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20-Hydroxyeicosatetraenoic acid inhibits the apoptotic responses in pulmonary artery smooth muscle cells

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Abstract

20-Hydroxyeicosatetraenoic acid (20-HETE), a ω-hydroxylation product of arachidonic acid catalyzed by cytochrome P450 4A (CYP4A), plays a role in vascular smooth muscle remodeling. Although its effects on angiogenic responses are known, it remains unclear whether 20-HETE acts on apoptosis of pulmonary arterial smooth muscle cells (PASMC), an important step in PASMC remodeling, and what pathways are involved in the process. Here we show evidence for the missing information. The effect of 20-HETE on PASMC apoptosis and the apoptosis-associated signaling pathways were determined with cell viability assay, Annexin V and propidium idodide binding, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), mitochondrial potentials assay, caspase activity assay and Western blots. We found that exogenous 20-HETE suppressed the serum deprivation-induced loss of bovine PASMCs and prevented Annexin V binding, DNA nick end labeling and chromatin condensation. The effect was worsened by 17-octadecynoic acid (17-ODYA),

which inhibited the production of endogenous 20-HETE. Furthermore, 20-HETE induced the

expression of bcl-2, maintained the stability of mitochondria membrane, and relieved the

activation of caspase-9 and caspase-3. Such effects were reversed in the presence of

17-ODYA. Thus, these findings indicate that 20-HETE protects PASMCs against apoptosis

by acting on, at least in part, the intrinsic apoptotic pathway.

1. Introduction

20-hydroxyeicosatetraenoic acid (20-HETE), a ω-hydroxylation product of arachidonic

acid catalyzed by cytochrome P450 4A (CYP 4A), is a paracrine and autocrine mediators of

numerous cellular processes (Williams et al., 2007; Kroetz and Xu, 2005; Kroetz and Zeldin,

2002; Roman, 2002). It is produced in renal, cerebral, pulmonary, mesenteric, and skeletal

muscle microvascular beds and acts on the microvasculature and kidney tubules (Imig et al.,

1996; Gebremedhin et al., 2000; Kunert et al., 2001; Wang et al., 2001; Zhu et al., 2002).

Accumulating experimental evidence indicates that 20-HETE affects angiogenic responses.

It is known that the growth of new blood vessels depends on the formation of both

capillary-like tubes of endothelial cells and the subsequent infiltration of vascular smooth

muscle cells (VSMCs) (Voelkel et al., 2007). 20-HETE could stimulate the proliferation of

endothelial cells. It plays a role in the angiotensin II-induced neointimal formation (Yaghini

et al., 2005), and such angiogenic effect relies on the increased expression of vascular

endothelium growth factor (Guo et al., 2007). Over-expression of CYP4A in vascular smooth

muscle cells promotes endothelial sprouting in renal arterioles (Jiang et al., 2004). Meanwhile,

proliferation and migration of the VSMCs is another crucial step in angiogenic responses. 20-HETE promotes VSMC migration via platelet derived growth factor (Stec et al., 2007), and it also acts as an important mitogen mediating the VSMC proliferation produced via small GTP binding proteins (Muthalif et al., 2001). Although the role of 20-HETE on proliferation and migration of the VSMCs had been investigated in systemic circulation in some degree, its effects on pulmonary vasculature remodeling remain to be determined.

Similar to cell proliferation, apoptosis is another cellular process responsible for maintaining normal function of tissues and organs. The regulation of apoptosis is one of the cellular mechanisms for neonatal vascular remodeling (Kim et al., 2000). Impaired apoptosis often causes the loss of control of VSMC growth and inflammation (Gulbins et al., 2000), leading to intimal media thickness and persistent constriction of the pulmonary arteries (Stenmark et al., 2006). It is possible that 20-HETE affects the vascular remodeling by protecting PASMCs from apoptosis in addition to its effects on VSMC proliferation and migration. To test this hypothesis, we examined the effect of exogenous 20-HETE on the viability of bovine PASMCs in vitro. We used serum deprivation (SD) to induce PASMC apoptosis and quantified annexin V binding, DNA nick end labeling, mitochondrial potentials and caspase-9 and caspase-3 activities. Our results showed that 20-HETE suppressed the serum deprivation-induced loss of PASMCs and was capable of attenuating the apoptotic response. These effects appear to be mediated through specific apoptotic pathways.

