Silencing of Sodium–Hydrogen Exchanger 1 Attenuates the Proliferation, Hypertrophy, and Migration of Pulmonary Artery Smooth Muscle Cells via E2F1

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We previously found that deficiency of the sodium–hydrogen exchanger 1 (NHE1) gene prevented hypoxia-induced pulmonary hypertension and vascular remodeling in mice, which were accompanied by a significantly reduced proliferation of pulmonary artery smooth muscle cells (PASMCs), and which decreased the medial-wall thickness of pulmonary arteries. That finding indicated the involvement of NHE1 in the proliferation and hypertrophy of PASMCs, but the underlying mechanism was not fully understood. To define the mechanism by which the inhibition of NHE1 decreases hypoxic pulmonary hypertension and vascular remodeling, we investigated the role of E2F1, a nuclear transcription factor, in silencing the NHE1 gene–induced inhibition of the proliferation, hypertrophy, and migration of human PASMCs. We found that: (1) silencing of NHE1 by short, interfering RNA (siRNA) significantly inhibited PASC proliferation and cell cycle progression, decreased hypoxia-induced hypertrophy (in terms of cell size and protein/DNA ratio) and migration (in terms of the wound-healing and migration chamber assays); (2) hypoxia induced the expression of E2F1, which was reversed by NHE1 siRNA; and (3) the overexpression of E2F1 blocked the inhibitory effect of NHE1 siRNA on the proliferation, hypertrophy, and migration of PASMCs. The present study determined that silencing the NHE1 gene significantly inhibited the hypoxia-induced proliferation, hypertrophy, and migration of human PASMCs via repression of the nuclear transcription factor E2F1. This study revealed a novel mechanism underlying the regulation of hypoxic pulmonary hypertension and vascular remodeling via NHE1.

Keywords: sodium–hydrogen exchanger 1; E2F1; PASMC proliferation; hypertrophy; hypoxia

Pulmonary hypertension is caused by many chronic lung diseases associated with prolonged hypoxia, and can result in right ventricular hypertrophy and heart failure. An important pathological feature of pulmonary hypertension involves an increase in medial thickening of pulmonary arteries, resulting from the hyperplasia and hypertrophy of pulmonary artery smooth muscle cells (PASMCs) (1–3). The Na⁺–H⁺ exchanger (NHE), a protein localized to plasma and the mitochondrial inner membrane (4), has nine isoforms (5), of which NHE isoform 1 (NHE1) is ubiquitously expressed. We previously reported on the inhibitory effect of reduced NHE activity in the proliferation of PASMCs (6) and on chronic hypoxia-induced pulmonary hypertension and vascular remodeling (7). Increased expression of the NHE1 gene was evident in animals with hypoxia-induced pulmonary hypertension and vascular remodeling (6–10). Moreover, we recently found that deficiency of the NHE1 gene prevented

CLINICAL RELEVANCE

This study determined that silencing the sodium–hydrogen exchanger 1 (NHE1) gene significantly inhibited the hypoxia-induced proliferation, hypertrophy, and migration of human pulmonary artery smooth muscle cells via the repression of nuclear transcription factor E2F1. This study revealed a novel mechanism underlying the regulation by NHE1 of hypoxic pulmonary hypertension and vascular remodeling.

hypoxia-induced pulmonary hypertension and vascular remodeling in mice (11), accompanied by a significantly reduced proliferation of PASMCs and decreased medial wall thickness of the pulmonary arteries. Our findings indicated the involvement of NHE1 in the proliferation and hypertrophy of PASMCs. However, the mechanism by which NHE1 regulated the proliferation and hypertrophy of PASMCs was not fully understood.

The E2F family is essential for cell-cycle progression. E2F1, one member of the family and a downstream factor of cell-cycle modulators and a nuclear transcription factor, plays an important role in governing nuclear transcription factors that regulate the synthesis of DNA (12). Reports indicate that E2F1 is involved in regulating the proliferation of different cells (13–16). We previously determined that p27, a cyclin-dependent kinase inhibitor and an upstream factor of E2F1, plays a critical role in inhibiting the proliferation of PASMCs and the pulmonary hypertension induced by hypoxia (17–19). We also observed a significantly decreased expression of cyclin D1, a downstream factor of p27 and upstream factor of E2F1, in PASMCs isolated from NHE1-deficient mice with decreased hypoxic pulmonary hypertension and vascular remodeling (11). We therefore assumed that the inhibition of NHE1 caused a reduction of PASMC proliferation via the E2F1 regulatory pathway.

