Role of histone deacetylases in regulation of phenotype of ovine newborn pulmonary arterial smooth muscle cells

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Abstract

Objective: Pulmonary arterial hypertension, characterized by pulmonary vascular remodelling and vasoconstriction, is associated with excessive proliferative changes in pulmonary vascular walls. However, the role of HDACs in the phenotypic alteration of pulmonary arterial smooth muscle cells (PASMC) is largely unknown.

Material and methods: Pulmonary arterial smooth muscle cells were isolated from newborn sheep. Cell cycle analysis was performed by flow cytometry. mRNA and protein expression were measured by real-time PCR and Western blot analysis. Wound-healing scratch assay was used to measure cell migration. Contractility of newborn PASMCs was determined by gel contraction assay. Chromatin immunoprecipitation was used to examine histone modifications along the *p21* promoter region. Global DNA methylation was measured by liquid chromatography-mass spectroscopy.

Results: Inhibition of class I and class II HDACs by apicidin and HDACi VIII suppressed proliferation of newborn PASMC and induced cell cycle arrest in G_1 phase. Acetyl H3 levels were higher in newborn PASMC treated with apicidin and HDACi VIII. This was accompanied by increased expression of *p21* and reduced expression of *CCND1* but not *p53*. HDAC inhibition altered histone codes around the *p21* promoter region in NPASMC. Apicidin inhibited serum-induced cell migration, and modulated profiling of expression of genes encoding pro-oxidant and antioxidant enzymes. Contractility and global DNA methylation levels of newborn PASMCs were also markedly modulated by apicidin.

Conclusion: Our results demonstrate that class I HDACs are clearly involved in phenotypic alteration of newborn PASMC.

Introduction

Pulmonary arterial hypertension (PAH), characterized by pulmonary vascular remodelling and vasoconstriction, is associated with proliferative changes in the pulmonary vascular wall (1). Structural alterations within the vessel wall are mainly caused by proliferation and migration of pulmonary arterial smooth muscle cells (PASMC) (2). Although the overall 5-year survival for PAH patients has significantly increased over the past number of years, none of the approved therapies has shown an ability to cure the disease. Current available treatments are limited, expensive, and often associated with side effects. As such, to further our understanding of mechanisms underlying PAH development, it is important to further our understanding of mechanisms underlying PAH development, so that the improvement of patient management and outcome can occur.

It is being increasingly recognized that environmental factors during prenatal and early postnatal periods influence developmental programming of homoeostatic mechanisms that profoundly impact susceptibility to diseases later in life (3–6). Neonatal nociception raises basal blood pressure level and attenuates cardiovascular responsiveness to nociceptive stress in adult rats (7). Effects of perinatal insults have most frequently been assessed in animal models of intrauterine growth retardation. Impaired foetal growth is a major cause of perinatal morbidity and has long-term clinical consequences (8–10). Perinatal hypoxia increases severity of pulmo-

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nary hypertension after reexposure to hypoxia in infant rats (4). It also triggers alterations in K^+ channels of adult pulmonary artery smooth muscle cells (5).

Epigenetics can explain that heritable changes in gene expression or cell phenotype are caused by mechanisms other than changes in the underlying DNA sequence. Although knowledge of relationships between PAH and epigenetics is quite limited (11), there are recent studies that show that epigenetic mechanisms are involved in lung development and pulmonary vascular dysfunction (10,12–18). Undernutrition during gestation induces pulmonary vascular dysfunction in the offspring through alterations in DNA methylation, whereas administration of HDAC inhibitors to offsprings of restrictive diet during pregnancy normalized pulmonary DNA methylation and vascular function (10). In the rat lung, intrauterine growth retardation induces epigenetic modifications to the *PPARr* gene, a member of a nuclear receptor family of transcription factors that contributes to epithelial–mesenchymal interactions that are crucial for lung development (15–17). Moreover, HDAC inhibition has been shown to suppress hypoxia-induced cardiopulmonary remodelling, improve pulmonary artery acceleration time and reduce systolic notching of the pulmonary artery flow envelope (19,20). We have recently reported that levels of global DNA methylation and histone acetylation in pulmonary arteries from foetal lung acclimatized to high-altitude long-term hypoxia were altered (21).

In this study, we investigated the effects of the inhibition of HDACs using their inhibitors, apicidin, HDACi VIII and Tenovin-1, on ovine NPASMC proliferation, migration, and expression of cell cycle related genes such as *p21*, *CCND1*, *CDK4* and *p53*. Expression of genes encoding pro-oxidant and antioxidant enzymes was measured. We also determined the effects of inhibition of class I HDACs on NPASMC contractility and global DNA methylation.

Materials and methods

Reagents

Apicidin, HDACi VIII, and Tenovin-1 were purchased from EMD Millipore, Billerica, MA, USA. Propidium iodide was purchased from Sigma, St. Louis, MO, USA.

Preparation of PASMC from ovine newborn lung

Intrapulmonary arteries, third to fourth generation, were dissected free of parenchyma and kept in ice-cold modified Krebs-Ringer bicarbonate buffer, and primary ovine newborn PASMC (NPASMC) were isolated from pulmonary arteries as described previously (22). Cells were maintained in DMEM containing 10% heat-inactivated FBS and antibiotics. All experiments were performed with cells at passages 4–8.

Cell number and cell viability

Cell number was determined by counting using a haemocytometer. Trypan blue staining was used to distinguish dead from live cells.

