective effects of gene delivery with cytochrome P450 2J2 (CYP2J2) on monocrotaline-induced PAH in rats (20). In the present study, porcine pulmonary artery endothelial cells (PAECs) and PASMCs were used to evaluate the effects of exogenous EETs or CYP2J2 overexpression on TNF-α-induced PAEC apoptosis and TGF-β1-induced PASMC proliferation and migration. In other words, this model was utilized to examine the hypothesis that exogenous EETs or CYP2J2 overexpression may attenuate pulmonary vascular remodeling.

Materials and methods

Experimental reagents. FBS, DMEM and trypsin were purchased from Invitrogen (Carlsbad, CA, USA). Collagenase II and the cell counting kit-8 (CCK-8) were purchased from Beyotime Biotechnology (Shanghai, China). TNF-α, TGF-β1, phosphoinositide 3-kinase (PI3K) inhibitor (LY294002), mitogen-activated protein kinase (MAPK) inhibitor (apigenin) and extracellular signal-regulated kinase (ERK) inhibitor (PD98059) were obtained from Sigma-Aldrich (St. Louis, MO, USA). EETs were from Cayman Chemical Co. (Ann Arbor, MI, USA). Antibody against CYP2J2 was purchased from Abcam Inc. (Cambridge, MA, USA). Antibody directed against PI3K was obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Other antibodies used in this study were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The Annexin V-FITC Apoptosis Detection Kit was purchased from KeyGen Biotech, Co. (Nanjing, China), and the Annexin V PE Apoptosis Detection Kit was from eBioscience, Inc. (San Diego, CA, USA). All other reagents were purchased from standard commercial suppliers unless otherwise indicated.

Cell culture and experimental incubation. PAECs and PASMCs were cultured from porcine pulmonary arteries as previously described (18,21). PAECs and PASMCs, which were used between passages 2 and 6, were isolated from porcine pulmonary arteries obtained from a local abattoir within 1 h of slaughter. Endothelial cells were digested with 1.7 mg/ml collagenase II, then the pulmonary arteries were cut open along the longitudinal axis, and the residual endothelium was gently removed by scraping the luminal surface and washed away with phosphate-buffered saline (PBS). The adventitia layer was removed by blunt dissection, and the medial smooth muscle tissue was minced into 1 mm^3 explant pieces. PAECs and the explant pieces of smooth muscle were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 U/ml), 5 mM L-glutamine and 1.5 g/l sodium bicarbonate. The medium was changed every 2 days. The cells were cultured in an incubator at 37˚C and maintained in a humidified atmosphere with 5% CO_2. The purity and identity of the endothelial cells and smooth muscle cells were verified by their typical morphological patterns and by immunofluorescent staining for factor VIII-related antigen and α-smooth muscle actin (α-SMA), respectively (16,21). All passages were performed using 0.05% trypsin and 0.02% EDTA. Cell viability was assessed using the CCK-8 assay.

Treatment of PAECs and PASMCs. Prior to each experiment, the PAECs and PASMCs were quiesced for 24 h in DMEM without serum. The PASMCs were treated with TGF-β1 in low serum (0.4%), and PAECs were treated with TNF-α in 10% FBS in the presence/absence of a physiologically relevant concentration [as previously described (22)] of EETs (250 nM) for 24 h. Alternatively, the PAECs were infected with recombinant adeno-associated viral vector (rAAV)-green fluorescent protein (GFP) or rAAV-CYP2J2 for 5 days to obtain maximal expression and then incubated with TNF-α. To investigate the anti-apoptotic effects of CYP2J2 or EETs and the possible signaling mechanisms involved, the infected PAECs were treated for 45 min with/without PI3K inhibitor (LY294002, 20 μM), ERK inhibitor (PD98059, 20 μM) or MAPK inhibitor (apigenin, 20 μM), followed by incubation with TNF-α (10 ng/ml) for 24 h. Alternatively, following pre-treatment with/without LY294002, PD98059 or apigenin, the cells were incubated with 14,15-EET (250 nM) for 30 min prior to treatment with TNF-α.

Recombinant adeno-associated virus. The PAECs were infected with rAAV-CYP2J2 or rAAV-GFP (~50 virions/cell), packed and purified as previously described (16,18), and grown for 5 days to obtain maximal expression. Recombinant adeno-associated viral vectors containing GFP or CYP2J2, pcDNA3.1-GFP plasmids and pcDNA3.1-CYP2J2 plasmids were prepared as previously described (23). The transfection efficiency of rAAV-GFP was detected by flow cytometry and fluorescence microscopy at 5 days post-infection.

Cell viability assay. Cell viability was measured using the CCK-8 assay, in which cellular dehydrogenase activity in the living cells was detected. Cell viability was expressed as a percentage of the value in the untreated control culture (24,25). All experiments were performed in triplicate on 3 separate occasions.

Flow cytometric analysis of apoptosis. As previously described (26,27), apoptotic responses were assessed by flow cytometry (Annexin V-FITC, propidium iodide and binding buffer) following the treatment of PAECs with 14,15-EET. As the emission wavelength of GFP and Annexin V-FITC are similar, we used the eBioscience Annexin V PE apoptosis detection kit (Annexin V-PE, 7-AAD viability staining solution, and binding buffer) to detect the apoptosis of virus-infected PAECs. All flow cytometry analyses were performed using commercially-available CellQuest software (BD Biosciences, San Jose, CA, USA).

Western blot analysis. The total expression levels and phosphorylation levels of signal transduction molecules were measured by western blot analysis as previously described (20). Protein expression was quantified by densitometry and normalized to β-actin expression.