Reports indicate that E2F1 is involved in the regulation of hypertrophy (20–22). Studies also showed that the inhibition of NHE1 by inhibitors attenuated the hypertrophy of cardiomyocytes (23–27). Because we also found a significant decrease in the medial wall thickness of pulmonary arteries in NHE1-deficient mice with decreased hypoxic pulmonary hypertension and vascular remodeling (11), we hypothesized that the effects of NHE1 on hypoxia-induced PASMC hypertrophy were also mediated via E2F1. In addition, the migration of PASMCs is reportedly involved in the pathogenesis of pulmonary hypertension (28), and hypoxia and smooth muscle growth-stimulating factors were shown to affect the migration of PASMCs (29–31). Therefore, we investigated the involvement of E2F1 in the regulation by NHE1 of the hypoxia-induced migration of PASMCs.

MATERIALS AND METHODS

Cells

Human pulmonary artery smooth muscle cells (HPASMCs), human pulmonary artery endothelial cells (HPAECs; Lonza, Inc., Walkers-

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ville, MD), and human pulmonary artery fibroblasts (HPAFs; ScienCell Research Laboratories, Carlsbad, CA) were used in this study.

Hypoxia and Hypoxia Chamber

Air gases (1, 2, 3, 5, and 10% O₂ in 5% CO₂ balance nitrogen; Airgas East, Cambridge, MA) and a hypoxia chamber were used. The hypoxia chamber was placed in a regular CO₂ incubator and maintained at 37°C. The concentration of oxygen in the chamber was monitored with an oxygen analyzer, and remained stable, as indicated in the cylinder (19).

NHE1 Short, Interfering RNA and E2F1 cDNA

Two sets of NHE1 short, interfering RNA (siRNA) and scrambled siRNA were obtained from Sigma (St. Louis, MO). The sequences (5' to 3') were sense CUGUCUUUGAGGAAAUUCA and antisense UGAAUUUCCUCAAGACAG for NHE1 siRNA set 1 (NHE1 siRNA1), and sense CCUGUUAUACAUCGUGCA and antisense UGACGGAUGAUUACAGG for NHE1 siRNA set 2 (NHE1 siRNA2). E2F1 human cDNA (pCMV6-XL6-E2F1) was purchased from Origene (Rockville, MD), and purified according to our previously described methods (17, 19).

siRNA and cDNA Transfection

Transfection agent Lipofectamine-2000 was obtained from Invitrogen (Carlsbad, CA). The procedures for NHE1 siRNA transfection and cotransfection with E2F1 cDNA were performed as described in our previous studies (17, 18).

Exposure to Hypoxia

To implement exposure to hypoxia, PASCs transfected with siRNA and cDNA were placed in the hypoxia chamber (19). After 24 hours, cells were harvested for the cell proliferation assay and cell-cycle analysis.

Cell Proliferation Assay and Cell-Cycle Analysis

We assayed cell proliferation by using a direct cell count and the bromo-2'-deoxyuridine (BrdU) incorporation assay (32). Cell cycles were analyzed using flow cytometry (32).

Measurement of Cell Hypertrophy

Cell hypertrophy was determined by measuring cell size and by calculating the ratio of total protein to DNA content. For the measurement of cell size, PASCs were plated on chamber slides, and NHE1 siRNA and E2F1 cDNA were transfected. After exposure to hypoxia for 24 hours, cells were washed, fixed, and stained with smooth muscle-specific α -actin (SM α -actin). After hematoxylin counterstaining, SM α -actin-positive cells were chosen from slides, and captured using a digital camera. The size of PASCs in different groups was measured using an imaging analysis system (IPLab scientific imaging software; Scanalytics, Inc., Fairfax, VA). For the analysis of protein/DNA ratios, PASCs were plated in six-well plates. After the transfection of NHE1 siRNA and exposure to hypoxia, PASCs were harvested, and the protein/DNA ratio was determined by measuring total DNA content and total protein content (33, 34).

