Pulmonary artery smooth muscle cell proliferation and migration in fetal lambs acclimatized to high-altitude long-term hypoxia: role of histone acetylation

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Yang Q, Lu Z, Ramchandran R, Longo LD, Raj JU. Pulmonary artery smooth muscle cell proliferation and migration in fetal lambs acclimatized to high-altitude long-term hypoxia: role of histone acetylation. Am J Physiol Lung Cell Mol Physiol 303: L1001–L1010, 2012.—High-altitude long-term hypoxia (LTH) is known to induce pulmonary arterial smooth muscle cell (PASMC) proliferation in the fetus, leading to pulmonary arterial remodeling and pulmonary hypertension of the newborn. The mechanisms underlying these conditions remain enigmatic however. We hypothesized that epigenetic alterations in fetal PASMC induced by high-altitude LTH may play an important role in modulating their proliferation during pulmonary arterial remodeling. To test this hypothesis, we have analyzed epigenetic alterations in the pulmonary vasculature of fetal lambs exposed to high-altitude LTH [pregnant ewes were kept at 3,801 m altitude from ~40 to 145 days gestation] or to sea level atmosphere. Intrapulmonary arteries were isolated, and fetal PASMC were cultured from both control and LTH fetuses. Compared with controls, in LTH fetus pulmonary arteries measurements of histone acetylation and global DNA methylation demonstrated reduced levels of global histone acetylation and DNA methylation, accompanied by the loss of the cyclin-dependent kinase inhibitor p21. Treatment of LTH fetal PASMCs with histone deacetylase (HDAC) inhibitor trichostatin A decreased their proliferation rate, in part because of altered expression of p21 at both RNA and protein level. In PASMC of LTH fetuses, HDAC inhibition also decreased PDGF-induced cell migration and ERK1/2 activation and modulated global DNA methylation. On the basis of these observations, we propose that epigenetic alterations (reduced histone acetylation and DNA methylation) caused by chronic hypoxia leads to fetal PASMC proliferation and vessel remodeling associated with vascular proliferative disease and that this process is regulated by p21.

Histone deacetylase; PDGF; high-altitude long-term hypoxia; epigenetics; p21

PULMONARY ARTERIAL HYPERTENSION (PAH), characterized by pulmonary vascular remodeling and vasoconstriction, is associated with proliferative changes in the pulmonary vascular wall (10). The structural alterations within the vessel are caused mainly by proliferation and migration of pulmonary arterial smooth muscle cells (PASMC) (29). Several growth factors including PDGF, basic FGF, and EGF have been implicated in the abnormal proliferation and migration of PASMC. PDGF acts as a potent mitogen and chemotactic for smooth muscle cells (SMC) (41), and it is involved in the progression of pulmonary hypertension. PDGF consists of dimers that include two genetically distinct but structurally similar polypeptides (A chain and B chain) (15, 31). PDGF stimulates cell growth through activation of its cell surface receptors α and β. The PDGF receptors belong to a family of transmembrane receptor tyrosine kinases and are believed to be conjoined by bivalent PDGF ligands (2, 26). In a high-altitude long-term hypoxia (LTH) sheep model, previously we have shown that LTH causes pulmonary vascular wall thickening by inducing PASMC proliferation (3, 27). Additionally, PASMC isolated from LTH fetuses displayed greater proliferative potential compared with cells from control fetal lambs (3).

The endogenous CDK inhibitor p21 plays a critical role in PASMC proliferation and has been identified as a key regulator of cell progression exposed to hypoxia. p53 gene deficiency leading to decreased p21 expression level promotes hypoxia-induced pulmonary hypertension and vascular remodeling in mice (19). p53 as an upstream mediator of p21 may suppress hypoxic pulmonary arterial remodeling and pulmonary arterial SMC proliferation by interacting with p21 and HIF-1α under hypoxia. In addition to p53-dependent regulation of p21, PDGF has been reported to induce p21 promoter activity independent of p53, suggesting the complexity of regulation of p21 expression relevance to hypoxia (40).

Although the molecular mechanisms underlying excessive cell proliferation induced by chronic hypoxia are largely unknown, recently epigenetic mechanisms have been reported to be involved in pulmonary vascular dysfunction (1, 24, 35). Restrictive diet during pregnancy in mice induces pulmonary vascular dysfunction in the offspring through alterations in DNA methylation, whereas administration of histone deacetylase (HDAC) inhibitors to offspring of mice fed a restrictive diet during pregnancy normalized pulmonary DNA methylation and vascular function (24). Superoxide dismutase-2 (SOD2) is a gatekeeper that regulates physiological production of H2O2. SOD2 deficiency due to epigenetic alteration has been shown to initiate and sustain a heritable form of PAH by impairing redox signaling and creating a proliferative, apoptosis-resistant PASMC (1). In vascular endothelial cells, endothelial nitric oxide (eNOS) is responsible for the production of the majority of NO and a decreased level of eNOS expression is associated with PAH. Altered expression of eNOS in a newborn rat model of PAH was reported to be due to histone modification (35). Little is known, however, regarding the association between high-altitude LTH and epigenetic alterations leading to vascular remodeling.