2. Materials and methods

2.1. Materials

20-HETE dissolved in ethanol was obtained from Cayman Chemical (Ann Arbor, USA) and stored at -20℃ under nitrogen. 17-octadecynoic acid (17-ODYA) was purchased from Sigma-Aldrich Co. (St. Louis, USA), reconstituted in ethanol and stored at -20℃. Antibodies against bcl-2, procaspase-3 and actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, USA). Polyclonal antibodies against CYP4A and CYP2J2 were purchased from Affinity Bioreagents (Golden, USA). The annexin V-FITC kit was purchased from BD Biosciences (San Jose, USA). The terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) cell apoptosis detection kit, JC-1 probe, caspase-9 and caspase-3 activity assay kit and lactate dehydrogenase (LDH) activity kit were provided by Beyotime Institute of Biotechnology (Haimen, China). Enhanced chemiluminesence (ECL) reagents were from Amersham International (Amersham, UK). All other reagents were purchased from Sigma.

2.2. Cell Culture

Calf was used in the studies in full compliance with the Ethical Committee of Laboratory Animals at Harbin Medical University. Primary cultures of PASMCs were prepared as previously described (Zhu et al., 2002). In brief, pulmonary conduct arteries were isolated from lungs of calf obtained at a local abattoir, slit open along their lengths, and washed with phosphate buffered saline (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 ($\sim$ 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). Passages 2-5 were used for further experimentation.

2.3. MTT Assay

PASMCs were cultured in 60-mm dishes ($\sim 1 \times 10^6$ cells) to $\sim$ 80% confluency, and then the cells 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 42-48 h. The samples were treated with 20-HETE (10-1000 nM), 17-ODYA (1 μM), indomethacin (1 μM) or ethanol (vehicle). Ethanol and other agents at the indicated concentration was added every 24 h. The concentration of ethanol in the medium was less than 0.1% (v/v). At the end of the incubation period, 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 viable cells. The MTT reaction was terminated by adding dimethyl sulphoxide to the medium followed by incubation for 10 min at 37℃. The absorbance was read at 540 nm in a spectrophotometer.

2.4. LDH Release

Cytotoxicity was quantified by measuring the activity of lactate dehydrogenase (LDH) released into culture medium. Percentages of injured cells in cultures are represented by the

LDH activities of medium relative to total LDH activity after complete cell lysis. Briefly, a portion of culture medium was reacted with an equal volume of LDH substrate solution for 30 min. The reaction was stopped by adding 5 volume of 0.1 M NaOH, and absorbance was then measured at 440 nm in a spectrophotometer. Sister cultures were treated with 1/100 volume of 10% Triton X-100 and incubated at 37℃ for 30 min. Total LDH activities were determined using medium containing Triton-lysed cellular supernatant.

2.5. Hoechst Staining

PASMCs were cultured in four-well chamber slides to ~70% confluency. The cells were treated as described for the MTT assay. Then the cells were stained with 1 µl of Hoechst 33342 (5 mg/ml, Sigma) in 1 ml basal medium and incubated for 30 min. Stained cells were washed twice with PBS and imaged under a fluorescent microscope by using 350-nm stimulation and 460-nm emission.

2.6. Annexin Ⅴ and propidium iodide (PI) staining

An apoptosis detection kit was used for annexin Ⅴ and PI staining. PASMCs were cultured in 60-mm dishes to ~80% confluency, washed twice, and then incubated with basal medium for 24 h. Subsequently, vehicle, 17-ODYA (1 µM) or 20-HETE (300 nM) was added. After treatment for 24 h, the cells were washed with PBS and resuspended in the binding buffer. The fluorescein-conjugated annexin Ⅴ (10 µl) and PI reagent (5 µl) were added to cell suspensions. Then the cells were incubated in dark for 15 min at room temperature, and the percentage of apoptotic cells and necrotic cells were assessed by FACScan flow cytometry.

2.7. TUNEL

The TUNEL method was performed to lable 3'-end of fragmented DNA of the apoptotic

PASMCs. The cells treated as indicated were fixed with 4% paraform phosphate buffer saline, rinsed with PBS, then permeabilized by 0.1% Triton X-100 for FITC end-labeling the fragmented DNA of the apoptotic PASMCs using TUNEL cell apoptosis detection kit. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscopy by using 488-nm excitation and 530-nm emission.

2.8. Mitochondrial membrane potentials assay

JC-1 probe was employed to measure mitochondrial depolarization in PASMCs. Briefly, Cells cultured in six-well plates after indicated treatments were incubated with an equal volume of JC-1 staining solution (5 μg/ml) at 37℃ 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 an Olympus fluorescent microscope under Argon-ion 488 nm laser excitation. Mitochondrial depolarization is indicated by an increase in the green/red fluorescence intensity ratio.