Cell cycle analysis

Cell cycle distribution was determined by flow cytometric analysis as previously described (23). Briefly, NPASMC (5×10^5 cells) were cultured in serum-free DMEM medium for 48 h. After starvation, medium containing 10% serum was replaced, and apicidin, HDACi VIII, and Tenovin-1 were added. Cells were treated for 24 h, then washed in PBS, fixed in 70% ethanol and hypotonically lysed in 500 μl of DNA staining solution [0.05 mg/ml PI (Sigma), 0.1 mg/ml RNase A, and 0.05% Triton X-100]. NPASMC were incubated, while protected from light at 37 °C for 40 min. Stained cells were washed in PBS and suspended in 300 μl of PBS before analysis. Cell cycle data were analysed using an Epics XL-MCL flow cytometer with System II (version 3.0) software (Beckman Coulter, Miami, FL, USA). Additional analysis of cell cycle distribution was determined using Modfit LT (Verity Software House, Topsham, ME, USA).

Migration assay

Newborn PASMC were grown to confluence on 35 mm petri dishes. Cells were growth-arrested for 24 h in DMEM containing 0.1% FBS. A scratch was made in each cell monolayer, medium was replaced by 0.1% FBS medium, baseline (0 h time point) images were captured, and 2.5 μg/ml of apicidin was added for 30 min prior to addition of 10% serum. Photomicrographs were taken at 0 and 24 h, and cell migration distance was determined by subtracting values obtained at 0 h from 24 h. Migration distances were expressed as percentages over control values.

cDNA synthesis and SYBR green real-time PCR

RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed using *Superscript* III (Invitrogen) and 50 μM oligo(dT)$_{20}$ at 50 °C for 50 min. SYBR green real-time PCR reactions were set up containing 1x Power SYBR Green Master Mix (Applied Biosystems, Foster City,

Table 1. Ovine primer sequences

Gene symbol	Assay	Sense	Antisense	Accession
SOD1	Q-PCR	TCATGGGTTCCACGTCCAT	GAGGGCCTGCACTGGTACAG	NM_001145185
SOD2	Q-PCR	CAGGATCCCCTGCAAGGA	CATGCTCCCACACGTCAATC	GQ221055
SOD3	Q-PCR	TCACCTTGATTTTTCTCCTTCCTT	TGGCAGAAGTGGTACTCCAGAGT	XM_004009740
PRDX5	Q-PCR	AAAAGGAGCCCGGGAACA	GCACTCCCTTCTTGCCTTTG	JX889614
DUOX1	Q-PCR	ACCCCGTTTCCACATCTTCTT	ACCAGCTTGTCCCCCACATA	XM_004011029
DUOX2	Q-PCR	AGAACTACCGGCGGCACAT	CAAACAGTCCGGCACAGATG	NM_001190392
NOX4	Q-PCR	GCTGGAGGCATTGGAGTCA	TTTCCAGTCATCCAGCAGAGTGT	EF369489
p21	Q-PCR	CCAGACCAGCATGACAGATTTC	GCTTCCTCTTGGAGCAGATCAG	EE754405
CCND1	Q-PCR	TCGAGCACTTCCTCTCCAAAA	GTTTGCGGATGATCTGCTTGT	EU525165
p53	Q-PCR	TCTGGGACTTAGTGCCTTTTATGG	CAGTCAGAAACTGTCAAATCATCCA	X81705
cdk4	Q-PCR	GCTTGCCAGTGGAGACCATAAA	ATGAAGGAAATCCAGGCCTCTT	NM_001127269
β-actin	Q-PCR	GCAGATGTGGATCAGCAAGCA	AGCATTTGCGGTGGACGAT	NM_001009784
p21	ChIP/Q-PCR	GAGAGGGAGCGTCCGACTT	TGGCTGGAACACAAGGAAGAC	

CA, USA) and 250 nM forward and reverse primers in a 20-μl reaction. All assays were carried out in 96-well format. Real-time fluorescence detection of PCR products was performed using StepOne Plus Real-Time PCR System (Applied Biosystems) using the following thermocycling conditions: 1 cycle of 95 °C for 10 min; 40 cycles of 95 °C for 30 s, and 60 °C for 1 min. Sequences of primers were designed using Primer Express software (Applied Biosystems). Ovine primer sequences are shown in Table 1. β-actin was used as an endogenous control for gene expression. For data analysis, the comparative method ($\Delta\Delta Ct$) was used to calculate relative quantities of a nucleic acid sequence.

Western blot analysis

Total proteins of NPASMC were extracted after lysing cells in cell lysis buffer containing protease and phosphatase inhibitor cocktails (Sigma, St. Louis, MO, USA); protein concentration was determined using a Bradford protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of total protein (10–25 μg) from cells were subjected to SDS-PAGE. Proteins were transferred to nitrocellulose membrane for 90 min at 100 V. Membranes were blocked for 1 h at room temperature in Tris-buffered saline containing 5% non-fat powdered milk, and probed with primary antibody in Tris-buffered saline with 2.5% non-fat powdered milk at concentrations from 1:500 to 1:20 000, pre-incubated overnight according to the manufacturer's instructions, for each antibody. In all cases, secondary antibody labelled with horseradish peroxidase (GE Healthcare Bio-Sciences, Piscataway, NJ, USA) was used at concentrations from 1:2000 to 1:20 000 for 1 h at room temperature. Immuno-reactive bands were detected using SuperSignal West Pico Chemi-luminescent Substrate (Pierce, Rockford, IL, USA) and recorded on photosensitive film. Relative intensities of immuno-reactive bands detected by Western blot analysis were quantified by densitometry using NIH Image J software, and normalized to β-actin levels. Primary antibodies used for this study include anti-Ac-histone H3 (Millipore), anti-p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-β-actin (Santa Cruz Biotechnology).

Gel contraction assay

Collagen contraction assay was performed as described previously (24). Briefly, collagen gels were prepared according to the manufacturer's instructions to a final collagen concentration of 1.5 mg/ml (Bacton Dickinson, Franklin Lanes, NJ, USA). NPASMC were seeded into gel mixtures at 2×10^5/ml in the presence or absence of apicidin, and gels were allowed to polymerize for 20 min at 37 °C in 24-well plates. Thereafter, gels were mechanically loosened from the sides of wells. Three gels were analysed for each condition in each individual experiment.

Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assay was performed as previously described (25). Briefly, NPASMC (5×10^6) were treated with 1% formaldehyde for 10 min to cross-link histones to DNA. After washing in cold PBS, cell pellets were resuspended in cell lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 0.2% NP40). Nuclei were resuspended in nuclei lysis buffer (50 mM Tris pH 8.0, 10 mM EDTA, 1% SDS) and sonicated. Soluble chromatin fractions were collected, and 5 μl antibody of acetyl-H3 (Millipore), acetyl-H4 (Abcam, Cambridge, MA, USA), or normal rabbit IgG (Santa Cruz Biotechnology) was added. After incubation, chromatin-antibody complexes were collected using salmon sperm DNA/protein A/G agarose beads (Millipore). After washing, immunoprecipitated DNA was treated with

RNase and crosslinks were reversed by heating samples at 65 °C for 6 h. DNA was extracted using QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA) and analysed by SYBR green real-time PCR. The primer pair used for ChIP assay is shown in Table 1.

Liquid chromatography-mass spectroscopy (LC/MS)

Total cytosine methylation was performed by LC/MS as described previously (26). Briefly, DNA was hydrolysed to nucleosides by adding 5 U nuclease P1 (Sigma) at 37 °C for 2 h, 0.002 units venom phosphodiesterase I (Sigma) at 37 °C for 2 h, 0.5 units alkaline phosphatase at 37 °C for 1 h. Stock solutions of 2′-deoxycytidine (Sigma) and 5-methyl-2′-deoxycytidine (ChemGenes, Wilmington, MA, USA) were prepared in water. Eight-point standard stock mixture was carefully prepared to provide exact known concentration ratio of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine. The concentration of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine in each sample was calculated from the standard curve. Each DNA sample was analysed in triplicate. A quantity of 25 μl (80 ng) per sample was injected into LC and run through an Atlantis DC18 sillica column (Waters Corporation, Milford, MA, USA). Identification of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine was obtained by mass spectra of chromatographic peaks.

Statistical analysis

Statistical analysis of the data was performed using standard two-sample Student's *t*-test, assuming unequal variances of the two data sets. Statistical significance was determined by two-tailed distribution assumption and was set at 5% level ($P < 0.05$).

Results

Effect of HDAC inhibition on cell proliferation and cell cycle arrest in NPASMC

To determine which class or classes of HDACs are involved in NPASMC proliferation, HDAC inhibitors mainly targeting HDAC class I (apicidin), class II (HDACi VIII) and class III (Tenovin-1), were used individually to treat NPASMC. As shown in Fig. 1a, cell accounting assay showed that apicidin, an HDAC inhibitor mainly targeting class I, strongly inhibited NPASMC proliferation at concentrations ranging from 0.5 to 10 μg/ml. In addition, HDACi VIII also inhibited cell proliferation in a dose-dependent manner (Fig. 1b), indicating that class I and class II HDACs play important roles in contributing to pulmonary vascular SMC proliferation. However, Tenovin-1 had no marked effect on proliferation of NPASMC (Fig. 1c).

Next, we investigated whether HDAC inhibitors reduced cell proliferation *via* cell cycle arrest. We resorted to flow cytometry to determine if HDAC inhibition caused a fall in cell proliferation of PASMC from newborn sheep lung. After 48 h serum starvation, NPASMC were cultured for 24 h in 10% FBS with or without 2.5 μg/ml apicidin, HDACi VIII or Tenovine-1. Cells were stained with propidium iodide to study the cell cycle progression. As shown in Fig. 1d and 1e, treatment of NPASMC with apicidin and HDACi VIII induced cell cycle arrest in G_1 phase. Apicidin showed more potent cell cycle arrest at G_1. However, Tenovine-1 had no effect on cell cycle arrest (Fig. 1f).

Effect of HDAC inhibition on expression of cell cycle-related genes and histone H3 acetylation levels

To determine which genes were involved in apicidin- and HDACi VIII-induced cell cycle arrest, expression of p21, a potent cyclin-dependent kinase inhibitor, was initially examined (Fig. 2). As shown in Fig. 2a, both apicidin and HDACi VIII markedly increased p21 expression. Higher levels of p21 were induced in apicidin-treated NPASMC compared to HDACi VIII-treated ones. Also, treatment of NPASMC with apicidin and HDACi VIII increased accumulation of hyperacetylated histone H3 (Fig. 2a), suggesting that histone hyperacetylation modulated by apicidin and HDACi VIII was responsible for inhibitory effects of pulmonary vascular SMC proliferation *via* selective induction of cell cycle-related genes such as *p21*. Induced *p21* expression was not seen in Tenovine-1-treated NPASMC, as is shown in Fig. 2b.