In this study, we investigated the epigenetic regulation of pulmonary artery smooth muscle phenotype in high-altitude...
LTH-exposed fetal lambs. We also studied the effect of inhibition of HDAC on PASMC proliferation and migration.

MATERIALS AND METHODS

Animals. For high-altitude LTH, pregnant ewes were kept at an altitude of 3,801 m (12,470 ft) from day 40 to day 145 of gestation (term being 150 days). The ewes were brought to sea level and euthanized the following day. The fetuses were then removed and their lungs harvested for use in our studies. For the control group, the pregnant ewes were kept at sea level for their entire gestation period and euthanized at a comparable gestational age to the LTH group. All procedures and protocols used in the present study were approved by the Animal Research Care Committees of the University of Illinois at Chicago and Loma Linda University.

Preparation of PASMC from high-altitude LTH fetuses. Intrapulmonary arteries, third to fourth generation, were dissected free of parenchyma and kept in ice-cold modified Krebs-Ringer bicarbonate buffer, and primary ovine fetal PASMC were isolated from pulmonary arteries as described previously (11). Cells were maintained in DMEM containing 10% heat-inactivated FBS and antibiotics. Primary ovine fetal PASMC were confirmed to be SMCs by their typical “hill and valley” morphology and by α-smooth muscle actin immunofluorescent staining. Contamination with endothelial cells was ruled out by negative immunofluorescent staining with an anti-von Willebrand factor VIII antibody. All experiments were performed with cells at passages 4 to 8.

Cell number, cell viability, and BrdU incorporation. Cell number was determined by use of a hemocytometer. Trypan blue staining was used to determine the dead and live cells. Cell proliferation was measured by 5-bromo-2-deoxyuridine (BrdU) incorporation by using a proliferation assay kit (Millipore, Billerica, MA) according to the manufacturer’s instructions. Briefly, PASMC of LTH fetuses were plated in 96-well plates. PASMC of LTH fetuses were starved for 24 h at 0.1% serum-containing medium. PDGF-BB (Millipore) was added for 24 h at the indicated concentrations in the presence or absence of inhibitors. In other experiments, PASMC of LTH fetuses were plated in 10% FBS DMEM medium overnight. Then the cells were treated with trichostatin A (TSA; 0.5, 5, 50, 500, 5,000 ng/ml), apicidin (0.05, 0.5 μg/ml, Millipore), HDAC inhibitor VIII-382175 (0.05, 0.5 μg/ml, Millipore), and tenovin-1 (0.05, 0.5 μg/ml Millipore) for 24 h. BrdU labeling solution (Millipore) was added to each well 18 h prior to the analysis. Denaturing solution was added to each well for 30 min at room temperature after removal of the well contents. Then, anti-BrdU antibody was added to each well and incubated for 1 h and peroxidase goat anti-mouse IgG horseradish peroxidase (HRP) conjugate was added in the well for 30 min at room temperature. The absorbance was read at 450–540 nm on a Glomax Multiple Detection System (Promega, Madison, WI).

Cell cycle analysis. Cell cycle distribution was determined by flow cytometric analysis as previously described (39). Briefly, PASMC from control and LTH fetuses were cultured in serum-free DMEM medium for 24 h. After starvation, medium containing 10% serum was replaced and the HDAC inhibitor TSA (Sigma, St. Louis, MO) was added at the concentration of 500 ng/ml. Cells were treated for 24 h, then washed with PBS, fixed in 70% ethanol, and hypotonically lysed in 500 μl of DNA staining solution [0.05 mg/ml phosphatidyl-inositol (Sigma), 0.1 mg/ml RNase A, and 0.05% Triton X-100]. While protected from light, the cells were incubated at 37°C for 40 min. Stained cells were washed with PBS and suspended in 300 μl of PBS before analysis. The cell cycle data were analyzed with an Epics XL-MCL flow cytometer with System II (version 3.0) software (Beckman Coulter, Miami, FL). Additional analysis of cell cycle distribution was determined by using Modfit LT (Verity Software House, Topsham, ME).

