Deficiency of the NHE1 Gene Prevents Hypoxia-induced Pulmonary Hypertension and Vascular Remodeling

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***Rationale*: Our previous studies found that Na^+/H^+ exchanger (NHE) activity played an essential role in pulmonary artery smooth muscle cell (PASMC) proliferation and in the development of hypoxia-induced pulmonary hypertension and vascular remodeling. Other investigators recently observed increased expression of the NHE isoform 1 (NHE1) gene in rodents with pulmonary hypertension induced by hypoxia. However, a causal role for the NHE1 gene in pulmonary hypertension has not been determined.**

***Objectives*: To determine the causal role of the NHE1 gene in pulmonary hypertension and vascular remodeling.**

***Methods*: We used NHE1-null mice to define the role of the NHE1 gene in the development of pulmonary hypertension and remodeling induced by hypoxia and to delineate the NHE1 regulatory pathway.**

***Measurements and Main Results*: After 2 weeks of exposure to hypoxia, in contrast to wild-type hypoxic littermates, there was no significant increase in right ventricular systolic pressure, in the ratio of right ventricular to left ventricular plus septal weight [RV/(LV + S)], or in medial wall thickness of the pulmonary arterioles in homozygous mice ($NHE1^{-/-}$). There was a significant decrease in Rho kinase (ROCK1 and ROCK2) expression, accompanied by an increase in p27 expression in $NHE1^{-/-}$ mice.**

***Conclusions*: Our study demonstrated that deficiency of the NHE1 gene prevented the development of hypoxia-induced pulmonary hypertension and vascular remodeling in mice and revealed a novel regulatory pathway associated with NHE1 signaling.**

Keywords: Na^+/H^+ exchanger isoform 1; pulmonary hypertension; vascular remodeling; hypoxia; mouse

The Na^+/H^+ exchanger (NHE) expressed in many mammalian cell types is a protein localized to the plasma and the mitochondrial inner membrane (1). The function of NHE is to regulate intracellular pH and cell volume by extruding H^+ from and taking up Na^+ into cells (2). Since NHE was first analyzed in 1976 (3) and the first NHE cDNA was cloned in 1989 (4), nine NHE isoforms have been identified and cloned (2), of which NHE isoform 1 (NHE1) is ubiquitously expressed.

Pulmonary hypertension caused by many chronic lung diseases associated with prolonged hypoxia can result in right ventricular hypertrophy and heart failure (5). An important pathological feature of pulmonary hypertension is increased medial thickening of the pulmonary artery resulting from hypertrophy and hyperplasia of the pulmonary artery smooth muscle cells (PASMCs) (6, 7). In previous studies, we reported that NHE activity played a significant role in regulation of intracellular pH in PASMCs (8) and found that the inhibition of NHE activity was related to the inhibition of bovine PASMC proliferation induced by growth factors (9, 10). We also found that inhibition of NHE activity significantly reduced pulmonary hypertension and vascular remodeling induced by chronic hypoxia in rats (11). Our studies suggested that NHE activity might play an important role in development of pulmonary hypertension and vascular remodeling.

Recently, increased NHE1 gene expression was observed in the lung in hypoxia- induced pulmonary hypertension in rats (12). Rios and colleagues investigated NHE activity and NHE gene expression in hypoxic animals and found an increase in NHE activity and NHE1 mRNA and protein expression in PASMCs isolated from the intrapulmonary arteries of mice with hypoxia-induced pulmonary hypertension (13, 14). These findings suggested that the NHE1 gene might contribute to the development of hypoxia-induced pulmonary hypertension. However, the causal role of NHE1 gene in pulmonary hypertension and vascular remodeling has not been fully defined.

Pederson has shown an involvement of NHE1 in stress-induced cell signaling regulation (15). However, the signaling pathway that NHE1 uses to regulate PASMC proliferation and pulmonary hypertension is unclear. We previously have determined that p27, a cyclin-dependent kinase inhibitor, played a critical role in inhibition of PASMC proliferation and pulmonary hypertension induced by hypoxia (16). Several studies have indicated that RhoA kinase (ROCK) was an upstream factor of p27 that regulated p27 activity by degrading p27 protein (17–19), although some reports have suggested that p27 regulates RhoA/ROCK (20, 21). We therefore also investigated RhoA/ROCK expression in $NHE1^{-/-}$ mice to clarify the regulatory pathway between NHE1 and RhoA/ROCK in hypoxia-induced pulmonary hypertension.

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Na^+/H^+ exchanger (NHE) activity plays an essential role in pulmonary artery smooth muscle cell proliferation and development of hypoxia-induced pulmonary hypertension and vascular remodeling, but a causal role for the NHE gene in pulmonary hypertension has not been determined.

What This Study Adds to the Field

We demonstrated that the NHE1 gene is a key determinant in development of hypoxia-induced pulmonary hypertension and vascular remodeling and revealed a novel regulatory pathway for NHE1.

METHODS

Animals

Animal experiments were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. The NHE1 homozygous ($NHE1^{-/-}$), heterozygous ($NHE1^{+/-}$), and wild-type ($NHE1^{+/+}$) male mice were bred from three breeding pairs of heterozygous NHE1

(*Received in original form October 15, 2007; accepted in final form February 26, 2008*)

Supported by National Institutes of Health grant HL039150 (C.A.H.).

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Am J Respir Crit Care Med Vol 177. pp 1276–1284, 2008
Originally Published in Press as DOI: 10.1164/rccm.200710-1522OC on February 28, 2008
Internet address: www.atsjournals.org

mice (mixed genetic background of C57BL/6 and Black Swiss strains; kind gifts of Dr. Gary Shull, University of Cincinnati College of Medicine, Cincinnati, OH). The offspring genotype of the mice was determined by polymerase chain reaction (PCR) analysis of genomic DNA from tail biopsies (22) (Figure 1).

Chronic Hypoxia Exposure

Hypoxia exposure was performed as previously described (16). Briefly, the mice (8- to 10-week-old littermates) were weighed and placed in a tightly sealed hypoxic chamber or exposed to normoxia in the same chamber for 2 weeks. Oxygen concentration was maintained at 10% by controlling the flow rates of compressed air and N_2. Cage concentration of O_2 was checked daily.

Measurement of Right Ventricular Pressure

The measurement for right ventricular pressure was performed as previously described (16). After 14 days in the chamber, the animals were removed and anesthetized with intraperitoneal ketamine (80 mg/kg) and diazepam (5 mg/kg). Animals were placed on a warming blanket to maintain body temperature at 37°C. Right ventricular systolic pressure (RVSP) was measured via a catheter (0.012" × 0.021" silicone tubing) passed through the right external jugular vein. Once the RVSP was obtained, the animals were killed with 200 mg/kg of pentobarbital and used immediately for the determination of right ventricular hypertrophy, hematocrit, lung pathology, and gene expression.

Measurement of Right Ventricle Hypertrophy

The ventricles and septum of the animals were collected, and the wet and dry ventricle and septal weight was obtained by drying them for 24 hours at 60°C. A ratio of right ventricle to left ventricle plus septum weight [RV/(LV+S)] was calculated for determination of right ventricular hypertrophy.

Hematocrit Analysis

Blood samples were centrifuged in microcapillary tubes for 3 minutes, and the hematocrit was read directly.

Measurement of Pulmonary Vascular Remodeling

Elastic fibers in pulmonary arteries were stained for measurement of medial wall thickness of pulmonary arteries. Percent wall thickness (%WT) and percent thick-walled vessels were used for evaluation of pulmonary artery remodeling as previously described (16). %WT was landmarked to terminal bronchial arterioles (%WT-TA) and the intraacinous arterioles (%WT-IA). It was calculated as the average diameter of the external elastic lamina minus the average diameter of internal elastic lamina divided by the average diameter of external elastic lamina. Percent thickness was calculated as percentage of thick-walled vessels as a fraction of total intraacinous vessels. A computer imaging analysis was applied for measurement of wall thickness. Images of individual pulmonary arteries were captured using a digital camera, mounted on a light microscope, and linked to a computer. The detail on measurement of wall thickness was described previously (11, 23).

