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15-Hydroxyeicosatetraenoic acid (15-HETE) protects pulmonary artery smooth muscle cells against apoptosis via HSP90

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ABSTRACT

Aims: 15-Hydroxyeicosatetraenoic acid (15-HETE), generated by hypoxia, is a product of arachidonic acid and mainly catalyzed by 15-lipoxygenase (15-LO) in pulmonary artery. As HSP90 is known to be involved in apoptosis in other tissues and cells, we aim to test whether anti-apoptotic effect of 15-HETE is regulated by the molecular chaperone in pulmonary artery smooth muscle cells.
Main methods: To test this hypothesis, we performed cell viability analysis, mitochondrial potential assay, caspase-3 activity measurement, Western blot, and terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling with and without HSP90 inhibitor.
Key findings: Our results showed that both exogenous and endogenous 15-HETE up-regulated HSP90 expression and prevented PASMC from serum deprivation-induced apoptosis. Serum deprivation lead to mitochondrial membrane depolarization, decreased expression of Bcl-2 and enhanced expression of Bax, and activation of caspase-3 and caspase-9 in PASMCs. 15-HETE reversed all these effects in a HSP90-dependent manner.
Significance: This study establishes the factor involved in 15-HETE-protecting PASMC from apoptosis and the regulation of HSP90 by 15-HETE may be an important mechanism underlying the treatment of pulmonary artery hypertension and provide a novel therapeutic target in future.

Introduction

Pulmonary arterial hypertension (PAH) is characterized by an elevated pulmonary vascular resistance, smooth muscle remodeling and apoptosis, leading to right heart failure and death (McMurtry et al. 2004; Brevnova et al. 2004). Lumen narrowing and medial hypertrophy of small-sized pulmonary arteries are hallmarks of the pulmonary vascular remodeling processes that are mainly due to increased number of pulmonary artery smooth muscle cells (PASMCs) (Fantozzi et al. 2006; Krick et al. 2001; Suzuki et al. 2007). The imbalance between apoptosis and proliferation results in an augmentation of the number of PASMCs (McMurtry et al. 2004). Chronic hypoxia, an important cause of PAH, inhibits apoptosis in many cells including PASMCs through certain unknown mechanisms (Malhotra et al. 2001; Matsushita et al. 2000; Caretti et al. 2008). Understanding these mechanisms may lead to information for PAH treatment.

Heat shock protein 90 (HSP90) plays a role in apoptosis. It functions as a molecular chaperone to ensure the correct conformation, activity, intracellular localization and proteolytic turnover of a range of proteins that are involved in cell growth, differentiation, activity and survival (Whitesell and Lindquist 2005). The HSP90 regulates many oncogenic client proteins, such as transcriptional factors (e.g., aryl hydrocarbon receptor, glucocorticoid receptor, Myo D, mutant p53 and HIF-1α), kinases (e.g., AKT, ERBB2, C-RAF, B-RAF, v-Src and CDK4), steroid hormone receptors (estrogen and androgen), survivin and telomerase hTERT (Powers and Workman 2006; Isaacs et al. 2002; Sharp et al. 2007), which contribute to the cancer development. Inhibition of HSP90 has been shown to cause degradation of client proteins via the ubiquitin-proteasome pathway (Connell et al. 2001). HSP90 inhibitors, 17-allylamino-17-demethoxygeldanamycin (17AAG) and geldanamycin, induce apoptosis in several cells types, but not including PASMCs (Holmes et al. 2008; Hostein et al. 2001). In hypoxic condition, HSP90 is involved in a mechanism for hypoxia attenuates cellular respiration exposed bovine aortic endothelial cells (BAECs) to extreme hypoxic condition (1–5% O_2) (Presley et al. 2008). But the function of HSP90 in PAH induced by hypoxia remains unknown.

In our previous studies, we have found that chronic hypoxia up-regulates 15-lipoxygenase (15-LO) and catalyzes arachidonic acid

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metabolism to produce 15-hydroxyeicosatetraenoic acid (15-HETE) (Zhu et al. 2003). Both endogenous and exogenous 15-HETE attenuate apoptosis in PASMCs (Li et al. 2009). Hypoxia affects many HSP90 client proteins (AKT, HIF-1α, p53, etc.) in PASMCs, in which 15-HETE plays an important role (Wang et al. 2006; Horstman et al. 2002; Belaiba et al. 2007; Graeber et al. 1994). Therefore, it is possible that 15-HETE protects against apoptosis in PASMCs via HSP90 and its client proteins. To test this hypothesis, we performed Western blot analysis and examined the relationship among 15-HETE, hypoxia and the HSP90 signaling system. Our results show that hypoxia up-regulates the HSP90 expression through 15-HETE, and 15-HETE attenuates apoptosis in PASMCs via HSP90 (Fig. 1).

Materials and methods

Materials

15-HETE dissolved in ethanol was obtained from Cayman Chemical (Ann Arbor, Michigan, USA) and was stored at −20 °C under nitrogen. CCT018159, Radicicol, Cinnamyl 3, 4-dihydroxy-[alpha]-cyanocinnamate (CDC) and nordihydro-guiairetic acid (NDGA) were purchased from Cayman Chemical Company (Ann Arbor, Michigan, USA), reconstituted in ethanol and stored at −20 °C. Antibodies against procaspase-3, bcl-2, bax, caspase-9 and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA). Monoclonal antibody against HSP90, caspase-3 activity kit, JC-1 probe, and the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) cell apoptosis detection kit were from Beyotime Institute of Biotechnology (Haimen, China). Enhanced chemiluminescence (ECL) reagents were obtained from Amersham International (Amersham, UK). All other reagents were from common commercial sources.

Animals

Male Wistar rats (150–200 g) were used in the study. The rats were housed in the Animal Research Center of Harbin Medical University, which is fully accredited by the Institutional Animal Care and Use Committee (IACUC), at a controlled ambient temperature of 22 ± 2 °C with (50 ± 10)% relative humidity and at a 12-h light–dark cycle (lights on at 8:00 AM).