2.9. Caspase-3 and caspase-9 activity assay

Caspase-3 and caspase-9 activity were measured by cleavage of chromogenic caspase substrates, Ac-DEVD-pNA (acetyl-Asp-Glu-Val-Asp p-nitroanilide) and Ac-LEHD-pNA (acetyl-Leu-Glu-His-Asp p-nitroanilide). Approximately 50 μg of total protein was added to reaction buffer containing Ac-DEVD-pNA (2 mM) and Ac-LEHD-pNA (2 mM), incubated for 2 h at 37℃, and the absorbance of yellow pNA cleavaged from its corresponding precursors were measured using a spectrometer at 405 nm. The specific caspase 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.

2.10. Western blot analysis

Cultured PASMCs were initially treated with the chemicals as described for annexin Ⅴ

and PI staining. After treatment for 24 h, the cells were washed three times with ice-cold PBS.

Proteins were solubilized and extracted with 300 µl lysis buffer (Tris 50 mM, pH 7.4, NaCl

150 mM, Triton X-100 1%, EDTA 1 mM, and PMSF 2 mM). The lysates were used to

estimate their protein content with Bradford protein assay. Equal amounts of protein (10 µg)

from each sample were subjected to electrophoresis on a SDS-polyacrylamide gel, transferred

onto nitrocellulose membrane (Millipore, USA), and allowed to react with appropriate

antibodies in Tris-buffered saline-Tween 20 (Tris 20 mM, pH 7.6, NaCl 150 mM, and Tween

20 0.1%). Blots were then incubated with horseradish peroxidase-conjugated secondary

antibodies and enhanced chemiluminescence reagents.

2.11. Statistical analysis

Results are presented as means ± S.E.M.. Comparisons between multiple groups were

performed using the one-way ANOVA followed by Dunnett's test. Differences were

considered to be significant at $P \leq 0.05$.

3. Results

3.1. 20-HETE improved cell viability, attenuated cell toxicity and prevented chromatin condensation following serum deprivation

PASMCs were cultured in DMEM containing 10% FBS. The cells remained adherent after completely removal of serum from the culture medium. The cell viability was determined by measuring colorimetric conversion of MTT to formazan. The cell toxicity was evaluated by assaying LDH release into the culture medium. Serum deprivation caused a marked decrease in cell viability. The viable PASMCs dropped to $77.7 \pm 2.6\%$ (n = 6) of the control level within 48 h of serum removal, consistent with previous reports (Aoki et al., 1997). In the presence of 1 μM exogenous 20-HETE, the cell viability was partly recovered to $93.5 \pm 5.3\%$ (n = 6, $P < 0.01$ in comparison to the serum deprivation group) (Fig. 1A). The significant improvement of cell viability occurred with graded increase in 20-HETE concentrations from 10 nM to 1000 nM (Fig. 1B). Meanwhile, 17-ODYA, which inhibited the formation of endogenous 20-HETE, further decreased the cell viability and caused more LDH to release, while indomethacin, an inhibitor of cyclooxygenase, had no significant effect on MTT convention and LDH release compared with that in the serum deprivation group (Fig. 1C-D).

We also examined chromatin condensation after serum deprivation by staining with Hoechst 33342. Serum deprivation resulted in clear changes in chromatin morphology such as crenation, condensation and fragmentation, and the changes were significantly attenuated by 20-HETE (300 nM) (Fig. 2).

3.2. 20-HETE relieved PASMC apoptosis during serum deprivation

Flow cytometric analysis with annexin Ⅴ and PI stainings and TUNEL assay were undertaken to determine the effect of 20-HETE on PASMC apoptosis. As shown in Figure 3, about 40% of counted cells became apoptotic after 24 h of serum deprivation. Of them about $16.2 \pm 1.7\%$ cells were necrotic. A treatment with 17-ODYA resulted in necrosis of about $32.7 \pm 2.0 \%$ of counted cells, although the total number of apoptotic cells changed little. The percentage of apoptotic cells was reduced to $21.9 \pm 0.4\%$ after treatment with 20-HETE, and the necrotic cells to $4.3 \pm 0.8\%$, effects that differ significantly from that of serum deprivation group ($n = 4$, $P < 0.05$). On the other hand, the number of TUNEL-positive cells significantly increased in the serum deprivation group, and the effect was strengthened by treatment with 17-ODYA. In contrast, 20-HETE significantly decreased TUNEL-positive cells in serum deprivation conditions (Fig. 4, $n = 10$, $P < 0.05$).