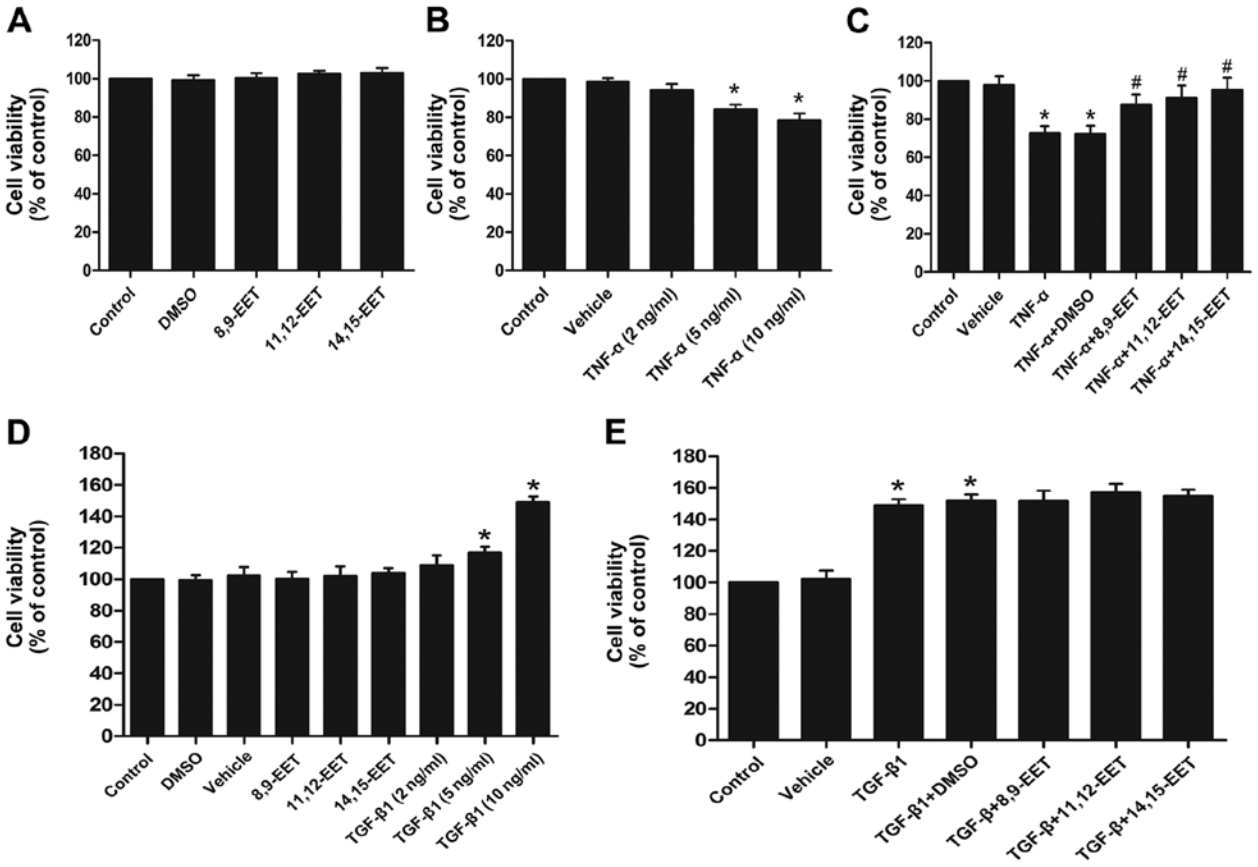

Figure 1. Effects of exogenous epoxyeicosatrienoic acids (EETs) on the viability of pulmonary artery endothelial cells (PAECs) and pulmonary artery smooth muscle cells (PASMCs). Cell viability was measured using the cell counting kit-8 (CCK-8) assay. (A) Effects of EETs on the cell viability of PAECs. (B) Effect of tumor necrosis factor-α (TNF-α) on cell viability of PAECs. (C) Effect of the combination of EETs and TNF-α on cell viability of PAECs. (D) Effect of EETs or transforming growth factor-β1 (TGF-β1) on cell viability of PASMCs. (E) Effect of the combination of EETs and TGF-β1 on cell viability of PASMCs. Data are presented as a percentage of untreated controls ± SEM (n=5); (*P<0.05 vs. control: #P<0.05 vs. TNF-α or vs. TNF-α + DMSO).

Ki-67 staining. The PASMCs were seeded on a 12-well plate to 80% confluence, then incubated with TGF-β1 and EETs as indicated above. After 24 h, the PASMCs were probed with anti-Ki-67 antibody as described previously (28). Hematoxylin was used to counterstain the nuclei. In all assays, negative controls were prepared using PBS instead of anti-Ki-67 antibody in order to exclude non-specific staining.

Cell migration assay. Migration assay was performed using Transwell chambers (Corning, Lowell, MA, USA) with a pore size of 8 μm (19,29,30). Briefly, the PASMCs were suspended in DMEM with 0.4% FBS at a concentration of $5x10^5$ cells/ml, and 0.2 ml aliquots of the cell suspension ($1x10^5$ cells) were added to the upper chambers. The cells in the upper chamber were incubated with EETs at 37˚C for 30 min, then TGF-β1 was added to the medium of the lower chamber that contained 0.6 ml of DMEM, 0.4% FBS. The migration lasted for 4 h at 37˚C in a CO_2 incubator. In order to determine the number of migrated cells, the cells on the upper surface of the filters were carefully scraped off with a cotton swab. The cells that had migrated through the filters were fixed to the membrane using 4% paraformaldehyde for 30 min, then stained with 0.1% crystal violet for 10 min at room temperature, and finally examined and photographed under a microscope (x200 magnification). The quantification of migrated cells was performed (5 randomly selected fields/Transwell from at least 3 Transwells/experiment).

Statistical analysis. All data was presented as the means ± SEM. Statistically significant differences between 2 groups were calculated using the Student's t-test, and one-way ANOVA was used to certify statistical differences among 3 groups; a P-value <0.05 was considered to indicate a statistically significant difference.

Results

Effects of exogenous treatment with EETs on viability of PAECs and PASMCs. Proliferation and apoptosis were measured using the CCK-8 assay. We wished to determine the effects of exogenous EETs on the viability of PAECs and PASMCs by comparing the responses of the 2 primary cell types to apoptosis or proliferation. In the PAECs, the physiologically relevant concentration of EETs (250 nM) had no significant effects on the cell viability of the control PAECs (Fig. 1A). TNF-α (5-10 ng/ml) induced a significant reduction in the number of viable cells (Fig. 1B), and EETs reversed the reduction in TNF-α (10 ng/ml)-induced cell viability (Fig. 1C). In the PASMCs, TGF-β1 (5-10 ng/ml) induced a significant increase in the number of viable cells (Fig. 1D). However, EETs

