HIF-1 regulates hypoxic induction of NHE1 expression and alkanilization of intracellular pH in pulmonary arterial myocytes

Larissa A. Shimoda,1 Michele Fallon,1 Sarah Pisarcik,1 Jian Wang,1 and Gregg L. Semenza2

1Division of Pulmonary and Critical Care Medicine, Department of Medicine, Johns Hopkins University School of Medicine; 2Institute for Cell Engineering, McKusick-Nathans Institute of Genetic Medicine, and Departments of Pediatrics, Medicine, Oncology, and Radiation Oncology, Johns Hopkins University School of Medicine, Baltimore, Maryland

Submitted 15 December 2005; accepted in final form 5 June 2006

Shimoda, Larissa A., Michele Fallon, Sarah Pisarcik, Jian Wang, and Gregg L. Semenza. HIF-1 regulates hypoxic induction of NHE1 expression and alkanilization of intracellular pH in pulmonary arterial myocytes. Am J Physiol Lung Cell Mol Physiol 291: L941–L949, 2006.—Vascular remodeling resulting from altered pulmonary arterial smooth muscle cell (PASMC) growth is a contributing factor to the pathogenesis of hypoxic pulmonary hypertension. PASMC growth requires an alkaline shift in intracellular pH (pHi) and we previously showed that PASMCs isolated from mice exposed to chronic hypoxia exhibit increased Na+/H+ (NHE) expression and activity, which resulted in increased pHi. However, the mechanism by which hypoxia caused these changes was unknown. In this study we tested the hypothesis that hypoxia-induced changes in PASMCs pHi homeostasis are mediated by the transcriptional regulator hypoxia-inducible factor 1 (HIF-1). Consistent with previous results, increased HIF-1α mRNA and protein, enhanced NHE activity, and an alkaline shift in pHi, were observed in PASMCs isolated from wild-type mice exposed to chronic hypoxia (3 wk at 10% O2). In contrast, these changes were absent in PASMCs isolated from chronically hypoxic mice with partial deficiency for HIF-1. Exposure of PASMCs to hypoxia ex vivo (48 h at 4% O2) or overexpression of HIF-1 in the absence of hypoxia also increased HIF-1α mRNA and protein expression. Our results indicate that full expression of HIF-1 is essential for hypoxic induction of NHE1 expression and changes in PASMC pHi homeostasis and suggest a novel mechanism by which HIF-1 mediates pulmonary vascular remodeling during the pathogenesis of hypoxic pulmonary hypertension.

Chronic reduction of alveolar oxygen tension is a consequence of many lung diseases that induces structural remodeling of the pulmonary vasculature, which is characterized by smooth muscle cell proliferation, intimal thickening, and extension of smooth muscle into previously nonmuscular arterioles (12, 24). In addition, vascular smooth muscle tone is increased, as evidenced by acute reduction in pulmonary arterial pressure in response to vasodilatory agents (16, 27, 32). For patients with chronic obstructive pulmonary disease, these hypoxia-induced structural and functional changes in the pulmonary vasculature correlate with the development of pulmonary hypertension and increased mortality. Although previous studies have characterized some of the morphological and functional changes that occur in the pulmonary vasculature in response to chronic hypoxia (CH), the cellular and molecular mechanisms underlying these changes remain poorly understood.

Regulation of Na+/H+ exchange and intracellular pH (pHi) is vital for maintaining cell viability. pHi modulates a number of important cell functions, including signal transduction pathways involved in the regulation of cell size and proliferation (5, 36, 37). Alterations in pHi are also associated with hypoxic pulmonary vasoconstriction (20, 21, 23). Pulmonary arterial smooth muscle cells (PASMCs) proliferate in response to growth factors (37) and contribute to pulmonary vascular remodeling during CH (38). We recently demonstrated that baseline pHi is more alkaline in PASMCs isolated from chronically hypoxic animals and that this elevation in basal pHi is accompanied by an increase in Na+/H+ exchange activity, secondary to increased expression of Na+/H+ exchanger (NHE) isoform 1 (NHE1) (39).

