Apoptosis (2009) 14:42–51
DOI 10.1007/s10495-008-0286-6

ORIGINAL PAPER

15-HETE suppresses K^+ channel activity and inhibits apoptosis in pulmonary artery smooth muscle cells

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Published online: 10 December 2008

Abstract 15-Hydroxyeicosatetraenoic acid (15-HETE) is an important hypoxic product from arachidonic acid (AA) in the wall of pulmonary vessels. Although its effects on pulmonary artery constriction are well known, it remains unclear whether 15-HETE acts on the apoptotic responses in pulmonary artery smooth muscle cells (PASMCs) and whether K^+ channels participate in this process. These hypothesises were validated by cell viability assay, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling, mitochondrial potentials assay, caspase activity assay and western blot. We found that 15-HETE enhanced cell survival, suppressed the expression and activity of caspase-3, upregulated bcl-2 and attenuated mitochondrial depolarization, prevented chromatin condensation and partly reversed K^+ channel opener-induced apoptosis in PASMCs under serum-deprived conditions. Our data indicated that 15-HETE inhibits the apoptosis in PASMCs through, at least in part, inactivating K^+ channels.

Keywords 15-Hydroxyeicosatetraenoic acid · Pulmonary artery muscle smooth cells · Hypoxia-induced pulmonary hypertension · Apoptosis · K^+ channel

Yumei Li and Qian Li contributed equally to this work.

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Abbreviations

15-HETE	15-Hydroxyeicosatetraenoic acid
PASMCs	Pulmonary artery smooth muscle cells
AA	Arachidonic acid
15-LO	15-Lipoxygenase
HPV	Hypoxic pulmonary vasoconstriction
PVR	Pulmonary vascular remodeling
NDGA	Nordihydroguaiaretic acid

Introduction

Chronic hypoxic exposure induces many pathophysiological changes, such as sustained pulmonary vasoconstriction, pulmonary arteries medial muscularization, artherosclerosis and diffuse interstitial fibrosis [1–3]. Chronic hypoxia is also an important contributor to the pulmonary vascular remodeling (characterized with medial and intimal hypertrophy), a critical pathological alteration in pulmonary hypertension. However, the mechanism of pulmonary vascular remodeling (PVR) and pulmonary hypertension is still unknown. Some investigators suggested that PASMC proliferation was the main reason for the vascular remodeling in lung [4]. But some studies reported that hypoxia-induced proliferation of medial PASMCs was not observed, instead of the inhibition of apoptotic responses in SMC [5, 6]. Therefore, it's believable that the disturbance between apoptosis and proliferation in PASMC under hypoxic conditions plays an important role in the progression of PVR. Compared with a large amount of reports on hypoxia-induced proliferation in pulmonary vasculature [7–9], the effects of chronic exposure on the apoptotic responses in PASMCs still need to be determined.

Hypoxia inhibited the whole cell K^+ currents (I_k) and suppressed the expression of potassium channel, such as Kv1.2, Kv1.5, Kv3.4 in PASMC [10, 11]. The subsequent decrease in I_k and intra-cellular K^+ concentration [$(K^+)_i$] inhibited the apoptotic responses by inducing the expression of bcl-2 and depressing the activity of caspase-family proteases [12]. However, whether hypoxia suppresses the apoptosis in PASMCs directly or through some mediators, such as bcl-2 or Kv channel, is still unknown.

K^+ channels play an important role in the apoptosis of PASMCs. Activation of K^+ channels induced the apoptosis in PASMCs, and inactivation of K^+ channels partly inhibited the apoptosis [13–15]. Lots of reports indicated that the K^+ channels in PASMCs were involved in the development of pulmonary vascular medial hypertrophy and pulmonary hypertension [16–18]. According to the statements above, it is very likely that, through selectively downregulating the activity and expression of some subtypes of the K^+ channels, hypoxic exposure elevated $[K^+]_i$, inhibits the caspase-cascade, and ultimately suppressed the apoptosis in PASMC.

In previous studies, we found that hypoxic exposure promoted the expression of 15-lipoxygenase (15-LO), which catalyzed the production of 15-HETE [3]. 15-HETE played a significant role in hypoxia-induced pulmonary vasoconstriction. What is more, 15-HETE suppressed the expression of $K_V1.5$ and $K_V3.4$, decreased K_V currents in PASMCs in vitro [1, 11, 19]. These data give a clue that 15-HETE might suppresses the apoptotic responses in PASMCs through inhibiting Kv channels, which leads to medial thickness of pulmonary artery and PVR. To test the hypothesis, we used serum deprivation (SD) to induce cell apoptosis, our results show that 15-HETE enhanced cell survival, inhibited the expression and activity of caspase-3, up-regulated bcl-2, relieved mitochondrial depolarization, suppressed DNA fragmentation and nuclear shrinkage. Moreover, pinacidil (Pin), an opener of K^+channel, attenuated the inhibitive effects of 15-HETE on PASMCs apoptosis. We propose that 15-HETE suppressed the SD-induced PASMCs apoptosis partly through inactivating K^+ channels.

Materials and methods

Materials

15-HETE dissolved in ethanol was obtained from Cayman Chemical (Ann Arbor, USA) and stored at −80°C under nitrogen. NDGA was purchased from Sigma-Aldrich Co. (St. Louis, USA), reconstituted in ethanol and stored at −80°C. Antibodies against bcl-2, caspase-3 and β-actin, JC-1 probe, the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) cell apoptosis detection kit, caspase-3 activity kit and lactate dehydrogenase (LDH) activity kit were provided by Beyotime Institute of Biotechnology (Haimen, China). Enhanced chemiluminescence (ECL) reagents were from Amersham International (Amersham, UK). All other reagents were purchased from Sigma-Aldrich Co. (St. Louis, USA).