3.3. 20-HETE relieved mitochondrial depolarization and induced bcl-2 expression in PASMCs after serum deprivation

The protective effect of 20-HETE on serum deprivation-induced cell death was further studied by examining mitochondrial depolarization. Normal PASMCs stained with JC-1 emitted mitochondrial orange–red fluorescence with a little green fluorescence (Fig. 5A). This aggregated JC-1 within normal mitochondria was dispersed to the monomeric form (green fluorescence) after removal of serum in culture medium for 24 h (Fig. 5B). In comparison with that in the serum deprivation group, 17-ODYA further decreased mitochondrial potentials and produced obvious green fluorescence (Fig. 5C). However, 20-HETE relieved the serum deprivation-induced mitochondrial depolarization as shown in the fluorescent color changes from green to orange–red (Fig. 5D) ($n = 10$, $P < 0.05$).

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As bcl-2 is an anti-apoptotic protein and protects the integrity of mitochondria (Cory et al., 2003), the regulation of its expression is associated with mitochondrial function. Thus, we investigated the effect of 20-HETE on the expression of bcl-2 in this study. The results showed that bcl-2 was downregulated in serum deprivation group and further decreased in 17-ODYA treated group. Meanwhile, 20-HETE induced the expression of bcl-2 significantly compared with both the serum deprivation group and the control group (Fig. 6, n = 3, $P <$ 0.05).

3.4. 20-HETE suppressed the activation of caspase-3 and caspase-9 induced by serum deprivation

Caspase-3 is one of the downstream effectors of the caspase family, and is involved in both the mitochondrial apoptotic pathway and the death receptor pathway. Caspase-3 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 (Degterev, et al., 2003). Serum deprivation promoted the cleavage activation of procaspase-3 compared with that in normal culture medium (control). 17-ODYA (1 µM) further increased the activation of procaspase-3 while 20-HETE (300 nM) inhibited its cleavage, although it did not restore the amount of procaspase-3 completely to the control level (Fig. 7A). A similar result was acquired in the activity assay of caspase-3 (Fig. 7B). To determine whether uptream apoptotic events are also affected by 20-HETE, we assayed caspase-9 activity in PASMCs. Serum deprivation promoted a 1.9 ± 0.1 fold increase in caspase-9 activity (n = 3, $P < 0.05$ in comparison to the control group). The activity of caspase-9 was strengthened by 17-ODYA but suppressed by 20-HETE (Fig. 7C, n = 3, $P <$

0.05).

3.5. 17-ODYA inhibited the expression of CYP4A and CYP2J2 in PASMCs after serum

deprivation

17-ODYA is not a specific inhibitor for 20-HETE formation; it also inhibits the formation

of epoxyeicosatrienoic acids (EET) (Miyata et al., 2001). Therefore, we analyzed whether

EET synthase cytochrome P450 2J2 (CYP2J2), besides 20-HETE synthase cytochrome P450

4A (CYP4A), was expressed in PASMCs and how 17-ODYA and 20-HETE affected their

expressions in serum deprivation conditions. As shown in Fig. 8, the expressions of both

CYP4A and CYP2J2 were detected in PASMCs, and their expressions were suppressed after

serum deprivation (n = 3, $P < 0.05$). 17-ODYA inhibited the expressions of both CYP4A and

CYP2J2, and 20-HETE diminished the expression of CYP4A but exerted a significant

induction on the expression of CYP2J2 (n = 3, $P < 0.05$ in comparison to the serum

deprivation group).

4. Discussion

20-HETE plays a critical role in angiogenic responses and vasculature remodeling (Chen et al., 2005). However, whether 20-HETE acts on PASMC apoptosis remains unclear. In this study, we presented new evidence that 20-HETE appears to protect PASMCs against apoptosis by acting, at least in part, on the intrinsic apoptotic pathway.

The effect of 20-HETE on PASMC apoptosis may have impact on pulmonary vascular remodeling and pulmonary hypertension (Voelkel et al., 2007). Although it is believed that pulmonary hypertension is related to the distal pulmonary circulation, there is experimental evidence suggesting that structural changes in larger vessels may contribute directly to hemodynamics and distal vascular remodeling (Stenmark et al., 2006). In large mammals (calf, pig, and human), the thickening in the media layer of arteries appears early and more dramatic than that in the intima and adventitia (Belknap et al., 1997; Stenmark and Mecham, 1997). The media of large conducting pulmonary vessels is composed of various smooth muscle cells, which are the main reservoir of eicosatetraenoic acid and in which the 20-HETE synthesis is localized (Harder et al., 1997). An exceptional observation in pulmonary circulation suggests that the main production of 20-HETE in the vascular endothelium is not smooth muscle-original (Zhu et al., 2002). It is unclear, however, whether the specific location of 20-HETE in pulmonary circulation is related to the progression of intimal media thickness. In the present study, we demonstrated that 20-HETE inhibited the apoptotic responses in PASMCs. And the inhibition of apoptotic cell death can result in the release of intracellular materials into the extracellular space leading to inflammatory responses (Gulbins et al., 2000), which may recruit circulating progenitor cells and inflammatory cells to

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promote persistent vasoconstriction and the structural remodeling of the pulmonary circulation.