To determine whether increased p21 protein expression is regulated at transcriptional level in NPASMC after treatment with apicidin and HDACi VIII, RNA expression of p21 was also determined. As shown in Fig. 3a, RNA expression of p21 was markedly increased in a dose-dependent manner in NPASMC treated with both apicidin and HDACi VIII. Higher levels of p21 RNA were observed in apicidin-treated NPASMC compared to HDACi VIII-treated NPASMC. There was no marked induction of p21 expression in NPASMC treated with Tenovin-1. In addition, RNA expression of cyclinD1, which forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, was reduced in NPASMC treated with apicidin and HDACi VIII; however, there was no remarkable change in cyclinD1 expression in NPASMC treated with Tenovin-1 (Fig. 3b). Moreover, RNA expression of p53 and CDK4 were not markedly altered in NPASMC treated with apicidin, HDACi VIII, or Tenovin-1 (Fig. 3c,d).
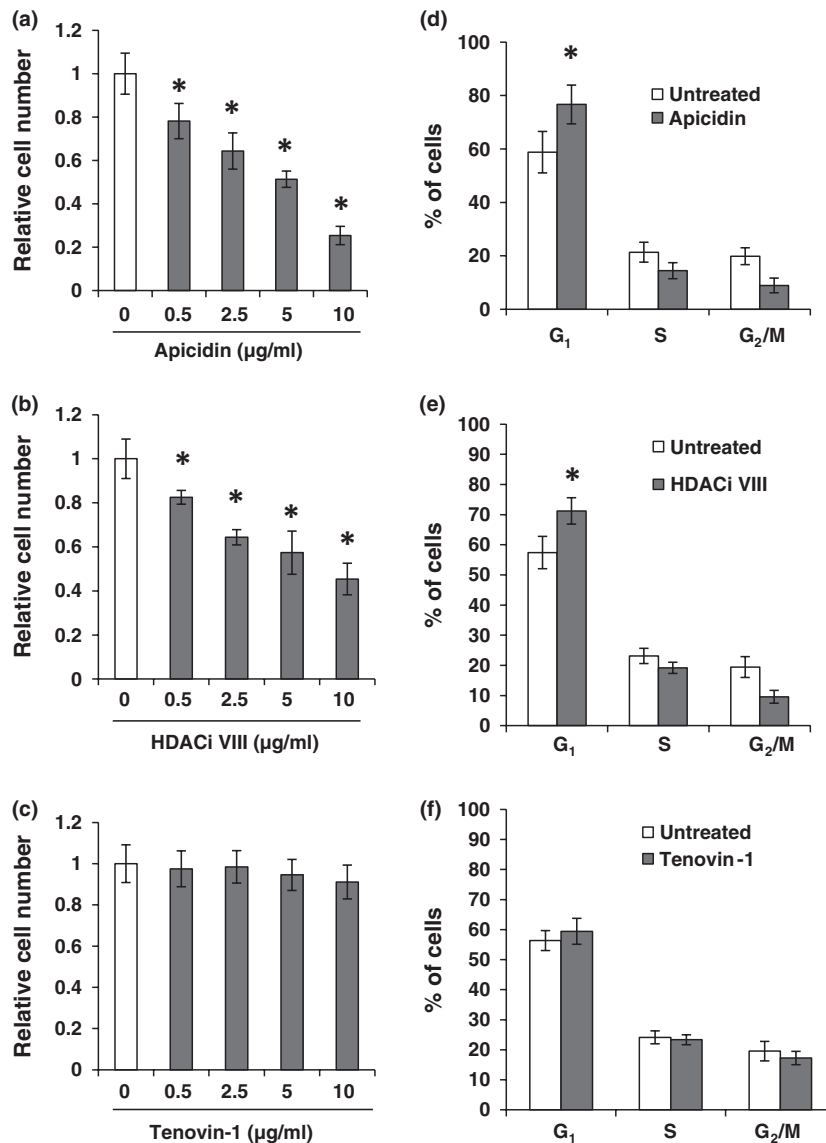
Figure 1. Effect of HDAC inhibition on proliferation and cell cycle of NPASMC. Cell proliferation was assessed by cell counting. NPASMC (2×10^5) were treated with various concentrations of apicidin (a), HDACi VIII (b), and Tenovin-1 (c) for 24 h. All experiments were performed in triplicate and mean $\pm$ SEM of three independent experiments is shown. Results are expressed as a ratio of untreated cells. *$P < 0.05$ compared to untreated cells. For cell cycle analysis, PASMC (5×10^5 cells) of newborn sheep were serum-starved for 48 h and treated with or without 5 μg/ml apicidin (d), HDACi VIII (e) or Tenovine-1 (f) for 24 h in the presence of 10% serum, and subjected to flow cytometric analysis to determine cell cycle distribution. *$P < 0.05$ compared to untreated PASMC of newborn sheep.

HDAC inhibition altered histone modifications around the p21 promoter

To determine the relationship between p21 expression and histone modifications, we tested effects of apicidin, HDACi VIII and Tenovin-1 on histone marks (AcH3 and AcH4) around the *p21* promoter region. As shown in Fig. 4a and 4b, apicidin and HDACi VIII modulated histone codes. Following treatment of the NPASMC with apicidin, levels of AcH3 and AcH4 along the *p21* promoter region were markedly enriched after 2 d treatment with apicidin. In addition, enrichment of AcH3 and AcH4 was also observed in cells treated with HDACi VIII (Fig. 4a,b). Moreover, more enrichment of AcH3-bound DNA was observed in apicidin-treated ovine NPASMC compared to HDACi VIII-treated ones. However, levels of AcH3 and AcH4 along the *p21* promoter were not markedly changed in NPASMC treated with Tenovin-1. In addition, enrichment of IgG-bound
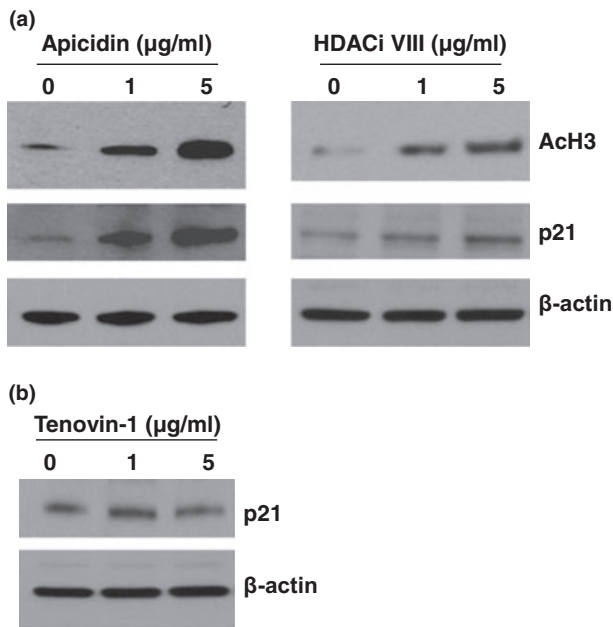
Figure 2. Effect of HDAC inhibition on protein expression level of cell cycle-related genes and acetyl-H3. (a) Cell lysates from untreated and apicidin- and HDACi VIII-treated NPASMC were prepared. Levels of p21 and AcH3 were examined by Western blot analysis using antibodies against p21 and AcH3, respectively. β-actin was used as an endogenous control. (b) Cell lysates from untreated and Tenovine-1-treated NPASMC were prepared. Levels of p21 were examined by Western blot analysis using antibody against p21. β-actin was used as an endogenous control.