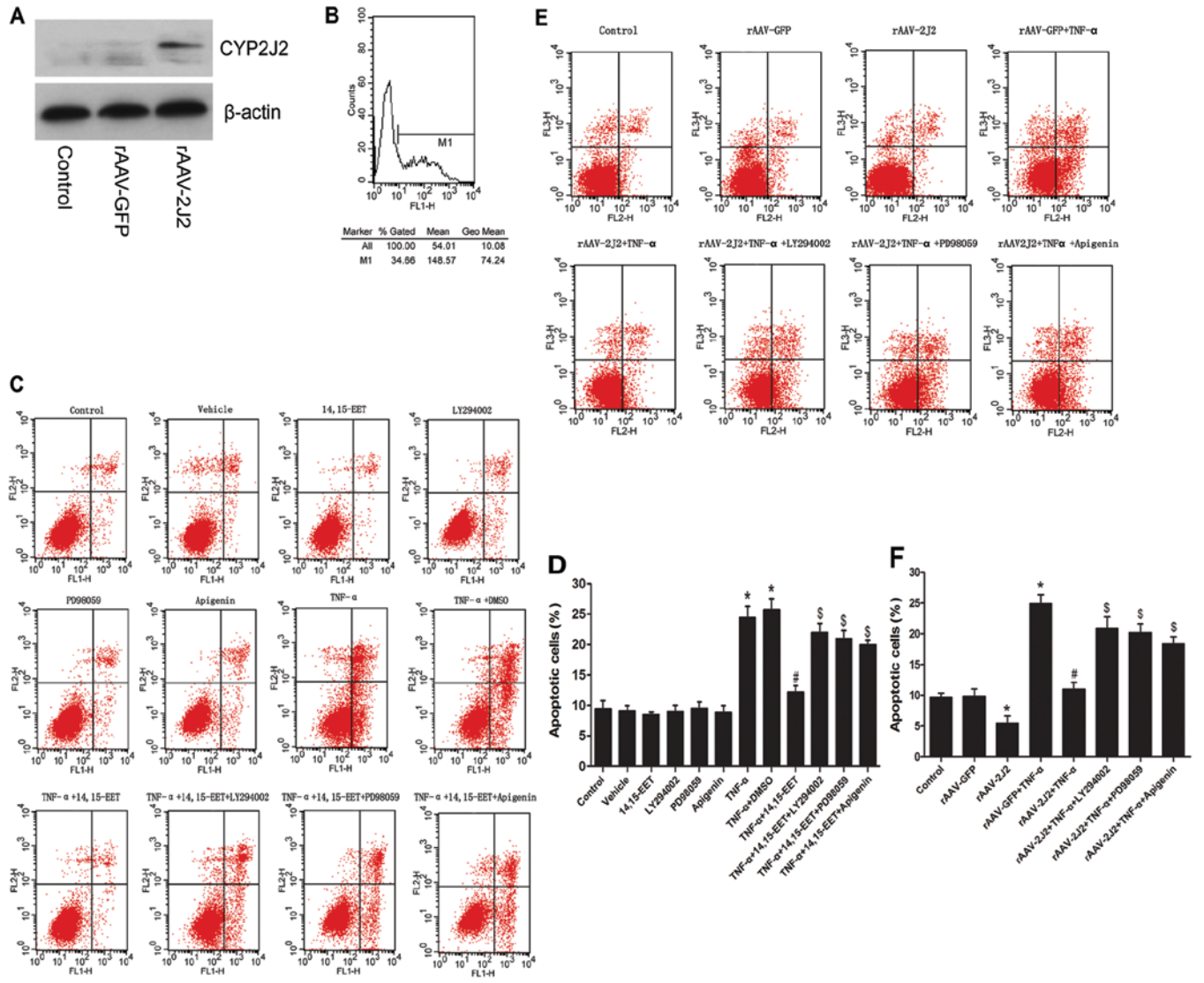

Figure 2. Effects of epoxyeicosatrienoic acids (EETs) and CYP2J2 overexpression on the apoptosis of pulmonary artery endothelial cells (PAECs). (A) CYP2J2 expression was measured by western blot analysis. (B) The transfection efficiency was maintained over 34% for 5 days post-transfection. (C) Density plots of Annexin V-FITC/PI staining based on flow cytometry. (D) Graph represents the mean number of apoptotic cells as a percentage of the control untreated cells ± SEM (n=3). The data are representative of 3 independent assays. (E) Density plots of Annexin V PE/7-AAD staining based on flow cytometry. (F) Graph represents the mean number of apoptotic cells as a percentage of the control untreated cells ± SEM. The data are representative of 3 independent assays (*P<0.05 vs. control or vs. rAAV-GFP; $^{\#}$P<0.05 vs. TNF-α + DMSO or vs. TNF-α + rAAV-GFP; $^{\$}$P<0.05 vs. TNF-α + 14:15-EET or vs. rAAV-CYP2J2 + TNF-α). TNF-α, tumor necrosis factor-α; rAAV, recombinant adeno-associated viral vector; GFP, green fluorescent protein.

(250 nM) did not reverse the increase in TGF-β1-induced cell viability (Fig. 1E). These data suggest that EETs inhibit PAEC apoptosis, but do not suppress PASMC proliferation.

EETs and CYP2J2 overexpression inhibits TNF-α-induced apoptosis of PAECs. To determine the transfection efficiency of rAAV-CYP2J2 in PAECs, the uptake of fluorescently labeled rAAV-GFP was detected by flow cytometry at 5 days post-transfection. The results revealed a high transfection efficiency (>34% cells displayed green fluorescence with rAAV-GFP) (Fig. 2B). The protein expression of CYP2J2 was examined by western blot analysis (Fig. 2A). CYP2J2 expression induced a marked increase in the protein levels in the rAAV-CYP2J2 group compared with rAAV-GFP. We also evaluated the effects of exogenous EETs and CYP2J2 overexpression on the apoptosis of PAECs by flow cytometry. Our results indicated that 14,15-EET partially abolished the apoptosis of PAECs induced by TNF-α (Fig. 2C and D). Likewise, the rAAV-CYP2J2 + TNF-α group showed significantly reduced cell apoptosis compared with the rAAV-GFP + TNF-α group (Fig. 2E and F). The anti-apoptotic effects of 14,15-EET or CYP2J2 overexpression were attenuated by LY294002 (PI3K inhibitor), PD98059 (ERK inhibitor) or apigenin (MAPK inhibitor) (Fig. 2C-F). These results demonstrate that the anti-apoptotic effects of EETs and CYP2J2 overexpression are mediated, at least in part, through the activation of PI3K, ERK and MAPK.

Effects of 14,15-EET and CYP2J2 overexpression on apoptosis-regulating protein expression in PAECs. The incubation of PAECs with a physiologically relevant concentration of