The pulmonary vascular remodeling is determined largely by a balance between cell proliferation and cell apoptosis. Although apoptosis is genetically controlled, it is also subject to physiologic and pathophysiologic regulations (Letai and Scorrano, 2006). In this study we adopted a simple and well-defined experimental system serum starvation for 24 h to produce apoptosis (Aoki et al., 1997; Duttaroy et al., 1997). It is known that serum deprivation triggers the apoptotic responses through mitochondrial pathways (Zhang et al., 2000), which is also demonstrated in the present study by bcl-2 expression reduction, mitochondria dysfunction, caspase-9 and caspase-3 activation. Two of the apoptotic indications, Annexin V binding and DNA nick end labeling in PASMCs have been examined in this study. The effects of serum deprivation on both indications are attenuated with exogenous 20-HETE. These findings suggest that 20-HETE inhibits PASMC apoptosis. Furthermore, 17-ODYA, an inhibitor of endogenous 20-HETE production, promotes fragmented DNA end labeling, suggesting a role of endogenous 20-HETE in the serum deprivation-induced apoptosis. However, 17-ODYA promotes mostly necrosis and has less effect on apoptosis in the Annexin V binding assay. A possible explanation is that the viability of adherent PASMCs was injured precariously when collected by trypsin digestion before Annexin V binding assay. Trypsin digestion decomposed the macromolecular proteins on cell membrane, increased membrane permeability and caused the cell injury if its action was prolonged (Daniels, et al., 1996).

It is noteworthy that the inhibitive effect of 17-ODYA is not specific for 20-HETE synthases but it also inhibits EET synthesis (Miyata et al., 2001). Both the expressions of

20-HETE synthase CYP4A and EET synthase CYP2J2 have been detected in PASMCs in this study. Their expressions are downregulated in serum deprivation conditions and over-downregulated after treatment with 17-ODYA. These findings indicate that the expressions of both 20-HETE synthase CYP4A and EET synthase CYP2J2 in PASMCs are inhibited by 17-ODYA. In the present study, we found that several apoptotic events were promoted by 17-ODYA, including mitochondrial dysfunction, caspase-9 and caspase-3 activation, and DNA fragmentation. It was reported that EET had protective effect on human endothelial cells from the pulmonary vasculature (Dhanasekaran, et al., 2006). All the information presented here gives a clue that both endogenous 20-HETE and EET have inhibitive effect on apoptotic response in PASMCs.

As mentioned above, serum deprivation-induced apoptosis leads to mitochondrial dysfunction. In this study, a significant reduction of mitochondrial potentials was observed after serum depletion, and exogenous 20-HETE attenuated the decrease of the potentials. The anti-apoptotic bcl-2, which resided in the outer mitochondrial wall and controlled mitochondrial permeability, was sharply induced by exogenous 20-HETE. On the contrary, the mitochondria dysfunction and bcl-2 expression reduction caused by serum deprivation were worsened by 17-ODYA. These findings indicate that 20-HETE protects PASMCs through relieving mitochondrial dysfunction. Mitochondria dysfunction plays a key role in the activation of the caspase cascade (Degterev, et al., 2003). Consistent with the study of Degterev, we also observed the activation of caspase-9 and caspase-3 induced by serum deprivation. The induction was promoted by 17-ODYA and suppressed by 20-HETE. In general, these data together with the result of Annexin V binding and TUNEL, suggest that

20-HETE inhibits the apoptosis of PASMCs by, at least in part, acting on the intrinsic apoptotic pathway through attenuating a series of events involving mitochondrial dysfunction, caspase-9 and caspase-3 activation and DNA fragmentation.

Although our study indicates that 20-HETE contribute to inhibit the apoptois of PASMC through the intrinsic pathway, the extrinsic death receptor pathway is an important alternative in the apoptotic process in PASMCs. Clearly further studies are needed to ascertain the signaling cascades of the extrinsic pathway associated with specific effects of 20-HETE in PASMCs.

In conclusion, our results have shown that 20-HETE protects PASMC against serum deprivation-induced apoptosis, and such an effect is likely to be mediated through the intrinsic apoptotic pathway. These findings together with previous reports indicate that 20-HETE is an important player in pulmonary vessel remodeling by acting on not only PASMC proliferation and migration but also apoptosis.