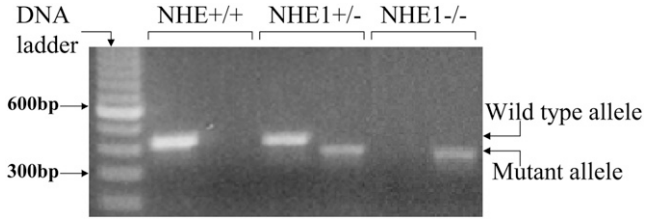

Figure 1. Representative of genotyping for NHE1 mice. DNA was isolated from the tails of the NHE1 mice, and polymerase chain reaction was performed to determine the genotype. Primers for the wild-type allele and mutant allele were used for identification of different genotypes. Wild-type mice ($NHE1^{+/+}$) only expressed wild-type allele, and homozygous NHE1 gene knockout mice ($NHE1^{-/-}$) only expressed mutant allele, but heterozygous NHE1 mice ($NHE1^{+/-}$) expressed both alleles.

Measurement of Vascular Muscularization

Immunohistochemical staining with anti–α-smooth muscle–specific actin antibody (mouse monoclonal; Calbiochem, San Diego, CA) was performed for additional evaluation of pulmonary vascular remodeling. A modified vessel category of muscularization was used (24, 25). Muscularized vessels were defined as presence of smooth muscle cells stained brown with anti–α-smooth muscle–specific actin in 50% or more of the vessel circumference of intraacinous arterioles (24). Muscularized vessel number were quantified and expressed per 100 alveoli (25).

Measurement of Ki67 Expression in Medial Wall of Pulmonary Arteries

Immunohistochemical staining with Ki67, a cell proliferation marker, was performed to analyze cell proliferation in the medial wall of pulmonary arterioles as described previously (16). A percentage of Ki-67–positive cells expressed as Ki67 proliferative index was estimated by calculating the ratio of Ki67 expressing cell nuclei to the total number of cell nuclei in the vessel wall of cross-sections of 10 terminal bronchial arterioles per slide.

Isolation of Pulmonary Artery and PASMCs from NHE1-Null Mice

Pulmonary artery and PASMCs were used for further biological analysis. Pulmonary arteries, including main pulmonary and left and right branches, were isolated under the anatomic microscope. NHE1 mouse PASMC isolation and culture were performed according to a method described previously (14). The PASMCs were characterized by the presence of anti–α-smooth muscle–specific actin antibody.

Reverse Transcription–PCR

Total RNA (2 μg), extracted from mouse lungs, pulmonary arteries, and isolated PASMCs, was used to carry out reverse transcription (RT)–PCR to measure mRNA expression with Ready-To-Go You-Prime First-strand beads for RT (GE Healthcare, Buckinghamshire, UK) and PCR Master Mix for PCR (Promega, Madson, WI). Primer pairs for RhoA, ROCK1 and ROCK2 (26), p27 (16), cyclin D1 (27), and for the housekeeping gene GAPDH were purchased from Sigma Genosys (Woodlands, TX).

Western Blot

Total cell lysates were obtained from mouse lungs. Antibodies included RhoA, cyclin D1, MYPT1 (myosin phosphatase target subunit 1), total MYPT1 and phospho-MYPT1 (Thr696) (Santa Cruz Biotechnology, Santa Cruz, CA), ROCK1 (ROCK-I/ROKβ), ROCK2 (ROCK-II/ROKα), p27 mouse monoclonal antibody (clone 57) (BD Biosciences Pharmingen, San Diego, CA), and GAPDH mouse monoclonal antibody (clone 6C5) (Research Diagnostics, Inc., Flanders, NJ). Western blot was performed as described previously (28).

Statistical Analysis

Statistics were performed using the computer program Statview (SAS Institute, Inc., Cary, NC) with factorial analysis of variance. All values were expressed as the mean ± SEM. Significance was set at $P < 0.05$.

RESULTS

NHE1 Null Mice Were Protected against Pulmonary Hypertension Induced by Chronic Hypoxia

During normoxia, $NHE1^{+/+}$, $NHE1^{+/-}$, and $NHE1^{-/-}$ mice had similar hemodynamic parameters. After 2 weeks exposure to hypoxia, $NHE1^{+/+}$ mice developed pulmonary hypertension with a significant increase in RVSP as compared with their normoxic littermates; in hypoxic $NHE1^{+/-}$ mice, the RVSP value was significantly lower than in the hypoxic $NHE1^{+/+}$ mice, although the pressure was still higher than that in the hypoxic $NHE1^{-/-}$ mice and the normoxic littermates. There was no significant increase in RVSP observed in $NHE1^{-/-}$ hypoxic mice when compared with normoxic mice (Figures 2A and 2B).