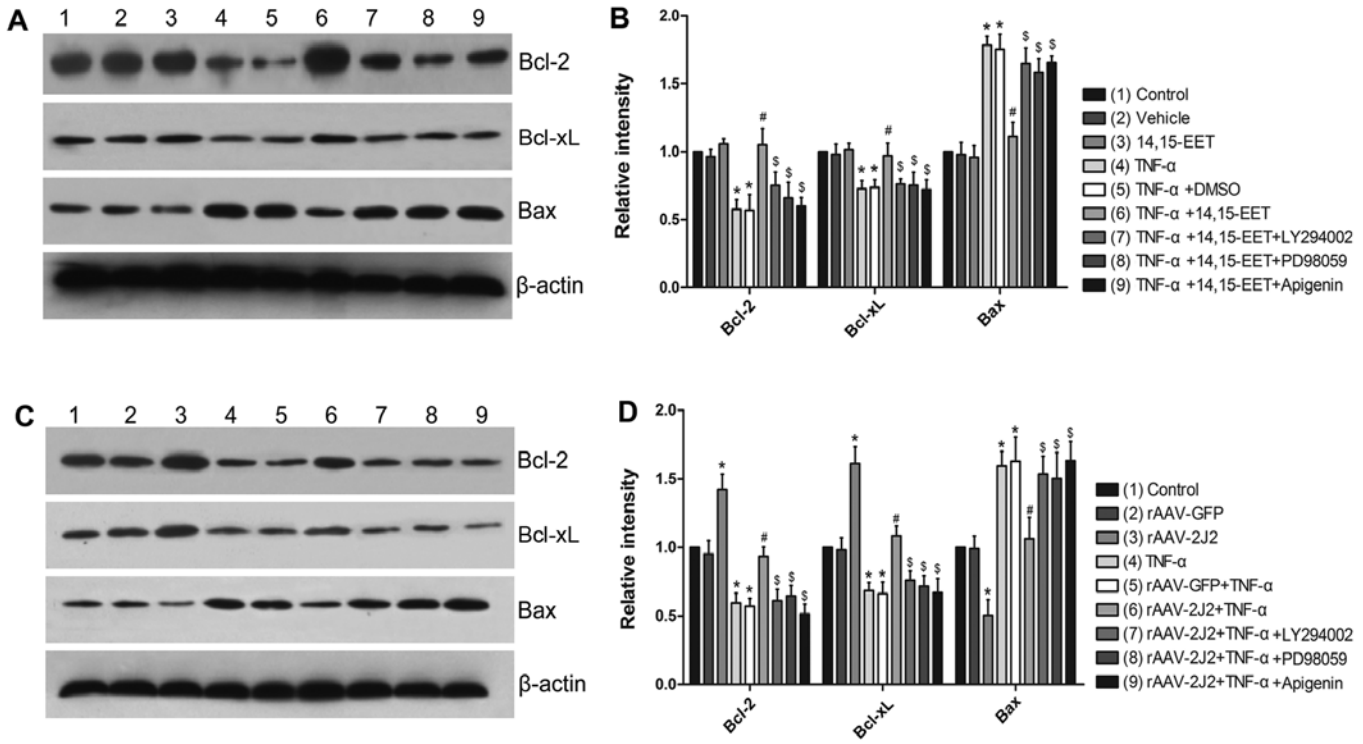

Figure 3. Effects of 14:15-EET and CYP2J2 overexpression on apoptosis-regulating protein expression in pulmonary artery endothelial cells (PAECs). (A and B) Representative western blots and densitometry results showing altered levels of Bcl-2, Bcl-xL and Bax following the exogenous administration of 14,15-EET, and the effects of LY294002, PD98059 or apigenin. (C and D) Representative western blots and densitometry results showing the altered levels of Bcl-2, Bcl-xL and Bax following CYP2J2 overexpression, and the effects of LY294002, PD98059 or apigenin (*P<0.05 vs. control or vs. rAAV-GFP; $^{\#}$P<0.05 vs. TNF-α + DMSO or vs. rAAV-GFP + TNF-α; $^{\$}$P<0.05 vs. TNF-α + 14:15-EET or vs. rAAV-CYP2J2 + TNF-α). TNF-α, tumor necrosis factor-α; rAAV, recombinant adeno-associated viral vector; GFP, green fluorescent protein.

14,15-EET had no effects on the baseline levels of Bcl-2, Bcl-xL and Bax, but significantly attenuated the TNF-α-induced downregulation of Bcl-2 and Bcl-xL expression, and prevented the upregulation of Bax (Fig. 3A and B). The overexpression of CYP2J2 in the PAECs induced a significant increase in the expression Bcl-2 and Bcl-xL, as well as a significant decrease in the baseline levels of Bax. Moreover, it also significantly inhibited the TNF-α-induced downregulation of Bcl-2, Bcl-xL and the TNF-α-induced upregulation of Bax (Fig. 3C and D). Furthermore, the effects of 14,15-EET and CYP2J2 overexpression were attenuated by LY294002, PD98059 or apigenin (Fig. 3), supporting the role of the ERK and PI3K/Akt signaling pathways in the EET-mediated regulation of apoptosis.

Effects of 14,15-EET and CYP2J2 overexpression on PI3K-dependent Akt phosphorylation and ERK1/2 pathway in PAECs. The treatment of PAECs with TNF-α inhibited the expression of PI3K and PI3K-dependent Akt phosphorylation; 14,15-EET did not significantly increase PI3K protein expression and Akt phosphorylation in the control cells, whereas 14,15-EET significantly increased PI3K protein expression and Akt phosphorylation in the presence of TNF-α (Fig. 4A and B). The effects of 14,15-EET were inhibited when the cells were pre-treated with LY294002 (a PI3K inhibitor) (Fig. 4A and B). Similarly, the overexpression of CYP2J2 significantly increased the expression of PI3K protein and Akt phosphorylation in the presence and absence of TNF-α, compared with the uninfected and rAAV-GFP-infected control cells. This effect was blocked by LY294002 (Fig. 4C and D). TNF-α also significantly reduced the phosphorylation of ERK1/2 in the PAECs. 14,15-EET did not significantly induce ERK1/2 phosphorylation, while 14,15-EET significantly induced ERK1/2 phosphorylation in the presence of TNF-α (Fig. 4A and B). The overexpression of CYP2J2 significantly increased ERK1/2 phosphorylation in the presence and absence of TNF-α (Fig. 4C and D). Furthermore, the effects of 14,15-EET and CYP2J2 overexpression on ERK1/2 phosphorylation were inhibited by PD98059 (an ERK inhibitor) and apigenin (a MAPK inhibitor) (Fig. 4C and D). Taken together, these results suggest that exogenous 14,15-EET and CYP2J2 overexpression activates the PI3K/Akt and ERK signaling pathways, which may contribute to the anti-apoptotic effects observed in the PAECs.

Effect of Ki-67 staining following stimulation of PASMCs with TGF-β1. There was a small percentage (~20%) of PASMCs entering the cell cycle in the untreated control cell culture (Fig. 5). However, there was a significant increase (~50%) in the number of Ki-67-positive cells after TGF-β1 (10 ng/ml) was administered for 24 h. However, EETs (250 nM) did not reverse the increase in the number of TGF-β1-induced Ki-67-positive cells (Fig. 5). These findings suggest that EETs does not suppress PASMC proliferation.