DNA was not markedly changed between treated and untreated groups around the *p21* promoter region (Fig. 4a). Our data suggest that elevated levels of *p21* expression is due to recruitment of AcH3 and AcH4 to the *p21* promoter region in these ovine NPASMC.

Class I HDAC inhibition attenuated serum-induced migration of NPASMC

As migration of vascular smooth muscle cells is involved in vascular remodelling, in the next experiment, we examined if class I HDAC inhibition reduced NPASMC migration induced by serum, as class I HDAC inhibition showed more potent cell cycle arrest. The effect of class I HDAC inhibition on serum-induced NPASMC migration using the wound-healing model/scratch assay was performed. As shown in Fig. 5a and 5b, there was minimal migration of NPASMC observed in medium containing 0.1% serum at 24 h compared to the 0 h time point; 10% serum caused over 2.8-fold increase in cell migration compared to untreated controls. However, inhibition of HDAC activity with 2.5 µg/ml apicidin resulted in ∼25% reduction in serum-induced cell migration ($P < 0.05$).
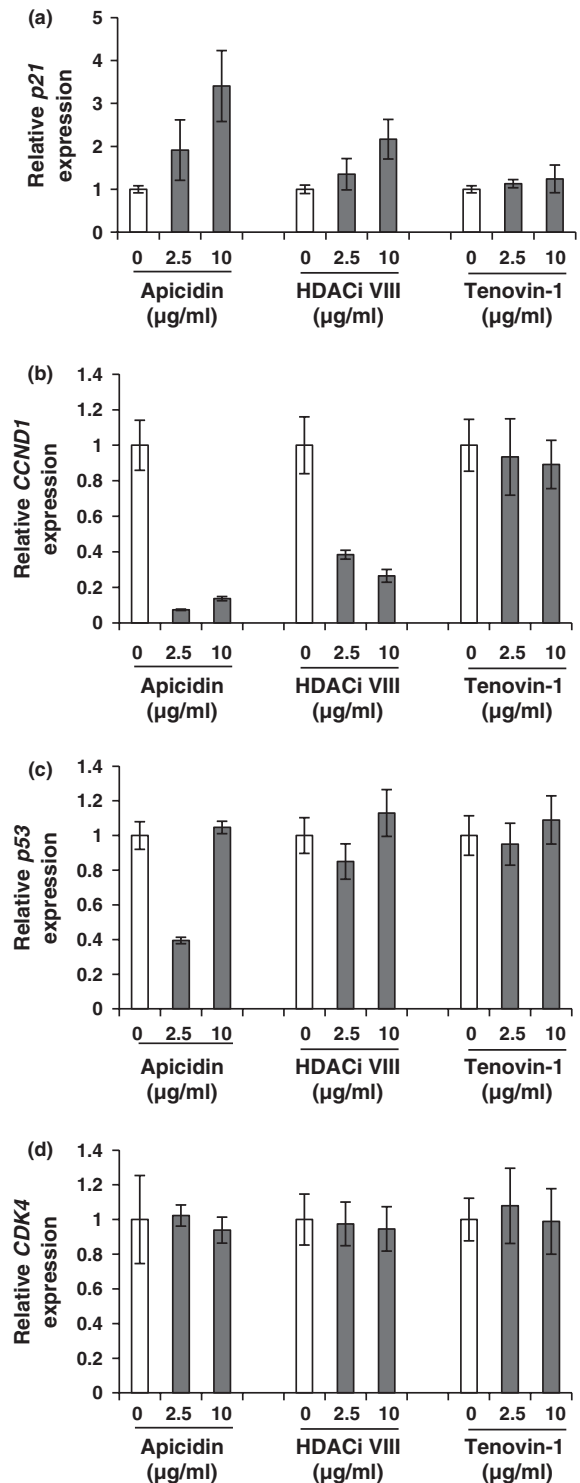

Figure 3. Effect of HDAC inhibitors on RNA expression of cell cycle-related genes in NPASMCs. NPASMCs were treated with 2.5 and 10 µg/ml of apicidin, HDACi VIII, and Tenovin-1 for 24 h. RNA was isolated using Trizol reagent. cDNA was synthesized and subjected to quantitative RNA expression of *p21* (a), *CCND1* (b), *p53* (c) and *CDK4* (d) by SYBR green q-PCR. β-actin was used as an endogenous control.
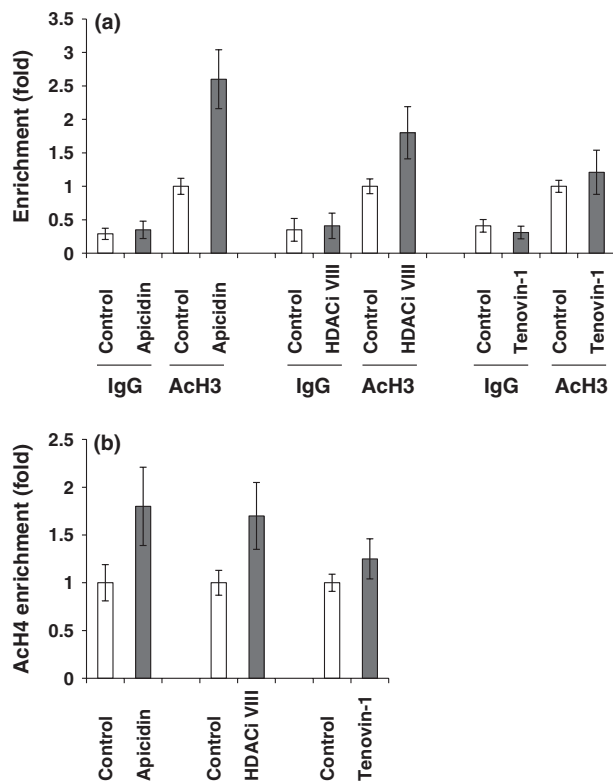
Figure 4. Alteration of histone marks around the *p21* promoter region. ChIP/quantitative PCR was performed around the *p21* promoter region in NPASMC in the presence or absence of apicidin, HDACi VIII, or Tenovin-1. (a) ChIP/quantitative PCR was performed to determine AcH3 levels around the *p21* promoter region in apicidin and HDACi VIII-treated and non-treated cells. Normal rabbit IgG was used as negative control. (b) ChIP/quantitative PCR was performed to determine the AcH4 levels around the *p21* promoter region in apicidin and HDACi VIII-treated and non-treated NPASMCs.