Cell preparation and experimental protocol

Experiments were in full compliance with the animal Ethical Committee of Harbin Medical University. Primary cultures of PASMCs were prepared as previously described [3, 11]. Briefly, pulmonary arteries were isolated from fresh lungs of calf, slit open along their lengths, and washed with phosphate buffered solution (PBS) to remove blood. The vessels were dissected free of fat and excess adventitial tissue, and the endothelial lining was removed by scraping of the luminal surface. Small fragments (~ 1 cm^2) were transferred to a flask. After adhered for 30 min, the arteries were covered with media dulbecco's modified eagle's medium (DMEM) containing 20% fetal bovine serum (FBS) and allowed to grow for 4 days in a tissue culture incubator. Tissue pieces were then lifted out of the medium, and adherent smooth muscle cells were allowed to proliferate. The purity and identity of PASMCs were verified by immunocytochemistry staining using specific mouse monoclonal antibodies against smooth muscle cell α-actin (Sigma), The cells, detected in positive rate >80% and with typical hill-and-valley morphology, were prepare for experiments. Passage 2–4 cells (primary culture = passage zero) at 80% confluence were used in all reported experiments. The cells were exposed to less than 2% O_2 in an incubator at 37°C for hypoxic-induced. The apoptosis was induced by serum deprivation (more details see references [3, 11, 20, 21].

MTT

PASMCs were cultured in 96 well culture clusters (about 1×10^4 per well), and then the cells were treated with Pin (1 μM) or Pin (1 μM) plus 15-HETE (1 μM) in serum deprivation conditions. Another batch of cells was exposed to hypoxia (<2% O_2) in absence or presence with NDGA (30 μM). The cells cultured in complete medium were considered as control. NDGA or 15-HETE at the indicated concentration was added every 24 h. The concentration of ethanol in the medium was less than 0.1% (v/v). After the indicated treatments for 48 h, the cells were incubated for 4 h in a medium containing 0.5% 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl- tetrazolium bromide (MTT), the yellow mitochondrial dye. The amount of blue formazan dye formed from MTT is proportional to the number of survival

cells. The MTT reaction was terminated by adding DMSO to the medium followed by incubation for 10 min at room temperature. The absorbance was read at 540 nm in a spectrophotometer.

Western blot analysis

The cells in 6 well culture clusters were added vehicle, 15-HETE (1 μM), NDGA (30 μM), Pin (1 μM) or 15-HETE (1 μM) plus Pin (1 μM) in serum deprivation conditions. The cells cultured in complete medium were considered as control. After the treatment for 24 h, the cells were lysed by 100 μl lysis buffer (Tris 50 mM, pH 7.4, NaCl 150 mM, Triton X-100 1%, EDTA 1 mM, and PMSF 2 mM) and the protein in the cell extracts were determined by the Coomassie protein assay with bovine serum albumin as a standard. Protein samples (20 μg) were subjected to 12% (caspase-3) or 15% (bcl-2) SDS–PAGE and then transferred to nitrocellulose membranes. After incubation for 1 h at 22–24°C in a blocking buffer (Tris 20 mM, pH 7.6, NaCl 150 mM, and Tween 20 0.1%) containing 5% nonfat dry milk powder, the membranes were reacted with anti-caspase-3 antibody overnight at 4°C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents.

Mitochondrial depolarization assay

The cells in 6 well culture clusters were treated with vehicle, 15-HETE (1 μM), NDGA (30 μM), Pin (1 μM) or 15-HETE (1 μM) plus Pin (1 μM) in serum deprivation conditions for 48 h. Then the cells were stained with JC-1 probe for measuring the depolarization of mitochondrial membrane. Briefly, the treated cells were incubated with an equal volume of 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 or dual emissions from both mitochondrial JC-1 monomers and aggregates using an Olympus fluorescent microscope under Argon-ion 488 nm laser excitation. Mitochondrial depolarization was indicated by an increase in the ratio of green/red fluorescence intensity.

Nuclear morphology determination

Cells treated for mitochondrial depolarization assay were washed with PBS for two times, and stained with acridine orange (AO) for 8 min at 24°C. The AO-stained cells were imaged with a fluorescent microscope under 488 nm laser excitation and 405 nm emission. For each well, 15–25 shot were randomly selected to determine the percentage of apoptotic cells in total cells based on the morphological characteristics of apoptosis. Cells with nuclear crenation, nuclear condensation and nuclear fractionation were defined as apoptotic cells.

TUNEL

TdT-UTP nick end labeling (TUNEL) assays were performed with the one step TUNEL kit according to the manufacturer's instructions. Cells grown in 6 well culture clusters were treated as mentioned in mitochondrial depolarization assay. Briefly, the cells were permeabilized with 0.1% Triton X-100 for 2 min on ice followed by TUNEL for 1 h at 37°C. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscope by using 488 nm excitation and 530 nm emission. The cells with green fluorescence were defined as apoptotic cells.

Statistics

The composite data are expressed as means ± SEM. Statistical analysis was performed with one-way ANOVA followed by Dunnett's test where appropriate. Differences were considered to be significant at $P \leq 0.05$.