Effect of EETs on TGF-β1-induced PASMC migration. To investigate the effects of EETs on TGF-β1-induced PASMC

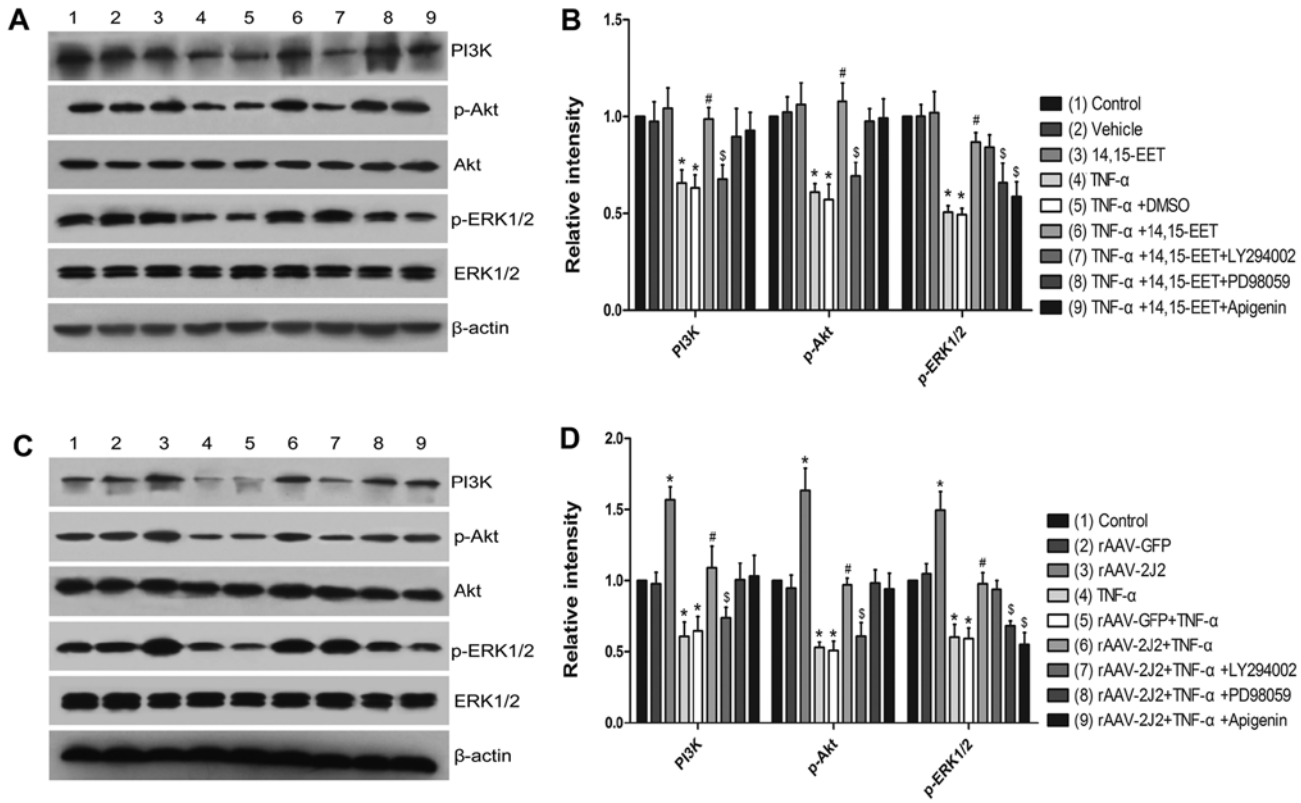

Figure 4. Effects of 14,15-EET and CYP2J2 overexpression on the activity of PI3K/Akt and extracellular signal-regulated kinase (ERK)1/2 in pulmonary artery endothelial cells (PAECs). (A and B) Representative western blots and densitometry results of PI3K/β-actin, phospho-Akt/Akt and phospho-ERK1/2/ERK1/2 upon 14,15-EET with or without LY294002, PD98059 or apigenin. (C and D) Representative western blots and densitometry results of PI3K/β-actin, phospho-Akt/Akt and phospho-ERK1/2/ERK1/2 upon CYP2J2 overexpression with or without LY294002, PD98059 or apigenin (*P<0.05 vs. control or vs. rAAV-GFP; $^{\#}$P<0.05 vs. TNF-α + DMSO or vs. rAAV-GFP + TNF-α; $^{\$}$P<0.05 vs. TNF-α + 14:15-EET or vs. rAAV-CYP2J2 + TNF-α). TNF-α, tumor necrosis factor-α; rAAV, recombinant adeno-associated viral vector; GFP, green fluorescent protein.

migration, a modified Transwell apparatus was used. The stimulation of the PASMCs with TGF-β1 (10 ng/ml) caused a ~3-fold increase in PASMC migration (Fig. 6B). At a concentration of 250 nM, 11,12-EET and 14,15-EET significantly inhibited the TGF-β1-induced PASMC migration. By contrast, 8,9-EET had no significant effect (Fig. 6B). These findings suggest that 11,12-EET and 14,15-EET attenuate the migration potential of PASMCs *in vitro*.

Discussion

Pulmonary vascular remodeling is characterized by endothelial dysfunction, abnormal muscularization of pulmonary arterioles, the upregulation of inflammatory cytokines and leukocyte infiltration (31,32). Inflammation plays an important role in various types of human PAH (33). TNF-α has been demonstrated as a key pro-inflammatory cytokine in severe pulmonary hypertension. Fujita *et al* (34) demonstrated that the overexpression of TNF-α results in severe pulmonary hypertension in mice. During the early stages of vascular remodeling, inflammatory cells infiltration directly participates in the apoptosis of endothelial cells. A body of evidence has demonstrated that the apoptosis of endothelial cells is an initiating mechanism for pulmonary vascular remodeling, and directly leads to microvascular obliteration (35,36). Moreover, increasing evidence suggests that pulmonary vascular remodeling may be reversed by inhibiting the apoptosis of endothelial cells during the early stages of PAH (37-39). In another study, Sakao *et al* (6) reported that the apoptosis of endothelial cells induces the release of mediators, in particular TGF-β1, which activates the proliferation and migration of vascular smooth muscle cells. Liu *et al* (40) showed that TGF-β1 increased the progression of cells from the G0/G1 phase to the G2/M + S phase and regulated the cell cycle progression of PASMCs. Considerable experimental evidence suggests that TGF-β1 mediates human PASMC proliferation in persistent hypoxia-induced PAH (41). However, it is not clear whether EETs exert anti-apoptotic effects on PAECs, particularly in the case of apoptosis induced by inflammatory cytokines (such as TNF-α). Moreover, the anti-proliferative and anti-migratory effects of EET on PASMCs remain elusive. Thus, in this study, we sought to evaluate the potential effects of exogenous EETs and CYP2J2 overexpression on TNF-α-induced PAEC apoptosis and TGF-β1-induced PASMC proliferation and migration.