The transcriptional regulation of the gene encoding NHE1 (NHE1 or SLC9A1) is just beginning to be explored and much is yet to be learned with respect to the factors involved in the process. Published reports identify a number of important binding sites in the promoter region of NHE1 including recognition sequences for AP-2, Sp1, and CREB (7, 8, 25), of which AP-2 has been shown to induce transcriptional activation of NHE1 (7). Examination of the promoter region of the NHE1 gene revealed candidate-binding sites for hypoxia-inducible factor 1 (HIF-1), which has been demonstrated to mediate numerous physiological and pathophysiological responses to hypoxia. HIF-1 exists as a heterodimer composed of HIF-1α and HIF-1β subunits (15). HIF-1α confers sensitivity and specificity for hypoxic induction as HIF-1β is ubiquitously expressed under normoxic conditions, whereas HIF-1α is ubiquitinated and rapidly degraded (40, 45). During hypoxia, stabilization of HIF-1α allows translocation to the nucleus (17) where it dimerizes with HIF-1β and initiates the transcription of numerous target genes (41).

Targeted homozygous disruption of the Hif1a locus encoding HIF-1α (Hif1a−/−) revealed a critical role for HIF-1 in development as these animals died midgestation (14). In contrast, mice that were heterozygous for a null allele (Hif1a+/−) were viable and phenotypically indistinguishable from their wild-type littermates under normoxic conditions (14). Utilizing Hif1a−/− mice, we have examined the role of HIF-1 in mediating (patho)physiological responses to hypoxia. In the lung, we found that the development of pulmonary hypertension and vascular remodeling was blunted and that the hypoxia-induced

Address for reprint requests and other correspondence: L. A. Shimoda, Division of Pulmonary and Critical Care Medicine, Johns Hopkins Univ., 5501 Hopkins Bayview Circle, JHAAC 4A.52, Baltimore, MD 21224 (e-mail: shimodal@welch.jhu.edu).

Published: June 9, 2006; doi:10.1152/ajplung.00528.2005.

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changes in PASMC membrane potential, K^+ channel activity, and Ca^{2+} homeostasis were markedly attenuated or absent in Hif1a^{-/-} mice exposed to CH (42, 48, 50). Although the promoter of the gene encoding NHE1 contains putative HIF-1-binding sites, the regulation of NHE1 by HIF-1 has not been studied. In this study, we tested the hypothesis that hypoxic induction of NHE1 expression and subsequent alterations in pH homeostasis in PASMCs are mediated by HIF-1.

**METHODS**

**Chronic hypoxic exposure.** All procedures were approved by the Animal Care and Use Committee of the Johns Hopkins University. Adult male Wistar rats and C57BL/6 mice were placed in a hypoxic chamber for 21 days. The chamber was continuously flushed with a mixture of room air and N_2 (10 ± 5% O_2) to maintain low CO_2 (≤0.5%). Chamber O_2 concentration was continuously monitored (PRO-OX; RCI Hudson, Anaheim, CA). The animals were exposed to 10% room air for 10 min twice a week to clean the cages and replenish food and water supplies. At the end of hypoxic exposure, animals were injected with heparin and anesthetized with pentobarbital (Nembutal; 100 mg/kg i.p.) and the septum (LV + RV) of the heart was separated from the left ventricle and the septum (LV + S) following removal of the atria, and the two portions were blotted dry and weighed.