Effect of class I HDAC inhibition on NPASMC-mediated collagen gel contraction

As apicidin showed more potent anti-proliferation effects, we next determined the consequences of apicidin on contractility of NPASMC using a collagen gel contraction assay. Surface area of 24-well dishes containing no cells was defined as 100%. In the presence of 10% FBS, untreated NPASMCs had significant collagen gel contractility after 24 h culture (Fig. 6a,b, *P < 0.01 compared to the group without cells). Contractility of NPASMCs was significantly attenuated by apicidin at concentrations of 0.5 and 5 µg/ml (#P < 0.05 compared to untreated group).

Effect of class I HDAC inhibition on expression of genes encoding pro-oxidant and antioxidant enzymes

Previous studies have documented that HDACs are involved in oxidative stress-induced pulmonary hyper-
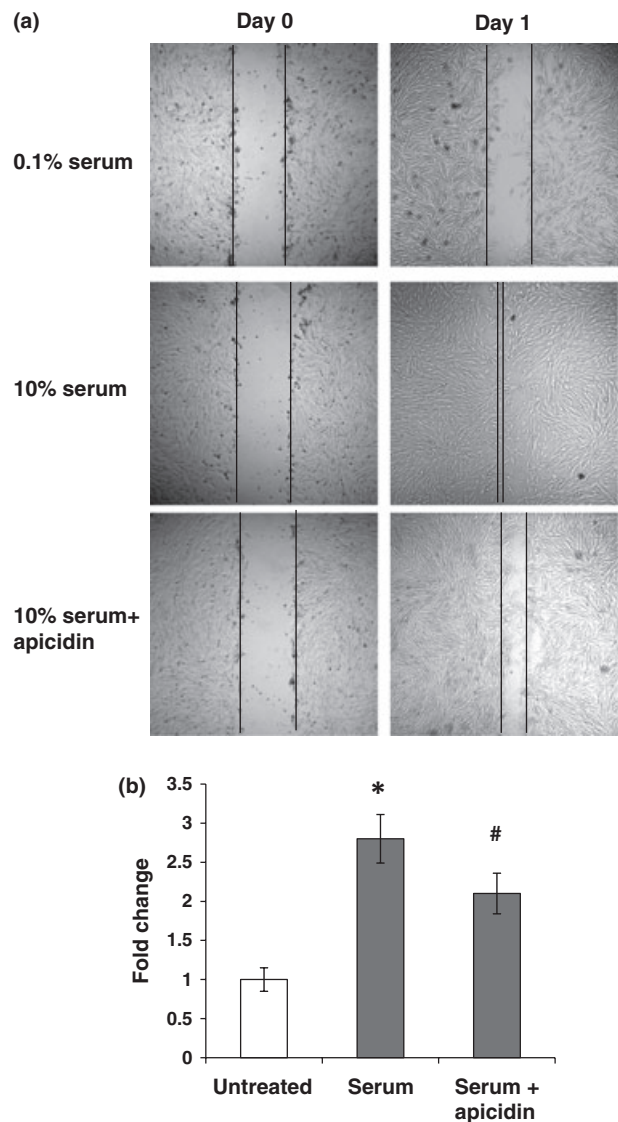

Figure 5. Effect of class I HDAC inhibition on serum-induced migration of NPASMCs. (a) Representative images of serum-induced NPASMC migration in the presence and absence of apicidin. (b) Cell migration into the wound areas was examined using microscopy, and photomicrographs were taken at 0 and 24 h. Cell migration distance was determined by subtracting values obtained at 0 h from 24 h. Migration distances were expressed as percentages over control values. *P < 0.05 compared to untreated control. #P < 0.05 compared to serum group.