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Figure Legends

Fig. 1. The survival rate of cultured pulmonary artery smooth muscle cells after serum deprivation was studied in the presence or absence of 20-HETE. Cells were growth-arrested for 24 h and stimulated with vehicle, 17-ODYA (1 μM), indomethacin (1 μM) or increasing concentrations of 20-HETE for the next 48 h under serum-depleted conditions. Control for the serum-deprived (SD) cells were cultured in DMEM (10% FBS) for the next 48 h after growth arrest. Cell viability was determined using the MTT assay (A-C) and cell toxicity was measured using LDH release assay (D). All values are denoted as means ± S.E.M. from three or more independent batches of cells. *$P<0.05$ compared with control cells cultured in complete medium; #$P<0.05$ compared with serum deprived cells in presence of vehicle.

Fig. 2. Morphological changes in chromatins after induction of apoptosis and 20-HETE treatment. The cells were serum-deprived (SD) in presence of vehicle or 20-HETE for 24 h. Control cells were cultured in DMEM containing 10% FBS for 24 h. PASMCs were stained with Hoechst reagent, and nuclei were imaged. There are visible crenation of nuclei (B1), condensation (B2) and fractionation (B3) of chromatin in serum-deprived cells (marked with arrows, B), which are absent in control cells (A) and less visible in cells pretreated with 20-HETE (300 nM) (C). A-C, scale bars = 20 μm; A1, B1-B3, scale bars = 5 μm. D, 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 Hoechst reagent from ten independent photographs shot in each group. *$P<0.05$ compared with control cells cultured in complete medium; #$P<0.05$ compared with serum deprived cells in presence of vehicle.

Fig. 3. 20-HETE protected PASMCs from apoptosis induced by serum deprivation. PASMCs were serum-deprived (SD) and then treated with vehicle, 17-ODYA (1 μM) or 20-HETE (300 nM) for 24 h,

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respectively. The number of apoptotic cells and necrotic cells were quantified by FACScan flow cytometry

after cells were stained with annexin Vand PI. Apoptotic cells were determined by counting the percentage

of annexin V(+), PI(-) cells and the percentage of annexin V(+), PI(+) cells. The number of necrotic cells

was obtained by counting the percentage annexin V(+), PI(+) cells. A, representative photographs of

annexin V and PI staining in different groups. B-C, quantitative analysis of the number of apoptotic cells

and that of necrotic cells. Data are shown as mean ± S.E.M. from at least three separate experiments.

*P<0.05 compared with control cells cultured in complete medium; #P<0.05 compared with serum

deprived cells in presence of vehicle.

Fig. 4. 20-HETE relieved the increase of TUNEL-positive cells induced by serum deprivation. PASMCs

were treated the same way as in Fig. 3 before TUNEL staining and fluorescent imaging. The content of

TUNEL-positive cells (marked with arrows) was calculated as the ratio of TUNEL-positive cells to the

total number of PASMCs. A-D, representative photographs of TUNEL staining in different groups. E,

quantitative analysis of TUNEL positive cells content among groups. All values are denoted as means ±

S.E.M. from ten independent photographs shot in each group. *P<0.05 compared with control cells

cultured in complete medium; #P<0.05 compared with serum deprived cells in presence of vehicle.

Fig. 5. 20-HETE attenuated mitochondrial potentials reduction in PASMCs induced by serum deprivation.

PASMCs were treated the same way as in Fig. 3. Then 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. An increase in the bar indicates a shift in the fluorescence ratio

correlating with an increase in mitochondrial depolarization. A-D, representative photographs of JC-1

staining in different groups. E, quantitative analysis of the shift of mitochondrial orange–red fluorescence

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to green fluorescence among groups. All values are denoted as means ± S.E.M. from ten independent

photographs shot in each group. Significant differences are indicated as: *$P<0.05$ compared with control

cells cultured in complete medium; #$P<0.05$ compared with serum deprived cells in presence of vehicle.

Fig. 6. The expression of bcl-2 was significantly induced in PASMCs by 20-HETE. PASMCs were grown

to ~80% confluency and serum starved for 24 h. Then the cells were cultured in DMEM (10% FBS) as

control or kept serum deprived (SD) and treated with vehicle, 17-ODYA or 20-HETE for another 24 h. The

cells were lysed in the presence of protease inhibitors, and the lysates were analyzed by Western blot with

antibody to bcl-2 to determine if 20-HETE induced the expression of bcl-2 in serum deprivation conditions.

Results are expressed as the mean ± S.E.M. from at least three separate experiments. *$P<0.05$ compared

with control cells cultured in complete medium; #$P<0.05$ compared with serum deprived cells in presence

of vehicle.