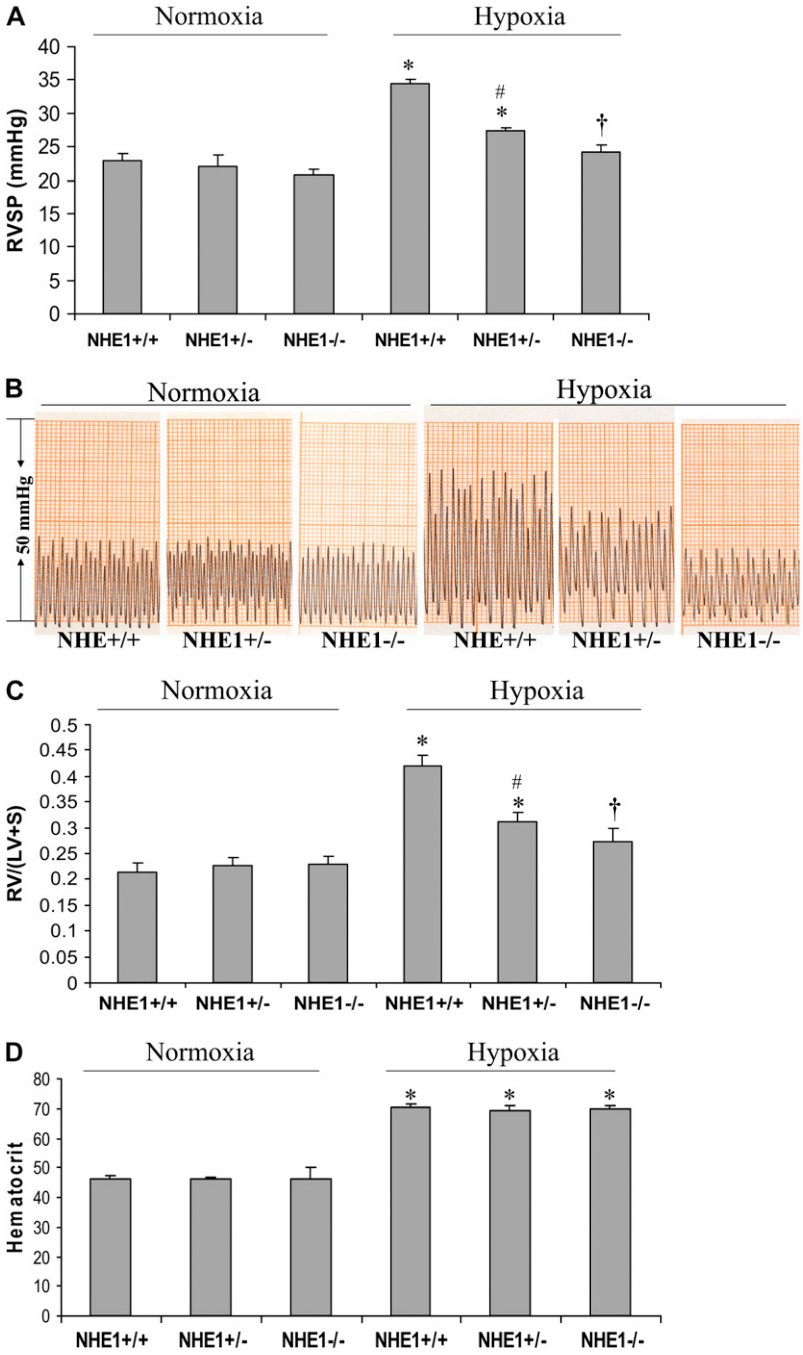

Figure 2. NHE1 gene knockout and pulmonary hypertension induced by chronic hypoxia in mice. (*A*) Right ventricular systolic pressure (RVSP) in NHE1-null mice. (*B*) Representative tracings of RVSP. (*C*) Ratio of right ventricular to left ventricular plus septal weight, RV/(LV + S), in NHE1-null mice. (*D*) Hematocrit. $NHE1^{+/+}$ (wild-type mice), $NHE1^{+/-}$ (heterozygous NHE1 mice), $NHE1^{-/-}$ (homozygous NHE1 mice). n = 6 to 8 mice for each group. $^{*}P < 0.05$ as compared with littermate normoxic mice; $^{\#}P < 0.05$ as compared with hypoxic $NHE1^{+/+}$ mice; $^{\dagger}P < 0.05$ as compared with hypoxic $NHE1^{+/-}$ mice.

NHE1 Null Mice Were Protected against Right Ventricular Hypertrophy Induced by Chronic Hypoxia

Two weeks of hypoxic exposure significantly induced right ventricular hypertrophy in $NHE1^{+/+}$ and $NHE1^{+/-}$ mice as indicated by the increase in the ratio of right ventricular weight to left ventricular plus septum weight, RV/(LV + S), but the ratio was significantly smaller in $NHE1^{+/-}$ mice than that in $NHE1^{+/+}$ mice. There was not a significant increase in the ratio of RV/(LV + S) in $NHE1^{-/-}$ hypoxic mice (Figure 2C) when compared with normoxic mice. $NHE1^{-/-}$ mice also did not show any difference in RV/(LV + S) from $NHE1^{+/+}$ mice in normoxia.

Hematocrit Value

Hypoxia significantly increased hematocrit values in all NHE1 mice as compared with their normoxic littermates. No significant difference was observed between $NHE1^{+/+}$, $NHE1^{+/-}$, and $NHE1^{-/-}$ mice (Figure 2D).

NHE1-Null Mice Were Protected against Pulmonary Vascular Remodeling Induced by Hypoxia

In $NHE1^{+/+}$ mice, the medial wall thickness of pulmonary arterioles at the level of the terminal bronchioles (%WT-TA) and at the level of the intraacinous arterioles (%WT-IA) as well as the percent thick, were significantly increased after 2 weeks exposure to hypoxia. In $NHE1^{+/-}$ mice, there was an increase in %WT-TA but significantly less than that in $NHE1^{+/+}$ mice. No significant increase in %WT-IA was seen in $NHE1^{+/-}$ mice, and there was a significantly lower %WT-IA in hypoxic $NHE1^{+/-}$ mice than that in hypoxic $NHE1^{+/+}$ mice. Consistent with the lack of rise in RVSP and RV/(LV + S) in $NHE1^{-/-}$

mice, there was no significant change in pulmonary vascular remodeling (Figure 3A–3C).

Vascular Muscularization Was Diminished in NHE1-Null Mice under Hypoxia

Hypoxia significantly increased muscularized vessel number in $NHE1^{+/+}$ mouse lungs as compared with their normoxic littermates, but no significant change was seen in $NHE1^{-/-}$ mice. Although increased vascular muscularization was observed in hypoxic $NHE1^{+/-}$ mice when compared with their normoxic littermates, the increase was significantly less than that in hypoxic $NHE^{+/+}$ mice (Figures 4A and 4B).

Cell Proliferation Was Decreased in the Medial Wall of Pulmonary Vessels in NHE1-Null Mice

Consistent with the results we observed, exposure to hypoxia significantly increased Ki67 proliferative index in $NHE1^{+/+}$ and $NHE1^{+/-}$ mice, but $NHE1^{+/-}$ mice had significant lower index than $NHE1^{+/+}$ mice. We did not observe an increase in Ki67 proliferative index in hypoxic $NHE1^{-/-}$ mice (Figures 4C and 4D).

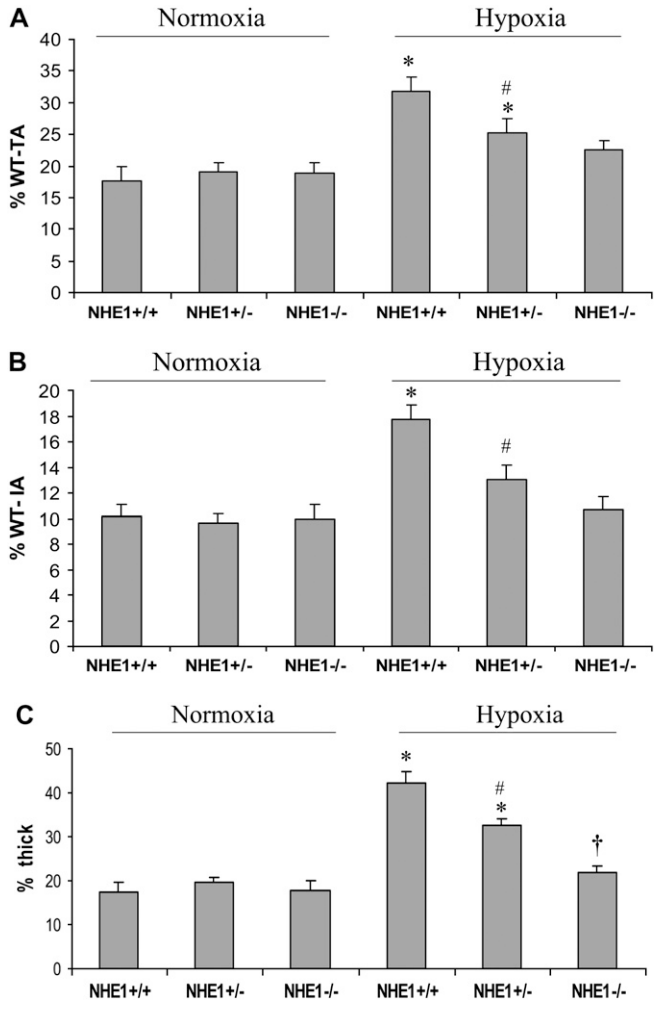

Figure 3. NHE1 gene knockout and pulmonary vascular remodeling induced by chronic hypoxia. (*A*) Percentage of wall thickness landmarked to terminal bronchial arterioles (%WT-TA), (*B*) percentage of wall thickness at the level of the intraacinous arterioles (%WT-IA), and (*C*) percentage of thickness (%thick) in NHE1-null mice. $NHE1^{+/+}$ (wild-type mice), $NHE1^{+/-}$ (heterozygous NHE1 mice), $NHE1^{-/-}$(homozygous NHE1 mice). n = 6 to 8 mice for each group. $^*P < 0.05$ as compared with littermate normoxic mice; $^{\#}P < 0.05$ as compared with hypoxic $NHE1^{+/+}$ mice $^{\dagger}P < 0.05$ as compared with hypoxic $NHE1^{+/-}$ mice.