Cell Migration Assay

A wound-healing assay and migration chamber assay were used to determine the effects of NHE1 on the migration of PASC, and were performed according to published methods (35, 36).

RT-PCR and Western Blotting

RT-PCR and Western blotting were performed according to previous studies (11, 17–19).

Statistical Analyses

Statistical analyses were performed using Statview (SAS Institute, Inc., Cary, NC), with factorial ANOVA. If the results of ANOVA were significant, multiple comparisons were performed among groups, using the Fisher protected least significant difference test. All values were expressed as mean $\pm$ SEM. Significance was set at $P < 0.05$.

RESULTS

Silencing of NHE1 Significantly Inhibited the Proliferation of Human PASCs under Normoxia

To confirm and compare the inhibitory effects of NHE1 siRNA on the proliferation of PASCs, we used two sets of NHE1 siRNAs to treat human PASCs separately. We found that the proliferation of PASCs was significantly decreased by either set of siRNA, compared with the control group or the scrambled siRNA group, demonstrating an approximately 35% inhibition of growth (Figure 1A). In the meantime, to obtain the maximal inhibition of PASC proliferation, we simultaneously used two sets of the NHE1 siRNA in one transfection. However, the combination of two sets of siRNA did not significantly change the inhibitory effects of NHE1 in the proliferation of PASCs, although slightly greater inhibition was evident in the group involving two sets of NHE1 siRNA.

Silencing of NHE1 Significantly Decreased the Proliferation of Human PASCs under Hypoxia

After we found that NHE1 siRNA significantly inhibited the proliferation of PASCs under normoxia, we investigated the effects of NHE1 siRNA on the proliferation of PASCs under hypoxia. First, we studied the effects of hypoxia on the proliferation of PASCs by using different concentrations of oxygen. We found a significant increase in cell proliferation in PASCs exposed to 1, 2, and 3% oxygen, either through direct cell count or the BrdU incorporation assay (Figure 1B). Although a slight stimulation of proliferation was evident in cells exposed to 5 and 10% oxygen, the change was not significant. Because a slightly greater stimulation of PASC growth was observed in cells exposed to 2% oxygen, we chose 2% oxygen for this study. We found that the silencing of NHE1 siRNA also significantly inhibited the proliferation of PASCs under hypoxia (Figure 1C), for an inhibition of approximately 45%. No significant difference was evident between the two sets of siRNAs, and the simultaneous use of two sets of siRNAs also did not significantly increase the inhibitory effect under hypoxia.

Silencing of NHE1 Significantly Reduced the Cell-Cycle Progression of Human PASCs

After we observed a significant inhibition in the proliferation of PASCs with NHE1 siRNA, we investigated whether that inhibition was involved in the change in cell-cycle progression. Flow cytometry was performed to analyze cell phases. We found that silencing the NHE1 gene with siRNA significantly inhibited the cell-cycle progression of PASCs under normoxia and hypoxia, with a significant decrease in the S phase and G2/M phase, and an increase in the G0/G1 phase in cells treated with NHE1 siRNA (Figure 2).

Overexpression of E2F1 Blocked the Inhibition by NHE1 of Hypoxia-Induced Proliferation of Human PASCs

To determine if E2F1 was involved in the regulation by NHE1 of PASC proliferation, we analyzed the expression of E2F1 and found that hypoxia significantly increased that expression, and NHE1 siRNA reversed the increase (Figure 3A). We subsequently overexpressed E2F1 via the transfection of E2F1 cDNA into PASCs, and found that the overexpression of E2F1 completely blocked the inhibitory effects of NHE1 siRNA on the hypoxia-induced proliferation of PASCs (Figures 3B and 3C). As expected, NHE1 siRNA transfection significantly decreased the hypoxia-induced expression of E2F1, but E2F1 cDNA did not affect the expression of NHE1 (Figure 3D).

