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15-HETE protects rat pulmonary arterial smooth muscle cells from apoptosis via the PI3K/Akt pathway

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ABSTRACT

15-Hydroxyeicosatetraenoic acid (15-HETE), a metabolic product of arachidonic acid (AA), plays an important role in pulmonary vascular smooth muscle remodeling. Although its effects on the apoptotic responses are known, the underlying mechanisms are still poorly understood. Since Akt is a critical regulator of cell survival and vascular remodeling, there may be a crosstalk between 15-HETE anti-apoptotic process and PI3K/Akt survival effect in rat pulmonary arterial smooth muscle cells (PASMCs). To test this hypothesis, we studied the effect of 15-HETE on cell survival and apoptosis using Western blot, cell viability measurement, nuclear morphology determination, TUNEL assay and mitochondrial potential analysis. We found that activation of the PI3K/Akt signaling system was necessary for the 15-HETE to suppress PASMC apoptosis and improve cell survival. Our results indicated that 15-HETE inhibited the apoptotic responses of PASMCs, including morphological alterations, mitochondrial depolarization and the expression apoptosis-specific proteins. These effects were likely to be mediated through the activation of PI3K/Akt. Two downstream signal molecules of PI3K/Akt were identified. Both FasL and Bad were down-regulated by 15-HETE and 15-HETE phosphorylated Bad. These changes depended on the PI3K/Akt signaling pathway in PASMCs. Thus a signal transduction pathway was demonstrated which is necessary for the effects of 15-HETE in protection PASMCs from apoptosis.

1. Introduction

Chronic exposure to moderate hypoxia results in the development of pulmonary hypertension (PH) [1,2]. The initial event is pulmonary vasoconstriction, followed by structural alterations in pulmonary arteries (PA) [3,4]. Remodeling of pulmonary arterial smooth muscle cell (PASMC) is a key feature known to result from an imbalance between apoptosis and cell proliferation [5].

Hypoxia up-regulates activity of 15-lipoxygenase (15-LO) that catalyzes the production of 15-hydroxyeicosatetrienoic acid (15-HETE) from arachidonic acid. 15-HETE contributes to pulmonary vascular remodeling and pulmonary vascular resistance by regulating not only proliferation but also apoptosis [6,7]. However, the mechanisms underlying the anti-apoptotic effects of 15-HETE are not clear.

It is known that Akt regulates cell growth and survival. The survival effects of Akt are mediated by inhibition of several pro-apoptotic proteins, including FasL, Bad and caspase-9 [8–11]. The potential role of Akt in the anti-apoptotic effect of 15-HETE has not been explored; it is unclear whether Akt plays a role in regulating PASMC fate, and the downstream elements of the Akt signaling remain to be determined. To address these issues, we carried out these studies. Our results suggested that 15-HETE inhibits apoptosis via activation of the PI3K/Akt signaling that modulates the pro-apoptotic mediators FasL and Bad and protects PASMCs against hypoxic injury.

2. Materials and methods

2.1. Materials

5-HETE, 12-HETE, 15-HETE obtained from Cayman Chemical (Ann Arbor, USA) were dissolved in ethanol and stored at a nitrogen freezer. Nordihydroguaiaretic acid (NDGA) and N,N-diisopropylethylamine were purchased from Sigma–Aldrich

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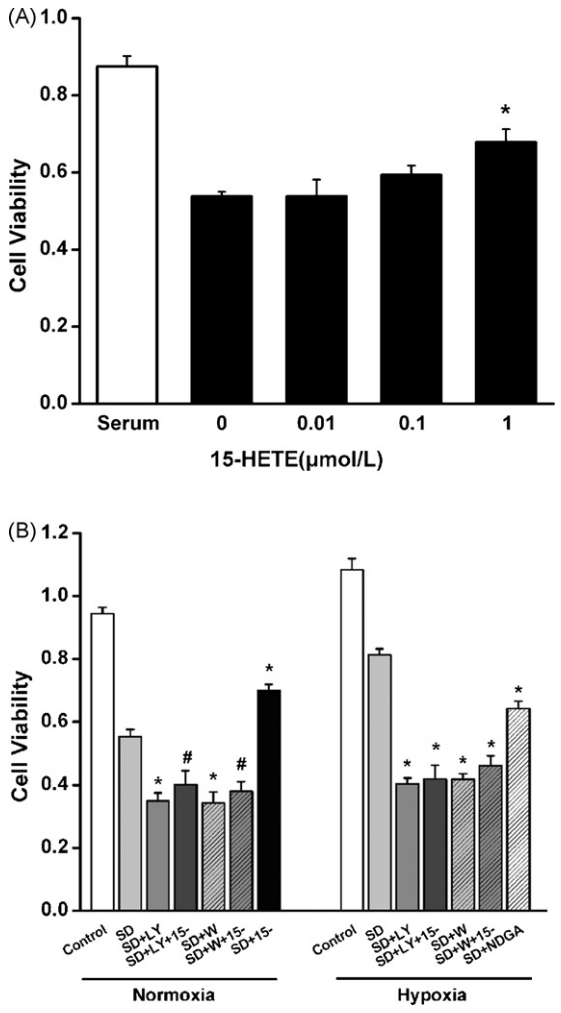

Fig. 1. (A) Effect of exogenous 15-HETE on the pulmonary artery smooth muscle cell (PASMC) survival. Cells were growth-arrested for 24 h and then treated with 15-HETE (0.01–1 μM) subjected to serum withdrawal. (B) 15-HETE promoted the survival of PASMCs in Akt-dependent manner. Cells were growth-arrested for 24 h and then exposured to normoxia or hypoxia in presence with 15-HETE (1 μM), LY294002 (20 μM), 15-HETE plus LY294002 (20 μM), wortmannin (10 μM), 15-HETE plus wortmannin (10 μM) or NDGA (30 μM), which were added every 24 h under serum-depleted conditions. Control cells were cultured in complete medium (DMEM with 10% FBS). Cell viability was determined by MTT assay after the indicated treatments for 48 h. All values are denoted as means ± S.E.M. from three or more independent batches of cells. "SD" means serum deprivation, "15-" means 15-HETE, "LY" means LY294002, "W" means wortmannin. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with serum deprived cells in the presence of 15-HETE.