tension. To determine whether HDAC inhibition alters expression of pro-oxidant and antioxidant enzymes which regulate the redox state, real-time PCR analysis was performed; our data reveal that apicidin modulated mRNA levels of pro-oxidant and antioxidant enzymes. As shown in Fig. 7a, apicidin treatment exhibited marked reduction in expression of genes encoding pro-oxidant enzymes, including *DUOX1* and *DUOX2*. On
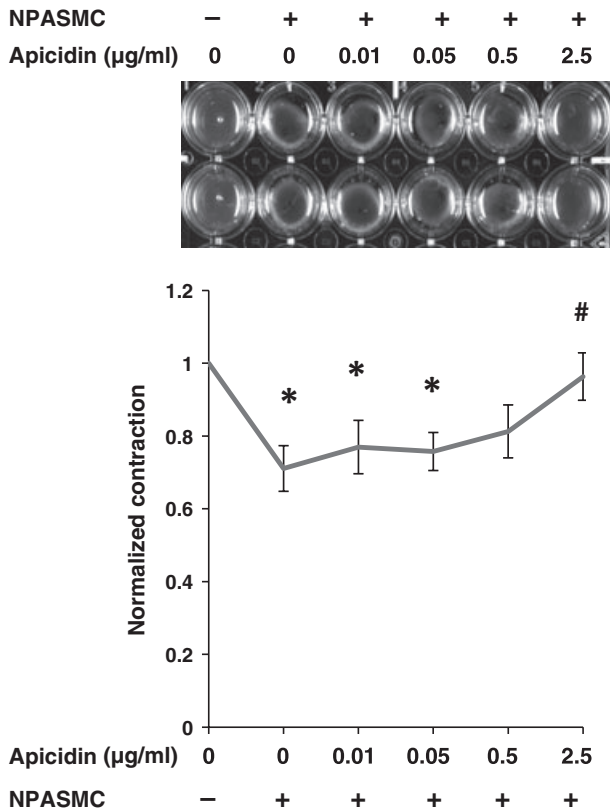
Figure 6. Effect of class I HDAC inhibition on contractility of NPASMC as assessed by collagen gel contraction assay. NPASMC were cultured in 3-D collagen gels for 24 h in the presence of various concentrations of apicidin. NPASMC-mediated collagen gel contraction was assessed by measuring gel surface area using Imagine J software. (a) Collagen gels were photographed. (b) Contractility of collagen gel without NPASMC was defined as 1. Relative contractility of apicidin-treated and untreated NPASMCs was calculated. *$P < 0.05$ compared to no cells group; #$P < 0.05$ compared to untreated cells group.

the other hand, apicidin treatment exhibited increased expression of genes encoding antioxidant enzymes including *SOD2* and *SOD3* as shown in Fig. 7b. Increased expression of SOD1 and PRDX5 proteins was not seen. Our studies suggest that HDAC inhibition may confer substantial protection against oxidative stress.

Class I HDAC inhibition modulated global DNA methylation in NPASMC

It has been reported that interplay between histone modification and DNA methylation occurs in a variety of cells (25,27–29), thus, we determined whether class I HDAC inhibition altered overall DNA methylation in NPASMC. The cells were treated with 0.05, 0.5 and 2.5 µg/ml apicidin for 48 h, and global DNA methylation was measured by LC/MS. As shown in Fig. 8, treatment of NPASMC by apicidin at concentrations of

0.5 and 2.5 µg/ml resulted in over 2-fold increase in overall 5-methylcytosine methylation in a dose-dependent manner ($P < 0.05$). However, apicidin at a lower concentration (0.05 µg/ml) reduced global DNA methylation levels ($P < 0.05$), indicating that the pattern of global DNA methylation was modulated by apicidin in a dose-dependent manner.

Discussion

Excessive cell proliferation of PASMC contributes to vascular remodelling in patients with PAH (30). In this study, we demonstrated that inhibition of HDACs reduced serum-induced PASMC proliferation. Apicidin is able to block serum-stimulated cell proliferation in a dose-dependent manner. It is possible that inhibitory effects of apicidin were exerted by changing the balance of CDK-cyclin and CDK inhibitors. CDK-cyclin complex and CDK inhibitor play a central role in regulation of cell proliferation during vascular remodelling (21,31). In this study, we examined expression level of *p21*, *CDK4*, *p53* and *cyclinD1* in NPASMC treated with HDACi. Expression of CDK inhibitor *p21* was markedly increased in cells treated with apicidin and HDACi VIII compared to vehicle control, which correlated with increased expression of acetylated histone H3. Inhibition of HDAC activity by apicidin and HDACi VIII significantly induced *p21* expression, but without induction of *p53*, indicating that apicidin- and HDACi VIII-induced *p21* induction is *p53*-independent. This is consistent with previous reports showing that apicidin arrests cancer cell population growth through induction of *p21* (32,33). On the other hand, apicidin reduced expression of *cyclinD1* in the NPASMC. Although the molecular mechanism underlying apicidin-induced reduction of *cyclinD1* expression here needs to be investigated, our ChIP data suggest that *p21* upregulation in apicidin and HDACi VIII-treated cells is due to recruitment of AcH3 and AcH4 to the *p21* promoter region in ovine PASMC.