NHE1 Knockout Mice Had Down-regulated ROCK1 and ROCK2 in the Lungs

To determine whether RhoA/ROCK were altered along with the failure to develop pulmonary hypertension in hypoxia by NHE1 gene knockout mice, we analyzed mRNA and protein expression of RhoA, ROCK1, and ROCK2 in the mouse lungs. We found that the expression of ROCK1 and ROCK2 mRNA and protein were significantly down-regulated in the lungs from hypoxic and normoxic $NHE1^{-/-}$ mice (Figures 5A–5C). Hypoxia increased RhoA mRNA expression in $NHE1^{+/+}$but not in $NHE1^{-/-}$ mice (Figure 5A).

NHE1 Knockout Mice Had Down-regulated ROCKs in Pulmonary Arteries and PASMCs

Because a significant decrease in ROCK was found in $NHE1^{-/-}$ mouse lung, we isolated pulmonary artery and PASMCs from NHE1-null mice for further analysis of ROCK expression. We confirmed the significant down-regulation of ROCK1 and ROCK2 in the pulmonary arteries (Figures 5D and 5E) and PASMCs (Figures 5F and 5G) from $NHE1^{-/-}$ mice as compared with $NHE1^{+/+}$ mice.

NHE1 Knockout Mice Had Decreased ROCK Activity in PASMCs

To examine if the reduction of ROCK expression by NHE1 gene knockout affected ROCK activity, we measured MYPT1, a direct downstream effector of ROCK. We found that ROCK activity was significantly decreased, showing an increase in phospho-MYPT1 in the PASMCs from $NHE1^{+/+}$ mice (Figure 6A).

NHE1 Knockout Mice Had Decreased ROCK Expression in Heart, Liver, and Kidney

To determine if the down-regulation of ROCKs by NHE1 knockout occurred in other organs, we measured RhoA and ROCKs in heart, liver, and kidney in $NHE1^{-/-}$ animals. Similar to the lungs, a significant decrease in expression of ROCKs, but not of RhoA, was observed in the three tissues (Figure 6B).

NHE1 Knockout Mice Had Increased p27 and Decreased Cyclin D1 Expression in the Lungs, Pulmonary Arteries, and PASMCs

Because p27 is a downstream factor for ROCK and plays an important role in regulation of cell proliferation, we analyzed p27 and its downstream factor, cyclin D1. A significant increase in p27 expression (Figures 7A–7C) and a decrease in cyclin D1 expression (Figures 7D–7F) were observed in the lungs, pulmonary arteries, and PASMCs from NHE1-null mice.

DISCUSSION

Mice with homozygous deficiency for the NHE1 gene (*see* Figure 1) did not respond to chronic hypoxia with a rise in RVSP (*see* Figures 2A and 2B), right ventricular hypertrophy (*see* Figure 2C), or pulmonary vascular remodeling (*see* Figures 3A–3C) compared with hypoxic wild-type littermates. Heterozygous NHE1-deficient mice made a modest but significant response to hypoxia, although much less than wild-type littermate hypoxic control mice. ROCK1 and 2, which have been shown to be part of the signaling cascade through p27 and cyclin D for the proliferation of normoxic vascular smooth muscle cells, had a significantly reduced expression in lung (*see* Figures 5A–5C) and pulmonary artery (*see* Figures 5D and 5E) and

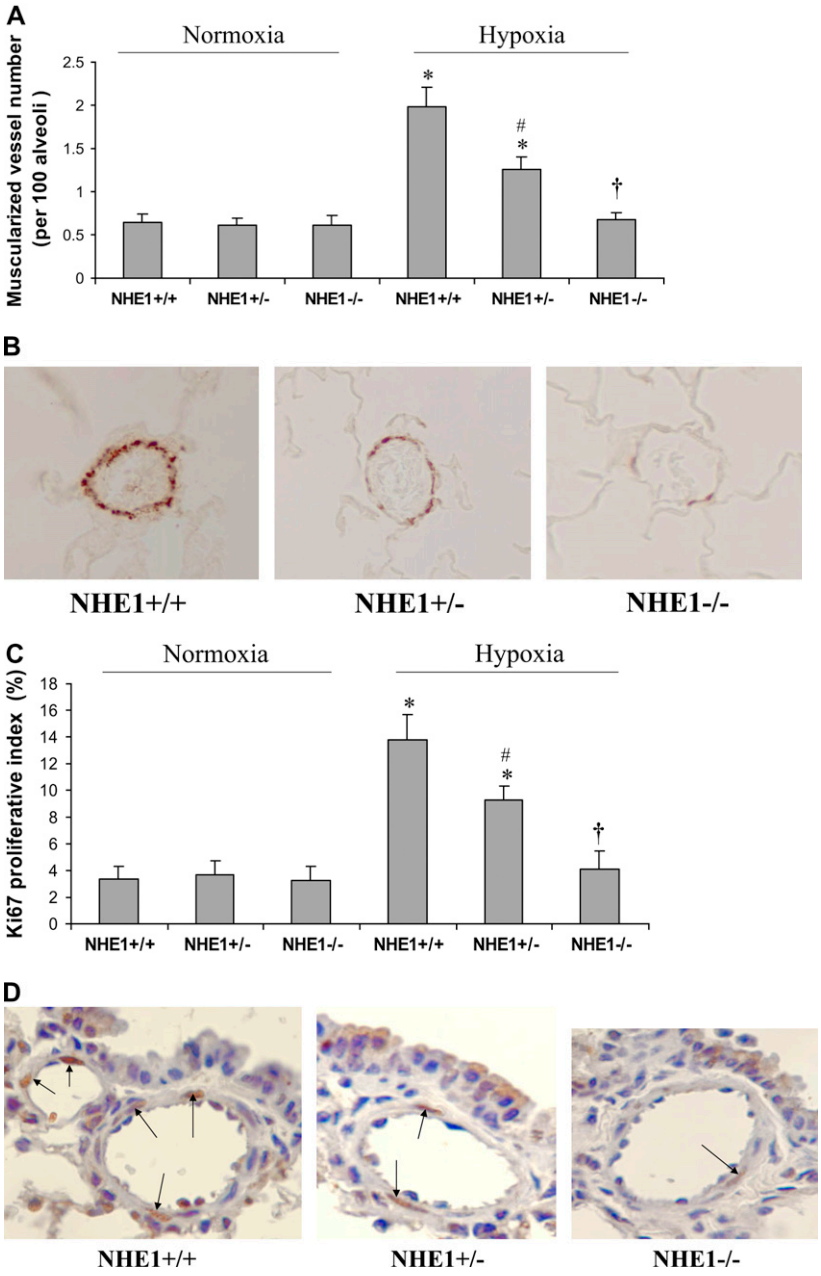

Figure 4. Immunohistochemical stain α–smooth muscle (α-SM) actin and Ki67 in the pulmonary artery from NHE1-null hypoxic mice. (*A*) Percentage of muscularized vessel number per 100 alveoli. (*B*) Representative photomicrographs showing α-SM actin staining of vessels from hypoxic NHE1 mouse lungs. $NHE1^{+/+}$ mice showed brown-stained, thick-walled intraacinous arteriole in full circumference, and $NHE1^{+/-}$ mice showed brown-stained, intraacinous arteriole in almost the full circumference, but no brown stain was seen in most of the vessel circumference in $NHE1^{-/-}$ mice. (*C*) Ki67 proliferative index in the wall of terminal bronchial arteriole cells. (*D*) Representative photomicrographs showing immunohistochemical staining for Ki67 from hypoxic mice. *Arrows* indicate Ki67-positive nuclei of the cells in the medial wall of terminal bronchial arterioles. $NHE1^{+/+}$ (wild-type mice), $NHE1^{+/-}$ (heterozygous NHE1 mice), $NHE1^{-/-}$ (homozygous NHE1 mice). n = 6 to 8 mice for each group. $^{*}P < 0.05$ as compared with littermate normoxic mice; $^{\#}P < 0.05$ as compared with hypoxic $NHE1^{+/+}$ mice; $^{\dagger}P < 0.05$ as compared with hypoxic $NHE1^{+/-}$ mice.