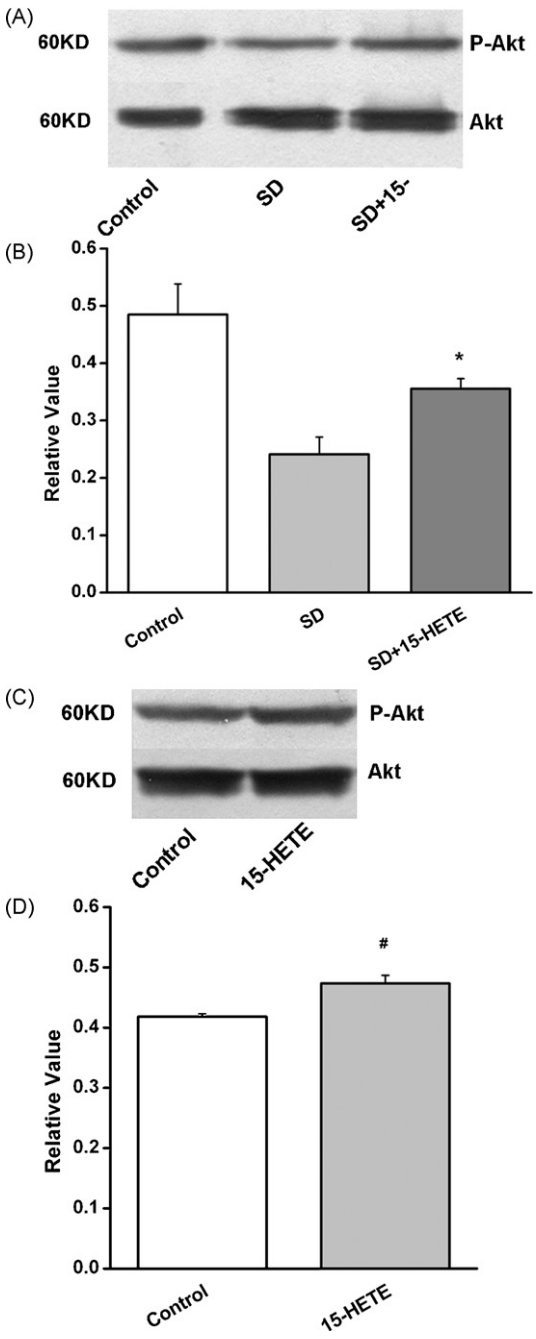

Fig. 2. 15-HETE induced activation of Akt in rat pulmonary artery smooth muscle cells (PASMCs). (A) 15-HETE up-regulated the expression of phospho-Akt in apoptotic PASMCs. Cells were growth-arrested for 24 h and then stimulated with 15-HETE (1 μM) under serum-free conditions. (B) Densitometric analysis of the Western blot assays. (C) 15-HETE increased phospho-Akt expression in non-apoptotic PASMCs. Cells were growth-arrested for 24 h and then treated with 15-HETE (1 μM) in serum-containing medium. (D) Densitometric analysis of the Western blot assays. Results are expressed as mean ± S.E.M. from at least three separate experiments. "SD" means serum deprivation, "15-" means 15-HETE. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with control cells cultured in complete medium.

Co. (Missouri, USA). 2-(2,3-naphthalimino)ethyl trifluoromethanesulfonate was purchased from Invitrogen Inc. (Oregon, USA). 20-5(Z),14(Z)-hydroxyeicosadienoic acid (WIT-002) was synthesized by Taisho Pharmaceutical (Saitama, Japan). Antibodies against Akt, procaspase-3 and FasL were purchased from Santa Cruz Biotechnology Inc. (California, USA). Antibodies specific for phospho-Akt, cleaved caspase-3 and phospho-Bad were from Cell Signaling Technology (Massachusetts, USA). Polyclonal antibody against Bad was purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). The TUNEL cell apoptosis detection kit and JC-1 probe were provided by Beyotime Institute of Biotechnology (Haimen, China). Enhanced chemiluminesence (ECL) reagents were purchased from Amersham International (Amersham, UK). All other reagents were from Sigma–Aldrich Co. (Missouri, USA).

2.2. Animals

Experiments were in full compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Adult male Wistar rats with a mean weight of 200 g were from the Animal Research Center of Harbin Medical University, which is fully accredited by the Institutional Animal Care and Use Committee (IACUC).

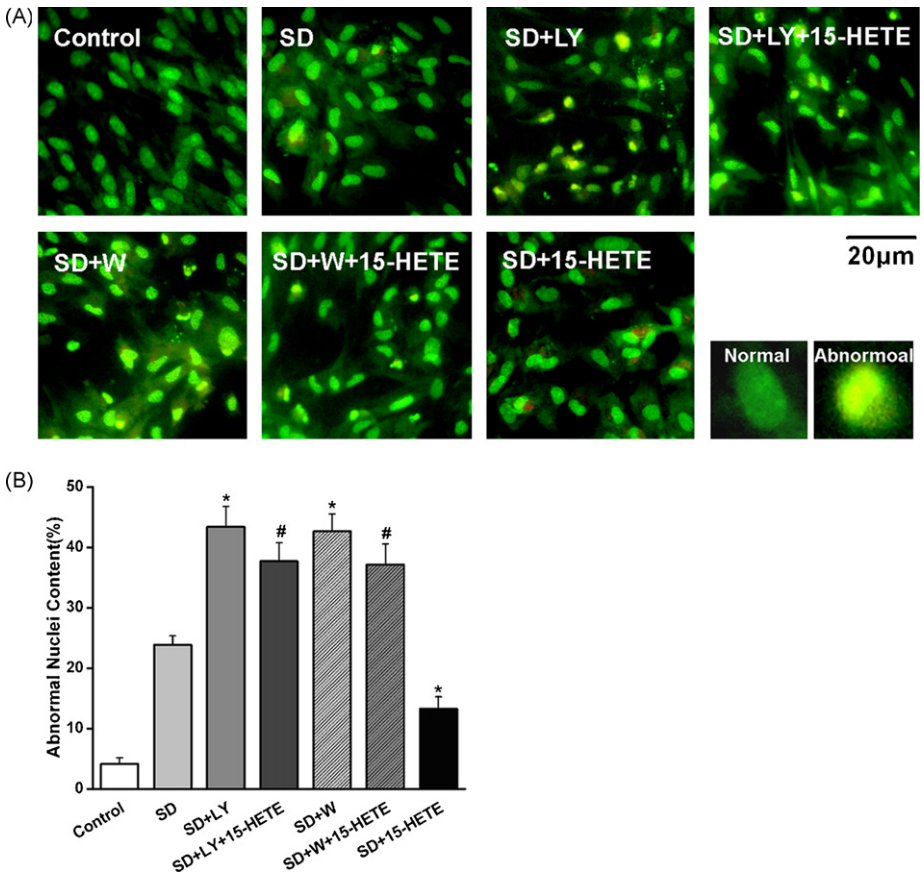

Fig. 3. Morphological changes in nucleus after induction of apoptosis and 15-HETE treatment. PASMCs were treated with 15-HETE (1 μM), LY294002 (20 μM), 15-HETE plus LY294002 (20 μM), wortmannin (10 μM) or 15-HETE plus wortmannin (10 μM), which were added every 24 h under serum-depleted conditions. Then the cells were stained with acridine orange (AO) and imaged by fluorescent microscope. (A) Representative photographs of AO staining in different groups. Scale bar = 20 μm. (B) Quantitative analysis of abnormal nuclei content among groups. All values are denoted as means ± S.E.M. from five independent photographs shot in each group. "SD" means serum deprivation, "LY" means LY294002, "W" means wortmannin. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with serum deprived cells in the presence of 15-HETE.