Results

Hypoxia enhanced PASMCs survival in serum-depleted culture via 15-LO/15-HETE pathway

PASMCs were cultured in DMEM containing 10% FBS. Cells remained adherent after complete removal of serum from the culture medium. The cell viability was determined by measuring colorimetric conversion of MTT to formazan. Serum deprivation caused a marked decrease in cell viability in normoxic conditions. 15-HETE (1 μM) exposure led to an increase in cell viability. Pin (1 μM), a potassium activator, enheanced the apoptosis effect of serum-deprived and attenuated 15-HETE-induced increase in viable cell amount (Fig. 1, $n = 8$, $P < 0.05$) in comparison to the serum deprivation group in normoxic conditions.

In separate study, PASMCs were divided into four groups and cultured under noroxic, hypoxic condition, hypoxia with 15-HETE, hypoxia with NDGA (30 μM) which was used to inhibit the formation of endogenous 15-HETE. The results showed that hypoxia prevented the decrease of cell viability caused by serum deprivation. Whereas, the inhibition of the formation of the endogenous 15-HETE decreased the cell viability, however, hypoxia with 15-HETE shown no significant difference compared to hypoxia alone (Fig. 2, $n = 8$, $P < 0.05$ in comparison to the hypoxia group). The data present here indicate that preventive effect of hypoxia on PASMCs survival against

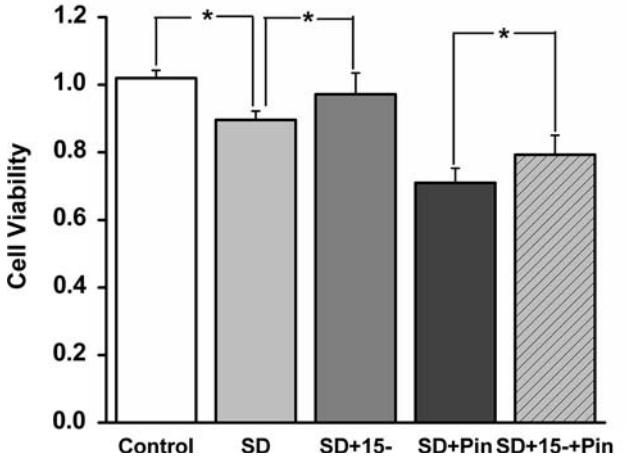

Fig. 1 15-HETE promoted the survival of pulmonary artery smooth muscle cells (PASMCs) in serum-deprived conditions. Cells were growth-arrested for 24 h and then exposed to serum deprivation in presence with 15-HETE (1 μM), Pin(1 μM) or 15-HETE plus Pin (1 μM), which were added every 24 h. The cells cultured in complete medium (DMEM with 10% FBS) were considered as control. The survival cell amount was measured by MTT assay after the indicated treatments for 48 h

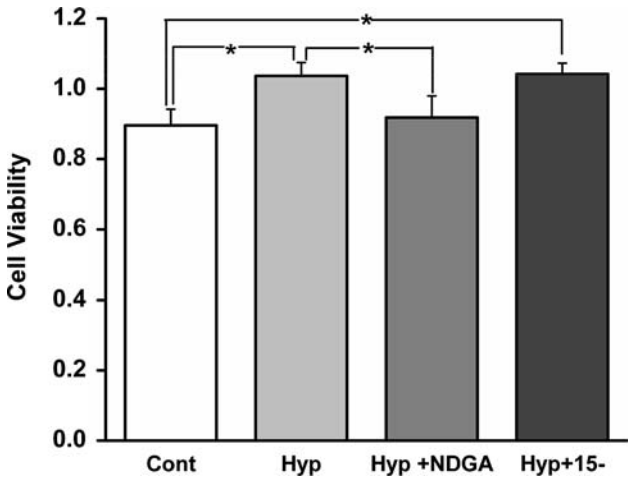

Fig. 2 Effect of NDGA on the proliferation of PASMCs under hypoxic condition. Cells were growth-arrested for 24 h and then exposed to hypoxia in absence or presence with NDGA (30 μM) and 15-HETE (1 μM). NDGA and 15-HETE were added every 24 h. The survival cell amount was measured after the indicated treatments for 48 h. "Con" means control, "15-" means 15-HETE, "Hyp" means hypoxia. $*P < 0.05$ compared with each other. All values are denoted as means ± SEM from three or more independent batches of cells

serum deprivation is partly dependent on 15-HETE and may be through the inhibition of K^+ channels.

15-HETE decreased caspase-3 activity and cleavage activation

Cleavage of procaspase-3 to generate the active effectors caspase-3 is an important step of chromatin degradation and apoptosis [14]. Thus, we measured caspase-3 activity by spectrometer and determined its expression by western blot in the cell extract samples. Serum deprivation caused a remarkable increase in caspase-3 activity. Exogenous 15-HETE (1 μM) inhibited caspase-3 activity induced by serum deprivation. The inhibitory effect of 15-HETE on caspase-3 activity was reversed by NDGA (30 μM), an inhibitor for the formation of endogenous 15-HETE. We further used Pin, a potassium activator, to test whether caspase-3 activity inhibited by 15-HETE was through K^+ channels in serum free cultured PASMCs. PASMCs were treated with Pin (1 μM) or Pin (1 μM) plus 15-HETE (1 μM) in serum deprivation conditions. The results showed that the Pin-induced increase in caspase-3 activity was suppressed by exogenous 15-HETE (Fig. 3, $n = 3$, $P < 0.05$), suggesting that K^+ channels involved in the procedure of 15-HETE inhibiting caspase-3 activity.