Migration assay. PASMC of LTH fetuses were grown to confluence on 35-mm petri dishes. Cells were grown-arrested for 24 h in DMEM containing 0.1% FBS. A scratch was made in the cell monolayer, medium was replaced by 0.1% FBS serum-free medium, baseline (0-h time point) images were captured, and 500 ng/ml of TSA was added to the medium for 30 min prior to addition of PDGF at concentration of 25 ng/ml. 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 by use of Trizol reagent (Invitrogen, Carlsbad, CA). Reverse transcription was performed using Superscript III (Invitrogen) and 50 μM oligo(dT)12 at 50°C for 50 min. SYBR Green real-time PCR reactions were set up containing 1× Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 250 nM forward and reverse primers in a 20-μl reaction. All assays were carried out in a 96-well format. Real-time fluorescent detection of PCR products was performed with an StepOne Plus Real-Time PCR System (Applied Biosystems) with 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. The sequences of the primers were designed by use of the Primer Express software (Applied Biosystems). β-Actin was used as an endogenous control for gene expression. For data analysis, the comparative method (ΔΔCt) was used to calculate relative quantities of a nucleic acid sequence.

siRNA transfection. All small interfering RNA (siRNA) sequences were designed and purchased from Dharmacon (Lafayette, CO). p2i siRNA sequences are CAGACCAGCAUGACAGAUUUU (sense-1), AAUCUGCAUGCGUGUCUU (antisense-1), GCUCAGAGGAGCCCUAUUU (sense-2), UAGGGCUUCCUUGGAGCUU (antisense-2). D-001210-03-05 was used as the nontargeting control. Subconfluent fetal PASMCs were transfected with combination of equal amounts of siRNA-1 and siRNA-2 by using 1 μl of siRNA/2.5 μl of Lipofectamine 2000 (Invitrogen) in DMEM containing 0.1% FBS without antibiotics for 6 h. siRNAs were first resuspended in Opti-MEM I medium (Invitrogen) and then mixed together for 20 min before the transfection. After 6 h, the complete medium was added and incubated for a further 48 h.

Western blot analysis. Total proteins of fetal pulmonary arteries or fetal PASMCs were extracted after the cells were lysed in cell lysis buffer containing protease and phosphatase inhibitor cocktails (Sigma-Aldrich), and protein concentration was determined by use of the Bradford protein assay kit (Bio-Rad, Hercules, CA). 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 (TBS) containing 5% nonfat powdered milk and probed with primary antibody in TBS with 2.5% nonfat powdered milk at concentrations from 1:500 to 1:20,000 dilutions and preincubated for overnight according to the manufacturer’s instructions for each antibody. In all cases, a secondary antibody labeled with HRP (GE Healthcare Bio-Sciences, Piscataway, NJ) was used at concentrations of 1:2,000 to 1:20,000 for 1 h at room temperature, and immunoreactive bands were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and recorded on photosensitive film. The relative intensities of immunoreactive bands detected by Western blot analysis in cells were quantified by densitometry using NIH Image J software (NIH) and normalized with β-actin or tubulin levels. The primary antibodies used for this study include anti-acetyl histone H4 (Abcam), anti-pERK1/2 (Cell Signaling, Danvers, MA), anti-ERK1/2 (Cell Signaling), anti-p21 (Santa Cruz), anti-p53 (Santa Cruz), anti-tubulin (Sigma), and anti-β-actin (Santa Cruz).

LC/MS. Total cytosine methylation level was measured by liquid chromatography-mass spectroscopy (LC/MS) as described previously (37). Briefly, DNA was hydrolyzed to nucleosides by adding 5 units of nuclease P1 (Sigma) at 37°C for 2 h, 0.002 units of venom phosphodiesterase I (Sigma) at 37°C for 2 h, and 0.5 units of alkaline phosphatase at 37°C for 1 h. Stock solutions of 2′-deoxycytidine...
(Sigma) and 5-methyl-2′-deoxycytidine (ChemGenes, Wilmington, MA) were prepared in water. An eight-point stock mixture of a standard was carefully prepared to give an 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 analyzed in triplicate; 25 μl (80 ng) of sample was injected into the liquid chromatography and run through an Atlantis DC18 silica column (Waters, Milford, MA). 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 by a standard two-sample Student’s t-test assuming unequal variances of the two data sets. Statistical significance was determined with a two-tailed distribution assumption and was set at 5% level (P < 0.05).

RESULTS

High-altitude LTH decreased the level of histone H4 acetylation, global DNA methylation, and p21 expression in pulmonary arteries of fetal lambs. To determine whether high-altitude LTH altered the histone H4 acetylation level, pulmonary artery lysates from LTH and control fetuses were prepared. The acetylation level of histone H4 was determined by Western blot analysis using an antibody against AcH4. As shown in Fig. 1A, global AcH4 was significantly decreased in LTH pulmonary arteries compared with control pulmonary arteries.

We determined the extent to which high-altitude LTH modulated the level of global DNA methylation in fetal pulmonary arteries. Genomic DNA was isolated from LTH and control fetal pulmonary arteries. LC/MS analysis was performed to determine the percentage of cytosine methylation in LTH and control arteries. Figure 1B shows the standard curve average of 5-methylcytosine measured by LC/MS.