PASMCs (*see* Figures 5F and 5G), which was accompanied by a decrease in ROCK activity (*see* Figure 6A) and an increase in p27 (Figures 7A–7C) and a decrease in cyclin D1 (Figures 7D and 7F).

Abnormal growth of smooth muscle cells in the medial wall of pulmonary arterioles is associated with an increase in pulmonary artery pressure and pulmonary vascular remodeling. NHE1 controls intracellular pH and cell volume by extruding H^+ from and taking up Na^+ into cells. This function seems to play an important role in regulating vascular smooth muscle cell proliferation (29). A wide range of mitogenic factors can stimulate cell proliferation and activate NHE (30), including platelet-derived growth factor (PDGF) (9, 31) and epidermal growth factor (EGF) (9, 32, 33). PDGF is one of the growth factors released by endothelial cells exposed to hypoxia (6, 23). Our previous study found that in cultured bovine PASMCs, PDGF increased NHE activity, causing a rise in intracellular pH, which was associated with stimulation of cell proliferation. We also found in PASMCs that inhibition of NHE activity with the inhibitor dimethyl amiloride was associated with the loss of intracellular alkalization and of proliferation in response to growth factors such as PDGF (11). We found that the NHE inhibitors dimethyl amiloride and ethylisopropyl amiloride (EIPA) significantly reduced pulmonary artery pressure and total pulmonary vascular resistance index and pulmonary vascular remodeling in rats exposed to chronic hypoxia (11). Rios and colleagues observed that PASMCs isolated from chronically hypoxic mice exhibited elevated basal intracellular pH value and increased NHE activity and expression of NHE1 mRNA and protein (13). Yao and colleagues also reported elevated intracellular pH and increased expression of NHE1 mRNA in PASMCs isolated from chronically hypoxic rats (12). These studies and others showing a relationship between NHE activity and PASMC proliferation (8–10, 13, 14) support our hypothesis that NHE, and in particular NHE1, plays a major role in the development of hypoxia-induced pulmonary artery hypertension and pulmonary vascular remodeling.

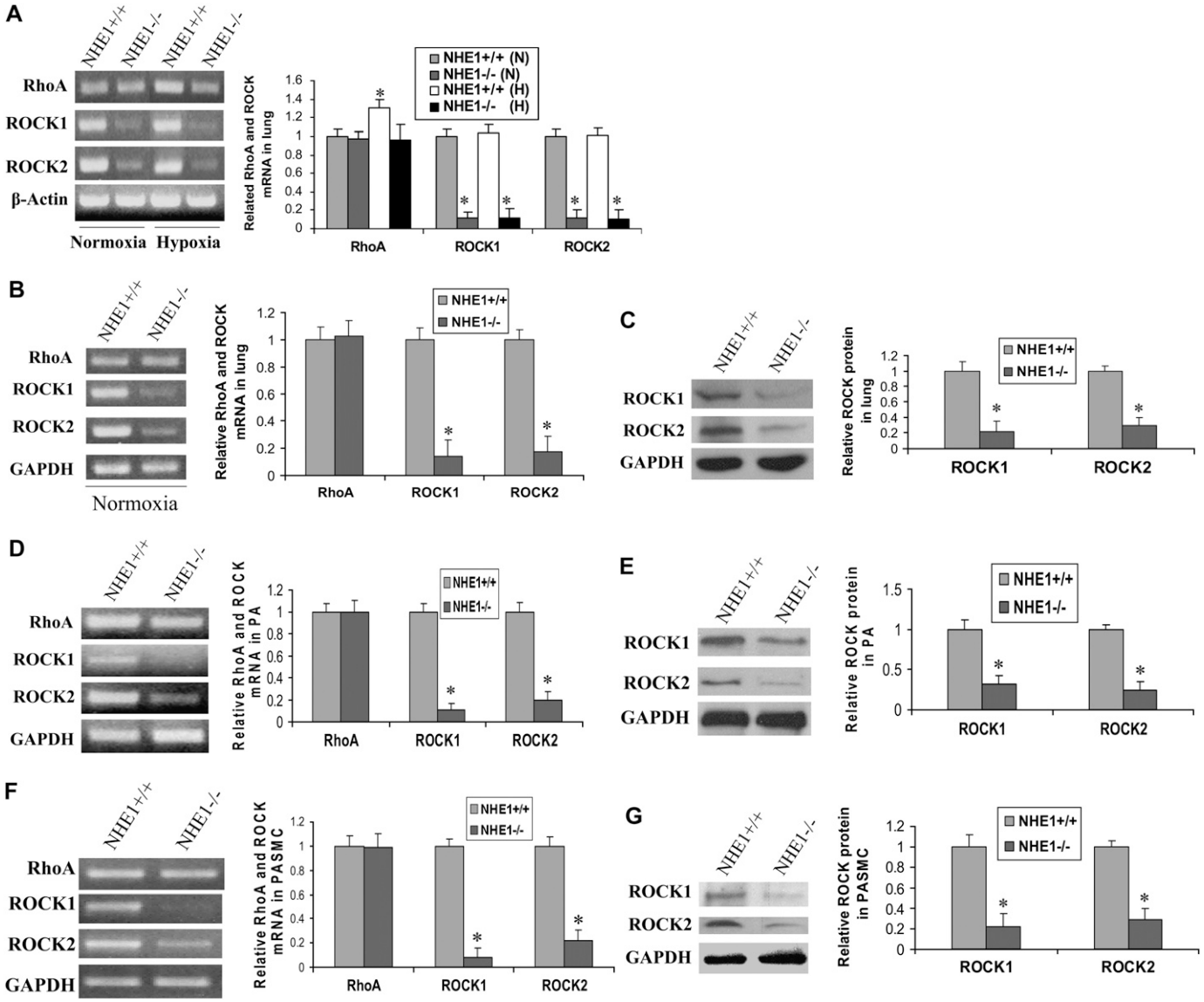

Figure 5. Expression of ROCK mRNA and protein in NHE1-null mice. Total RNA and protein from the mouse whole lung tissue, the pulmonary arteries, and pulmonary artery smooth muscle cells (PASMCs) was isolated and subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blot analysis. GAPDH was used for equal loading control. mRNA expression of ROCKs in NHE1-null mice under hypoxia (*A*) (β-actin was used for this equal loading control) and normoxia (*B*). (*C*) Protein expression of ROCKs in NHE1-null mice under normoxia. Expression of RhoA and ROCK mRNA and protein in NHE1-null mouse pulmonary arteries (PA) (*D*, *E*) and PASMCs (*F*, *G*). *Left panels* show representative images of RT-PCR and Western blot images. *Right panels* show quantification of RhoA and ROCKs expressed as relative expression and setting control as 1. $NHE1^{+/+}$ (wild-type mice), $NHE1^{-/-}$ (homozygous NHE1 mice). n = 3 for each group $^*P < 0.05$ as compared with $NHE1^{+/+}$ normoxic mice.

In this study, we observed that knockout of the NHE1 gene not only protected mice against hypoxia-induced pulmonary hypertension and vascular remodeling but also inhibited cell proliferation in the pulmonary artery medial wall as assessed by the Ki67 proliferative index consistent with our previous work showing that heparin can inhibit $Na^+/H+$ exchange (8, 9) and inhibit cell proliferation in the pulmonary vascular medial wall in $p27^{+/+}$ mice (16). However, the exact mechanism by which loss of the NHE1 gene inhibits cell proliferation is not clear. Wu and colleagues recently reported that a new NHE inhibitor, sabiporide, inhibited human PASMC proliferation and DNA synthesis and arrested the cell at the G0/G1 phase. Their finding demonstrated that NHE activation plays a permissive role in entrance of cells into cell cycle, suggesting that the NHE1 gene might be mediating cell proliferation through cell cycle regulating genes (34).