The effect of 15-HETE on the expressions of caspase-3 and bcl-2

Caspase-3 and bcl-2 play important roles in the process of apoptosis. Here, we examined the protein expression of caspase-3 and bcl-2 in PASMCs. As shown in Fig. 4, Exogenous 15-HETE suppressed the expression of caspase-3 protein induced by serum deprivation, which was significantly alleviated by 30 μM NDGA, suggesting that both endogenous and exogenous 15-HETE has the inhibitory effects on the expression of caspase-3 protein.

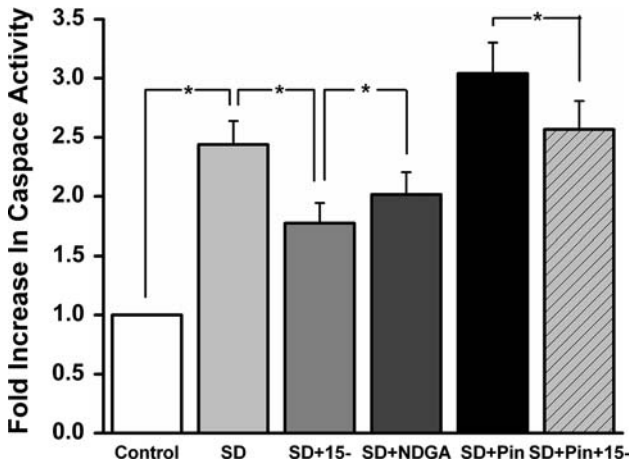

Fig. 3 15-HETE inhibited caspase-3 activity in PASMCs. PASMCs were grown to ~80% confluency and serum starved for 24 h. Then the cells were treated with 15-HETE (1 μM), NDGA (30 μM), Pin (1 μM) or 15-HETE (1 μM) plus Pin (1 μM) in serum deprivation conditions for another 24 h. After the treatment, the cells were lysed and the protein in the cell extracts were determined by a spectrometer. The cells cultured in complete medium were considered as control. "Con" means control, "15-" means 15-HETE. Results are expressed as mean ± SEM from at least three separate experiments. $*P < 0.05$ compared to each other

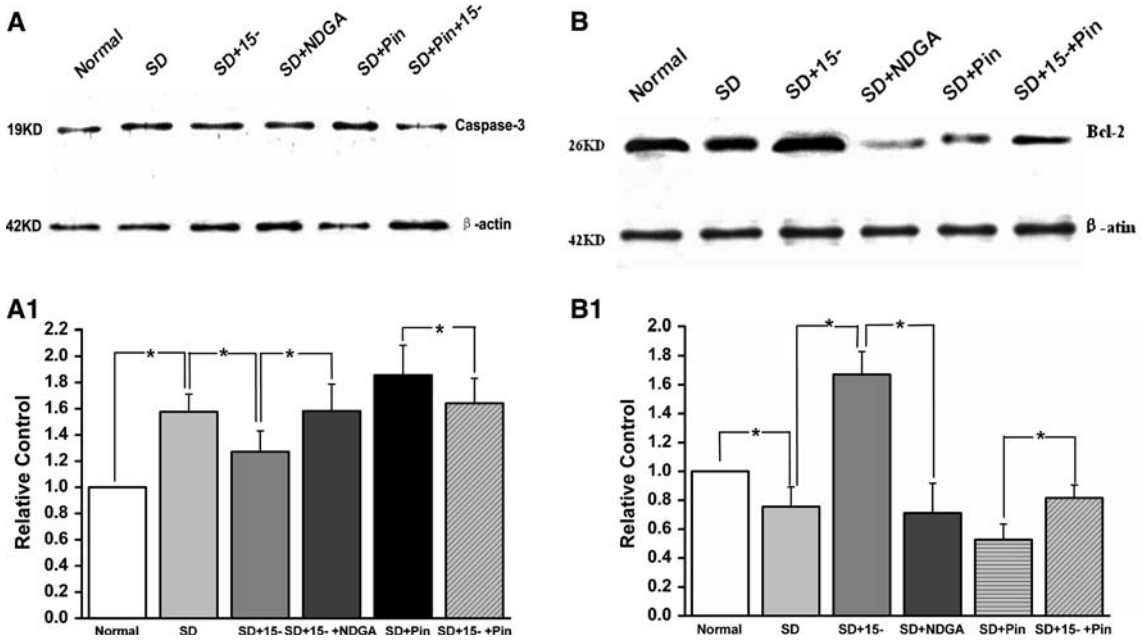

Fig. 4 **a** 15-HETE inhibited the expression of caspase-3 in PASMCs. The cells were treated the same way as in Fig. 3. The cell extracts were subjected to 12% SDS–PAGE and probed with anti-caspase-3 antibody to detect the cleaved fragments of caspase-3 specifically. **b** 15-HETE upregulated the expression of bcl-2 in PASMCs. The cells were treated the same way as in Fig. 3. The cell extracts were subjected to 15% SDS–PAGE and probed with anti-bcl-2 antibody to detect the bcl-2 specifically. **a1** and **b1** The immunoblots were measured by the software Quantity One. Results are expressed as mean ± SEM from at least three separate experiments. "Con" means control, "15-" means 15-HETE. *$P < 0.05$ compared to each other

Furthermore, Pin, a potassium activator, upregulated the expression of caspase-3 protein induced in serum free cultured PASMCs and 15-HETE significantly decreased the expression in response to Pin. These data implicated that the inhibitory effects of 15-HETE on the expression of caspase-3 protein is mediated through a mechanism of potassium activity.