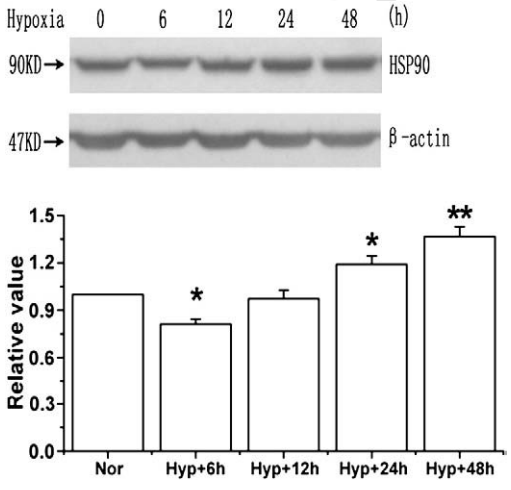

Fig. 1. Time-dependent changes of HSP90 expression. PASMCs were exposed to hypoxia for different time points to examine the expression changes of HSP90. After exposed to hypoxia for 48 h, the expression of HSP90 in rat PASMCs was up-regulated significantly. "Nor" means Normoxia, "Hyp" means Hypoxia. All values are denoted as means ± S.E.M. from six or more independent batches of cells. ($n = 6$, $^{*}P < 0.05$ compared with Normoxia, $^{**}P < 0.01$ compared with Normoxia).

Cell preparation and culture

PASMCs were collected according to our previously published protocol (Han et al. 2004). Cell viability as determined by Trypan Blue exclusion was consistently greater than 98%. The purity of PASMCs in the primary cultures was confirmed by the specific monoclonal antibody raised against smooth muscle α-actin (Boehringer Mannheim, Germany) and cells were cultured in 20% fetal bovine serum (FBS)-DMEM in a 37 °C, 5% CO_2 humidified incubator. Cells in hypoxic condition were incubated with a gas mixture composed of 3% O_2, 5% CO_2, and 92% N_2 for 48 h. Before each experiment, the apoptosis in PASMC was induced by serum deprivation, the cells were incubated in DMEM without serum for 24 h. Passages 2–5 were used for further experimentation.

MTT

PASMCs were cultured in 96-well microtitration plates (about 1×10^4 per well), and then the cells were subjected to growth arrest for 24 h before being placed in either complete medium (DMEM with 10% FBS) or switched to basal medium for the next 24 h. The cells were treated with CCT018159 (3.2 μM) or CCT018159 (3.2 μM) plus 15-HETE (1 μM) in serum deprivation conditions. All drugs (Ethanol, CCT018159, Radicicol and 15-HETE) at the indicated concentration were added every 24 h. The concentration of ethanol in the medium was less than 0.1% (v/v). After 48 h of the incubation in 37 °C, 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, which was prepared in PBS at a concentration of 5 g/L and 20 μL per well. MTT assay was employed in this study to quantitatively assess the viable cell numbers of rat pulmonary artery smooth muscle cells after treatment with 15-HETE, CCT018159 and Radicicol. The dye MTT was tested for possible inhibitory effects on the growth of PASMCs. The MTT reaction was terminated by adding DMSO to the medium followed by incubation for 10 min at 37 °C. The absorbance was read at 540 nm in a spectrophotometer.

Western blot analysis

The cells in 6 well culture clusters were growth-arrested for 24 h before adding vehicle, 15-HETE (1 μM), NDGA (30 μM), CDC (5 μM) and 15-HETE (1 μM) plus NDGA (30 μM) or CDC (5 μM) or vehicle, 15-HETE (1 μM), CCT018159 (3.2 μM), Radicicol (3 μM) or 15-HETE (1 μM) plus CCT018159 (3.2 μM) and Radicicol (3 μM) in serum deprivation medium in normoxic condition or hypoxic condition. The cells cultured in complete medium were considered as control. After the treatment for 48 h, the cells were lysed by 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 lysates were then sonicated and centrifuged at 16,099 g for 10 min, and the insoluble fraction was discarded. The protein concentrations in the supernatant were determined by the bicinchoninic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin (BSA) as a standard. Protein samples (50 μg) were subjected to 10% SDS-PAGE or 12% 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 appropriate antibodies (HSP90, procaspase-3, Bcl-2, Bax and Caspase-9) at a dilution of 1:1000 (HSP90) and 1:500 (procaspase-3, Bcl-2, Bax and Caspase-9) overnight at 4 °C. The blots were then incubated with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents. Immunoblots were scanned using a GS-800 densitometer and protein bands were quantified with Quantity One software (Bio-Rad Laboratories, Hercules, CA).

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Measurement of caspase-3 activity

Caspase-3 activity was measured by cleavage of chromogenic caspase substrates, Ac-DEVD-*p*NA (acetyl-Asp-Glu-Val-Asp p-nitroanilide), a caspase-3 substrate. The optical density value at 405 nm was used as indicative for the amount of caspase-3. The protein samples were prepared as indicated in western blot analysis. Then approximately 50 μg of total protein was added to the reaction buffer containing Ac-DEVD-*p*NA (2 mM), incubated for 4 h at 37 °C, and the absorbance of yellow *p*NA cleavaged from its corresponding precursors was measured using a spectrometer at 405 nm. The specific caspase-3 activity, normalized for total proteins of cell lysates, was then expressed as fold of the baseline caspase activity of control cells cultured in DMEM with 10% FBS.

Mitochondrial depolarization assay

Mitochondrial function was indirectly assessed by variation in mitochondrial transmembrane potential measured by JC-1 red fluorescence. Relative mitochondrial mass was measured by a fluorescence microscope using 5,5′,6,6′-tetrachloro-1,1,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1), analyzed for green fluorescence. After growth-arrested for 24 h, the cells in 6 well culture clusters were treated with vehicle, 15-HETE (1 μM), CCT018159 (3.2 μM) or 15-HETE (1 μM) plus CCT018159 (3.2 μM) in serum deprivation condition for 48 h. Then the cells were stained with JC-1 probe for measuring the depolarization of mitochondrial membrane. 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 green/red fluorescence intensity ratio.