**Cell isolation and culture.** The method for obtaining single PASMCs has been described previously (39, 43). Briefly, intrapulmonary arteries (200–500 μm OD) were isolated and cleaned of connective tissue. After the endothelium was disrupted by gentle rubbing of the luminal surface with a cotton swab, the arteries were allowed to recover for 30 min in cold (4°C) PSS, followed by 20 min in reduced Ca^{2+} PSS (20 μM CaCl_2) at room temperature. The tissue was digested in reduced Ca^{2+} PSS containing collagenase (type I; 1,750 U/ml), papain (9.5 U/ml), bovine serum albumin (2 mg/ml), and dithiothreitol (DTT; 1 mM) at 37°C for 10 min (murine PASMCs) or 25 min (rat PASMCs). Following digestion, single smooth muscle cells were dispersed by gentle trituration with a wide-bore transfer pipette in Ca^{2+}-free PSS, and the cell suspension was placed on 25-mm glass coverslips for pH measurements or grown to 80% confluence in 6-cm culture dishes for protein and RNA. Murine PASMCs were cultured in SmBm Complete Media (Clonetics) supplemented with 10% fetal calf serum (FCS) for 3–4 days and placed in serum-free media 24 h before experiments. Rat PASMCs were cultured in Ham’s F-12 media supplemented with 0.5% FCS and 1% serum-free media 24 h before experiments. Rat PASMCs were cultured in SmBm Complete Media (Clonetics) supplemented with 10% fetal calf serum (FCS) for 3–4 days and placed in serum-free media 24 h before experiments. Rat PASMCs were cultured in Ham’s F-12 media supplemented with 0.5% FCS and 1% penicillin/streptomycin. In some experiments, PASMCs were cultured under hypoxic conditions (4% O_2, 5% CO_2) in a modular incubator (Billups-Rothenberg) for 48 h.

**Intracellular pH measurements.** PASMCs were placed in a laminar flow cell chamber perfused with either Krebs bicarbonate solution containing (in mM): 118 NaCl, 4.7 KCl, 0.57 MgSO_4, 1.18 KH_2PO_4, 25 NaHCO_3, 2.5 CaCl_2, and 10 glucose gassed with 16% O_2-5% CO_2, or a HEPES-buffered PSS containing (in mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO_4, 25 NaHCO_3, 1.1 glucose, and 1.2 KH_2PO_4, pHi was measured in cells incubated with a membrane-permeant (acetoxymethyl ester) form of the pH-sensitive fluorescent dye BCECF-AM for 60 min at 37°C under examination via a ×20 fluorescence objective (Super Fluor 20, Nikon). Light emitted from the cell at 530 nm was returned through the objective and detected by a cooled CCD imaging camera. An electronic shutter (Sutter Instruments) was used to minimize photo-bleaching of dye. Protocols were executed and data were collected online with InCyte software (Intracellular Imaging). pHi was estimated from in situ calibration after each experiment. Cells were perfused with a solution containing (in mM): 105 KCl, 1 MgCl_2, 1.5 CaCl_2, 10 glucose, 20 HEPES-Tris, and 0.01 nigericin to allow pHi to equilibrate to external pH. A two-point calibration was created from fluorescence measured as pHi was adjusted with KOH from 6.5 to 7.5. Baseline pHi was measured in PASMCs for 3 min, and values were averaged to obtain a mean value for each cell. A standard ammonia pulse technique was used to measure Na^+ /H^+ exchange activity (Fig. 1A). PASMCs loaded with BCECF were placed on the fluorescence microscope and perfused at a rate of 2.5 μl/min with HEPES1 solution containing (in mM): 130 NaCl, 5 KCl, 1 MgCl_2, 1.5 CaCl_2, 10 glucose, and 20 HEPES with pH adjusted to 7.4 with NaOH at 37°C. Baseline pHi was measured for 2 min before cells were briefly exposed to NH_4Cl (ammonium pulse) by perfusing with HEPES2 solution containing (in mM): 110 NaCl, 20 NH_4Cl, 5 KCl, 1 MgCl_2, 1.5 CaCl_2, 10 glucose, and 20 HEPES at a pH of 7.4 using NaOH for 3 min. The ammonium pulse caused alkalization due to influx of NH_4^+ and buffering of intracellular H^+. Washout of NH_4Cl in the absence of extracellular Na^+ using a Na^- and NH_4^-free solution containing (in mM): 130 choline chloride, 5 KCl, 1 MgCl_2, 1.5 CaCl_2, 10 glucose, and 20 HEPES at a pH of 7.4 using KOH for 10 min allowed exchanger activity to resume. Recovery rates were measured by calculating the intracellular pH (pHi) change over a 2-min recovery period. B: representative traces demonstrating ammonia pulse and Na^+ /H^+ exchange activity in PASMCs cultured under control or hypoxic conditions. C and D: bar graphs illustrating average basal pHi (C) and Na^+ /H^+ exchange activity (D) measured in PASMCs cultured under control and hypoxic (n = 49 cells) conditions. *Significant difference from control value (P < 0.05).
resulted in acidification due to rapid diffusion and washout of NH₃, leaving behind H⁺ ions. The external solution was then switched back to HEPES1 solution for 10 min. Readdition of extracellular Na⁺ allowed activation of Na⁺/H⁺ exchange and recovery from acidification to basal levels. The rate of Na⁺-dependent recovery from intracellular acidification corresponds to Na⁺/H⁺ exchange activity.