In separate studies, bcl-2 expression bcl-2 expression was detected in PASMCs. As shown in Fig. 4b, the bcl-2 expression was decreased in serum free cultured PASMCs. 15-HETE recovered the downregulation of bcl-2 protein with further increase. NDGA and Pin were significantly down regulates bcl-2. Morever, 15-HETE partly reversed the down regulation of Bcl-2 induced by Pin. (Fig. 4. $n = 3$, $P < 0.05$).

15-HETE relieved serum deprivation or H_2O_2 induced mitochondrial depolarization

Loss of mitochondrial membrane potential is an important indicator of cell apoptosis. The changes in mitochondrial membrane potential were measured by the JC-1 probe, which was dispersed from aggregated form (red fluorescence) to the monomeric form (green fluorescence) when mitochondrial membrane potential was lost. (Fig. 5. $n = 10$, $P < 0.05$). The protocol was showed in part of materials and methods. The results obtained from each treatment were pooled, and the graphs were shown in Fig. 5 a1, b1. PASMCs cultured in serum-deprived conditions had a significant decrease in mitochondria membrane potential as indicated by a notable shift in the ratio of green/red fluorescence versus control. 15-HETE treatment partly restored the mitochondria depolarization. However, 15-HETE-induced increase in mitochondrial membrane potential can be reverse by NDGA, the endogenous 15-HETE blocker, suggesting that endogenous 15-HETE was also involved in the procedure of apoptosis. Pin opened K^+ channels and downregulated mitochondria membrane potential, and this effect was reduced by 15-HETE. (a1) 15-HETE not only attenuated the apoptosis effect of serum-deprived, but also inhibited the apoptotic responses of H_2O_2 (A2).

15-HETE prevented PASMCs from nuclear shrinkage

The alteration of nuclei conformation visualized by Acridine orange staining was applied to determine the percentage of apoptotic cells in PASMCs. The results obtained from each treatment were pooled, and the graphs were shown in Fig. 6. The results showed that percentage of apoptotic cells were increased in serum free cultured PASMCs. Treatment with 15-HETE significantly decreased that percentage of apoptotic cells caused by serum free culture. However, the effect of 15-HETE was