Nuclear morphology determination

Quantitative nuclear chromatin morphology was employed for the apoptosis counting. PASMCs were cultured in a six-well culture cluster to ~60% confluency. The cells were treated as described for the mitochondrial depolarization assay. Then the cells were stained with 5 μl of acridine orange (AO) (5 mg/ml, Sigma) in 1 ml basal medium and incubated for 10 min at room temperature (22–24 °C). Stained cells were washed three times with PBS and imaged under a fluorescent microscope at 488 nm laser excitation and 405 nm emission. For each well, 15–25 shots 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) method was performed to label 3′-end of fragmented DNA of the apoptotic PASMCs. The cells cultured in 6-well plates were treated as mentioned in mitochondrial depolarization assay, and fixed with 4% paraformaldehyde phosphate buffer saline, rinsed with PBS, then permeabilized by 0.1% Triton X-100 for 2 min on ice followed by TUNEL for 1 h at 37 °C. The FITC-

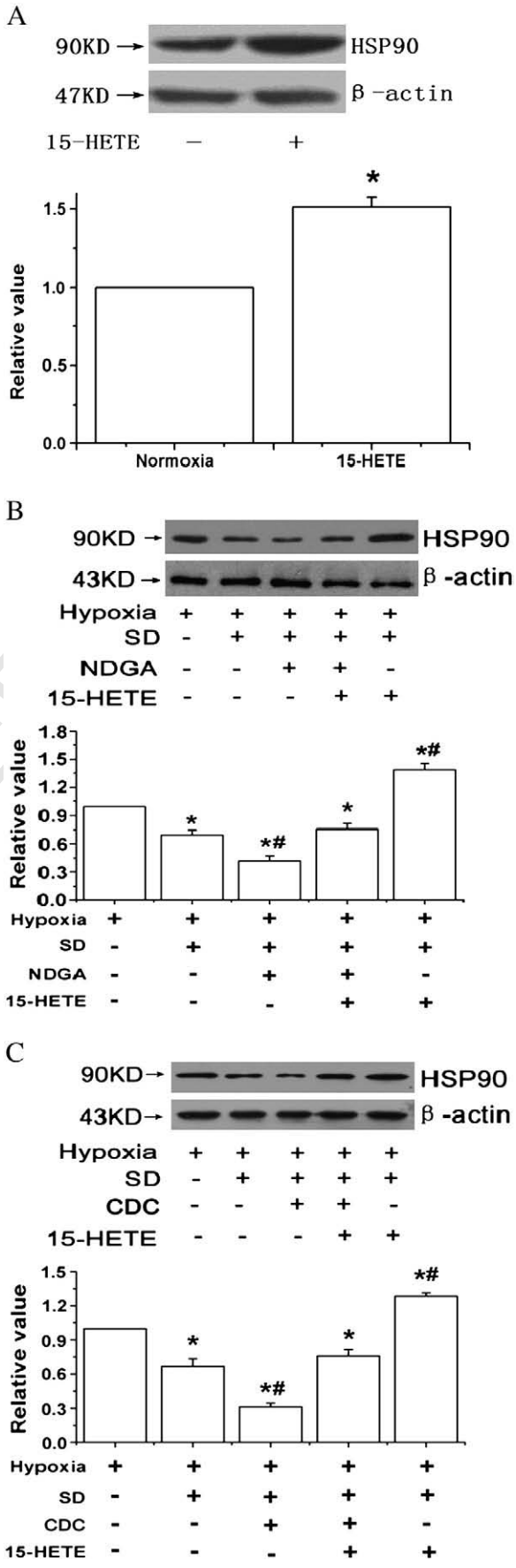

Fig. 2. Exogenous and endogenous 15-HETE promotes the expression of HSP90. A: The expression of HSP90 after adding 15-HETE under normoxic condition. B: The expression of HSP90 after blocking endogenous 15-HETE generation with NDGA under hypoxic condition. C: The expression of HSP90 after inhibiting endogenous 15-HETE with CDC under hypoxic condition. "SD" means serum deprivation. All values are denoted as means ± S.E.M. from six or more independent batches of cells. ($n = 6$, $^*p < 0.05$ compared with Control; #$p < 0.05$ compared with SD).

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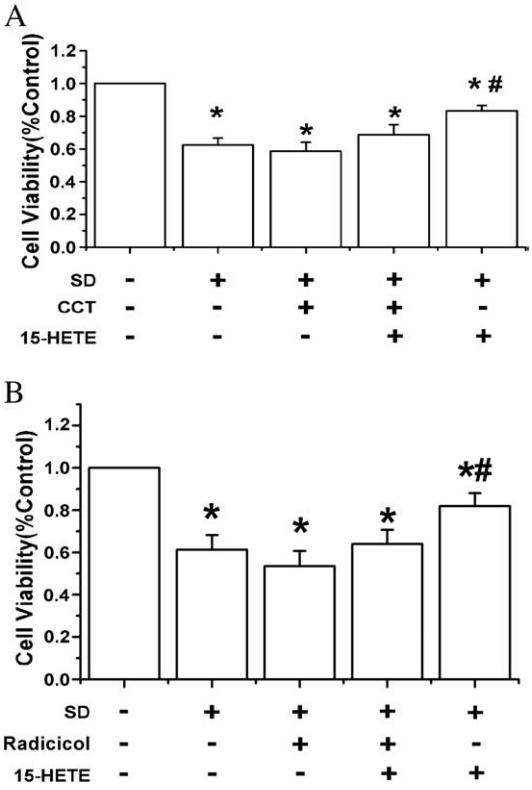

Fig. 3. The cell viability of cultured pulmonary artery smooth muscle cells after serum deprivation was examined by MTT. PASMCs were treated as the anticipated groups under normoxic condition for 48 h and cell viability was tested by the MTT assay. CCT018159 and Radicicol (HSP90 inhibitors) inhibited the protective effect of 15-HETE on cell viability in serum deprivation conditions. "SD" means serum deprivation, "CCT" means CCT018159. All values are denoted as means ± S.E.M. from six or more independent batches of cells. ($n = 6$, $^*p < 0.05$ compared with Control; $\#p < 0.05$ compared with SD).

labeled TUNEL-positive cells were imaged under a fluorescence microscopy at 488-nm excitation and 530-nm emission. The cells with green fluorescence were defined as apoptotic cells.