Mitogen-induced activation of NHE1 has been reported to involve Janus kinase 2 in rat vascular smooth muscle cells (35), and the inhibitory effect of NHE1 on cardiomyocyte hypertrophy has been associated with reduction of p38 and extracellular signal-regulated kinase 1 and 2 (ERK1/2) and mitogen-activated protein kinases (MAPKs) activation (36). An early study (37) showed that p38 and ERK1/2, but not Jun N-terminal kinase (JNK), were involved in the regulation of NHE1 activity mediated by angiotension II in vascular smooth muscle cells. A recent study observed that full expression of HIF1 was essential for hypoxic

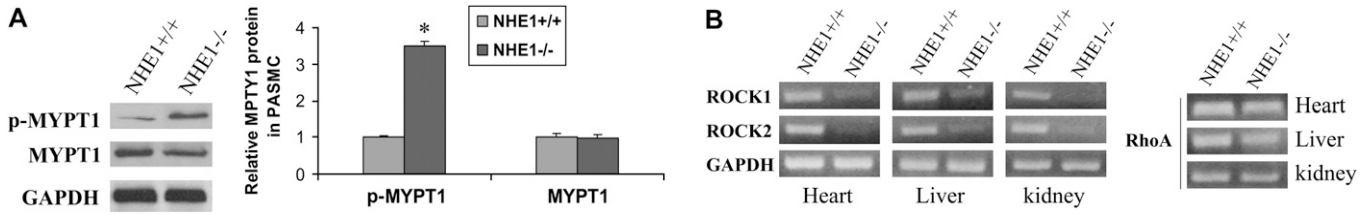

Figure 6. MYPT1 expression in pulmonary artery smooth muscle cells (PASMCs) and ROCK mRNA expression in heart, liver, and kidney in NHE1-null mice. Total protein was isolated from PASMCs for MYPT1 analysis using Western blot (*A*). *Left panels* show representatives of Western blot images. *Right panels* show quantification of MYPT1 expressed as relative expression and setting control as 1. Total RNA from the mouse heat, liver, and kidney was isolated and subjected to reverse transcriptase–polymerase chain reaction analysis for ROCK and RhoA (*B*). GAPDH was used for equal loading control. NHE1$^{+/+}$ (wild-type mice), NHE1$^{-/-}$ (homozygous NHE1 mice). n = 3 for each group. *$P < 0.05$ as compared with NHE1$^{+/+}$ normoxic mice.

induction of NHE1 expression and change in PASMC pH homeostasis (13). In addition, the effect of NHE1 on cell proliferation regulation involved the MAPK, protein kinase B (Akt/PKB) pathway (14). The full pathway for NHE1 signaling in the regulation of cell proliferation has not been determined.

The effects of RhoA/ROCK on mediating NHE1 activity have been reported. NHE1 has been shown to act as a downstream effecter of RhoA/ROCK (38–44). Tominaga and Barber (41) found that NHE1 activity was necessary for RhoA-induced formation of actin-stress fibers. It was observed that a low concentration of serum increased NHE1 activity by decreasing RhoA and increasing Rac in human mammary epithelial cancer cell line (43). Cardone found that NHE1 activity was regulated via PKA/RhoA/ROCK/p38MAPK signaling pathway (44). In the present study, we found that deficiency of NHE1 gene in mice decreased ROCK expression not only in PASMCs and pulmonary artery but also in lung and other organs. We also found that the decrease in ROCK expression was accompanied by an increase in phospho-MYPT1. This result demonstrated that reduction of ROCK expression affected its activity because ROCK can phosphorylate MYPT1 of smooth muscle myosin phosphatase, resulting in the inhibition of MYPT1 activity (45).

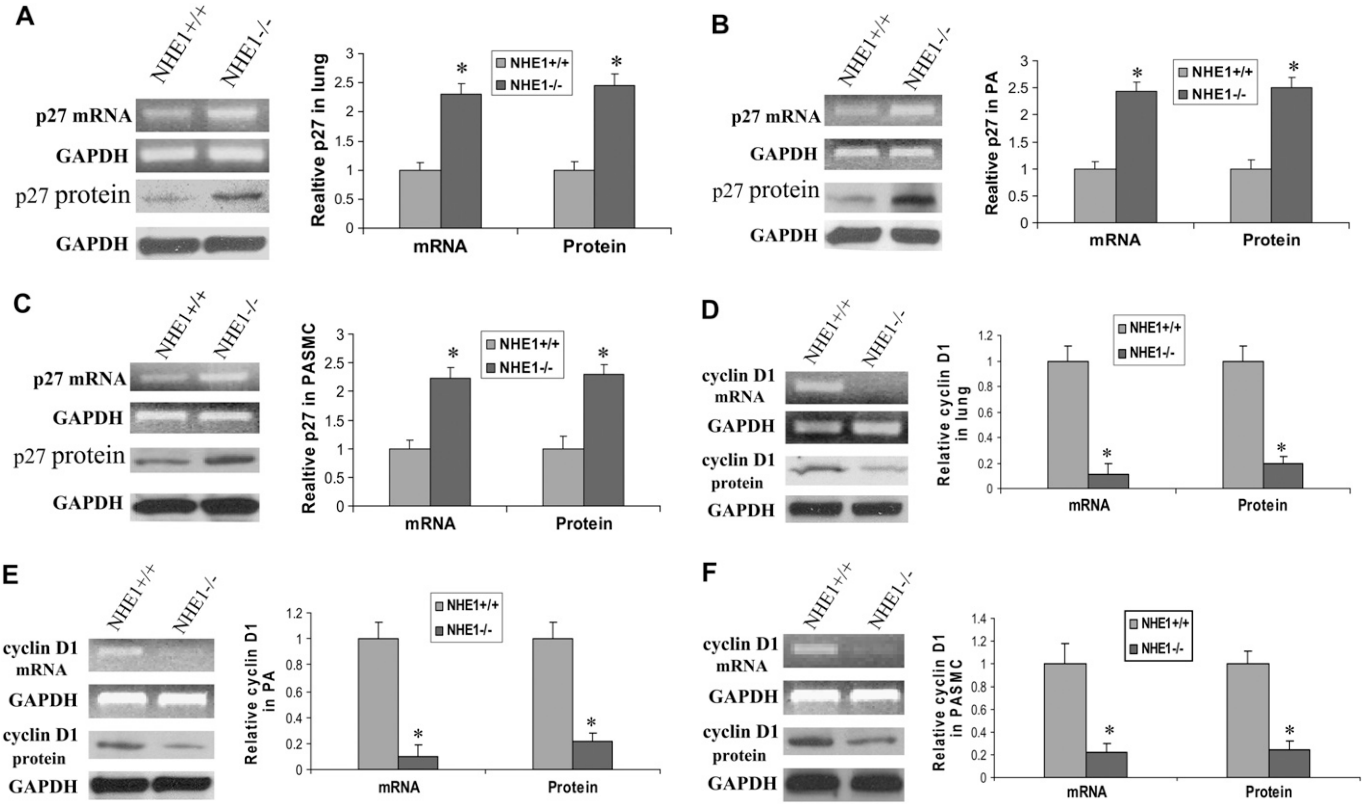

Figure 7. p27 and cyclin D1 expression in NHE1-null mouse lungs. Total RNA and protein from the mouse lung tissue, the pulmonary arteries, and pulmonary artery smooth muscle cells (PASMCs) was isolated and subjected to reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blot analysis. GAPDH was used for equal loading control. The results were representative of three separate experiments. p27 expression in the lungs (*A*), pulmonary arteries (PA) (*B*), PASMCs (C), and cyclin D1 expression in the lungs (*D*), pulmonary arteries (PA) (*E*), and PASMCs (*F*). *Left panels* show representatives of RT-PCR and Western blot images. *Right panels* show quantification expressed as relative expression, setting control as 1. n = 3 for each group. NHE1$^{+/+}$ (wild-type mice), NHE1$^{-/-}$ (homozygous NHE1 mice). *$P < 0.05$ as compared with NHE1$^{+/+}$ normoxic mice. The results were representative of thee separate experiments.