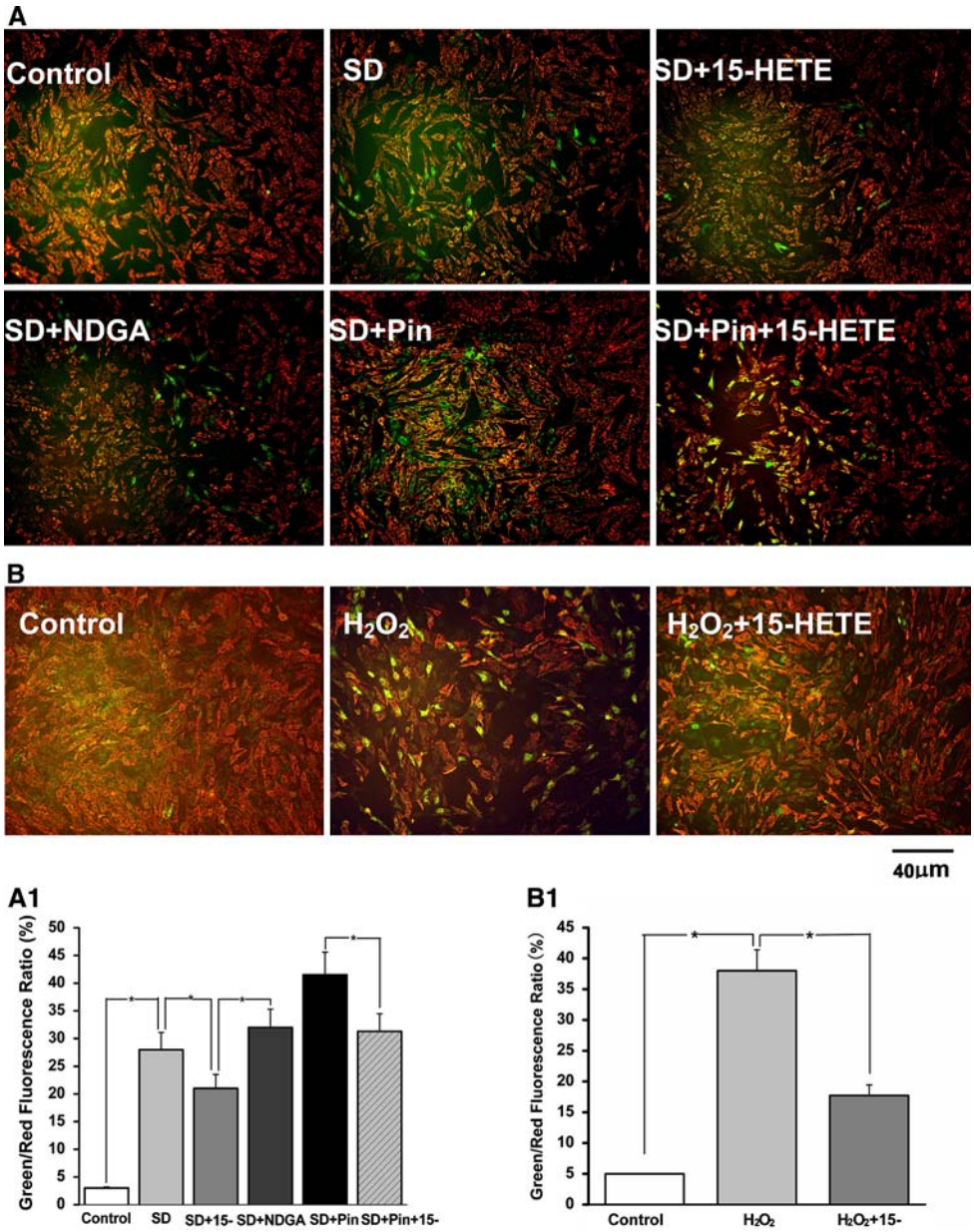

Fig. 5 **a** 15-HETE attenuated mitochondrial depolarization in PASMCs induced by serum deprivation or Pin. PASMCs were treated the same way as in Fig. 3. After indicated treatment for 48 h, the cells were stained with JC-1 probe and imaged by fluorescent microscope. Scale bars = 40 μm. **b** 15-HETE attenuated mitochondrial depolarization in PASMCs induced by H_2O_2. PASMCs were treated with normal, H_2O_2, H_2O_2 plus 15-HETE for 48 h. 15-HETE and H_2O_2 were added every 24 h. After the treatment, the cells were stained with JC-1 probe and imaged by fluorescent microscope. Scale bars = 40 μm. **a1** and **b1** quantitative analysis of the change of mitochondrial potentials among groups. An increase in the bar indicates a shift in the ratio of green/red fluorescence correlating with an increase in mitochondrial depolarization. "Con" means control, "15-" means 15-HETE. All values are denoted as means ± SEM from ten independent photographs shot in each group. $^*P < 0.05$ compared to each other

reverse by NDGA, the endogenous 15-HETE inhibitor. Furthermore, percentage of apoptotic cells induced by Pin treatment was nearly two times more than that in serum-depleted cells, which was partly suppressed by 15-HETE treatment. (Fig. 6b, $n = 10$, $P < 0.05$).

15-HETE suppressed DNA fragmentation in PASMCs

TUNEL assay with fluorescent microscopy were undertaken to determine the effect of 15-HETE on DNA fragmentation in PASMCs. As shown in Fig. 7. A, serum deprivation caused a significant increase in the number of TUNEL-positive cells ($n = 10$, $P < 0.05$ in comparison to the control group). Exogenous 15-HETE relieved the increase of TUNEL-positive cells induced by serum deprivation and Pin ($n = 10$, $P < 0.05$). However, NDGA inversed this protective effect of 15-HETE by increasing the number of TUNEL-positive cells (Fig. 7b, $n = 10$, $P < 0.05$ in comparison to the 15-HETE treated group).

Discussion

In this work, we found that 15-HETE affected a series of apoptotic events, such as maintaining the cell viability, decreasing the expression and activity of caspase-3, reducing the mitochondrial depolarization, suppressing DNA fragmentation and nuclear shrinkage in serum-deprived conditions. What is more, the inhibitive effect of 15-HETE on the apoptosis in PASMCs was relieved by K^+