As shown in Fig. 1C, chronic hypoxia exposure resulted in decreased global DNA methylation. Because p21, a cyclin-dependent kinase (CDK) inhibitor, plays an important role in proliferation associated with vascular remodeling (19), we compared p21 expression level between control and LTH fetal pulmonary arteries. As shown in Fig. 1D, p21 expression was significantly reduced in LTH arteries compared with controls.

HDAC inhibition suppresses the proliferation of fetal PASMCs exposed to high-altitude LTH. To determine the relationship between PASMC proliferation and histone acetylation, we isolated PASMC from LTH fetal lungs. PASMC of LTH fetuses were treated with HDAC inhibitor TSA at a concentration of 0.5 and 2.5 μg/ml. The lysates from untreated and TSA-treated PASMC of LTH fetuses were subjected to Western blot analysis. As shown in Fig. 2A, TSA at concen-

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Fig. 1. Effect of high-altitude long-term hypoxia (LTH) on histone acetylation, global DNA methylation, and p21 expression. A: cell lysates of pulmonary arteries from control or LTH fetuses were subjected to Western blot analysis using antibody against acetyl histone H4 (AcH4). β-Actin was used as a loading control. B: stock solutions of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine were prepared in water. An 8-point stock mixture of a standard was carefully prepared to give an exact known concentration ratio of 2′-deoxycytidine and 5-methyl-2′-deoxycytidine. Std, standard; Avg, average; AU, arbitrary units. C: genomic DNA was isolated from pulmonary arteries of control or LTH fetuses, and subjected to 5-methylcytosine quantitation analysis. *P < 0.05 compared with normoxic fetal arteries. D: total RNA was isolated from pulmonary arteries of control and LTH fetuses. After cDNA synthesis, expression of p21 was measured by real-time PCR. *P < 0.05 compared with normoxic fetal arteries.
tation of 0.5 and 2.5 μg/ml enhanced the AcH4 level. To determine the extent to which TSA impaired the proliferation of PASMC of LTH fetuses, PASMC of LTH fetuses were placed in DMEM medium containing 0.1% serum for 24 h to reduce basal proliferation, followed by the addition of TSA. The BrdU incorporation assay was performed to detect the incorporation of the thymidine analog BrdU by use of an immunochromed BrdU detection kit. As shown in Fig. 2B, TSA at concentration range between 50 and 5,000 ng/ml significantly decreased BrdU incorporation (P < 0.01), suggesting that TSA inhibited cell proliferation in LTH fetal PASMC. Trypan blue staining (Fig. 2C) exhibited no significant difference in cell viability up to 500 ng/ml TSA-treated cells, indicating that the prominent inhibitory effect of cell proliferation by TSA at concentration of 50–500 ng/ml is dependent on cell cycle regression.

To determine whether HDAC inhibition caused a decrease in cell proliferation of PASMC from both control and LTH fetal lungs via cell cycle arrest, we performed flow cytometry analysis. After 24 h of serum starvation, fetal PASMC were cultured for 24 h in 10% FBS with or without 500 ng/ml TSA. Cells were stained with propidium iodide to study the cell cycle progression. As shown in Fig. 2D, 61.82 ± 4.0% of control fetal PASMCs in untreated group were in G0/G1 phase, 19.92 ± 12.73% in S phase, and 18.26 ± 10.95% in G2/M. On the other hand, 76.32 ± 0.68% of control fetal PASMCs in the TSA-treated group were in G0/G1 phase, 12.73 ± 0.47% in G1 phase, and 10.95 ± 1.16% in G2/M. As shown in Fig. 2E, 57.16 ± 0.57% of LTH fetal PASMCs in untreated group were in G0/G1 phase, 22.91 ± 1.55% in S phase, and 19.93 ± 2.12% in G2/M. On the other hand, 88.47 ± 5.52% of LTH fetal PASMCs in the TSA-treated group were in G0/G1 phase, 3.22 ± 2.67 in S phase, and 8.32 ± 2.85% in G2/M. This indicated that specific HDAC inhibition can arrest growth of PASMC from both control and LTH fetal lambs, and more cell cycle arrest was observed in LTH fetal PASMC in response to TSA treatment.