It has been reported that HDACs modulate vascular smooth muscle cell migration induced by cyclic mechanical strain (34). Cyclic strain upregulates levels of acetylated histone H3 and HDAC7 while downregulating levels of HDAC3/4 in VSMCs. Furthermore, mechanically induced VSMC migration was diminished by treatment with tributyrin, another HDAC inhibitor (34). However, whether HDACs take part in modulating migration of newborn PVSMC is poorly understood. We have demonstrated that in addition to its inhibitory effect on PASMC proliferation, inhibition of class I HDACs attenuated serum-induced cell migration. Mechanisms of apicidin-induced inhibitory effects on migration of PASMC require further investigation; however, our pre-
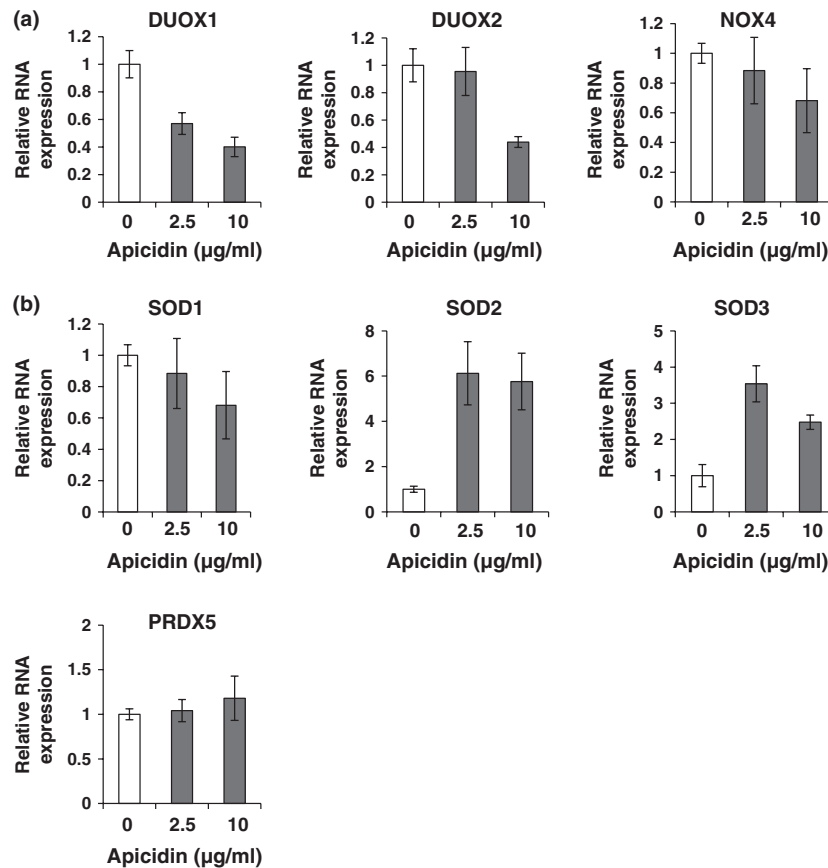
Figure 7. Effect of class I HDAC inhibition on expression of genes encoding pro-oxidant and antioxidant enzymes. (a) mRNA levels from genes encoding pro-oxidant enzymes including *DUOX1*, *DUOX2* and *NOX4* were measured by real-time PCR analysis. (b) mRNA levels from genes encoding antioxidant enzymes including *SOD1*, *SOD2*, *SOD3* and *PRDX5* were measured by real-time PCR analysis. β-actin was used as an endogenous control.
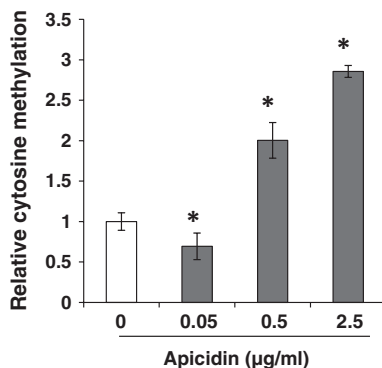

Figure 8. Effect of class I HDAC inhibition on global DNA methylation level in NPASMC. DNA methylation was determined by LC/MS. Genomic DNA was isolated from untreated and apicidin-treated NPASMC (0.05, 0.5 and 2.5 μg/ml for 48 h). DNA was then subjected to digestion with nuclease P1, venom phosphodiesterase I, and alkaline phosphatase, respectively. Concentration of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine in each sample was calculated from the standard curve. Each genomic DNA sample was analysed in triplicate. *$P < 0.05$ compared to untreated control.

vious studies have shown that Trichostatin A significantly reduced phosphorylation of ERK (21). These results provide convincing evidence that HDACs are involved in proliferation and migration of PASMC induced by serum through chromatin remodelling. Thus, inhibition of HDAC may be beneficial in preventing migration of PASMC in treating proliferative vascular diseases.

Oxidative stress is involved in contributing to the development and progression of PAH (35–37). In this study, we determined whether inhibition of class I HDAC altered expression of genes encoding pro-oxidant and antioxidant enzymes in NPASMC. Real-time PCR analysis revealed increased expression of genes encoding antioxidant enzymes including SOD2 and SOD3 proteins in cells treated with apicidin. In addition, apicidin reduced expression of genes encoding pro-oxidant enzymes including DUOX1. Our studies are in accordance with recently published results showing that β-hydroxybutyrate, an endogenous histone deacetylase

inhibitor, activated genes encoding oxidative stress resistance factors FOXO3A and MT$_2$. Treatment of cells with β-hydroxybutyrate increased histone acetylation at the *Foxo3a* and *Mt2* promoters, and both genes were activated by selective depletion of HDAC1 and HDAC2. Consistent with increased FOXO3A and MT2 activity, treatment of mice with βOHB conferred substantial protection against oxidative stress (38).

It has been considered that DNA methylation and histone modifications appear to act in a coordinated fashion (39,40). Although HDAC inhibition by apicidin in the range of 0.05–2.5 μg/ml is capable of modulating global DNA methylation levels in NPASMC, lower concentration of apicidin at 0.05 μg/ml reduced global DNA methylation level. On the other hand, relatively high concentrations of apicidin (0.5 and 2.5 μg/ml) increased global DNA methylation level, which is reverse-correlated with cell proliferation, suggesting that the dose-dependent effect of apicidin on alteration of DNA methylation pattern in the genome of NPASMC occurred. Our data provide evidence showing that the interplay between histone modification and DNA methylation may be involved in the process of gene transcription and aberrant silencing related to PASMC behaviour. One may expect that HDAC inhibition may modulate abnormal distribution of DNA methylation in PASMCs leading to increased risk of abnormal cell proliferation.

Taken together, inhibition of class I HDACs results in modulation of the epigenome in NPASMC, with changes in global acetylation and DNA methylation levels. HDAC inhibition is capable of inhibiting PASMC proliferation and cell cycle progression through chromatin remodelling. This change in NPASMC behaviour is associated with alteration in *p21* and *CCND1* expression. *p21* upregulation in apicidin- and HDACi VIII-treated NPASMC is due to recruitment of AcH3 and AcH4 to the *p21* promoter region in ovine PASMC. Moreover, HDAC inhibition attenuates migration of NPASMC in response to serum stimulation. HDAC inhibition modulates gel contraction ability and mRNA levels of pro-oxidant and antioxidant enzymes. Finally, HDAC inhibition modulated global DNA methylation in NPASMC. Our studies suggest that epigenetic mechanisms of histone acetylation may have significant mechanistic and therapeutic implications in human PAH, and histone acetylation modifiers may be considered as a new target for therapy for vascular disease.

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Disclosures

No conflicts of interest, financial or otherwise, are declared by the author(s).

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