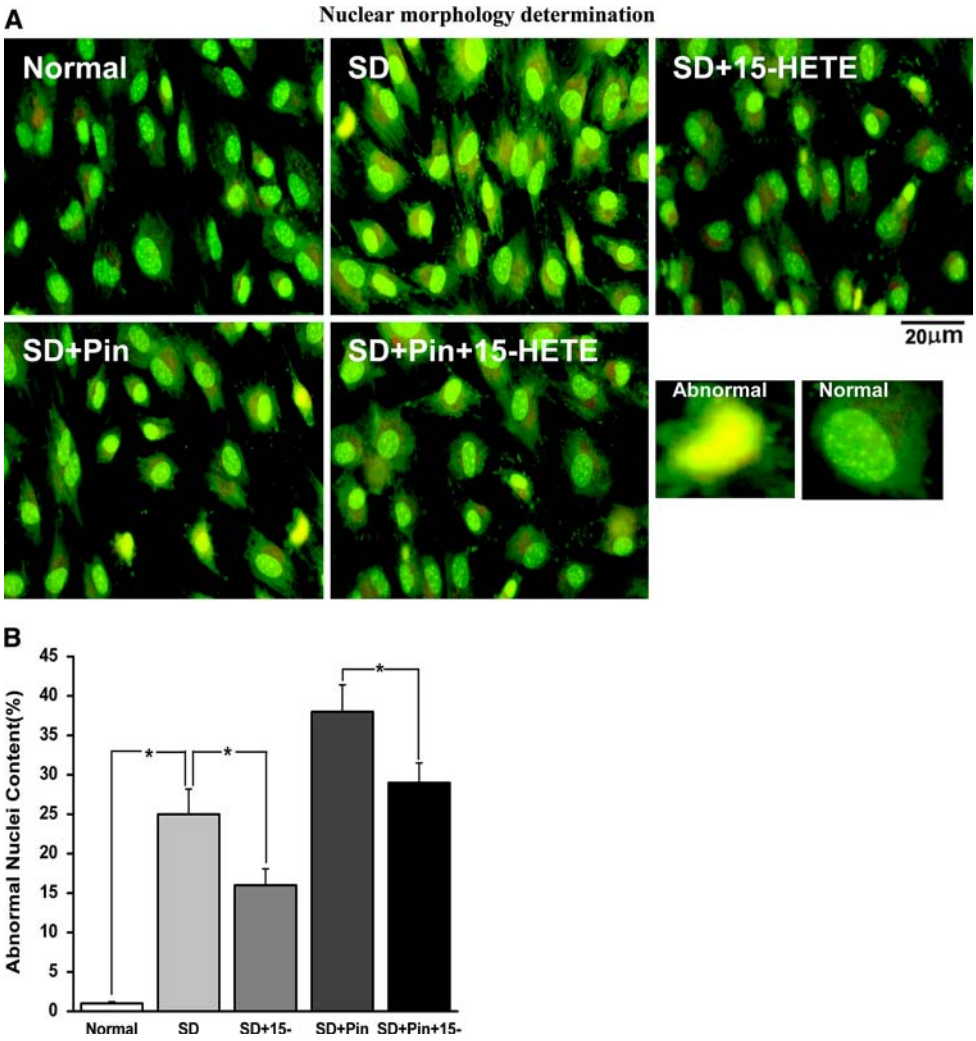

Fig. 6 **a** 15-HETE attenuated nuclear deformation in PASMCs induced by serum deprivation or Pin. A: PASMCs were treated the same way as in Fig. 3. After indicated treatment for 48 h, the number of apoptotic PASMCs were quantified by fluorescent microscope after AO (acridine orange) staining. Scale bars = 20 μm. **b** Quantitative analysis of abnormal nuclei content in different groups. The content was calculated as the ratio of abnormal nuclei (crenation, condensation and fractionation) to the total number of nuclei stained by AO reagent from ten independent photographs shot in each group. "Con" means control, "15-" means 15-HETE. $*P < 0.05$ compared to each other

channel opener Pin. These findings indicate that 15-HETE inhibits the apoptotic responses in PASMC through inactivation of the K^+ channel.

In previous study, we found that hypoxic exposure induced the expression of 15-LO in the pulmonary artery [3]. The 15-LO catalyzed the production of 15-HETE, which was an important mediator in regulating the tone of pulmonary vessels and hypoxic pulmonary vasoconstriction [11, 19]. Hypoxic pulmonary vasoconstriction is only one of the main pathological processes involved in the progression of hypoxic pulmonary hypertension. And the other pathological process is the hypoxic pulmonary remodeling. The disturbance between the growth and the apoptosis in PASMCs under hypoxic conditions is one of the mechanisms responsible for the development of hypoxic pulmonary remodeling [22, 23]. Our previous data showed that hypoxia enhanced 15-HETE is an important mediator of hypoxic pulmonary vasoconstriction. However, whether 15-HETE mediates the progression of hypoxic pulmonary remodeling is still poor understand. Many researches indicated that arachidonate lipoxygenases and its metabolites are essential regulators of cell survival and apoptosis [21, 24]. In the present studies, we found that both hypoxic exposure and exogenous 15-HETE promoted the cellular viability and inhibited the apoptotic responses in PASMCs. NDGA, which inhibited the formation of endogenous 15-HETE, promoted the apoptotic responses in PASMCs under hypoxic conditions. We also found that hypoxia significantly increased PA intimato-media ratios compared with that of normoxia in Sprague–Dawley rats, however, this increasing was attenuated with administration of NDGA to the hypoxic rats (data was not shown). These data suggested that hypoxia protected PASMCs from death through inducing the expression of 15-LO and augmenting the production of endogenous 15-HETE, implying that 15-LO pathway maybe a new target for HPV therapy.

Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors. In contrast, the intrinsic