Our findings demonstrated that EETs significantly reversed the TNF-α-induced reduction in cell viability. Moreover, EETs and CYP2J2 overexpression effectively protected the PAECs from TNF-α-induced apoptosis, as evaluated by FACS analysis. EETs and CYP2J2 overexpression significantly inhibited the downregulation of TNF-α-induced Bcl-2 and Bcl-xL protein expression, and the upregulation of TNF-α-induced Bax protein expression. These effects were attenuated by the addition of LY294002 (a PI3K inhibitor), PD98059 (an ERK inhibitor) and apigenin (a MAPK inhibitor). Furthermore,

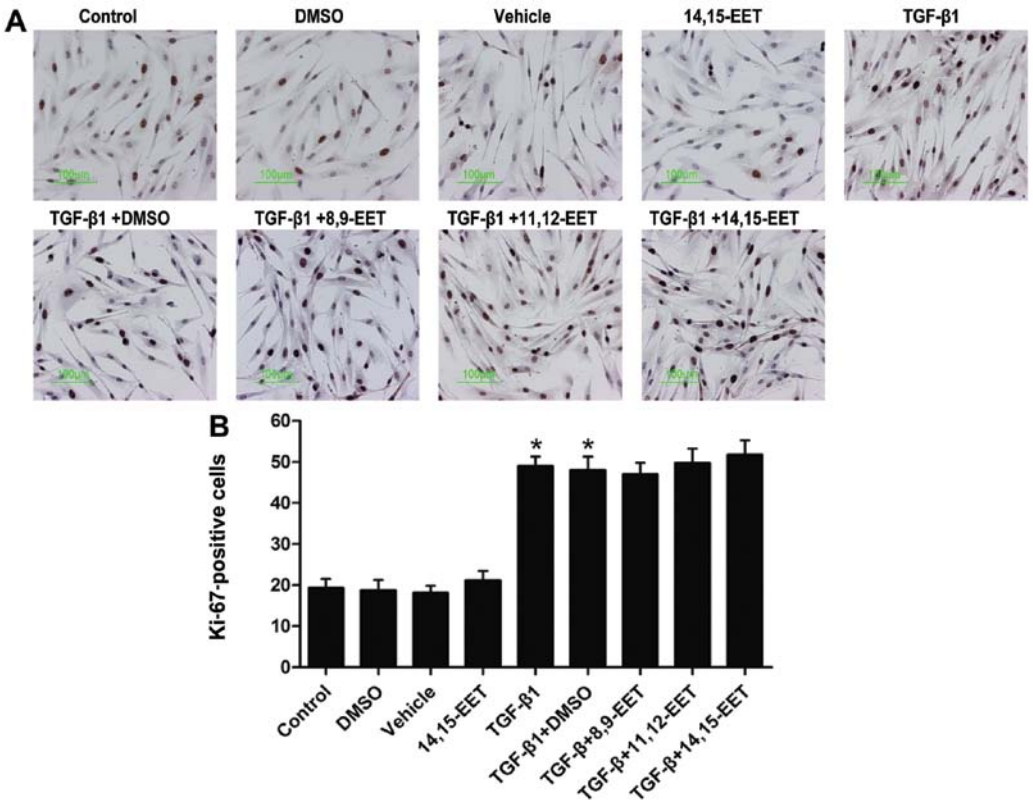

Figure 5. Effect of Ki-67 staining following the stimulation of pulmonary artery smooth muscle cells (PASMCs) with transforming growth factor-β1 (TGF-β1). (A) Representative Ki-67 immunohistochemical staining results of TGF-β1 stimulation of primary PASMCs. The specimens are shown at x200 magnification. (B) Data are presented as the means ± SEM (5 randomly selected fields/group). All experiments are performed in triplicate on 3 separate occasions (*P<0.05 vs. control or vs. vehicle).

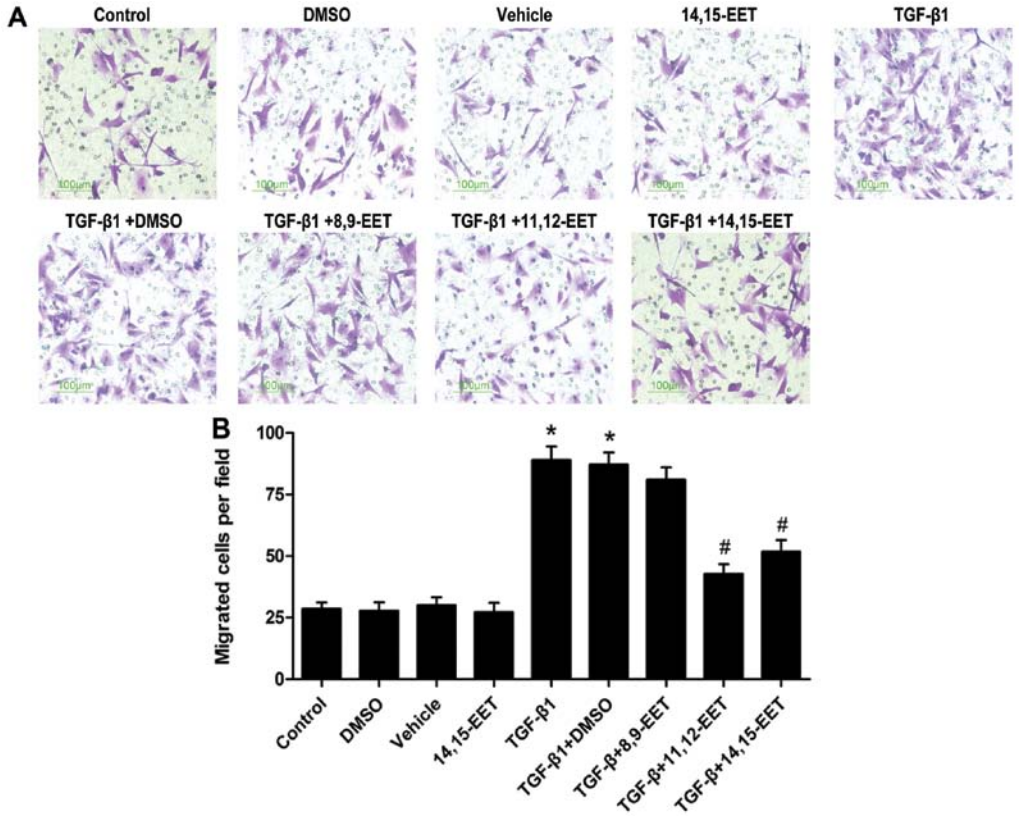

Figure 6. Effect of epoxyeicosatrienoic acids (EETs) on transforming growth factor-β1 (TGF-β1)-induced pulmonary artery smooth muscle cell (PASMC) migration. (A) 11,12-EET and 14,15-EET attenuated the TGF-β1-induced migration of PASMCs *in vitro*. Representative images of the mobility assay are shown (x200). (B) Bar graph of the mobility assay. Bar represents the mean number of the cells/field (*P<0.05 vs. control or vs. vehicle; $^{\#}$P<0.05 vs. TGF-β1 or vs. TGF-β1 + DMSO).