Figure 1. Effects of silencing the sodium–hydrogen exchanger 1 (NHE1) on the proliferation of human pulmonary artery smooth muscle cells (PASCs). After transfection with NHE1 short, interfering RNA (siRNA), PASCs were cultured in normoxia and hypoxia for 24 hours, and harvested for a cell count to assay the proliferation of cells. The effects of NHE1 siRNA on the proliferation of PASCs were determined under conditions of normoxia (A) and hypoxia (C), setting the control value as 100. * $P < 0.05$, compared with control and scrambled siRNA groups. # $P > 0.05$, compared with the NHE1 siRNA1 and siRNA2 groups ($n = 9$ for each group). (B) Proliferation of PASCs under different concentrations of hypoxia, setting 21% O_2 as 100. Direct cell count (above) and bromo-2'-deoxyuridine (BrdU) incorporation assay (below). * $P < 0.05$ and $\Delta P > 0.05$, compared with each control group ($n = 9$ for each group).

Silencing of NHE1 Significantly Inhibited the Hypoxia-Induced Hypertrophy of Human PASCs, and the Overexpression of E2F1 Prevented the Inhibition

Because the hypertrophy of PASCs is considered an important pathological feature in the thickening of pulmonary artery walls in hypoxia-induced pulmonary hypertension and vascular remodeling, we measured cell size and cell volume to evaluate the effects of NHE1 on the hypoxia-induced hypertrophy of PASCs. We

Figure 2. Effects of silencing of NHE1 on cell-cycle progression of human PASCs. After transfection with NHE1 siRNA, PASCs were cultured in normoxia or hypoxia for 24 hours, and harvested for cell-cycle analysis by flow cytometry. (A) Quantitative data on cell-cycle progression. (B) Representative depictions of flow cytometry. * $P < 0.05$, compared with the control group ($n = 3$ for each group). NC, normoxia control; N + siRNA, normoxia + NHE1 siRNA; HC, hypoxia control; H + siRNA, hypoxia + NHE1 siRNA.

observed a significant increase in cell size in the PASCs exposed to hypoxia (Figure 4A). Notably, NHE1 siRNA significantly decreased cell size and protein/DNA ratio, but the overexpression of E2F1 blocked the effects of NHE1 siRNA on the hypoxia-induced hypertrophy of PASCs (Figures 4B and 4C).

Silencing of NHE1 Significantly Inhibited the Migration of Human PASCs, and the Overexpression of E2F1 Blocked the Effect

Because NHE1 was reported to be involved in the regulation of cell migration, we investigated whether the migration of PASCs was mediated by the NHE1 gene. We found that hypoxia significantly increased the migration of PASCs, and that the silencing of NHE1 siRNA inhibited the migration under normoxia and hypoxia (Figure 5A). In the meantime, we found that the overexpression of E2F1 blocked the inhibition by NHE1 siRNA of hypoxia-induced PASC migration in the wound-healing assay (Figures 5B and 5C) and in the migration chamber assay (Figure 5D). As shown in Figure 5C, a clearly recognized gap (pipette tip-scratched area) was seen after 24 hours of exposure to hypoxia in the control group, but the gaps were completely filled with cells that resulted in unrecognizable gaps in other groups.

Figure 3. Effects of overexpression of E2F1 on inhibition by NHE1 siRNA of human PASMC proliferation. (A) Expression of E2F1. Protein was isolated from human PASMCs under conditions of normoxia, hypoxia, and hypoxia, and treated with NHE1 siRNA for Western blot analysis. *Left*, representative imaging. *Right*, quantitative data. * $P < 0.05$, compared with other groups. # $P > 0.05$, compared with normoxia. Cell proliferation data were obtained through direct cell counts (B) and the BrdU incorporation assay (C). After transfection with NHE1 siRNA or E2F1 cDNA, PASMCs were cultured in a hypoxia chamber for 24 hours and harvested for cell counts and a BrdU assay. * $P < 0.05$, compared with the control group ($n = 9$ for each group). (D) Quantitative data on the protein expression of NHE1 and E2F1. Proteins isolated from PASMCs treated with NHE1 siRNA and E2F1 cDNA were subjected to Western blot analysis, setting the control value as 1. Data were normalized to Glyceraldehyde-3 phosphate dehydrogenase (GAPDH). * $P < 0.05$, compared with each control group ($n = 3$ for each group). NHE1 + E2F1, NHE1 siRNA + E2F1 cDNA.