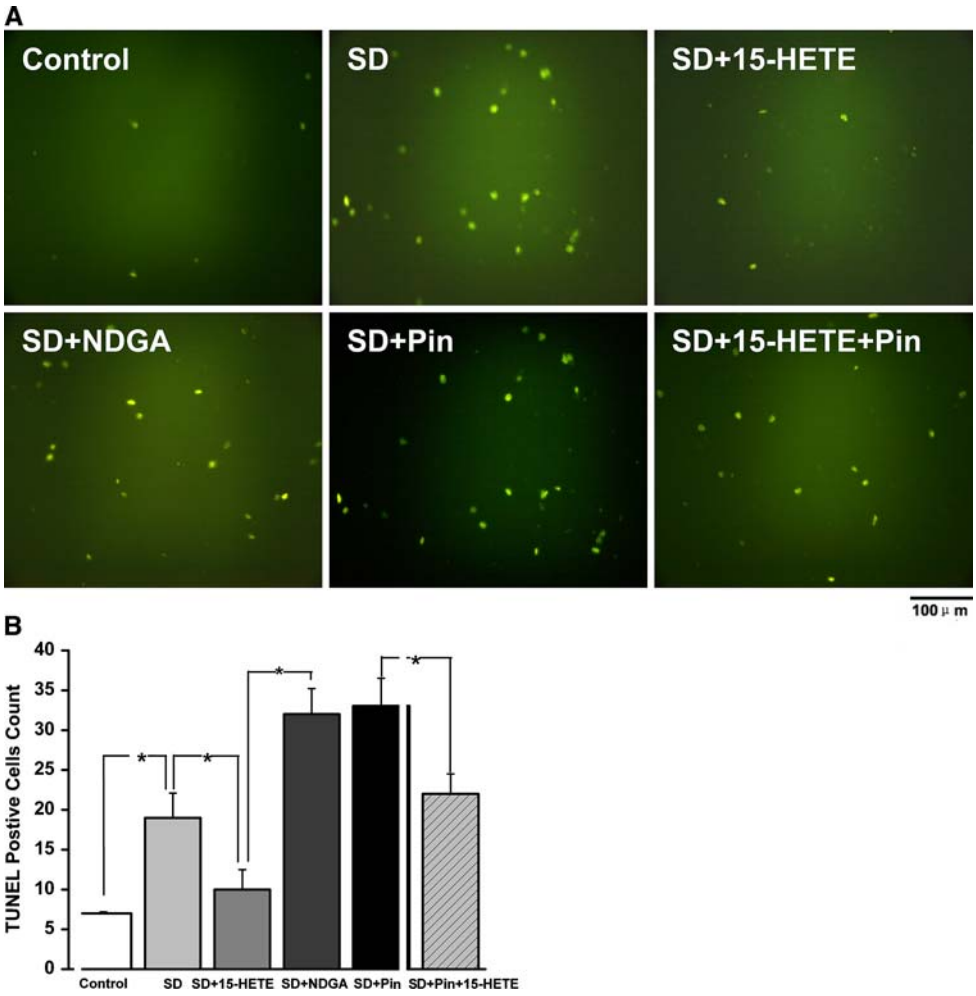

Fig. 7 **a** 15-HETE relieved the increase of TUNEL-positive cells induced by serum deprivation and Pin. The cells were treated the same way as in Fig. 3. **a** After indicated treatment for 48 h, the cells were TdT-UTP nick end labelled and imaged by fluorescent microscope. Scale bar = 100 μm. The content of TUNEL-positive cells was the number of green points in each photograph. **b** Quantitative analysis of TUNEL positive cells content among groups. "Con" means control, "15-" means 15-HETE. All values are denoted as means ± SEM from ten independent photographs shot in each group. $*P < 0.05$ compared to each other

pathway is initiated through the release of signal factors by mitochondria within the cell, which is triggered by many cellular abnormal events. It has been reported that hypoxia activates intracellular signalling pathways involved in apoptosis and cell survival. One pathway of particular importance is that hypoxia-induced mitochondrial membrane permeability, leading to subsequent release to activate caspase-3. Our MTT data showed that hypoxia and 15-HETE exposure leaded to an increase in cell viability, whereas, inhibition of the formation of endogenous 15-HETE decreased the cell viability caused by serum deprivation. As MTT test partly represents mitochondrial respiratory activity, our data may imply that the preventive effect of 15-HETE is related to mitochondrial pathway, for 15-HETE upregulated bcl-2, attenuated apoptotic effect of H_2O_2 on PASMCs. This implication is reinforced by the measurement of mitochondrial membrane potential. Our data showed 15-HETE induced an increase in mitochondrial membrane potential which can be reverse by NDGA, the endogenous 15-HETE blocker. Furthermore, our data indentified that exogenous 15-HETE inhibited the activity and expression of caspase-3 induced by serum deprivation, and this inhibitory effect of 15-HETE was reversed by inhibition of the formation of endogenous 15-HETE. Based on these data, we believe that inhibitory effect of 15-HETE on apoptosis is via keeping mitochondrial membrane potential and then inhibiting caspase-3.

However, the mechanism for the inhibitory effect of 15-HETE on the apoptosis in PASMCs and on the progression of hypoxic pulmonary remodeling is still unknown. K^+ channel is one of the candidates that mediate these specific functions of 15-HETE since 15-HETE downregulated the activity and expression of K^+ channels. In PASMCs, 15-HETE suppressed the whole cell 4-aminopyridine-sensitive K^+ current and downregulated some subtypes of K^+ channel, such as Kv1.5, Kv 2.1, Kv3.4 [11, 19, 20]. Meanwhile, we found that Pin, the opener of K^+ channel, attenuated the inhibitive effect of 15-HETE. It was validated by the decrease of cellular viability, the increase of the expression and the activity of caspase-3, the reduction of mitochondrial membrane potentials and the augmentation of DNA fragmentation and nuclear shrinkage compared with the 15-HETE-treated group in PASMCs. These findings indicate that K^+ channel, at least in part,

mediates the inhibitive effect of 15-HETE on the apoptosis in PASMCs.

It is well known that K^+ channel mediated the apoptotic responses in PASMCs through both receptor-mediated pathway and mitochondria-mediated pathway [12, 25, 26]. In this experiment, we found that 15-HETE attenuated mitochondrial dysfunction in PASMCs. Whereas Pin, the opener of K^+ channel, inhibited the protective role of 15-HETE on membrane potentials and promoted the mitochondrial dysfunction in PASMCs. Interestingly, 15-HETE also attenuated apoptosis effect of H_2O_2 in another experiment (Fig. 5b). Known to all of us that H_2O_2 is a special hypoxia-induced reactive oxygen species (ROS) product directly connect to mitochandrial oxidation-reduction system. ROS lead to the opening of mitochondrial transition pore and mitochondrial depolarization [27], therefore, the protective effect of 15-HETE on the apoptosis in PASMCs may be mediated through a mitochondria- and ROS- dependant way. Clearly, this hypothesis needs to be determined in our future work.

In conclusion, our results have shown that 15-HETE mediates the inhibitive effect of hypoxic exposure on the apoptosis in PASMCs. One mechanism for the specific effect of 15-HETE is through inactivating K^+ channel and promoting mitochondrial dysfunction in PASMCs. These findings together with previous reports indicate that 15-HETE is an important contributor in pulmonary vascular remodeling and pulmonary vascular resistance by regulating not only proliferation but also apoptosis of PASMCs.

Acknowledgments This work was supported by National Natural Science Foundation of China (No. 30870904 and 30470752), Graduate Innovation Foundation of Heilongjiang Province (YJSCX2008-121HLJ).

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