Statistics

The composite data were expressed as means ± S.E.M. Statistical analysis was performed with Student's *t*-test, *u*-test or one-way ANOVA followed by Dunnett's test where appropriate. Differences were considered to be significant at $P \leq 0.05$.

Results

Changes in HSP90 expression under sub-acute hypoxic condition

Previous studies have shown that hypoxia stimulates HSP90 protein expression in endothelial cells (Presley et al. 2008). To determine how the HSP90 in PASMCs is affected by hypoxia at different time points, we applied Western blot to examine the expression of HSP90 and found that hypoxia (PO_2 23 Torr) produced a biphasic response in cultured PASMCs. While the HSP90 protein expression was down-regulated with 6 h hypoxia, a clear up-regulation was seen with a longer hypoxic exposure. The protein levels were up-regulated by about 19% with 24 h hypoxia and 35% with 48 h hypoxia ($n = 6$, $p < 0.05$).

Exogenous and endogenous 15-HETE up-regulated HSP90 expression in cultured PASMCs

To demonstrate the influence of 15-HETE on the HSP90 expression, we studied the effect of exogenous 15-HETE on HSP90 expression under normoxic condition. Our results showed that the HSP90 protein expression was up-regulated when cells were treated with 15-HETE for 48 h. In separate studies, inhibiting the generation of endogenous 15-HETE with NDGA or CDC, two selective 15-LO inhibitors, relieved the up-regulation of HSP90 expression caused by hypoxia, suggesting that the up-regulation of the HSP90 expression induced by hypoxia is mediated by endogenous 15-HETE (Fig. 2A, B, and C).

15-HETE improved PASMC viability via HSP90

The cell viability was determined by measuring colorimetric conversion of MTT to formazan (Li et al. 2009), a method that we used to evaluate PASMC viability. We found that serum deprivation markedly decreased the PASMC viability, while 15-HETE had a protective role in cell viability after serum deprivation. Such protective role was significantly attenuated with 3.2 μM CCT018159 or 3 μM Radicicol (Fig. 3A and B, $n = 6$, $P < 0.05$).

15-HETE relieved mitochondrial depolarization, induced Bcl-2 expression and suppressed Bax expression via HSP90

An important indication of apoptosis is the mitochondrial membrane potential. To ascertain whether HSP90 is involved in the mitochondrial-dependent apoptosis and inhibits PASMC apoptosis, we performed the assay of the mitochondrial membrane potential using mitochondrial membrane potential kit and examined the expression of mitochondrial membrane proteins (Bcl-2 and Bax).

Normal PASMCs stained with JC-1 emitted mitochondrial orange-red fluorescence with a little green fluorescence, while in apoptotic PASMCs JC-1 was dispersed to the monomeric form (green fluorescence). The quantitative analysis of JC-1-stained cells revealed a significant decrease in the red (high $\Delta\Psi m$) to green (low $\Delta\Psi m$) ratio in SD-treated cells when compared with control cells, which were cultured in the presence of 20% FBS ($p < 0.05$, $n = 10$). A treatment of SD cells with 15-HETE significantly increased the red fluorescence. Exposure of SD with 15-HETE cells to CCT018159 suppressed the effect of 15-HETE without marked changes in $\Delta\Psi m$ compared to SD cells (Fig. 4A, $n = 10$, $P < 0.05$).

Bcl-2 and Bax, both of which are localized on mitochondrial membrane and associated with mitochondrial function, play important roles in cell apoptosis. Bcl-2 is an anti-apoptotic protein, while Bax is a pro-apoptotic protein. We found 15-HETE up-regulated the Bcl-2 expression and inhibited the Bax expression, while CCT018159