Inhibition of HDAC class I contributes to the suppression of cell proliferation with altered p21 expression. Since TSA inhibited HDAC class I and II activity, we further determined which class or classes of HDACs are involved in LTH fetal PASMC proliferation. HDAC inhibitors mainly targeting HDAC class I (apicidin), II (HDAC inhibitor VIII), and III (tenovin-1) were used individually to treat LTH fetal PASMC. BrdU incorporation assays indicated that tenovin-1 at a concentration of 0.5 μg/ml has no effect on proliferation of LTH fetal PASMC (Fig. 3A). HDAC inhibitor VIII inhibited cell proliferation at a concentration of 0.5 μg/ml (Fig. 3A) via the apoptosis pathway (data not shown). Treatment of LTH fetal PASMCs with apicidin-1 at a concentration of 0.05 and 0.5 μg/ml significantly inhibited the rate of their proliferation (Fig. 3A) accompanied with remarkable increase of p21 expression (Fig. 3B), suggesting that HDAC class I may play a major role in regulating proliferation of LTH fetal PASMC. In addition, induction of p53 in the LTH fetal PASMC treated with apicidin was not observed as shown in Fig. 3C.

p21 is required for TSA-induced inhibitory proliferation of fetal PASMC exposed to high-altitude LTH. In the following experiments, we determined the extent to which the expression of CDK inhibitor p21 and p53 was increased after TSA treatment of PASMC from control and LTH fetuses. Control and LTH fetal PASMC were cultured in the presence or absence of 500 ng/ml of TSA for 24 h, and expression of p21 and p53 was measured by Western blot using antibody against p21 and p53, respectively. As shown in Fig. 4A, induction of p21 was observed in both control and LTH fetal PASMC after treatment with TSA. However, more induction of p21 was observed in LTH fetal PASMC following 24-h TSA treatment. To determine whether the induction of p21 by TSA is via enhanced p21 transcriptional level, RNA expression of p21 and p53 was examined by quantitative PCR with ovine specific primers for CDKN1A and p53. Figure 4B and C, depicts the fold change of p21 and p53 expression after treatment of fetal PASMC with TSA. p21 expression showed a ~5.3-fold in-
shown in Fig. 4 indicated that si-p21 enhanced LTH PASMC proliferation as compared with NsRNA (Fig. 4D). BrdU incorporation assay was performed to determine the role of siRNA-induced p21 knockdown on cell proliferation upon TSA treatment. *P < 0.05 compared with untreated LTH fetal PASMC. B: RNA was isolated using TRIzol reagent. cDNA was synthesized and subjected to quantitative RNA expression of p21 by SYBR Green q-PCR. β-Actin was used as an endogenous control. *P < 0.05 compared with untreated cells. C: p53 RNA expression was examined by SYBR Green q-PCR. β-Actin was used as an endogenous control.

We next examined the causative role of p21 in the enhanced proliferative behavior of PASMC from fetal lungs acclimatized to high-altitude LTH. PASMC isolated from LTH fetal lungs were transfected with siRNA specific for p21 (si-p21) or a nontargeting control siRNA (NsRNA). At a concentration of 100 nM siRNA, expression of p21 was decreased by 80% compared with NsRNA (Fig. 4D). BrdU incorporation assay indicated that si-p21 enhanced LTH PASMC proliferation as shown in Fig. 4E (P < 0.05). To determine whether p21 is required for TSA-induced inhibitory effect on cell proliferation, LTH fetal PASMC were transfected with si-p21. After 24 h posttransfection, fetal PASMC were treated with TSA for 24 h, and BrdU incorporation was assessed. As shown in Fig. 4E, inhibitory effect of LTH fetal PASMC proliferation induced by TSA was attenuated by p21 reduction (P < 0.05), indicating that TSA inhibited LTH fetal PASMC proliferation via the p21 pathway.

HDAC inhibition attenuated PDGF-induced proliferation of LTH fetal PASMC. PDGF promotes vascular SMC proliferation, a key event during pulmonary vascular remodeling (2, 13, 26); therefore we examined the effect of TSA on PDGF-induced cell proliferation of PASMC from high-altitude LTH fetuses. First, we determined whether PDGF stimulated proliferation of PASMC from LTH fetuses. As shown in Fig. 5A, induction was not observed following TSA treatment.

We next examined the causative role of p21 in the enhanced proliferative behavior of PASMC from fetal lungs acclimatized to high-altitude LTH. PASMC isolated from LTH fetal lungs were transfected with siRNA specific for p21 (si-p21) or a nontargeting control siRNA (NsRNA). At a concentration of 100 nM siRNA, expression of p21 was decreased by 80% compared with NsRNA (Fig. 4D). BrdU incorporation assay indicated that si-p21 enhanced LTH PASMC proliferation as shown in Fig. 4E (P < 0.05). To determine whether p21 is required for TSA-induced inhibitory effect on cell proliferation, LTH fetal PASMC were transfected with si-p21. After 24 h posttransfection, fetal PASMC were treated with TSA for 24 h, and BrdU incorporation was assessed. As shown in Fig. 4E, inhibitory effect of LTH fetal PASMC proliferation induced by TSA was attenuated by p21 reduction (P < 0.05), indicating that TSA inhibited LTH fetal PASMC proliferation via the p21 pathway.