Silencing of NHE1 Also Significantly Diminished the Proliferation of Human PAECs and PAFs under Hypoxia

Because the proliferation of PAECs and PAFs is also reportedly involved in the development of pulmonary hypertension, we examined the effects of silencing NHE1 siRNA on the

Figure 4. Effects of NHE1 and E2F1 on hypertrophy of human PASMCs under condition of hypoxia. After transfection with NHE1 siRNA and growing under condition of hypoxia for 24 hours, PASMCs were removed from hypoxia, and a hypertrophy assay was performed. (A) Cell-size assay for cells treated with NHE1 siRNA. (B) NHE1 siRNA plus E2F1 cDNA under condition of hypoxia. (C) Protein/DNA ratio under condition of hypoxia. * $P < 0.05$, # $P > 0.05$ as compared with the normoxia control, and \$ $P < 0.05$ as compared with hypoxia control in A. * $P < 0.05$ as compared with the controls in B and C ($n = 9$ for each group). NHE1 + E2F1, NHE1 siRNA + E2F1 cDNA.

proliferation of PAECs and PAFs, using the same conditions as for PASMCs. We found that NHE1 was expressed in all three types of pulmonary artery cells (Figure 6A), and that the transfection of NHE1 siRNA significantly inhibited the proliferation of the three cell types (Figure 6B).

DISCUSSION

In this study, we found that the silencing of NHE1 by siRNA significantly inhibited the proliferation of human PASMCs (Figure 1) and the cell-cycle progression induced by hypoxia (Figure 2). The inhibitory effect of NHE1 siRNA was blocked by overexpressing E2F1 (Figure 3). We found that NHE1 siRNA attenuated the hypertrophy of human PASMCs (Figure 4) and the migration induced by hypoxia (Figure 5), and that the overexpression of E2F1 prevented these attenuations. NHE1 siRNA also decreased the hypoxia-induced proliferation of human PAECs and PAFs (Figure 6).

Figure 5. Effects of NHE1 and E2F1 on migration of human PASM C s under condition of hypoxia. After transfection with NHE1 siRNA and E2F1 cDNA and being cultured in 2% oxygen for 24 hours, a wound-healing assay and migration chamber assay were performed. (A) Wound-healing assay for cells treated with NHE1 siRNA. (B) NHE1 siRNA plus E2F1 cDNA under condition of hypoxia. (C) Representative imaging. (D) Migration chamber assay under condition of hypoxia. * $P < 0.05$, compared with control group. [#] $P < 0.05$, compared with other groups ($n = 9$ for each group). NHE1 + E2F1 = NHE1 siRNA + E2F1 cDNA. Gap = the scratched area by pipette tip.

The proliferation of PASM C s is considered an important pathological change in the development of the pulmonary hypertension and vascular remodeling induced by hypoxia. In previous studies, we reported that the NHE inhibitors dimethyl amiloride and 5-ethylisopropyl amiloride significantly inhibited the growth of bovine PASM C s (6) *in vitro*, and inhibited hypoxia-induced pulmonary hypertension in animals (7). However, those NHE inhibitors were not NHE1-specific, but also affected NHE2 and NHE3 (5). Wu and colleagues reported that a new NHE1 inhibitor, sabiporide, significantly inhibited the proliferation of human PASM C s and migration under normoxia (37). However, that inhibitor also affected other NHEs (5). Therefore, the role of NHE1 in the regulation of PASM C proliferation has not been specifically investigated. We recently found that NHE1 gene knockout prevented hypoxia-induced pulmonary hypertension and vascular remodeling in mice, accompanied by a significant decrease in the proliferation of PASM C s (11) and indicating specific

effects of the NHE1 gene in regulating the proliferation of PASM C s. In the present study, we used NHE1-specific siRNA to silence NHE1 in human PASM C s under normoxia and hypoxia, and found that silencing NHE1 not only inhibited the proliferation of PASM C s, but also reduced cell-cycle progression. These results demonstrate that NHE1 is a critical regulator of the hypoxia-induced proliferation of PASM C s.

Zhang and colleagues reported a significant increase in the inhibition of NHE1 expression by using two sets of NHE1 siRNA for one transfection (38). To obtain the maximal silencing of the NHE1 gene in PASM C s, we simultaneously transfected two sets of NHE1 siRNA into human PASM C s. However, we did not observe a significantly increased inhibition of PASM C proliferation under normoxia or hypoxia, although a slightly greater inhibition of cell growth was evident in the group with two sets of NHE1 siRNA.