Fig. 4. HSP90 involves in the inhibitory effect of 15-HETE on mitochondrial potentials reduction induced by serum deprivation in PASMCs. A: The cells were stained with JC-1 probe and imaged by fluorescent microscope. The individual red and green average fluorescence intensities are expressed as the ratio of green to red fluorescence. The increase of fluorescence ratio, which is represented in the bars, is correlating with an increase in mitochondrial depolarization A–E, representative photographs of JC-1 staining in different groups. Scale bars = 40 μm. B: quantitative analysis of the shift of mitochondrial red fluorescence to green fluorescence among groups. C: The expression of Bcl-2 in rat PASMCs under normoxic condition. The expression of Bcl-2 increased by exogenous 15-HETE is partly inhibited by CCT018159 (HSP90 inhibitor). D: The expression of Bax in rat PASMCs under normoxic condition. The expression of Bax decreased by exogenous 15-HETE is partly inhibited by CCT018159 (HSP90 inhibitor). E: The expression of Bcl-2 in rat PASMCs. The effect of 15-HETE on the expression of Bcl-2 was weakened after using the HSP90 inhibitor (Radicicol). F: The expression of Bax in rat PASMCs. Radicicol decreased the inhibitory effect of 15-HETE on Bax expression. "SD" means serum deprivation, "CCT" means CCT018159, "15-" mean 15-HETE. All values are denoted as means ± S.E.M. from six or more independent batches of cells or ten independent photographs shot in each group. ($n = 6$, $^*p < 0.05$ compared with Control; $\#p < 0.05$ compared with SD). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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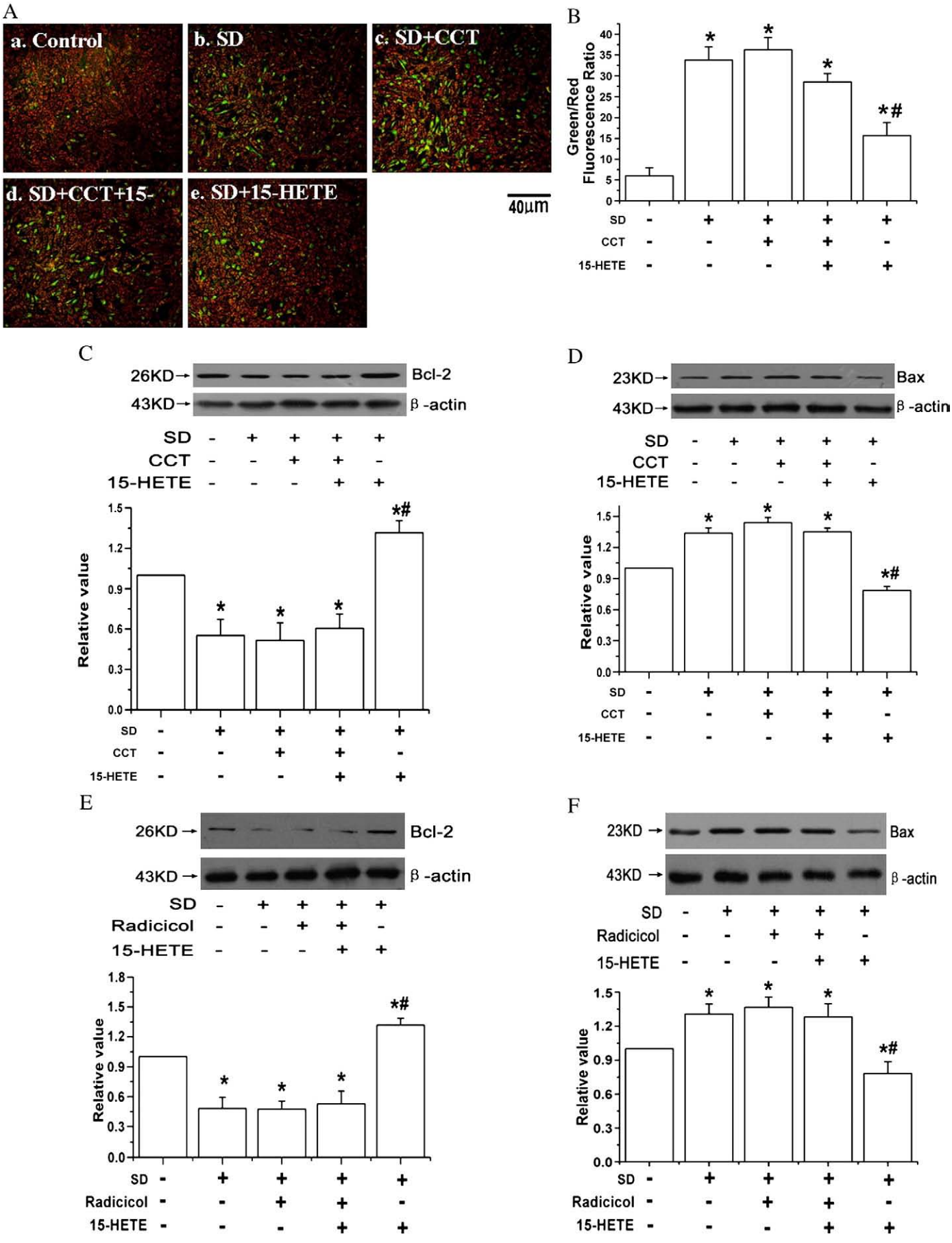

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partially attenuated these effects (Fig. 4C and D, $n=6$, $P<0.05$). Similar results were obtained after applying another HSP90 inhibitor (Radicicol) (Fig. 4E and F, $n=6$, $P<0.05$). All these results thus indicated that 15-HETE regulates pro- and anti-apoptotic proteins on mitochondrial membranes at least partly through HSP90, leading to better maintenance of mitochondrial integrity.

The inhibitory effects of 15-HETE on caspase-3 activation, caspase-9 expression and procaspase-3 cleavage were blocked by HSP90 inhibitors

Caspase-9 is another pro-apoptotic signaling molecule. To determine whether the 15-HETE signaling system affects caspase-9 activation, we examined caspase-9 expression with Western blot and found that SD activated caspase-9, while 15-HETE in SD condition partially inactivated caspase-9. The caspase-9 inactivation induced by 15-HETE was weakened after applying HSP90 inhibitor CCT018159 (Fig. 5B, $n=6$, $P<0.05$).

Caspase-3, a down-stream protein of caspase-9, is synthesized as a precursor protein procaspase-3 that undergoes cleavage in response to apoptotic stimuli by initiator caspases (including caspase-2, -8, -9, -10, -11 and -12) and then becomes activated. The caspase-3 is cleaved from procaspase-3 whose expression has been used to indicate caspase-3 activity (Chen et al. 2007). We found that the inhibitory effect of 15-HETE on the cleavage of procaspase-3 was partly blocked by HSP90 inhibitor (CCT018159). (Fig. 5A, $n=6$, $P<0.05$). Similar result was acquired in caspase-3 activity assay. 15-HETE decreased the caspase-3 activity and inhibited its activation through HSP90 (Fig. 5C, D, $n=6$, $P<0.05$).

Inhibition of HSP90 abolished the inhibitory effects of 15-HETE on nuclear shrinkage and DNA fragmentation in PASMCs

To study whether 15-HETE prevented PASMCs from nuclear shrinkage via HSP90, we examined the morphology of cell nuclear by staining with Acridine orange in PASMCs. The percentage alteration of nuclei conformation was used to determine the degree of apoptosis of PASMC. We found that exogenous 15-HETE significantly reduced the number of abnormal nuclei content cells (crenation, condensation and fractionation) generated by SD (Fig. 6). However, after inhibition of HSP90 by CCT018159, 15-HETE was no longer able to inhibit the alteration of nuclei morphology under SD condition in PASMCs (Fig. 6, $n=10$, $P<0.05$).