We also observed that ROCK down-regulation in NHE1-null mice was associated with increased p27 expression and decreased cyclin D1. This novel discovery suggested that NHE1 gene might act upstream of RhoA/ROCK in regulating PASMC proliferation and pulmonary hypertension by directly inhibiting ROCK gene expression. Putney and Barber (46) investigated gene expression in NHE1-null mouse fibroblasts by using a cDNA microarray. They observed changes in some genes, including cell proliferation regulating genes, but they did not report on RhoA/ROCK.

The best way to reflect gene expression for pulmonary hypertension is to use distal pulmonary arterioles or isolate PASMCs from the distal vessels because the most significant morphological changes in pulmonary lung during pulmonary hypertension occur in the distal arterioles. However, we have not found a way to obtain distal pulmonary arterioles or PASMCs from the arterioles from mice. Therefore, we used main and branch pulmonary arterioles and the PASMCs isolated from the vessels in this study.

We demonstrated a decrease in ROCK1 and ROCK2 but not RhoA in cardiac muscle of our $NHE^{-/-}$ mice (*see* Figure 6B). Sugiyama and colleagues have shown that Y-27632, an inhibitor of RhoA, mildly decreased myocardial contractibility in the isolated perfused dog heart (47). Inhibition of Rho/ROCK diabetic rats has been shown to enhance contractibility in the isolated working heart (48). These constitutive Rho/ROCK may influence cardiac contractibility and their cardiac output. We doubt, though, that the cardiac output in our $NHE1^{-/-}$ mice was an important factor in their response to 10% O_2 because baseline RVSP and pulmonary architecture was similar, as were the cardiac weights in our $NHE1^{+/+}$ mice and the $NHE1^{-/-}$ mice, suggesting baseline cardiac outputs were similar. We have previously shown in guinea pigs and rats (49, 50) that 10% O_2 did not change cardiac output. We therefore suspect a low cardiac output did contribute to the failure of the $NHE^{-/-}$ mice to develop pulmonary hypertension in chronic hypoxia.

In conclusion, in the present study, we found that the NHE1 gene knockout resulted in the loss of chronic hypoxia–induced pulmonary hypertension and vascular remodeling in mice. This result has demonstrated that the NHE1 gene is a key determinant in the development of pulmonary hypertension and vascular remodeling induced by hypoxia. In addition, in this study, we found that the NHE1 gene knockout significantly knocked down ROCKs and increased p27. This finding suggested that the inhibition of hypoxic pulmonary hypertension by NHE1 gene deficiency was associated with direct down-regulation of ROCK, which may provide a novel regulatory pathway for NHE1.

***Conflict of Interest Statement*:** None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

References

1. Orlowski J, Grinstein S. Na^+/H^+ exchangers of mammalian cells. *J Biol Chem* 1997;272:22373–22376.
2. Masereel B, Pochet L, Laeckmann D. An overview of inhibitors of Na(+)/H(+) exchanger. *Eur J Med Chem* 2003;38:547–554.
3. Murer H, Hopfer U, Kinne R. Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *Biochem J* 1976;154:597–604.
4. Sardet C, Franchi A, Pouysségur J. Molecular cloning, primary structure, and expression of the human growth factor-activatable Na+/H+ antiporter. *Cell* 1989;56:271–280.
5. Stenmark R, Fagan K, Frid MG. Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. *Circ Res* 2006;99: 675–691.
6. Jeffery TK, Wanstall JC. Pulmonary vascular remodeling: a target for therapeutic intervention in pulmonary hypertension. *Pharmacol Ther* 2001;92:1–20.
7. Rabinovitch M. Pulmonary vascular remodeling in hypoxic pulmonary hypertension. In: Yuan J, editor. Hypoxic pulmonary vasoconstriction: cellular and molecular mechanisms. Boston, MA: Kluwer Academic Publishers; 2004. pp. 403–418.
8. Quinn DA, Honeyman TW, Joseph PM, Thompson BT, Hales CA, Schied CR. Contribution of Na+/H+ exchange to pH regulation in pulmonary artery smooth muscle cells. *Am J Respir Cell Mol Biol* 1991;5:586–591.
9. Quinn DA, Dahlberg CG, Bonventre JV, Scheid CR, Honeyman TW, Joseph PM, Thompson BT, Hales CA. The role of Na+/H+ exchange and growth factors in pulmonary artery smooth muscle cell proliferation. *Am J Respir Cell Mol Biol* 1996;14:139–145.
10. Dahlberg CG, Thompson BT, Joseph PM, Garg HG, Spence CR, Qiunn DA, Bonvebtre JV, Hales CA. Differential effect of three commercial heparins on Na+/H+ exchange and growth of PASMC. *Am J Physiol* 1996;270:L260–L265.
11. Quinn DA, Du HK, Thompson BT, Hales CA. Amiloride analogs inhibit chronic hypoxic pulmonary hypertension. *Am J Respir Crit Care Med* 1998;157:1263–1268.
12. Yao W, Qian G, Yang X. Roles of NHE-1 in the proliferation and apoptosis of pulmonary artery smooth muscle cells in rats. *Chin Med J (Engl)* 2002;115:107–109.
13. Rios EJ, Fallon M, Wang J, Shimoda LA. Chronic hypoxia elevates intracellular pH and activates Na+/H+ exchange in pulmonary arterial smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* 2005; 289:L867–L874.
14. Shimoda LA, Fallon M, Pisarcik S, Wang J, Semenza GL. HIF-1 regulates hypoxic induction of NHE1 expression and alkalinzation of intracellular pH in pulmonary arterial myocytes. *Am J Physiol Lung Cell Mol Physiol* 2006;291:L941–L949.
15. Pedersen SF. The Na+/H+ exchanger NHE1 in stress-induced signal transduction: implications for cell proliferation and cell death. *Pflugers Arch* 2006;452:249–259.
16. Yu L, Quinn DA, Garg HG, Hales CA. Cyclin-dependent kinase inhibitor p27Kip1, but not p21WAF1/Cip1, is required for inhibition of hypoxia-induced pulmonary hypertension and remodeling by heparin in mice. *Circ Res* 2005;97:937–945.
17. Mammoto A, Huang S, Moore K, Oh P, Ingber DE. Role of RhoA, mDia, and ROCK in cell shape-dependent control of the Skp2-p27kip1 pathway and the G1/S transition. *J Biol Chem* 2004;279:26323–26330.
18. Vidal A, Millard SS, Miller JP, Koff A. Rho activity can alter the translation of p27 mRNA and is important for RasV12-induced transformation in a manner dependent on p27 status. *J Biol Chem* 2002;277:16433–16440.
19. Croft DR, Olson MF. The Rho GTPase effector ROCK regulates cyclin A, cyclin D1, and p27Kip1 levels by distinct mechanisms. *Mol Cell Biol* 2006;26:4612–4627.
20. Besson A, Gurian-West M, Schmidt A, Hall A, Roberts JM. p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes Dev* 2004;18:862–876.
21. Nguyen L, Besson A, Heng JI, Schuurmans C, Teboul L, Parras C, Philpott A, Roberts JM, Guillemot F. p27kip1 independently promotes neuronal differentiation and migration in the cerebral cortex. *Genes Dev* 2006;20:1511–1524.
22. Kintner DB, Su G, Lenart B, Ballard AJ, Meyer JW, Ng LL, Shull GE, Sun D. Increased tolerance to oxygen and glucose deprivation in astrocytes from Na(+)/H(+) exchanger isoform 1 null mice. *Am J Physiol Cell Physiol* 2004;287:C12–C21.
23. Hales CA, Kradin RL, Brandstetter RD, Zhu YJ. Impairment of hypoxic pulmonary artery remodeling by heparin in mice. *Am Rev Respir Dis* 1983;128:747–751.
24. Eddahibi S, Hanoun N, Lanfumey L, Lesch KP, Raffestin B, Hamon M, Adnot S. Attenuated hypoxic pulmonary hypertension in mice lacking the 5-hydroxytryptamine transporter gene. *J Clin Invest* 2000;105: 1555–1562.
25. Brusselmans K, Compernolle V, Tjwa M, Wiesener MS, Maxwell PH, Collen D, Carmeliet P. Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. *J Clin Invest* 2003; 111:1519–1527.
26. Wang G, Woods A, Sabari S, Pagnotta L, Stanton LA, Beier F. RhoA/ROCK signaling suppresses hypertrophic chondrocyte differentiation. *J Biol Chem* 2004;279:13205–13214.