NHE1 has been studied for years, but the mechanism by which NHE1 mediates the proliferation of PASM C s is not

Figure 6. Effects of silencing NHE1 on proliferation of human (HPASMCs), human pulmonary artery endothelial cells (HPAECs), and human pulmonary artery fibroblasts (HPAFs) under condition of hypoxia. (A) Representative RT-PCR data show expression of NHE1 mRNA in three cell types. RNA was isolated from HPASMCs, HPAECs, and HPAFs. RT-PCR was performed to measure the expression of NHE1. GAPDH was used as loading control. (B) Cell proliferation data. After transfection with NHE1 siRNA, HPASMCs, HPAECs, and HPAFs were cultured in a 2% oxygen chamber for 24 hours, and harvested for gene expression and cell counts to assay cell proliferation. * $P < 0.05$, compared with control group ($n = 9$ for each group).

well-understood. We previously found that NHE1 gene knock-out significantly decreased the expression of Rho-associated, coiled-coil containing protein kinase 1 or Rho-associated protein kinase 1 (ROCK1) and ROCK2, which was accompanied by the significantly increased expression of p27 and decreased expression of cyclin D1 (11). Because E2F1 is a downstream factor of p27 and cyclin D1, we hypothesized that E2F1 plays an important role in the inhibition by NHE1 siRNA of human PASC proliferation. The E2F family of transcription factors, composed of eight different members, regulates cell-cycle progression (12, 18). E2F1 is one of these members and is essential for cellular proliferation. E2F1 functions to activate nuclear transcription factors and promote cell-cycle progression, after its release from the Rb/E2F1 complex (12, 39). Bindra and colleagues (40) reported that E2Fs may be linked to the transcriptional response to hypoxia. Previous studies demonstrated the involvement of E2F1 in cell proliferation (13–16). Tammali and colleagues (13) found that the inhibition of high glucose-induced and TNF- α -induced rat aorta vascular smooth muscle cell proliferation by aldose reductase was accompanied by the decreased expression of E2F1. Grouwels and colleagues (14) found that the overexpression of E2F1 by an adenovirus E2F1 promoter increased the proliferation of primary β -cells isolated from the pancreas of rats. Amrani and colleagues (15) reported that interferon- γ inhibited the proliferation of airway smooth muscle cells by inhibiting the expression of the E2F1 gene. Goukassian and colleagues (16) reported that the overexpression of E2F1 negated the inhibitory effects of a potent repressor of cellular proliferation, PCA-4230, on the proliferation of human vascular smooth muscle cells. In this study, we found that the expression of E2F1 was induced in PASCs under hypoxia, and that NHE1 siRNA significantly decreased the induction of E2F1 in PASCs. We also found that the

overexpression of E2F1 completely blocked the inhibitory effects of NHE1 siRNA on the hypoxia-induced proliferation of PASCs. These results indicate that E2F1 signaling is essential for the regulation by NHE1 of PASC proliferation.

In addition to proliferation, hypertrophy is another pathological characteristic of PASCs in pulmonary hypertension (41, 42). Studies indicated the relationship between NHE1 and myocardial hypertrophy, showing that the inhibition of NHE1 activity by inhibitors reduced cardiac hypertrophy in animals and cultured cardiomyocytes (23–27). The increased medial wall thickness of pulmonary arteries in pulmonary hypertension and vascular remodeling is involved not only in the proliferation of PASCs, but also in the hypertrophy of PASCs. In our previous study, we found a decrease in medial wall thickness of the pulmonary arteries in NHE1-deficient mice. We therefore investigated the effects of NHE1 on the hypertrophy of PASCs in this study. We found that silencing the NHE1 gene significantly decreased the hypoxia-increased size of human PASCs. In addition to cell size, cell volume, established by calculating the protein/DNA ratio, was used for the analysis of cell hypertrophy (39), because research indicates that the stimulation of protein synthesis without DNA replication is a feature of hypertrophy (33). We found that silencing NHE1 significantly decreased the protein/DNA ratio in human PASCs cultured under hypoxia. E2F1 was shown to be involved in the pathogenesis of cardiac hypertrophy. Wohlschlaeger and colleagues (20) reported a significant increase in the expression of E2F1 in myocardial tissues from patients with chronic heart failure-associated cardiomyocyte hypertrophy. Hlaing and colleagues (21) found that E2F1 regulated the hypertrophy of skeletal myoblasts induced by angiotensin II. Vara and colleagues (22) showed that the inhibition of E2F1 function prevented the development of myocyte hypertrophy in rats. In this study, we found that the overexpression of E2F1 also blocked the inhibition by NHE1 siRNA of PASC hypertrophy, indicating the involvement of E2F1 in the regulation by NHE1 of PASC hypertrophy.