TUNEL assay was undertaken to determine whether HSP90 participated in the 15-HETE-inhibited DNA fragmentation in PASMCs. As shown in Fig. 7B ($n=10$, $P<0.05$), the number of TUNEL-positive cells was counted after SD for 48 h. We found 15-HETE significantly reduced the number of TUNEL-positive cells induced by SD. The protective effect of 15-HETE was weakened after blocking HSP90 with CCT018159.

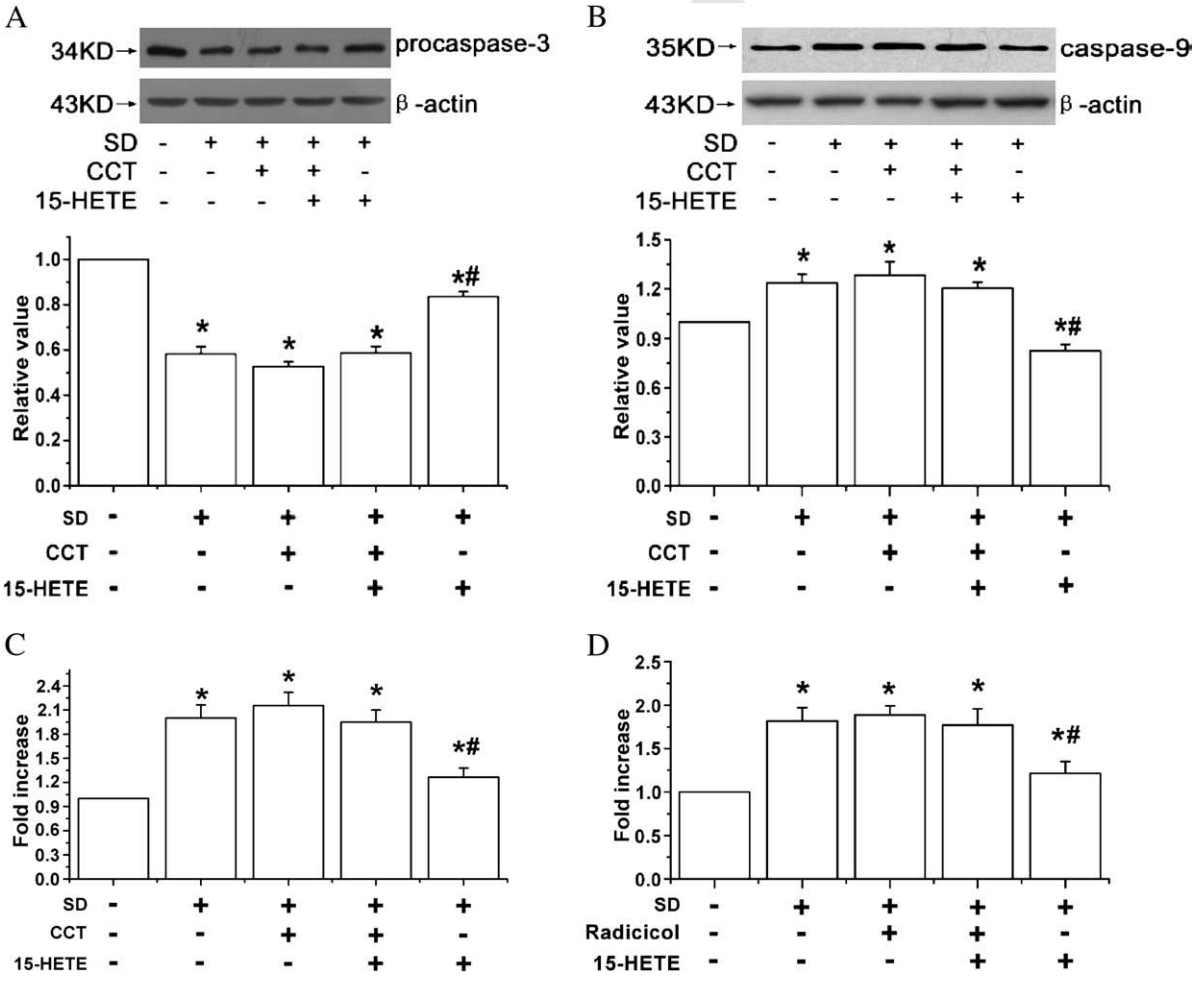

Fig. 5. Exogenous 15-HETE inhibits the cleavage of procaspase-3, the activity of caspase-3 and the expression of caspase-9 through HSP90. A: The expression of procaspase-3 in rat PASMCs under normoxic condition. B: The expression of caspase-9 in rat PASMCs under normoxic condition. C: Caspase-3 activity was measured by cleavage of the Ac-DEVD-pNA substrate to pNA. D: The inhibitory effect of 15-HETE on caspase-3 activity was abolished after blocking the HSP90 with Radicicol. 15-HETE decreases the caspase-3 activity and suppresses cleavage of procaspase-3 and the caspase-9 expression in PASMCs under serum deprivation condition via HSP90. "SD" means serum deprivation, "CCT" means CCT018159. All values are denoted as means ± S.E.M. from six or more independent batches of cells. ($n=6$, $^*p<0.05$ compared with Control; $\#p<0.05$ compared with SD).