**RT-PCR.** Total RNA was prepared from endothelium-denuded intalobar pulmonary arteries by TRIzol extraction. Two arteries each

### Table 1. Primers used for RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Accession No.</th>
<th>Source</th>
<th>Primer Pair Sequence (Sense/Antisense)</th>
<th>Product Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>NHE1 (mouse)</td>
<td>U5112</td>
<td>Mouse</td>
<td>5’-CTGTGTTGCTATATYGGCC-3’; 5’-TGGTTCTATAAGGGCAGT-3’</td>
<td>521</td>
</tr>
<tr>
<td>NHE1 (rat)</td>
<td>M85299</td>
<td>Rat</td>
<td>5’-GGCCGGGCAACTGGAAGCA-3’; 5’-TGCTCGGGGAGGGTAAGACATCA-3’</td>
<td>334</td>
</tr>
<tr>
<td>NHE2</td>
<td>L11004</td>
<td>Rat</td>
<td>5’-ATCACAGCTGCCATCGTT-3’; 5’-GACTCATCGTACTCCTG-3’</td>
<td>189</td>
</tr>
<tr>
<td>NHE3</td>
<td>M85300</td>
<td>Rat</td>
<td>5’-TCGGCTAGCGTGGAAGCATCA-3’; 5’-AGTGGACGAGCAGGGAGGAT-3’</td>
<td>412</td>
</tr>
<tr>
<td>β-actin</td>
<td>J00691</td>
<td>Rat</td>
<td>5’-AGCTGACTGCTAGCGTT-3’; 5’-GACTCATCGTACTCCTG-3’</td>
<td>244</td>
</tr>
</tbody>
</table>

Data analysis. Data are expressed as means ± SE; n is the number of experiments performed, or the number of cells in each experiment, as indicated. Statistical comparisons were performed using Student’s t-test (paired or unpaired) or ANOVA, as appropriate. Differences were considered to be significant when P < 0.05.

**RESULTS**

**Direct effect of hypoxia on pH homeostasis in PASMCs.** We previously demonstrated that exposure to CH in vivo resulted in an alkaline shift in PASMC pHₓ and increased NHE activity. However, in vivo, in addition to hypoxia, the vascular cells are also exposed to increased pressure and circulating factors that may influence cell function. To determine whether hypoxia exerted a direct effect on pH homeostasis, we examined the direct effect of hypoxia on pHₓ and Na⁺/H⁺ exchange activity in PASMCs isolated from normoxic rats and cultured under hypoxic conditions ex vivo. To isolate the change in pHₓ due to hypoxia-induced alterations in Na⁺/H⁺ exchange activity, PASMCs were superfused with bicarbonate-free (HEPES-buffered) extracellular solution, which eliminates contributions from the Cl⁻/HCO₃⁻ exchangers. Under these conditions, basal pHₓ was significantly greater in PASMCs exposed to 4% O₂ for 48 h (6.98 ± 0.02; n = 66 cells) compared with cells cultured under normoxic conditions (6.70 ± 0.03; n = 63 cells; Fig. 1C). Na⁺/H⁺ exchange activity, measured using the ammoneum pulse technique (Fig. 1B), was also greater in PASMCs cultured under hypoxic conditions (0.096 ± 0.01 pH U/min in 56 cells vs. 0.18 ± 0.01 pH U/min in 49 cells; Fig. 1D), indicating that hypoxia directly alters pH homeostasis in PASMCs, independent of changes in arterial pressure or circulating factors.