In addition to proliferation and hypertrophy, the migration of PASCs is also involved in the vascular remodeling underlying pulmonary hypertension. The extension of PASCs into nonmuscularized vessels suggests the involvement of smooth muscle cell migration in the pathogenesis of pulmonary hypertension (28). The migration of PASCs was reportedly induced by sustained hypoxia (29), endothelin-1 (30), serotonin (28), and other factors (31). NHE1 is reportedly involved in regulating the migration of different types of cells (40, 43), and the NHE1 inhibitor sabiporide also inhibited the migration of human PASCs under normoxia (38). In this study, we found that silencing NHE1 siRNA significantly diminished the migration of PASCs induced by hypoxia. Interestingly, we also found that the overexpression of the E2F1 gene prevented the inhibition by NHE1 siRNA of human PASC migration.

The effects of hypoxia on the proliferation of PASCs were studied in different laboratories, and inconsistent results were reported (44). To determine the best concentration of oxygen that stimulates the proliferation of human PASCs, we investigated the effects of different levels of oxygen (1, 2, 3, 5, and 10% O_2) on the proliferation of PASCs. We found that 1, 2, and 3% oxygen significantly stimulated the proliferation of PASCs. Although some investigators obtained controversial results (44), we did not find a significantly increased growth of PASCs under 5 and 10% oxygen. This result was consistent with the data we obtained in another study (32). The mechanisms underlying the reactions of PASCs to different oxygen concentrations have not been fully clarified. One report stated that cells in the medial wall of the artery normally exist under

conditions of low oxygen (45). Santilli and colleagues (45) measured the concentration of transarterial wall oxygen in rabbit aortas by using an oxygen microelectrode, and found the lowest oxygen concentrations in the medial wall of the artery, compared with the adventitia and lumen. The oxygen concentration was only approximately 4% in the center of the media, but at the same time, oxygen concentrations were around 12% in the lumen and 8% in the adventitia. Because 10% oxygen was widely used to induce pulmonary hypertension and vascular remodeling in rodents, then based on the findings of Santilli and colleagues (45), the oxygen concentration would be lower than 5 or 10% in the media of the pulmonary artery when animals were placed in a hypoxia chamber (10% O₂), which resulted in the proliferation and hypertrophy of smooth muscle cells in the medial walls of pulmonary arteries. Thus, a slight reduction of oxygen concentrations to 5 or 10% may not be enough to stimulate the proliferation of PASM C s, because cells in the medial walls of the artery were already under a low oxygen condition. This may explain why investigators generally use 1–3% oxygen for studies related to the proliferation of PASM C s (44, 46, 47).

Moreover, we investigated whether the regulation by E2F1 of NHE is NHE1-specific. We did not detect the expression of NHE2 in human PASM C s. Although the expression of NHE3 was evident in PASM C s, the proliferation of cells was not significantly affected by either the NHE3 inhibitor S-3226 or NHE3 siRNA. Previous reports showed that no expression of NHE2 and NHE3 was evident in human lung tissue (48) and pulmonary endothelial cells (49). Therefore, we did not investigate the effects of E2F1 on NHE2 and NHE3 in this study.

In conclusion, we demonstrated that the specific inhibition of the NHE1 gene by siRNA significantly inhibited the hypoxia-induced proliferation, hypertrophy, and migration of human PASM C s via the repression of nuclear transcription factor E2F1. This observation revealed a novel mechanism underlying the regulation by NHE1 of hypoxic pulmonary hypertension and vascular remodeling.

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