EETs and CYP2J2 overexpression significantly induced PI3K protein expression, Akt phosphorylation and ERK1/2 phosphorylation in the presence of TNF-α, and these effects were attenuated by the addition of LY294002, PD98059 and apigenin, respectively. These anti-apoptotic effects were significantly attenuated by the inhibition of the PI3K/Akt and MAPK signaling pathways, suggesting that the anti-apoptotic effects of EETS are mediated, at least in part, through the activation of the PI3K/Akt and MAPK signaling pathways.

The monocrotaline-induced primary pulmonary hypertension model, which has previously been used by Revermann *et al* (42), is highly toxic to endothelial cells in the lungs. It induced endothelial cell apoptosis, vascular inflammation, cellular proliferation and vascular remodeling. We have previously reported that CYP2J2 overexpression, which is known to increase EET biosynthesis, significantly ameliorated monocrotaline-induced PAH in rats (20). We found that CYP2J2 overexpression attenuated right ventricle systolic pressure (RVSP) and hypertrophy of the right ventricle (RV) and pulmonary vessel walls *in vivo*. However, our results did not favor any role of the anti-proliferative effects of EETs on TGF-β1-induced PASMCs *in vitro*, indicating that a more complex mechanism of inflammation may be present for PASMC proliferation. These results were supported by those from previous studies in cultured rat PASMCs, showing that 11,12-EET and soluble epoxide hydrolase (sEH) inhibition have no effect on platelet-derived growth factor (PDGF), serum or serotonin-induced smooth muscle cell proliferation (19). In addition, we also discovered that at a concentration of 250 nM 11,12-EET and 14,15-EET significantly inhibited the TGF-β1-induced PASMC migration. Taken together, these data indicate that EETs suppress pulmonary vascular remodeling mainly through the inhibition of PASMC migration, and not by inhibiting proliferation; it could also be speculated that the anti-proliferative effects of EETs *in vivo* are indirect in nature.

In conclusion, the present study reveals a novel role for the protective effects of EETs against pulmonary vascular remodeling. EETs exerted potent anti-apoptotic effects, markedly attenuating the TNF-α-induced apoptosis of pulmonary arterial endothelial cells. Two mechanisms were found to be involved in these important protective effects: firstly, EETs and CYP2J2 overexpression inhibited the decrease in the expression of the anti-apoptotic proteins, Bcl-2 and Bcl-xL, and the increase in the expression of the pro-apoptotic protein, Bax, mediated by TNF-α; secondly, EETs activated the PI3K/Akt and ERK signaling pathways. In addition, EETs inhibited the TGF-β1-induced PASMC migration. Our findings suggest that EETs may be a novel approach to the treatment of pulmonary vascular remodeling complications in diseases such as PAH.

Acknowledgments

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References

1. Stefanec T: Endothelial apoptosis: could it have a role in the pathogenesis and treatment of disease? Chest 117: 841-854, 2000.
2. Yang X, Long L, Reynolds PN and Morrell NW: Expression of mutant BMPR-II in pulmonary endothelial cells promotes apoptosis and a release of factors that stimulate proliferation of pulmonary arterial smooth muscle cells. Pulm Circ 1: 103-110, 2011.
3. Wort SJ, Ito M, Chou PC, *et al*: Synergistic induction of endothelin-1 by tumor necrosis factor alpha and interferon gamma is due to enhanced NF-kappaB binding and histone acetylation at specific kappaB sites. J Biol Chem 284: 24297-24305, 2009.
4. Soon E, Holmes AM, Treacy CM, *et al*: Elevated levels of inflammatory cytokines predict survival in idiopathic and familial pulmonary arterial hypertension. Circulation 122: 920-927, 2010.
5. Antonelli-Orlidge A, Saunders KB, Smith SR and D'Amore PA: An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. Proc Natl Acad Sci USA 86: 4544-4548, 1989.
6. Sakao S, Taraseviciene-Stewart L, Wood K, Cool CD and Voelkel NF: Apoptosis of pulmonary microvascular endothelial cells stimulates vascular smooth muscle cell growth. Am J Physiol Lung Cell Mol Physiol 291: L362-L368, 2006.
7. Sturrock A, Cahill B, Norman K, *et al*: Transforming growth factor-beta1 induces Nox4 NAD(P)H oxidase and reactive oxygen species-dependent proliferation in human pulmonary artery smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 290: L661-L673, 2006.
8. Ma W, Han W, Greer PA, *et al*: Calpain mediates pulmonary vascular remodeling in rodent models of pulmonary hypertension, and its inhibition attenuates pathologic features of disease. J Clin Invest 121: 4548-4566, 2011.
9. Wu S, Moomaw CR, Tomer KB, Falck JR and Zeldin DC: Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. J Biol Chem 271: 3460-3468, 1996.
10. Zeldin DC: Epoxygenase pathways of arachidonic acid metabolism. J Biol Chem 276: 36059-36062, 2001.
11. Node K, Huo Y, Ruan X, *et al*: Anti-inflammatory properties of cytochrome P450 epoxygenase-derived eicosanoids. Science 285: 1276-1279, 1999.
12. Pasceri V, Wu HD, Willerson JT and Yeh ET: Modulation of vascular inflammation in vitro and in vivo by peroxisome proliferator-activated receptor-gamma activators. Circulation 101: 235-238, 2000.
13. Wang N, Verna L, Chen NG, *et al*: Constitutive activation of peroxisome proliferator-activated receptor-gamma suppresses pro-inflammatory adhesion molecules in human vascular endothelial cells. J Biol Chem 277: 34176-34181, 2002.
14. Feige JN, Gelman L, Michalik L, Desvergne B and Wahli W: From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. Prog Lipid Res 45: 120-159, 2006.
15. Sacerdoti D, Colombrita C, Di Pascoli M, *et al*: 11,12-epoxyeicosatrienoic acid stimulates heme-oxygenase-1 in endothelial cells. Prostaglandins Other Lipid Mediat 82: 155-161, 2007.
16. Wang H, Lin L, Jiang J, *et al*: Up-regulation of endothelial nitric-oxide synthase by endothelium-derived hyperpolarizing factor involves mitogen-activated protein kinase and protein kinase C signaling pathways. J Pharmacol Exp Ther 307: 753-764, 2003.
17. Wang Y, Wei X, Xiao X, *et al*: Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways. J Pharmacol Exp Ther 314: 522-532, 2005.
18. Yang S, Lin L, Chen JX, *et al*: Cytochrome P-450 epoxygenases protect endothelial cells from apoptosis induced by tumor necrosis factor-alpha via MAPK and PI3K/Akt signaling pathways. Am J Physiol Heart Circ Physiol 293: H142-H151, 2007.
19. Sun J, Sui X, Bradbury JA, Zeldin DC, Conte MS and Liao JK: Inhibition of vascular smooth muscle cell migration by cytochrome p450 epoxygenase-derived eicosanoids. Circ Res 90: 1020-1027, 2002.
20. Zheng C, Wang L, Li R, *et al*: Gene delivery of cytochrome p450 epoxygenase ameliorates monocrotaline-induced pulmonary artery hypertension in rats. Am J Respir Cell Mol Biol 43: 740-749, 2010.
21. Tian X, Vroom C, Ghofrani HA, *et al*: Phosphodiesterase 10A upregulation contributes to pulmonary vascular remodeling. PLoS One 6: e18136, 2011.