**Direct effect of hypoxia on NHE1 expression in PASMCs.** To determine whether the hypoxia-induced changes in pHₓ and Na⁺/H⁺ exchange activity observed in PASMCs cultured under hypoxic conditions resulted from alterations in NHE1 expression, mRNA and protein levels were measured. To date, nine NHE isoforms have been characterized. NHE1 is ubiquitously expressed, whereas NHE2 and NHE3 are found predominately in the kidney and gastrointestinal epithelium (3, 35, 47, 49), although low-level expression of both isoforms in the lung has been reported (3, 49). Expression of NHE4 and NHE5 is restricted primarily to the gastrointestinal tract and brain, respectively (1, 33), and although very low expression of NHE6–9 has been reported in whole lung tissue, localization of these isoforms is restricted to subcellular organelles (10, 26, 28, 30, 31). Initial screening experiments using specific primers designed for rat NHE1, NHE2, and NHE3 were performed.

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to determine which NHE isoforms were expressed in rat PASMCs. Similar to our previous findings in murine PASMCs (39) and in smooth muscle cells from other vascular beds (22), we found that rat PASMCs only expressed NHE1 (Fig. 2A). The identity of our PCR product as NHE1 was confirmed by sequencing the band excised from the gel. The failure to detect NHE2 and NHE3 mRNA in PASMCs was not due to incorrect primer sequences or PCR conditions, as these mRNAs were readily detected in rat kidney (Fig. 2B). Both mRNA (Fig. 2C) and protein (Fig. 2D) levels of NHE1 were substantially greater in cells cultured under hypoxic conditions, compared with PASMCs from the same animals cultured under nonhypoxic conditions.

**Basal pH$_i$ in PASMCs from Hif1a$^{+/+}$ and Hif1a$^{-/-}$ mice.** We next examined whether HIF-1 mediated the hypoxia-induced changes in PASMC pH homeostasis using mice with partial deficiency for HIF-1a and their wild-type (Hif1a$^{+/+}$) littermates. We first verified development of RV hypertrophy in Hif1a$^{+/+}$ mice exposed to CH. In Hif1a$^{+/+}$ mice, 3 wk of hypoxia caused a significant increase in right, but not left, ventricle weight, leading to an increase in the RV/LV+S ratio (Table 2). RV and LV weights were not different in normoxic Hif1a$^{+/+}$ and Hif1a$^{-/-}$ mice; however, the CH-induced increase in both RV weight and RV/LV+S ratio was attenuated in mice that were partially deficient in HIF-1. These results are consistent with our previous findings (50).

Basal pH$_i$ was similar in PASMCs isolated from normoxic animals (6.82 ± 0.05 in Hif1a$^{+/+}$ mice vs. 6.78 ± 0.03 in Hif1a$^{-/-}$ mice; Fig. 3A). Following exposure to CH, basal pH$_i$ increased significantly in PASMCs from Hif1a$^{+/+}$ mice (7.04 ± 0.03), consistent with previous results (39). The CH-induced increase in pH$_i$ was markedly reduced in PASMCs isolated from mice partially deficient for HIF-1 (6.86 ± 0.02). Indeed, pH$_i$ in PASMCs isolated from normoxic and chronically hypoxic Hif1a$^{-/-}$ mice was not significantly different.