Twelve-hour light exposure cycles, standard rat chow, and water ad libitum were provided to all rats.

2.3. Cell culture

Primary cultures of cells were prepared as previously described from rat PAs [12]. The vessels were incubated in Hank's balanced salt solution containing 1.5 mg/ml collagenase (Worthington, Shanghai, P. R. China) for 20 min [13]. After incubation, a thin layer of the adventitia was carefully stripped off with fine forceps and the endothelium was removed by gently scratching the intimal surface with a fine cotton tip. Then the remaining smooth muscles were digested with 1.0 mg/ml of collagenase and 0.5 mg/ml elastase (Sigma) for 1 h at 37 °C. The primary cells were cultured in 20% fetal bovine serum (FBS)-containing DMEM. The purity and identity of rat PASMCs were verified by immunocytochemistry using specific mouse monoclonal antibodies against α-actin (Sigma). Cultured cells in passage 2–4 at 80% confluence and with typical hill-and-valley morphology were prepared for experiments. Before each experiment, the cells were synchronized by serum withdrawal for 24 h [14,15]. The cells were exposed to ~2% O_2 in an incubator at 37 °C for hypoxic induction.

2.4. MTT assay

PASMCs, grown in a 96-well culture plate, were subject to growth arrest for 24 h before being placed in either complete medium (DMEM with 10% FBS) or switched to serum deprivation medium for the next 48 h. The samples were treated with 5-HETE (1 μM), 12-HETE (1 μM), 15-HETE (0.1 μM to 1 μM), LY294002 (20 μM), wortmannin (10 μM), 15-HETE plus LY294002 or 15-HETE plus wortmannin in serum deprivation conditions. Another batch of cells was exposed to hypoxia (2% O_2) in the absence or presence of NDGA (30 μM). 15-HETE and other agents at the indicated concentrations were added every 24 h. The concentration of ethanol in the medium was less than 0.08% (v/v). At the end of the incubation period, the cells were incubated for 4 h in a medium containing the yellow 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT), final concentration 0.5 mg/ml. The amount of blue formazan dye formed from MTT was in proportion to the number of living cells. The MTT reaction was terminated by adding dimethyl sulphoxide to each well followed by incubation for 10 min at 37 °C. The spectrophotometric absorbance at 540 nm was measured.

2.5. Western blot analysis

Cultured rat PASMCs were treated with 15-HETE (1 μM), LY294002 (20 μM) and 15-HETE plus LY294002, the others were exposed to hypoxia in absence or presence of NDGA (30 μM). After treatment for 24 h, the cells were washed three times with ice-cold PBS, and then treated in 300 μl lysis buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, Triton X-100 1%, EDTA 1 mM, and PMSF 2 mM), and incubated for 30 min on ice. The protein concentrations were determined by the Bradford protein assay. The protocol for Western blot was similar to previously described [7].

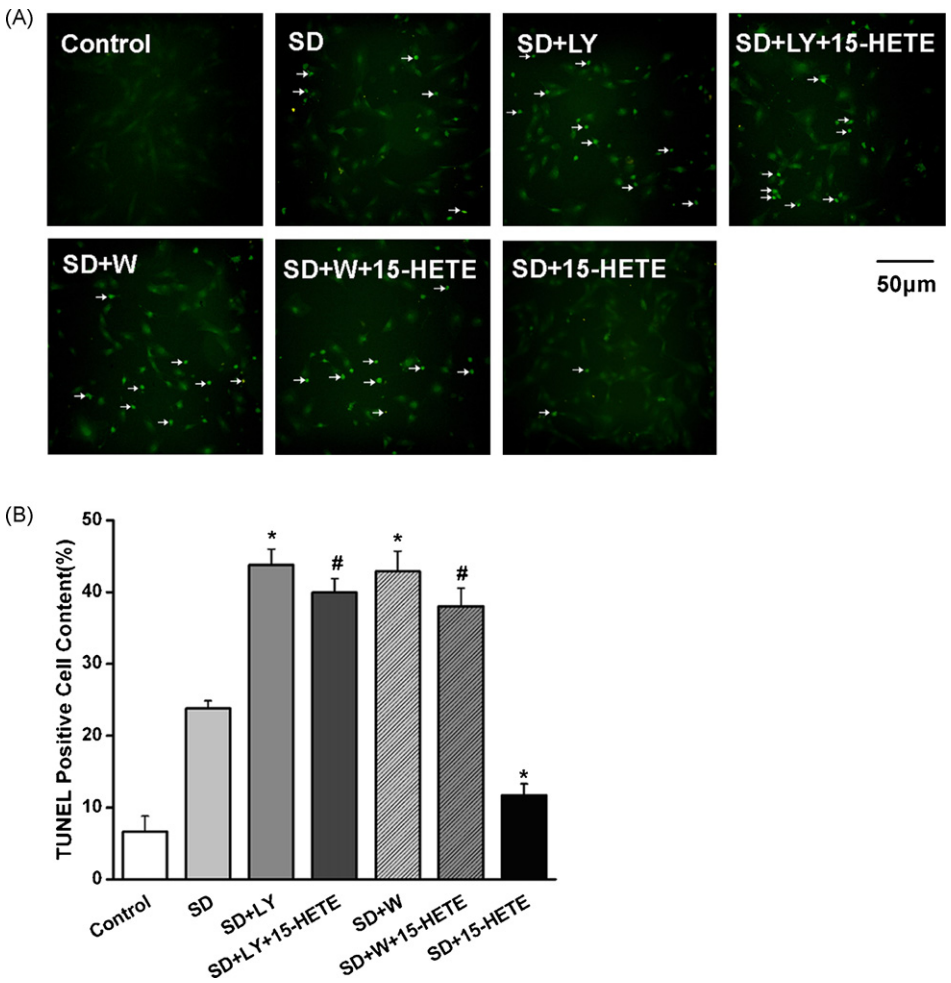

Fig. 4. 15-HETE attenuated serum deprivation-induced DNA strand breaks via the PI3K/Akt pathway. The cells were treated the same way as in Fig. 3. Then the cells were treated with TUNEL staining and imaged by fluorescent microscope. The content of TUNEL-positive cells was calculated as the ratio of TUNEL-positive cells to the total number of PASMCs. (A) Representative photographs of TUNEL staining in different groups. Scale bar = 50 μm. (B) Quantitative analysis of TUNEL-positive cells content in different groups. All values are denoted as means ± S.E.M. from five independent photographs shot in each group. "SD" means serum deprivation, "LY" means LY294002, "W" means wortmannin. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with serum deprived cells in the presence of 15-HETE.