22. Dhanasekaran A, Al-Saghir R, Lopez B, *et al*: Protective effects of epoxyeicosatrienoic acids on human endothelial cells from the pulmonary and coronary vasculature. Am J Physiol Heart Circ Physiol 291: H517-H531, 2006.
23. Yang B, Graham L, Dikalov S, *et al*: Overexpression of cytochrome P450 CYP2J2 protects against hypoxia-reoxygenation injury in cultured bovine aortic endothelial cells. Mol Pharmacol 60: 310-320, 2001.
24. Yuan P, Salvadore G, Li X, *et al*: Valproate activates the Notch3/c-FLIP signaling cascade: a strategy to attenuate white matter hyperintensities in bipolar disorder in late life? Bipolar Disord 11: 256-269, 2009.
25. Kim HY, Hwang JY, Kim SW, *et al*: The CXCR4 antagonist AMD3100 has dual effects on survival and proliferation of myeloma cells in vitro. Cancer Res Treat 42: 225-234, 2010.
26. Clark PE, Polosukhina DA, Gyabaah K, Moses HL, Thorburn A and Zent R: TRAIL and interferon-alpha act synergistically to induce renal cell carcinoma apoptosis. J Urol 184: 1166-1174, 2010.
27. Zhang M, He Z, Wen L, *et al*: Cadmium suppresses the proliferation of piglet Sertoli cells and causes their DNA damage, cell apoptosis and aberrant ultrastructure. Reprod Biol Endocrinol 8: 97, 2010.
28. He J and Bazan HE: Epidermal growth factor synergism with TGF-beta1 via PI-3 kinase activity in corneal keratocyte differentiation. Invest Ophthalmol Vis Sci 49: 2936-2945, 2008.
29. Merlo S, Frasca G, Canonico PL and Sortino MA: Differential involvement of estrogen receptor alpha and estrogen receptor beta in the healing promoting effect of estrogen in human keratinocytes. J Endocrinol 200: 189-197, 2009.
30. Xu C, Gui Q, Chen W, *et al*: Small interference RNA targeting tissue factor inhibits human lung adenocarcinoma growth in vitro and in vivo. J Exp Clin Cancer Res 30: 63, 2011.
31. Cool CD, Stewart JS, Werahera P, *et al*: Three-dimensional reconstruction of pulmonary arteries in plexiform pulmonary hypertension using cell-specific markers. Evidence for a dynamic and heterogeneous process of pulmonary endothelial cell growth. Am J Pathol 155: 411-419, 1999.
32. Yi ES, Kim H, Ahn H, *et al*: Distribution of obstructive intimal lesions and their cellular phenotypes in chronic pulmonary hypertension. A morphometric and immunohistochemical study. Am J Respir Crit Care Med 162: 1577-1586, 2000.
33. Wang Q, Zuo XR, Wang YY, Xie WP, Wang H and Zhang M: Monocrotaline-induced pulmonary arterial hypertension is attenuated by TNF-α antagonists via the suppression of TNF-α expression and NF-κB pathway in rats. Vascul Pharmacol 58: 71-77, 2013.
34. Fujita M, Shannon JM, Irvin CG, *et al*: Overexpression of tumor necrosis factor-alpha produces an increase in lung volumes and pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 280: L39-L49, 2001.
35. Zhao YD, Courtman DW, Deng Y, Kugathasan L, Zhang Q and Stewart DJ: Rescue of monocrotaline-induced pulmonary arterial hypertension using bone marrow-derived endothelial-like progenitor cells: efficacy of combined cell and eNOS gene therapy in established disease. Circ Res 96: 442-450, 2005.
36. Sage E, Mercier O, Van den Eyden F, *et al*: Endothelial cell apoptosis in chronically obstructed and reperfused pulmonary artery. Respir Res 9: 19, 2008.
37. Taraseviciene-Stewart L, Kasahara Y, Alger L, *et al*: Inhibition of the VEGF receptor 2 combined with chronic hypoxia causes cell death-dependent pulmonary endothelial cell proliferation and severe pulmonary hypertension. FASEB J 15: 427-438, 2001.
38. Teichert-Kuliszewska K, Kutryk MJ, Kuliszewski MA, *et al*: Bone morphogenetic protein receptor-2 signaling promotes pulmonary arterial endothelial cell survival: implications for loss-of-function mutations in the pathogenesis of pulmonary hypertension. Circ Res 98: 209-217, 2006.
39. Sun CK, Lee FY, Sheu JJ, *et al*: Early combined treatment with cilostazol and bone marrow-derived endothelial progenitor cells markedly attenuates pulmonary arterial hypertension in rats. J Pharmacol Exp Ther 330: 718-726, 2009.
40. Liu Y, Ma C, Zhang Q, *et al*: The key role of transforming growth factor-beta receptor I and 15-lipoxygenase in hypoxia-induced proliferation of pulmonary artery smooth muscle cells. Int J Biochem Cell Biol 44: 1184-1202, 2012.
41. Ismail S, Sturrock A, Wu P, *et al*: NOX4 mediates hypoxia-induced proliferation of human pulmonary artery smooth muscle cells: the role of autocrine production of transforming growth factor-{beta}1 and insulin-like growth factor binding protein-3. Am J Physiol Lung Cell Mol Physiol 296: L489-L499, 2009.
42. Revermann M, Barbosa-Sicard E, Dony E, *et al*: Inhibition of the soluble epoxide hydrolase attenuates monocrotaline-induced pulmonary hypertension in rats. J Hypertens 27: 322-331, 2009.