HISTONE ACETYLATION IN CELL PROLIFERATION AND MIGRATION

Fig. 3. Effect of apicidin, HDAC inhibitor VIII (HDAC VIII; 382175) and tenovin-1 on cell proliferation and expression of p21 and p53. A: PASMC of LTH fetuses were treated with 0.05 and 0.5 μg/ml of apicidin, HDAC inhibitor VIII (382175), and tenovin-1 for 24 h, and BrdU incorporation assay was performed. *P < 0.05 compared with untreated LTH fetal PASMC. B: RNA was isolated using TRIzol reagent. cDNA was synthesized and subjected to quantitative RNA expression of p21 by SYBR Green q-PCR. β-Actin was used as an endogenous control. *P < 0.05 compared with untreated cells. C: p53 RNA expression was examined by SYBR Green q-PCR. β-Actin was used as an endogenous control.

Fig. 4. Effect of HDAC inhibition on p21 and p53 expression and role of p21 in proliferation of PASMC of LTH fetuses. A: PASMC of control and LTH fetuses were treated with 500 ng/ml of TSA for 24 h. The cell lysates from untreated and trichostatin A-treated PASMC of control and LTH fetuses were prepared. The level of p21 and p53 was examined by Western blot analysis. β-Actin was used as an endogenous control. B: PASMC of LTH fetuses were treated with 500 ng/ml of TSA for 24 h. RNA was isolated using TRIzol reagent. cDNA was synthesized and subjected to quantitative RNA expression of p21 by SYBR Green q-PCR. β-Actin was used as an endogenous control. C: p53 expression was examined by SYBR Green q-PCR. β-Actin was used as an endogenous control. D: role of p21 in PASMC proliferation was determined by a small interfering RNA (siRNA) approach. Nontargeting oligo (NsRNA), 50 nM and 100 nM siRNA for p21, was introduced by Lipofectamine 2000. After 6 h, the complete medium was added and incubated for a further 48 h. Cells were collected for RNA isolation and cDNA synthesis. p21 expression was examined by q-PCR. E: BrdU incorporation was performed to determine the role of siRNA in cell proliferation. Mock RNA, NsRNA, and siRNA for p21 transfection was performed in 96-well plates. After 48-h transfection, BrdU incorporation assay was performed. Also 100 nM siRNA for p21 was introduced in PASMC by Lipofectamine 2000. After 24-h transfection, TSA at concentration of 500 ng/ml was also added to 96-well plates and treated for 24 h. BrdU incorporation was performed to determine the effect of siRNA-induced p21 knockdown on cell proliferation upon TSA treatment. *P < 0.05 compared with mock fetal PASMCs; #P < 0.05 compared with mock fetal PASMC; ##P < 0.05 compared with TSA-treated fetal PASMC.
PDGF promoted PASMC proliferation in a dose-dependent manner. At concentrations of 5, 10, 25, and 50 ng/ml of PDGF, BrdU incorporation was increased by ~15, ~50, ~120, and ~150%, respectively. Next, we determined whether TSA exhibited an inhibitory effect on PDGF-induced cell proliferation. As shown in Fig. 5B, in response to 25 ng/ml of PDGF, BrdU incorporation was decreased by ~70% following TSA treatment.

**HDAC inhibition attenuated PDGF-induced migration of LTH fetal PASMCs.** Because vascular SMC migration is involved in vascular remodeling, we examined the extent to which HDAC inhibition decreased LTH fetal PASMC migration induced by PDGF using the wound-healing model/scratch assay. As shown in Fig. 6, A and B, there was minimal migration of fetal PASMC observed in the medium containing 0.1% serum at 24 h compared with 0-h time point. PDGF at a concentration of 25 ng/ml caused over twofold increase in cell migration compared with untreated controls. Inhibition of HDAC activity with 500 ng/ml of TSA resulted in ~50% reduction of PDGF-induced cell migration, however (P < 0.05).

**HDAC inhibition attenuated PDGF-induced ERK signaling.** To examine the effect of HDAC inhibition on ERK signaling in fetal PASMCs induced by PDGF, we performed Western blot analysis using anti-ERK 1/2 and anti-pERK antibodies. Although there was no change in ERK phosphorylation after treatment with 2.5 μg/ml of TSA for 6 h (Fig. 7, A and B), a marked reduction of ERK phosphorylation was seen after 24 h (Fig. 7, C and D). At 0.5 μg/ml of TSA treatment for 6 and 24 h, no significant reduction of ERK phosphorylation was observed in PASMC of LTH fetuses.