2.6. Nuclear morphology determination

PASMCs were cultured in 6 well culture clusters, washed twice, and then incubated with basal medium for 24 h. Subsequently, LY294002 (20 μM), wortmannin (10 μM), 15-HETE (1 μM), 15-HETE plus LY294002 or 15-HETE plus wortmannin was added. After treatment for 48 h, the cells were washed with phosphate buffer saline (PBS) twice, and stained with acridine orange (AO) for 8 min at 24 °C. The AO-stained cells were imaged with a fluorescent microscope at 488 nm excitation and 405 nm emission. For each well, 15–25 shots were randomly selected to determine the percentage of apoptotic cells of total cells based on the morphological characteristics of apoptosis. Cells with nuclear shrinkage, nuclear condensation and nuclear fractionation were considered to be apoptotic cells.

2.7. TUNEL assay

The TUNEL method was employed to label 3′-end of fragmented DNA of the apoptotic PASMCs. Cells treated for TUNEL assay were fixed with 4% paraformaldehyde in PBS, rinsed with PBS twice, then permeabilized by 0.1% Triton X-100 for FITC end-labeling of the fragmented DNA of the apoptotic PASMCs using the TUNEL detection kit from Beyotime Institute of Biotechnology. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscopy at 488 nm excitation and 530 nm emission.

2.8. Mitochondrial membrane potential assay

The JC-1 probe was performed to measure mitochondrial depolarization in rat PASMCs. Briefly, cells were cultured in six-well plates. After indicated treatments, they were incubated with an equal volume of a JC-1 staining solution (5 μg/ml) at 37 °C for 20 min and rinsed twice with PBS. Mitochondrial membrane potentials were monitored by determining the relative amounts of dual emissions from mitochondrial JC-1 monomers or aggregates using a fluorescent microscope at 488 nm excitation. Mitochondrial depolarization was indicated by an increase in the green/red fluorescence intensity ratio.

2.9. Determination of endogenous 15-HETE with reverse-phase high pressure liquid chromatography (RP-HPLC) analysis

The contents of 15-HETE in PASMCs were analyzed by HPLC according to published method [16]. Briefly, acidic lipids in PASMCs were extracted with ethyl acetate which was acidified to pH 3.0 with formic acid. The samples were dried down under argon, reconstituted in 0.5 ml of 20% acetonitrile:water (pH 3.0), and applied to a Sep-Pak Vac that was prewashed with water followed by acetoni-

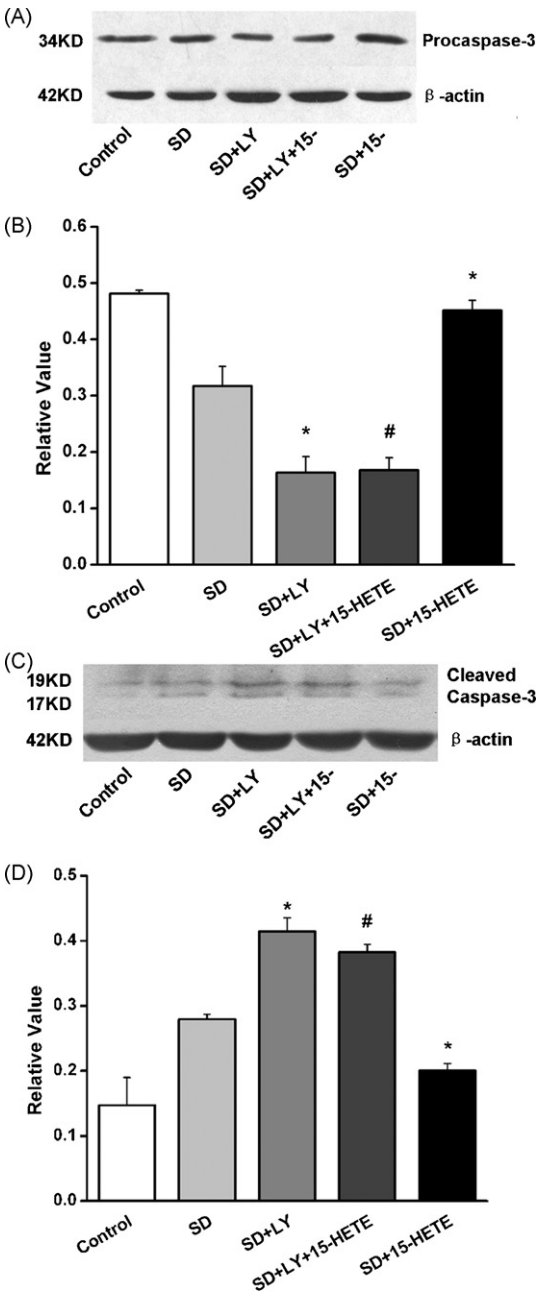

Fig. 5. 15-HETE suppressed the cleavage activation of procaspase-3. (A) 15-HETE up-regulated the expression of procaspase-3 in rat PASMCs. Cells were growth-arrested for 24 h and stimulated with LY294002 (20 μM), 15-HETE (1 μM) or 15-HETE plus LY294002 for another 24 h. (B) Densitometric analysis of the Western blot assays. (C) The effect of 15-HETE on the expression of cleaved caspase-3. (D) Densitometric analysis of the Western blot assays. Results are expressed as mean ± S.E.M. from at least three separate experiments. "SD" means serum deprivation, "LY" means LY294002, "15-" means 15-HETE. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with serum deprived cells in the presence of 15-HETE.