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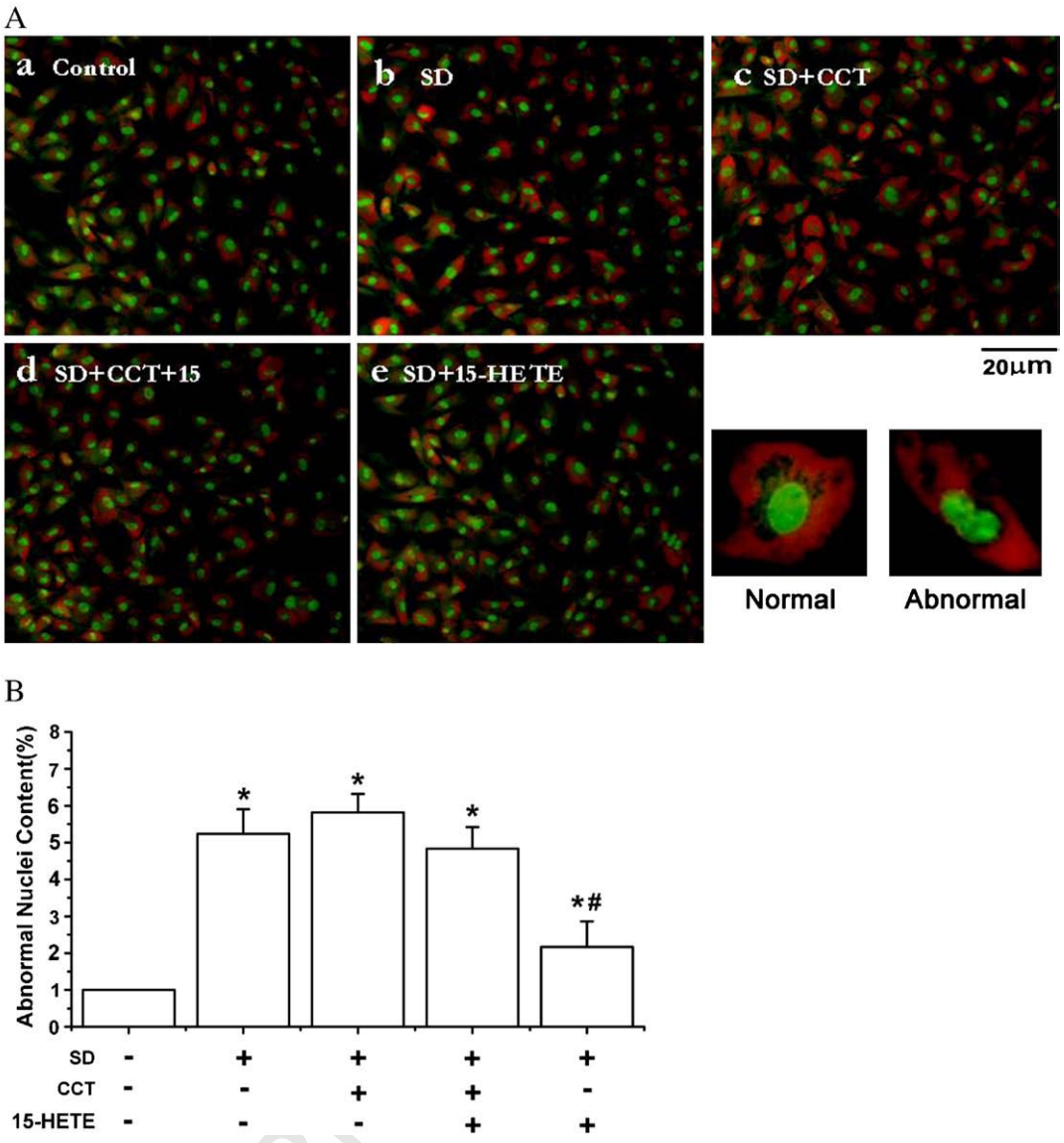

Fig. 6. 15-HETE suppresses the nuclear deformation and chromatin condensation via HSP90. A: PASMCs were treated the same way as Fig. 4A and then stained with AO (acridine orange). The number of apoptotic PASMCs were quantified by fluorescent microscope after staining. Scale bars = 20 μm. B: Quantitative analysis of abnormal nuclei content (crenation, condensation and fractionation) 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. "SD" means serum deprivation, "CCT" means CCT018159, "15" means 15-HETE. All values are denoted as means ± S.E.M. from ten independent photographs shot in each group. ($n = 10$, $^{*}p < 0.05$ compared with Control; $\#p < 0.05$ compared with SD).

Discussion

Pulmonary artery hypertension (PAH), containing two physiological processes (pulmonary vasoconstriction and vascular remodeling), is mainly caused by hypoxia. 15-HETE, as an important product induced by hypoxia, participates in both processes in hypoxic PAH. Our previous research show that hypoxia induces the generation of 15-HETE in pulmonary artery, and 15-HETE constricts pulmonary artery in a dose-dependent manner, and some cell signals mediates pulmonary vasoconstriction induced by 15-HETE, such as ERK1/2, RhoA/ROCK, and Kv channel. (Chu et al. 2009; Guo et al. 2008; Lu et al. 2006; Wang et al. 2010; Zhu et al. 2003). We also find 15-HETE mediates the pulmonary vascular remodeling (PVR) through inhibition of PASMC apoptosis and promotion of proliferation. NDGA (15-LO inhibitor) blocks PVR and decreases the blood pressure in PAH rat model (Ma et al. 2010). However, the factors participating in the processes for 15-HETE-induced inhibition of apoptosis are unclear. Our results from this study suggest that HSP90 is involved in the 15-HETE-induced inhibition of apoptosis in PASMCs.

The novel finding of this study is that 15-HETE protects PASMC from apoptosis via HSP90. To our knowledge, this is the first demonstration of the role of HSP90 in hypoxic pulmonary arterial hypertension. There are reports that hypoxia up-regulates the expression of HSP90, which is involved in regulating a wide range of fundamental cell functions (Presley et al. 2008; Isaacs et al. 2004). However, the mediators that participated in this process remain unknown. In our experiments, NDGA or CDC, 15-LO inhibitors, reverse the effects of hypoxia on the expression of HSP90, and exogenous 15-HETE significantly induces the expression of HSP90. The results show that hypoxia up-regulates the expression of HSP90 through generating endogenous 15-HETE.