**HDAC inhibition of LTH fetal PASMC increases global DNA methylation.** Because interplay between histone modification and DNA methylation has been reported in a variety of cells (14, 16, 17, 33, 37, 38), we determined the extent to which HDAC inhibition altered global DNA methylation in PASMC of LTH fetuses. The cells were treated with 500 ng/ml of TSA for 24 h, and the global DNA methylation was measured by LC/MS. As shown in Fig. 8, TSA treatment resulted in over twofold increase in total 5-methylcytosine methylation in PASMC of LTH fetuses. There is a statistically significant increase in total 5-methylcytosine levels in TSA-treated PASMC (P < 0.05) compared with controls, indicating that the pattern of methylation is modulated after inhibiting the activity of HDAC.

**DISCUSSION**

Reduction in oxygen levels below normal concentrations plays an important role in different normal and pathological conditions (23, 34). However, little is known about the role of chromatin remodeling in the response to hypoxia. In the present study, we used the high-altitude LTH pulmonary hypertension model in fetal lambs to investigate the effect of chronic hypoxia on epigenetic alterations. We have demonstrated for the first time that high-altitude LTH modulates the epigenome of pulmonary arteries of LTH fetuses and decreases CDK inhibitor p21 expression. This was associated with excessive proliferation of SMC in LTH fetal lungs, as we previously described (3). We also demonstrated that HDACs are involved in cell proliferation and migration in PASMC of high-altitude LTH fetuses.

Excessive cell proliferation of PASMCs contributes to vascular remodeling in patients with PAH (30). The CDK-cyclin complex and CDK inhibitor play a central role in regulation of cell proliferation during vascular remodeling. In this study, we examined p21 expression level in both LTH and control arteries. Expression of CDK inhibitor p21 is significantly low in LTH arteries compared with control arteries. This was correlated with decreased level of AcH4. Our data is consistent with a previous study showing that p53 gene deficiency with decrease of p21 expression promotes hypoxia-induced pulmonary hypertension and vascular remodeling in mice (19).

To determine the extent to which p21 expression is regulated by histone acetylation, we isolated PASMC from LTH and control fetuses and examined the expression level of p53 and p21 in PASMC treated with TSA. Inhibition of HDAC activity significantly induced p21 expression without induction of p53. This indicated that TSA-induced p21 induction is p53 independent. A siRNA-induced p21 knockdown experiment further demonstrated that p21 is required for TSA-induced inhibitory effect on the PASMC proliferation of LTH fetuses. In addition, more induction of p21 expression was observed in LTH fetal PASMC in the presence of TSA compared with induction level in control fetal PASMCs, suggesting that differential response of control and LTH fetal PASMC to TSA exists. Our study in fetal PASMC is consistent with a previous report showing that TSA inhibited cell proliferation of vascular SMC from rat thoracic aorta via p21 induction (22). We have further demonstrated that p21 is required for TSA-induced inhibitory effect of cell proliferation by p21 siRNA knockdown experiment. A recent study by others also indicated that hypoxia-induced cardiopulmonary remodeling can be suppressed by HDAC inhibition through an antiproliferation mechanism (5). In addition to targeting SMC, HDAC inhibition also altered the...
phenotype of fibroblasts in severe hypoxic pulmonary hypertension (18). However, suppression of HDAC by TSA and valproic acid showed detrimental effects on the pressure-overloaded right ventricle after pulmonary artery banding (4). The discrepancy between above reports may be due to different causes leading to ventricular dysfunction and variation in administration of HDAC inhibitors.

A number of signaling pathways have been reported to be involved in PASMC proliferation in response to hypoxia (6). The PDGF signaling pathway has been implicated in a broad range of diseases, such as vascular diseases, fibrosis, and cancer. Pulmonary hypertension is one of the major vascular disorders for which, in its pathogenesis, PDGF signaling has been incriminated (13, 32). PDGF was shown to induce expression of a set of immediate-early response genes critical for cell cycle entry. However, another report showed that PDGF induced p21 promoter activity independent of p53 (40). In the present study, we demonstrated that inhibitory effect of PDGF signaling by TSA is correlated with decreased proliferate rate in LTH fetal PASMC. Although we have not determined the differential level of p21 expression between PDGF and PDGF plus TSA-treated LTH fetal PASMC, our previous study showed that p21 was required for BIX-01294 (G9a inhibitor)-induced inhibitory effects on fetal PASMC proliferation, and p21 was upregulated in the presence of PDGF after treatment with BIX-01294 (37). Our result is also consistent with previ-

![Fig. 6. Effect of HDAC inhibition on PDGF-induced migration of LTH fetal PASMC.](http://ajplung.physiology.org/)

A: a and d represent control group (in low-serum medium), b and e represent the 25 ng/ml PDGF group, and c and f represent the 500 ng/ml TSA + PDGF group. Representative images are shown. B: cell migration into the wound areas were examined by microscopy, photomicrographs were taken at 0 and 24 h, and cell migration distance was determined by subtracting values obtained at 0 h from those obtained at 24 h. Migration distances were expressed as percentages over control values. *P < 0.05 compared with untreated control. #P < 0.05 compared with PDGF group.
deoxycytidine and 5-methyl-2,6-deoxycytidine in each sample treated PASMCs of LTH fetuses. DNA was then subjected to digestion with mass spectroscopy. Genomic DNA was isolated from untreated and TSA-LTH fetuses. DNA methylation was determined by liquid chromatography-mass spectrometry. Genomic DNA was isolated from untreated and TSA-LTH fetuses. DNA methylation occurred in response to TSA treatment, suggesting the interplay between histone modification and DNA methylation may be involved in the process of gene transcription and replication.