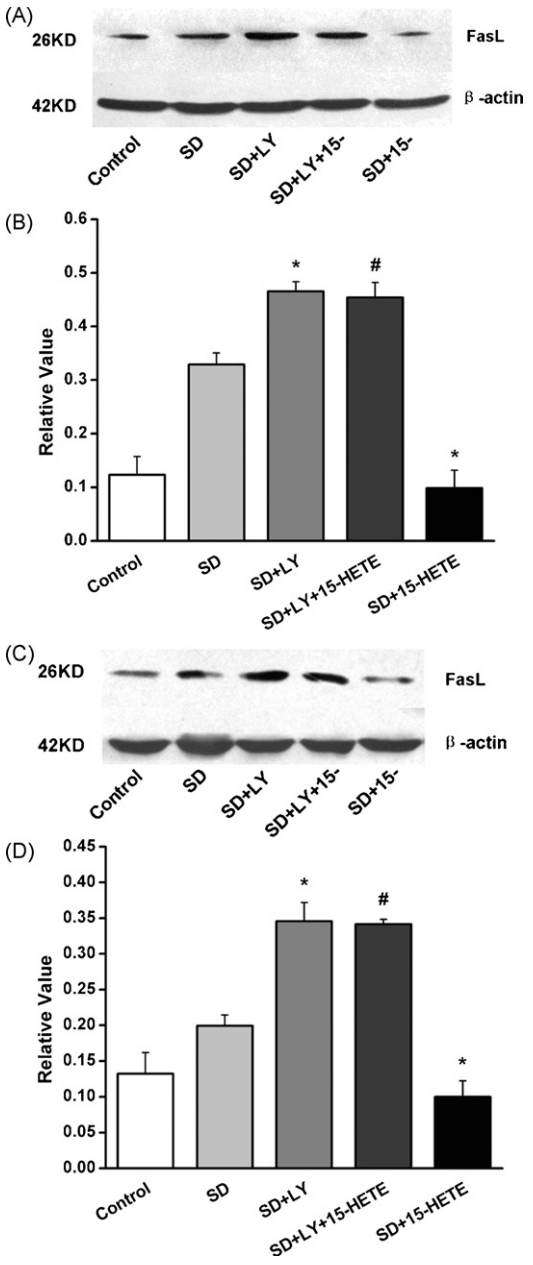

Fig. 6. The expression of FasL was decreased in rat PASMCs by 15-HETE. (A) 15-HETE inhibited the expression of FasL in PASMCs under normoxic conditions. The cells were treated the same way as in Fig. 5. The cell extracts were analyzed by Western blot with antibody to FasL. (B) Densitometric analysis of the Western blot assays. (C) 15-HETE inhibited the FasL expression in PASMCs under hypoxic conditions. Cells were growth-arrested for 24 h and then exposure to hypoxia in presence with LY294002 (20 μM), 15-HETE (1 μM) or 15-HETE plus LY294002 for another 24 h. (D) Densitometric analysis of the Western blot assays. All values are denoted as mean ± S.E.M. from at least three separate experiments. "SD" means serum deprivation, "LY" means LY294002, "15-" means 15-HETE. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with serum deprived cells in the presence of 15-HETE.

trile and water. The column was washed with different proportional acetonitrile:water to remove polar lipids and then was eluted with 500 μl of ethyl acetate to capture the free fatty acids. The sample was taken to dryness under argon. After extraction, the samples were labeled with 2-(2,3-naphthalimino)-ethyl trifluoromethanesulfonate (36.4 mmol/l). N,N-diisopropylethylamine was added to catalyze the reaction. Endogenous 15-HETE was separated on a ODS column (4.6 mm × 250 mm, 5 μm) at 1.3 ml/min isocratically with methanol/water/glacial acetic acid (80:20:0.01) and detected with fluorometer. WIT-002 (10 ng/μl) was used as an internal standard. Retention times of 15-HETE and WIT-002 were 45 and 72 min, respectively. The recovery was 93.6 ± 2.3% and the precision relative standard deviation was less than 12.7% (n = 6). Linearity of the samples in the range 1–200 ng/ml was good, $r^2 = 0.992$.

2.10. Statistical analysis

All data are presented as mean ± S.E.M. Comparisons between multiple groups were performed using the Mann–Whitney test and

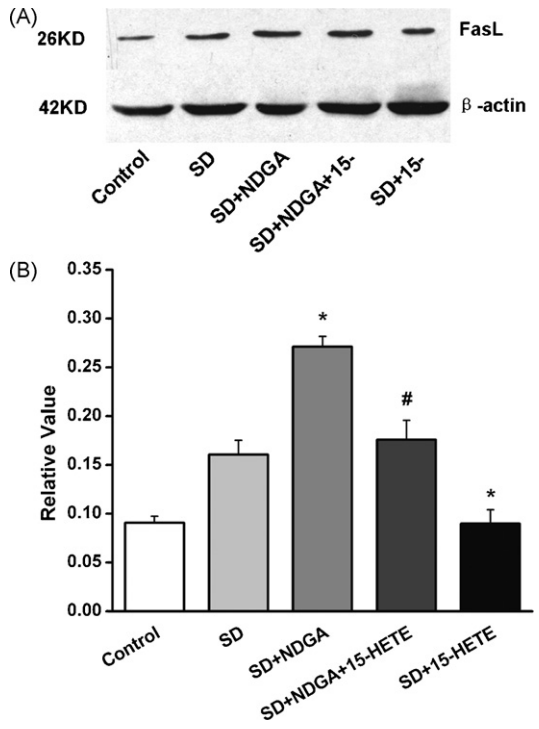

Fig. 7. Effect of endogenous 15-HETE on the expression of FasL under hypoxic condition. The cells were exposed to hypoxia in presence with NDGA (30 μM), 15-HETE (1 μM), or 15-HETE plus NDGA. The cell extracts were analyzed by Western blot with antibody to FasL. Results are expressed as mean ± S.E.M. from at least three separate experiments. "SD" means serum deprivation, "15-" means 15-HETE. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with serum deprived cells in the presence of NDGA.

the one-way ANOVA followed by Dunnett's and Student t test. Differences were considered to be significant at $P \leq 0.05$.

3. Results

3.1. 15-HETE improved PASMC viability via the PI3K/Akt survival pathway

To investigate the role of 15-HETE on PASMC viability, we examined the cell viability by measuring colorimetric conversion of MTT to formazan. Serum deprivation caused a marked decrease in PASMC viability that was partially prevented by a treatment with 15-HETE (1 μM). However, the addition of 15-HETE (0.01 and 0.1 μM) failed to rescue a significant portion of PASMCs committed to cell suicide (Fig. 1A). In separate studies, LY294002 (20 μM), a PI3K inhibitor, further decreased the cell viability when compared with the serum deprivation group. The effect of LY294002 was reversed in cells exposed to 15-HETE. Wortmannin (10 μM), another PI3K inhibitor, had a similar effect as LY294002. These results suggest that 15-HETE improves PASMC viability and PI3K/Akt signaling plays a role in rat PASMC viability (Fig. 1B).

We also studied the effect of endogenous 15-HETE on cell viability (Fig. 1B). A treatment with NDGA (30 μM), an inhibitor of the production of endogenous 15-HETE, decreased the cell viability significantly. All these data indicate that the survival-promoting effect of 15-HETE is likely to be mediated by the PI3K/Akt pathway.

3.2. 15-HETE phosphorylated Akt in rat PASMCs

Akt is a kinase known to promote cell survival and block apoptosis. To determine whether 15-HETE-inhibited apoptosis was mediated by activation of Akt, we used western blot to examine the influence of 15-HETE on the expression of phospho-Akt. The results showed that 15-HETE (1 μM) increased the Akt activity in comparison with the serum withdrawal group, which was assessed by the amount of phospho-ser473 in total Akt (Fig. 2A). Further exploration of the effect of 15-HETE on the Akt activity in serum-containing medium shows that 15-HETE induced PI3K/Akt pathway is not only present in apoptotic rat PASMCs but also in non-apoptotic cells (Fig. 2C).