It is reported that HSP90 can bind to p53, Akt and HIF-1α to modulate apoptosis and survival of cells (Isaacs et al. 2002; Sato et al. 2000; Blagosklonny et al. 1996). VEGF165 promotes survival of leukemic cells by HSP90-mediated apoptosis inhibition and inhibition HSP90 with 17-allylamino-17-demethoxygeldanamycin results in apoptosis (Hostein et al. 2001; Dias et al. 2002). Moreover, inhibition of apoptosis and promotion of proliferation in PASMCs likely result in

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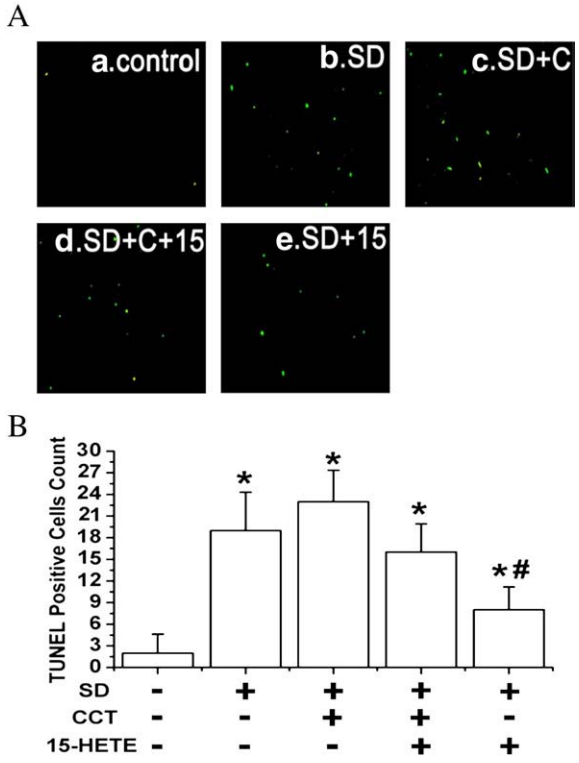

Fig. 7. HSP90 mediated the inhibitory effect of 15-HETE on the increase of TUNEL-positive cells induced by serum deprivation. PASMCs were treated the same way as Fig. 4A before TUNEL staining and fluorescent imaging. Cells undergoing apoptosis were positively stained with TUNEL reagent and are shown in green. A: Representative photographs of TUNEL staining in different groups. Scale bars = 100 μm. B: Quantitative analysis of TUNEL-positive cells content among groups. "SD" means serum deprivation, "C" means CCT018159, "15" means 15-HETE. All values are denoted as means ± S.E.M. from ten independent photographs shot in each group. ($n = 10$, $^*p < 0.05$ compared with Control; $\#p < 0.05$ compared with SD).

pulmonary vessel remoldeling and pulmonary artery medial hypertrophy, which is a major component in the development of hypoxic PAH (McMurtry et al. 2004; Fantozzi et al. 2006). Our previous studies have shown that 15-HETE, an important mediator during PAH induced by hypoxia, inhibits the apoptosis of PASMCs and induces pulmonary artery medial hypertrophy (Ma et al. 2010). All the above findings raise the possibility that 15-HETE inhibiting the apoptosis of PASMCs is mediated by HSP90. In our study, we have found that the protective effect of 15-HETE on cell viability is significantly blocked by the HSP90 inhibitors (CCT018159 and Radicicol). Two apoptotic indications, AO and DNA nick end-labeling (TUNEL), have also been examined. The effects of serum deprivation on both indications are attenuated by 15-HETE, and CCT018159 significantly inhibits the protective effects of 15-HETE. These findings are consistent with our hypothesis that HSP90 is involved in the process that 15-HETE inhibits the apoptosis of PASMCs and induces the PVR during hypoxic PAH.

We further explore the mechanism how HSP90 is involved in the process of 15-HETE protective effect on PASMCs apoptosis. There are reports that the apoptosis induced by serum deprivation causes the mitochondrial dysfunction (Wang et al. 2008), and the disruption of the mitochondrion leads to the release of cytochrome c into the cytosol, binding to Apaf-1 and activation of caspase-9 and caspase-3 to induce apoptosis (Samraj et al. 2007; Hengartner 2000). In our experiments, we have found 15-HETE inhibits the decrease of the mitochondrial potential and affects the expression of Bcl-2 and Bax, localized on the mitochondrial membranes and controlled the stabilization of mitochondrial membranes. However, the protective effect of 15-HETE is abolished after inhibiting HSP90 with CCT018159. Furthermore, HSP90 inhibitors (CCT018159 and Radicicol) reverse the inhibitory effects of 15-HETE on the activation of caspase-3 and the expression of caspase-9. All the above results indicate that 15-HETE inhibits the PASMCs apoptosis through the mitochondria-dependent ways, and HSP90 is involved in this process as an essential factor.

An interesting finding of our study is that hypoxia produces a biphasic response in the HSP90 protein expression in cultured PASMCs. The expression of HSP90 is inhibited after 6-h exposure of hypoxia, while hypoxia significantly induces the expression of HSP90 after 24 h. A possible explanation is that HSP90 is a stress protein. Short time hypoxia leads to acute stress response, which results in the inhibition of HSP90 expression. Similar results are reported by Yunchao Su (Su and Block 2000). However, the long treatment of hypoxia to PASMC for 48 h, induces a great amount of endogenous 15-HETE, and our results have shown that 15-HETE promotes the HSP90 expression, so exposure to hypoxia for 24 to 48 h significantly induces the expression of HSP90. We speculate that HSP90 may play a different pathophysiological role in the development stages of pulmonary arterial hypertension induced by sub-acute hypoxia. Clearly, this hypothesis needs to be determined in our future work.

Although our results imply that HSP90 participates in the inhibitory effect of 15-HETE on PASMC apoptosis, further studies should evaluate which proteins bind to HSP90 and the major subunit of HSP90 in the inhibitory processes. Our study mainly focuses on the intrinsic pathway. The extrinsic pathway, which is also important in the apoptotic process, should be addressed in future studies.

In conclusion, we have demonstrated that 15-HETE inhibits the apoptosis of PASMC via HSP90. Furthermore, HSP90, which plays an important role in the stress processes, may be a key factor in hypoxia-induced pulmonary artery hypertension, and it provides a new direction for future studying the pathogenesis of PAH.

Conclusion

HSP90 is involved in the process that 15-HETE protects PASMCs from apoptosis induced by serum deprivation via mitochondrial pathway.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Uncited reference

Zheng et al. 2008

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