Vascular smooth muscle cell (VSMC) migration is necessary for vascular remodeling. HDACs can modulate VSMC migration induced by cyclic mechanical strain (36). In VSMC, cyclic strain upregulated the levels of acetylated histone H3 and HDAC7, and downregulated the level of HDAC3/4. Mechanically induced VSMC migration was diminished by treatment with tributyrin, another HDAC inhibitor (36). However, whether HDACs take part in modulating migration of VSMC induced by PDGF is not known. In addition to its inhibitory effect on PASMC proliferation, we have demonstrated that inhibition of HDAC attenuated PDGF-induced cell migration. We also determined whether HDAC inhibition blocked PDGF-induced cell migration through ERK signaling. At 500 ng/ml of TSA treatment, ERK phosphorylation was unchanged, indicating that alternative pathway(s) may be involved in TSA-induced inhibitory effect on cell migration in response to PDGF stimulation. For instance, phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) have been reported to be involved in SMC migration and proliferation (12, 21), and both PI3K and MAPK can be activated by PDGF. Interestingly, our studies revealed that higher concentration of TSA (2.5 μg/ml) significantly decreased ERK phosphorylation. The mechanisms of TSA-induced inhibitory effect on migration of PASMC of LTH require further investigation. These results provide convincing evidence that HDACs are involved in the proliferation and migration of PASMC induced by PDGF through chromatin remodeling. Thus, in treating proliferative vascular diseases, inhibition of HDAC may be beneficial in preventing PASMC migration.

Major findings in PASMC of LTH fetuses are that the arteries exhibited low global DNA methylation levels, and HDAC inhibition was capable of increasing the global DNA methylation level, which is inversely correlated with cell proliferation. The association of global DNA methylation with cell proliferation and cancer progression has been reported in some types of cancers (7–9, 14). During the development of neoplasms, the degree of hypomethylation of genomic DNA increases as the lesion progresses from benign proliferation of cells to invasive cancer. During gastric carcinogenesis, global DNA methylation of gastric mucosa gradually decreased as normal mucosa progressed to Helicobacter pylori (Hp)-positive gastritis, indicating that global DNA hypomethylation is an early molecular event in Hp-related gastric carcinogenesis. The decrease of DNA methylation was mainly a consequence of hypomethylation of repetitive DNA sequences and demethylation of some coding regions and introns. It is possible that high-altitude LTH resulted in abnormal distribution of DNA methylation in PASMC, leading to an increased risk of abnormal cell proliferation.

In PASMC of LTH fetuses, we have demonstrated that both hyperacetylation of histone H4 and increase of global DNA methylation occurred in response to TSA treatment, suggesting the interplay between histone modification and DNA methylation may be involved in the process of gene transcription and replication.
aberrant silencing related to PASMC behavior. Thus our data strongly suggest that epigenetic histone acetylation modification offers the prospect of “reverse chromatin remodeling,” leading to a decrease in vascular remodeling. The observed decreased level of global DNA methylation in LTH arteries is in good agreement with published studies, which showed that the level of global DNA methylation was altered in blood of patients with essential hypertension (28). The level of 5-methylcytosine in the DNA of patients suffering from essential hypertension is lower than that of healthy controls and depends on the progression of hypertension. Our novel finding requires further confirmation by examining whether circulating DNA from LTH fetus lambs has similar lower methylation level as LTH arteries. This blood-based marker could provide exciting new opportunities for assessment of patients with PAH.

Taken together, our results demonstrate that high-altitude LTH results in modulation of the epigenome in fetal pulmonary arteries with a decrease of both global acetylation and DNA methylation. The abnormal PASMC proliferation is associated with decreased p21 expression in arteries of LTH fetuses. HDAC inhibition is capable of inhibiting PASMC proliferation, migration, and cell cycle progression through chromatin remodeling. Moreover, HDAC inhibition attenuates cell proliferation and migration in response to PDGF stimulation and modulates global DNA methylation. Our present studies suggest that epigenetic mechanisms of histone acetylation may have significant medicinal and therapeutic implications in human PAH and that histone acetylation modifiers may be used as a new target for therapy in vascular disease.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