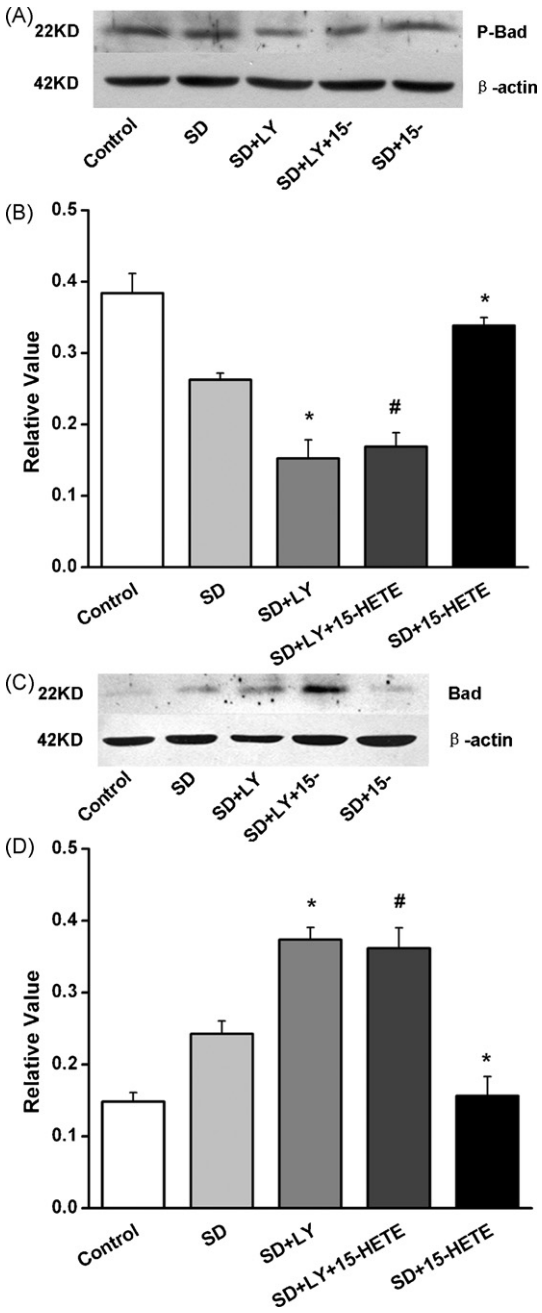

Fig. 8. (A) 15-HETE phosphorylated Bad in PASMCs. The cells were treated the same way as in Fig. 5. The cell extracts were analyzed by Western blot with phosphospecific antibody against Bad. (B) Densitometric analysis of the Western blot assays. (C) 15-HETE decreased the expression of Bad. The cell extracts were analyzed by Western blot with antibody to Bad. (D) Densitometric analysis of the Western blot assays. Results are expressed as mean ± S.E.M. from at least three separate experiments. "SD" means serum deprivation, "LY" means LY294002, "15-" means 15-HETE. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with serum deprived cells in the presence of 15-HETE.

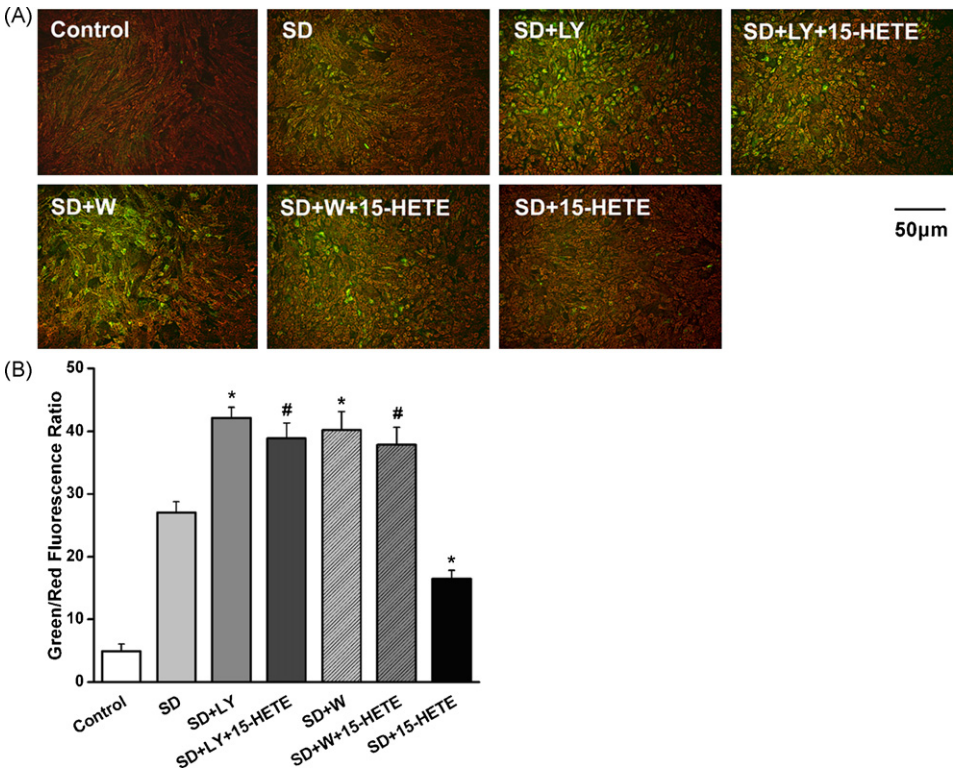

Fig. 9. 15-HETE relieved mitochondrial potentials reduction via the PI3K/Akt survival pathway in rat PASMCs. PASMCs were treated the same way as in Fig. 3 before JC-1 probe staining and fluorescent imaging. (A) Representative photographs of JC-1 staining in different groups. Scale bar = 50 μm. (B) Quantitative analysis of the change of mitochondrial red fluorescence to green fluorescence in different groups. "SD" means serum deprivation, "LY" means LY294002, "W" means wortmannin. $^{*}P < 0.05$ compared with serum deprived cells; $^{\#}P < 0.05$ compared with serum deprived cells in the presence of 15-HETE.

3.3. Activation of the PI3K/Akt pathway was necessary for 15-HETE to protect PASMCs from apoptosis

AO staining, TUNEL assay and caspase-3 expression analysis were undertaken to determine the effect of 15-HETE on PASMC apoptosis. As shown in Fig. 3, the addition of 15-HETE decreased the percentage of nuclear shrinkage when compared with the serum deprivation group. Either LY294002 or wortmannin treatment induced more nuclear fragmentation. On the other hand, 15-HETE relieved the increase of TUNEL-positive cells induced by serum deprivation but not LY294002 or wortmannin (Fig. 4).

Since caspase-3 plays an important role in the process of apoptosis, we examined the protein expression of procaspase-3 and cleaved caspase-3 which has been used to indicate caspase-3 activity. The results showed that the expression of procaspase-3 was up-regulated and the cleavage of procaspase-3 was inhibited in the 15-HETE-treated group in comparison with the serum deprivation group. Meanwhile, LY294002 promoted the cleavage of procaspase-3. Interestingly, 15-HETE did not seem to reverse the cleavage of procaspase-3 induced by LY294002 (Fig. 5). These results indicate that the effect of 15-HETE on PASMC apoptosis appears to be regulated by the PI3K/Akt signaling.

3.4. 15-HETE decreased the expression of FasL via PI3K/Akt survival dependent pathway

Several downstream signals of Akt, such as FasL, Bad and caspase-9 have been reported. To determine whether the activity of FasL is modulated by 15-HETE, we studied the FasL protein expression. As shown in Fig. 6A, 15-HETE suppressed the FasL expression, and this effect was blocked by LY294002. Similar experiments were performed under hypoxic condition, and similar results were observed (Fig. 6C).