27. Vacca A, Felli MP, Palermo R, Di Mario G, Calce A, Di Giovine M, Frati L, Gulino A, Screpanti I. Notch3 and pre-TCR interaction unveils distinct NF-kappaB pathways in T-cell development and leukemia. *EMBO J* 2006;25:1000–1008.
28. Yu L, Quinn DA, Garg HG, Hales CA. Gene expression of cyclin-dependent kinase inhibitors and effect of heparin on their expression in mice with hypoxia-induced pulmonary hypertension. *Biochem Biophys Res Commun* 2006;345:1565–1572.
29. LaPointe MS, Batlle DC. Na+/H+ exchange and vascular smooth muscle proliferation. *Am J Med Sci* 1994;307:S9–S16.
30. Lang F, Ritter M, Gamper N, Huber S, Fillon S, Tanneur V, Lepple-Wienhues A, Szabo I, Gulbins E. Cell volume in the regulation of cell proliferation and apoptotic cell death. *Cell Physiol Biochem* 2000;10: 417–428.
31. Di Sario A, Bendia E, Svegliati Baroni G, Ridolfi F, Bolognini L, Feliciangeli G, Jezequel AM, Orlandi F, Benedetti A. Intracellular pathways mediating Na+/H+ exchange activation by platelet-derived growth factor in rat hepatic stellate cells. *Gastroenterology* 1999;116: 1155–1166.
32. Vera F, Rozengurt E. Stimulation of Na+/H+ antiport activity by epidermal growth factor and insulin occurs without activation of protein kinase C. *Biochem Biophys Res Commun* 1985;130: 646–653.
33. Liaw YS, Yang PC, Yu CJ, Kuo SH, Luh KT, Lin YJ, Wu ML. PKC activation is required by EGF-stimulated Na+/H+ exchanger in human pleural mesothelial cells. *Am J Physiol* 1998;274:L665–L672.
34. Wu D, Doods H, Stassen JM. Inhibition of human artery smooth muscle cell proliferation and migration by sebiporide, a new specific NHE-1 inhibitor. *J Cardiovasc Pharmacol* 2006;48:34–40.
35. Gamovskaya MN, Mukhin YV, Turner JH, Vlasova TM, Ullian ME, Raymond JR. Mitogen-induced activation of Na+/H+ exchange in vascular smooth muscle cells involves janus kinase 2 and Ca^{2+}/calmodulin. *Biochemistry* 2003;42:7178–7187.
36. Javadov S, Baetz D, Rajapurohitam V, Zeidan A, Kirshenbaum LA, Karmazyn M. Antihypertrophic effect of Na+/H+ exchanger isoform 1 inhibition is mediated by reduced mitogen-activated protein kinase activation secondary to improved mitochondrial integrity and decreased generation of mitochondrial-derived reactive oxygen species. *J Pharmacol Exp Ther* 2006;317:1036–1043.
37. Kusuhara M, Takahashi E, Peterson TE, Abe J, Ishida M, Han J, Ulevitch R, Berk BC. p38 kinase is a negative regulator of angiotensin II signal transduction in vascular smooth muscle cells: effect of on Na+/H+ exchange and ERK1/2. *Circ Res* 1998;83:824–831.
38. Hooley R, Yu CY, Symons M, Barber DL. G alpha 13 stimulates Na+-H+ exchange through distinct Cdc42-dependent and RhoA-dependent pathways. *J Biol Chem* 1996;271:6152–6158.
39. Vexler ZS, Symons M, Barber DL. Activation of Na+-H+ exchange is necessary for RhoA-induced stress fiber formation. *J Biol Chem* 1996; 271:22281–22284.
40. Tominaga T, Ishizaki T, Narumiya S, Barber DL. p160ROCK mediates RhoA activation of Na-H exchange. *EMBO J* 1998;17:4712–4722.
41. Tominaga T, Barber DL. Na-H exchange acts downstream of RhoA to regulate integrin-induced cell adhesion and spreading. *Mol Biol Cell* 1998;9:2287–2303.
42. Wallert MA, Thronson HL, Korpi NL, Olmschenk SM, McCoy AC, Funfar MR, Provost JJ. Two G protein-coupled receptors activate Na+/H+ exchanger isoform 1 in Chinese hamster lung fibroblasts through an ERK-dependent pathway. *Cell Signal* 2005;17:231–242.
43. Paradiso A, Cardone RA, Bellizzi A, Bagorda A, Guerra L, Tommasino M, Casavola V, Reshkin SJ. The Na+-H+ exchanger-1 induces cytoskeletal changes involving reciprocal RhoA and Rac1 signaling, resulting in motility and invasion in MDA-MB-435 cells. *Breast Cancer Res* 2004;6:R616–R628.
44. Cardone RA, Bagorda A, Bellizzi A, Busco G, Guerra L, Paradiso A, Casavola V, Zaccolo M, Reshkin SJ. Protein kinase A gating of a pseudopodial-located RhoA/ROCK/p38/NHE1 signal module regulates invasion in breast cancer cell lines. *Mol Biol Cell* 2005;16:3117–3127.
45. Guilluy C, Sauzeau V, Rolli-Derkinderen M, Guérin P, Sagan C, Pacaud P, Loirand G. Inhibition of RhoA/Rho kinase pathway is involved in the beneficial effect of sildenafil on pulmonary hypertension. *Br J Pharmacol* 2005;146:1010–1018.
46. Putney LK, Barber DL. Expression profile of genes regulated by activity of the Na-H exchanger NHE1. *BMC Genomics* 2004;5:46.
47. Sugiyama A, Yatomi Y, Takahara A, Satoh Y, Hashimoto K. Cardiac effects of a selective rho-associated kinase inhibitor, Y-27632, assessed in canine isolated, blood-perfused heart preparations. *Jpn J Pharmacol* 2002;88:359–361.
48. Lin G, Craig GP, Zhang L, Yuen VG, Allard M, McNeill JH, MacLeod KM. Acute inhibition of Rho-kinase improves cardiac contractile function in streptozotocin-diabetic rats. *Cardiovasc Res* 2007;75:51–58.
49. Thompson BT, Hassoun PM, Kradin RL, Hales CA. Acute and chronic hypoxic pulmonary hypertension in guinea pigs. *J Appl Physiol* 1989; 66:920–928.
50. Janssens SP, Thompson BT, Spence CR, Hales CA. Functional and structural changes with hypoxia in pulmonary circulation of spontaneously hypertensive rats. *J Appl Physiol* 1994;77:1101–1107.