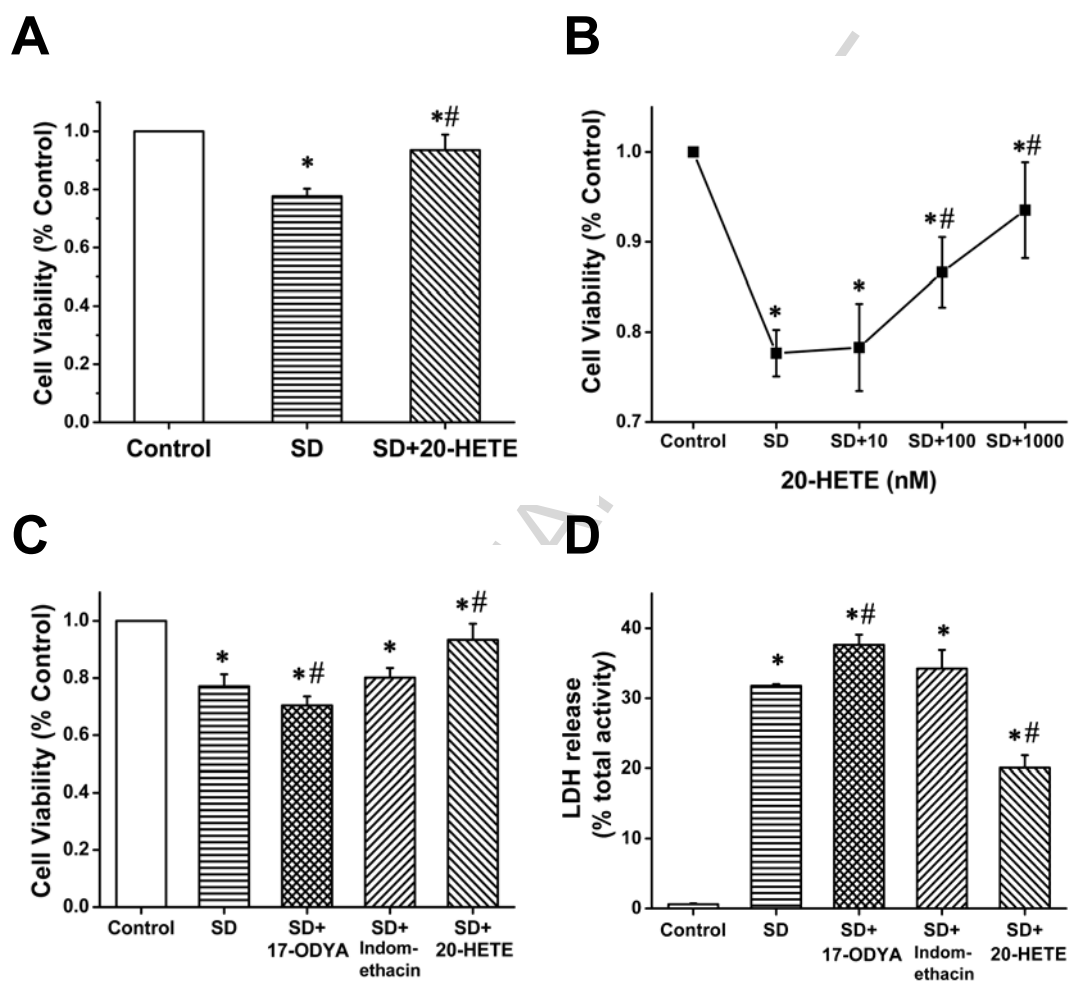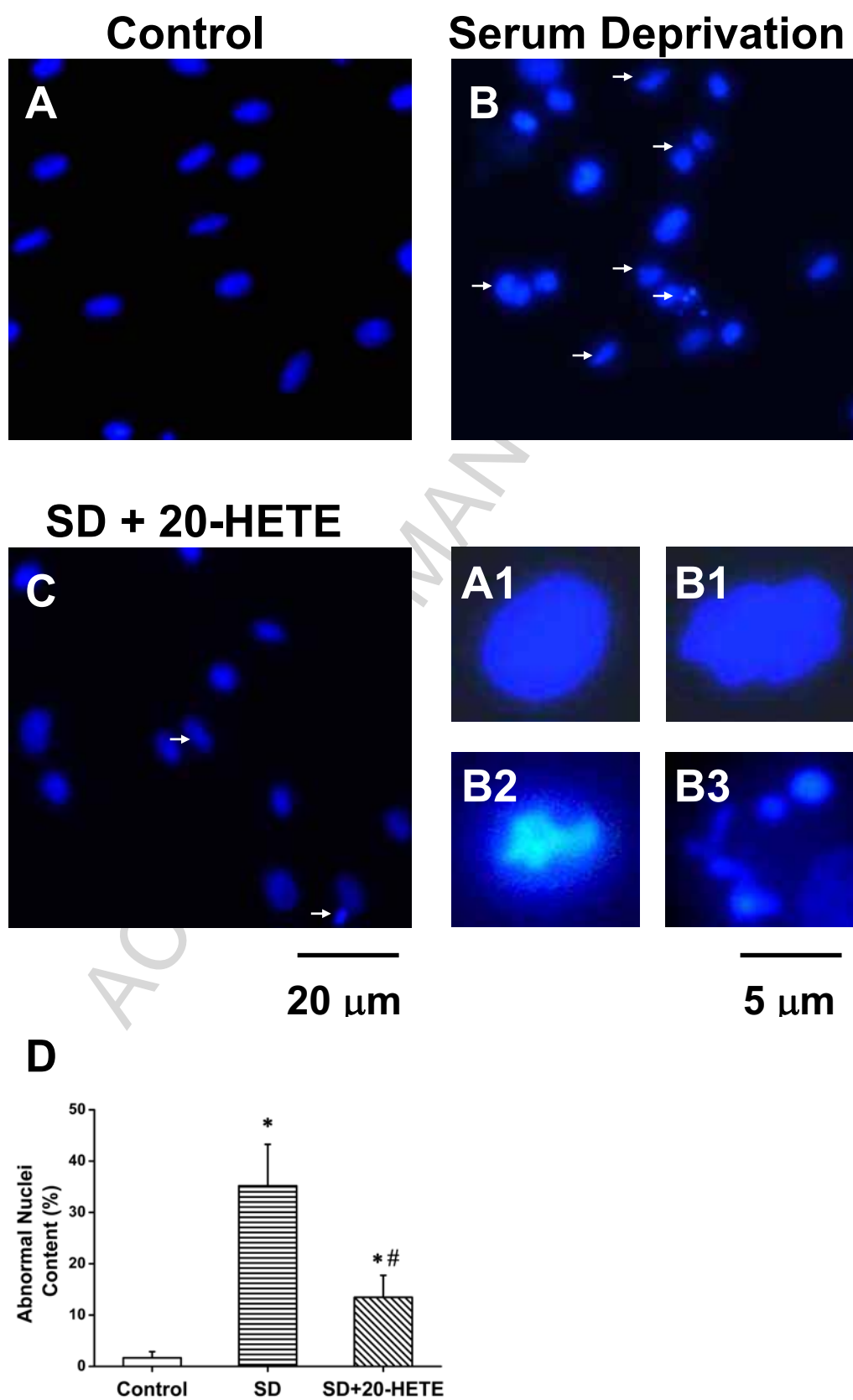Fig. 7. A, the cleavage activation of procaspase-3 by serum deprivation was suppressed in PASMCs treated