We further explored the role of endogenous 15-HETE on FasL expression. Fig. 7 shows that inhibition of endogenous 15-HETE with NDGA under hypoxia markedly up-regulated the FasL expression, and exogenous 15-HETE significantly decreased the expression. Without inhibiting endogenous 15-HETE formation, hypoxia plus exogenous 15-HETE produced even greater down-regulation of the FasL expression than either alone, suggesting that both endogenous and exogenous 15-HETE has inhibitory effects on the expression of FasL. These results therefore suggest that FasL is a downstream mediator of the 15-HETE-PI3K-Akt pathway in PASMCs.

3.5. Effect of 15-HETE on the expression of Bad

Having ideas about the role for 15-HETE in decreasing the expression of FasL via Akt, we further investigated whether 15-HETE suppresses the pro-apoptotic activity of Bad via phosphorylation. The results showed that 15-HETE treatment results in phosphorylation of Bad at Ser136 residue. Meanwhile, blockade of the PI3K/Akt activity with LY294002 attenuated the 15-HETE-induced phosphorylation of Bad (Fig. 8A). Moreover, 15-HETE inhibited the expression of Bad in comparison with the serum-free baseline. A treatment with LY294002 attenuated the inhibition of Bad expression in response to 15-HETE stimulation (Fig. 8C). Therefore, it is likely that Bad mediates the 15-HETE-induced anti-apoptotic events in rat PASMCs.

3.6. 15-HETE relieved mitochondrial depolarization via the PI3K/Akt pathway

An early event of apoptosis is the disruption of the mitochondrial membrane potential. The loss of mitochondrial membrane potentials can be detected with JC-1 that can be incorporated into healthy

mitochondria, aggregates and displays red fluorescence. When the mitochondrial membrane potentials collapse in apoptotic cells, the JC-1 resumes a monomeric form and fluoresces green. As shown in Fig. 9, serum deprivation-induced mitochondrial depolarization as indicated by reduced ratios of green/red fluorescence. A treatment of PASMCs with 15-HETE relieved the serum deprivation-induced mitochondrial depolarization. A treatment with LY294002 or wortmannin further worsened the mitochondrial potentials over the serum-free condition. Such an effect was not reduced by exogenous 15-HETE. Thus, these data provide evidence that action of the PI3K/Akt pathway is required for the protection of mitochondrial potentials.

3.7. Anti-apoptotic effects of other HETEs isoforms on PASMCs

The specificity of 15-HETE in the inhibition of PASMCs apoptosis was examined by using MTT and TUNEL. As shown in Fig. 10A, 5-HETE (1 μM) and 12-HETE (1 μM) had no noticeable protective effect on cell viability. Consistently, the number of TUNEL-positive cells was not significantly decreased after 5-HETE or 12-HETE administration (Fig. 10B). These findings suggested that 15-HETE is a potent arachidonic acid-derived lipid mediator involved in the PASMC survival.

3.8. Measurements of 15-HETE levels after serum deprivation

To investigate the effect of serum deprivation on 15-HETE, we measured endogenous 15-HETE levels in PASMCs by using RP-HPLC. The results showed that serum deprivation decreased the 15-HETE levels in PASMCs, suggesting that apoptosis of PASMCs may result in inhibiting formation of endogenous 15-HETE (Fig. 11).

4. Discussion

Hypoxia-induced pulmonary vasoconstriction and pulmonary remodeling are important factors related to both primary and secondary PH. The mechanisms are still largely unknown. We have recently reported that hypoxia enhances the 15-LO protein expression. Both exogenous and endogenous 15-HETE induces pulmonary vasoconstrictions [6,17,18]. Furthermore, hypoxia stimulates 15-HETE production, promoting PA remodeling through inactivation of K^+ channels [7]. In the present study, we have found evidence for the 15-HETE effect on PASMC apoptosis is via the PI3K/Akt survival pathway.

We have found that 15-HETE induced phosphorylation of Akt and inhibitors of either PI3K or Akt promoted the apoptotic responses of PASMCs, indicating that the PI3K/Akt pathway is required for the anti-apoptotic effects of 15-HETE in rat PASMCs. It is known that Akt is a serine/threonine protein kinase that is activated by a number of growth factors and cytokines in a PI3K-dependent manner [19]. Activation of the PI3K/Akt pathway has a major impact on cell survival and apoptosis [20]. Moreover, we found that 15-HETE also activated PI3K/Akt in non-apoptotic PASMCs. Studies are underway to determine the role of 15-HETE in non-apoptotic PASMC fate.

The survival effects of Akt are mediated by stimulation of signaling pathway for extrinsic cell death involving the down-regulation of membrane FasL [8,11,21]. In this study, we have shown that both hypoxic exposure and exogenous 15-HETE down-regulated FasL, whereas LY294002 inhibited the effect of 15-HETE on FasL in PASMCs. All the information presented leads to a clue that both endogenous and exogenous 15-HETE inhibits apoptosis through the PI3K-Akt-FasL signaling pathway.

Apoptosis not only involves the extrinsic pathway but also the intrinsic pathway [22,23]. One of them is manifested as mitochondria integrity that is regulated by the Bcl-2 family of proteins, including the pro-apoptotic family member Bad [24]. Bad is the first of Akt substrates to be identified [25,26]. It promotes cell death by interacting with anti-apoptotic Bcl-2 members such as Bcl-xL, which causes a release of apoptogenic molecules from mitochondria to the cytosol culminating into caspase activation and cell death. The mechanism of Bad promoting cell survival is via phosphorylation of Bad, which reduces Bad ability to bind Bcl-xL [27,28]. In this study, significant mitochondrial depolarization has been observed after the 15-HETE treatment, which was attenuated by PI3K inhibitor. Meanwhile, 15-HETE induced phosphorylation of Bad. Our data thus imply that the anti-apoptosis effect of 15-

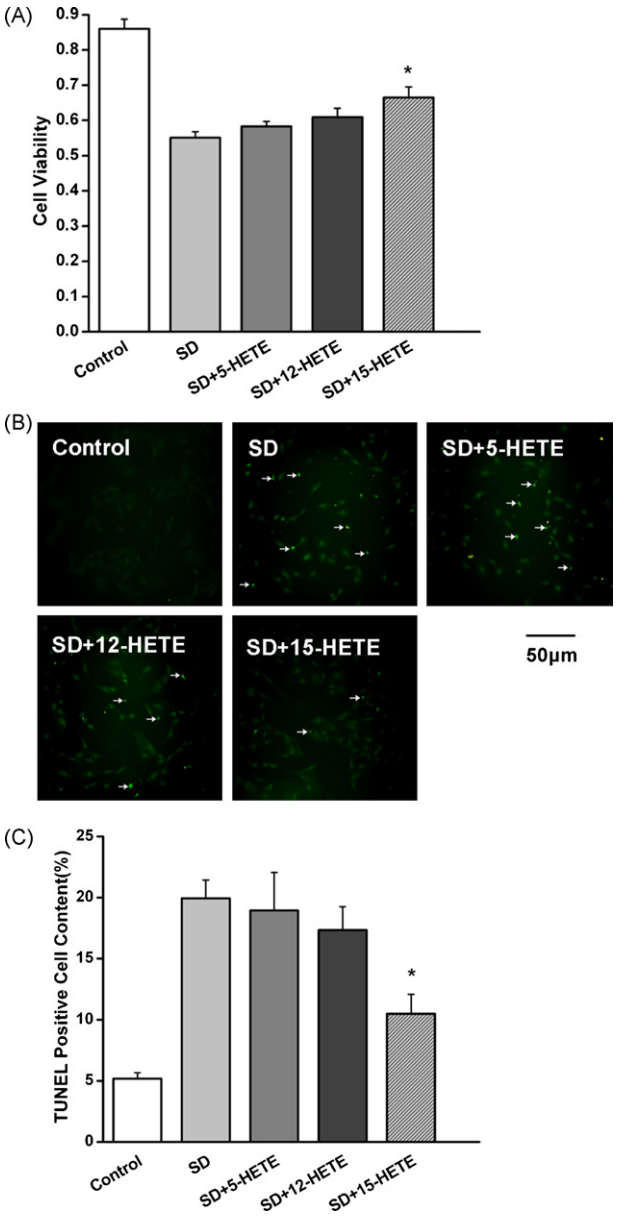

Fig. 10. (A) The survival rate of PASMCs following treatment of 5-HETE, 12-HETE and 15-HETE was studied by MTT. Cells were growth-arrested for 24 h and then stimulated with 5-HETE (1 μM), 12-HETE (1 μM), 15-HETE (1 μM), which were added every 24 h under serum-depleted conditions. Control cells were cultured in complete medium (DMEM with 10% FBS). (B) Effects of 5-HETE, 12-HETE and 15-HETE on PASMCs apoptosis. The cells were treated the same way as in (A). Then the cells were treated with TUNEL staining and imaged by fluorescent microscope. Scale bar = 50 μm. (C) Quantitative analysis of TUNEL-positive cells content in different groups. All values are denoted as means ± S.E.M. from five independent photographs shot in each group. "SD" means serum deprivation, $^*P < 0.05$ compared with serum deprived cells.

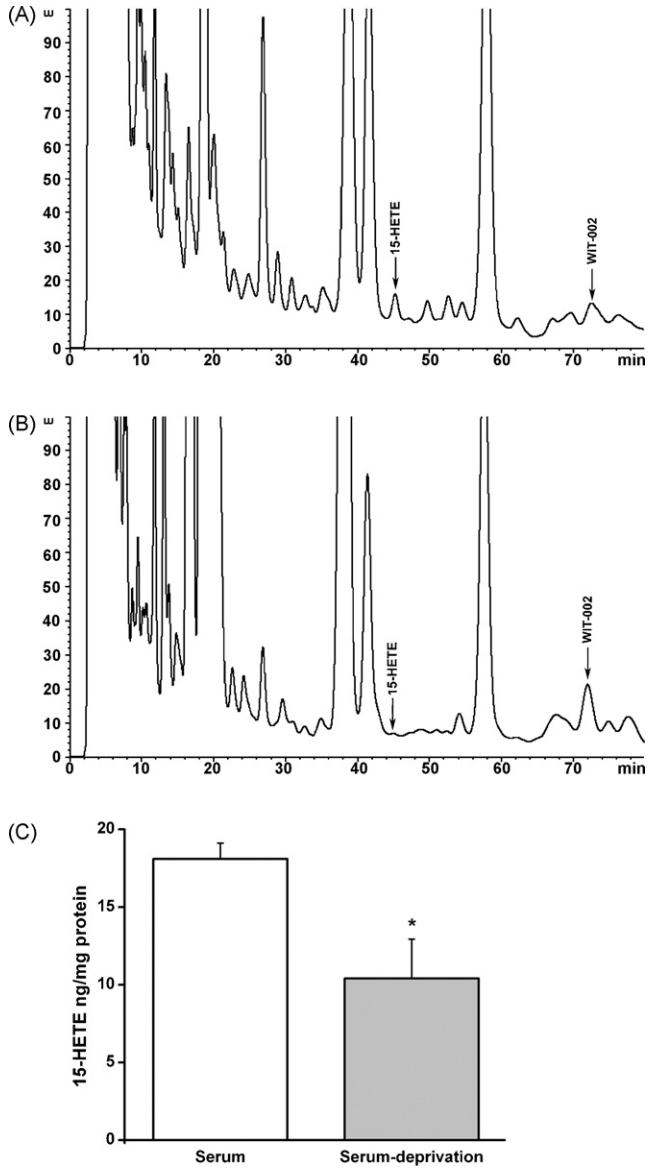

Fig. 11. RP-HPLC chromatograms showing the production of 15-HETE in PASMCs which were cultured in serum medium (A) and serum deprivation medium (B). (C) Quantitative analysis of 15-HETE levels in PASMCs exposed to serum and serum deprivation. Results are expressed as mean ± S.E.M. from at least four separate experiments. $^{*}P < 0.05$ compared with cells cultured in serum-containing medium.

HETE is due to the stimulation of an intrinsic cell death signaling pathway via Bad. However, whether the extrinsic pathway and the intrinsic pathway are linked or that molecules in one pathway can influence the other needs to be determined. In addition, we found that 15-HETE also had influence on total Bad via the PI3K/Akt pathway. It is possible that long term stimulation of PASMCs by 15-HETE results in suppression of Bad protein level. Yet the precise mechanism of how Bad protein level is changed remains to be determined. Therefore, further studies are needed to fully illustrate the signaling system. Nevertheless, our present study is remarkable as it opens avenue for therapeutical interventions to PH.

The monohydroxyeicosatetraenoic acids (HETEs) are a group of lipoxygenase products of AA metabolism that include 5-HETE, 12-HETE and 15-HETE [18,29,30]. Although 5-HETE and 12-HETE are potent survival factors for cancer cells, we found that, unlike 15-HETE, 5-HETE and 12-HETE exerted no noticeable anti-apoptotic effects in PASMCs. These data suggest that some actions of the various lipoxygenases are cell specific. It is well known that hypoxia and ischaemia can result in lipoxygenases activation, thereby leading to an increased production of HETEs [6,31]. In the present study, we surprisingly found that serum deprivation also had an effect on the production of endogenous 15-HETE. It seems that apoptosis is associated with levels of 15-HETE in PASMCs which remains to be further defined.

In conclusion, our results have shown that 15-HETE inhibits apoptosis of PASMCs via the PI3K/Akt pathway, and such an effect is likely to be mediated through both the extrinsic and the intrinsic apoptotic pathways. These findings have major implications for devising a strategy to limit in pulmonary vessel remodeling in pulmonary hypertension.

Acknowledgments

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