with 20-HETE. The cells were treated the same as in bcl-2 analysis. The cell lysates were analyzed by

Western blots with antibody to procaspase-3 to determine if the 20-HETE attenuated the activation induced

by serum deprivation. B-C, caspase-3 and caspase-9 activation in PASMCs were measured using the

chromogenic substrate Ac-DEVD-pNA and Ac-LEHD-pNA. The bar represents the fold increase of the

absorbance of pNA produced by cleaving its corresponding precursors in PASMCs. Data are expressed as

mean ± S.E.M. from at least three separate experiments. *$P<0.05$ compared with control cells cultured in

complete medium; #$P<0.05$ compared with serum deprived cells in presence of vehicle.

Fig. 8. The expressions of CYP4A and CYP2J2 in PASMCs were inhibited by 17-ODYA. PASMCs were

treated the same as in bcl-2 analysis. The lysates of the cells were analyzed by Western blot with

polyclonal antibodies to CYP4A and CYP2J2 to quantify their individual expressions in PASMCs. Data

are expressed as mean ± S.E.M. from at least three separate experiments. *$P<0.05$ compared with control

cells cultured in complete medium; #$P<0.05$ compared with serum deprived cells in presence of vehicle.

Figure 1

A

B

C

D

Figure 2

Control

A

Serum Deprivation

B

SD + 20-HETE

C

A1   B1

B2   B3

20 μm   5 μm

D

Figure 3

Figure 4

50 μm

Figure 5

A Control

B SD

C SD + 17-ODYA

D SD + 20-HETE

50 µm

E

Figure 6

Figure 7

A1

A2

B Activity of caspase-3

C Activity of caspase-9

Figure 8