Table 2. **Means ± SE values for heart weight measurement in normoxic and chronically hypoxic Hif1a$^{+/+}$ and Hif1a$^{-/-}$ mice**

<table>
<thead>
<tr>
<th>Condition</th>
<th>RV Weight, g</th>
<th>LV + S Weight, g</th>
<th>RV/LV + S Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic Hif1a$^{+/+}$ (n = 12)</td>
<td>0.0251 ± 0.002</td>
<td>0.1050 ± 0.005</td>
<td>0.239 ± 0.01</td>
</tr>
<tr>
<td>Hypoxic Hif1a$^{+/+}$ (n = 14)</td>
<td>0.0332 ± 0.002*</td>
<td>0.0988 ± 0.004</td>
<td>0.335 ± 0.009*</td>
</tr>
<tr>
<td>Normoxic Hif1a$^{-/-}$ (n = 15)</td>
<td>0.0267 ± 0.002</td>
<td>0.1077 ± 0.005</td>
<td>0.243 ± 0.007</td>
</tr>
<tr>
<td>Hypoxic Hif1a$^{-/-}$ (n = 11)</td>
<td>0.0286 ± 0.002†</td>
<td>0.0961 ± 0.005</td>
<td>0.296 ± 0.009†</td>
</tr>
</tbody>
</table>

RV, right ventricle; LV + S, left ventricle + septum; n = number of animals; *significant difference from normoxic Hif1a$^{+/+}$ value; †significant difference from normoxic Hif1a$^{-/-}$ values; ‡significant difference from hypoxic Hif1a$^{+/+}$ value.
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Effect of CH on Na⁺/H⁺ exchange activity in PASMCs from Hif1a<sup>+/+</sup> and Hif1a<sup>−/−</sup> mice. Na⁺/H⁺ exchange activity was similar in PASMCs isolated from normoxic Hif1a<sup>+/+</sup> and Hif1a<sup>−/−</sup> mice (0.104 ± 0.02 vs. 0.109 ± 0.01 pH U/min, respectively) (Fig. 3B). Consistent with our previous results, exposure to CH markedly increased Na⁺/H⁺ exchange activity in PASMCs isolated from Hif1a<sup>+/+</sup> mice (0.156 ± 0.02 pH U/min). In contrast, the CH-induced increase in Na⁺/H⁺ exchange activity was absent in PASMCs isolated from mice partially deficient for HIF-1 (0.088 ± 0.01 pH U/min).

Effect of CH on NHE1 expression in Hif1a<sup>+/+</sup> and Hif1a<sup>−/−</sup> mice. As anticipated, NHE1 mRNA expression was increased in endothelium-denuded pulmonary arteries isolated from Hif1a<sup>+/+</sup> mice exposed to CH compared with mRNA levels in pulmonary arteries isolated from normoxic Hif1a<sup>+/+</sup> (Fig. 4A). Immunoblot analysis revealed a similar increase in NHE1 protein expression in these animals (Fig. 4B). Neither mRNA nor protein expression of NHE1 increased in response to CH in pulmonary arteries from Hif1a<sup>−/−</sup> mice.

Effect of overexpression of HIF-1α on NHE1 expression. To verify that the hypoxia-induced increase in NHE1 expression was due to activation of HIF-1 and not an unrelated aspect of hypoxic exposure, HIF-1α was overexpressed in rat PASMCs isolated from normoxic animals and cultured under nonhypoxic conditions. Previous studies in this cell type demonstrated that transfection with an adenovirus containing a construct encoding AdCA5 increased HIF-1α protein levels and HIF-1 target gene expression under nonhypoxic conditions (18). Following transfection with AdCA5 for 48 h, an increase in NHE1 mRNA expression was observed compared with PASMCs transfected with AdLacZ (Fig. 5A). A similar increase in NHE1 protein expression was observed in PASMCs transfected with AdCA5 compared with AdLacZ (Fig. 5B).

Transfection alone did not significantly impact basal pH<sub>i</sub> or Na⁺/H⁺ exchange activity. Basal pH<sub>i</sub> was similar in control PASMCs and those transfected with AdLacZ (6.71 ± 0.02; n = 77 cells). However, basal pH<sub>i</sub> was significantly increased in PASMCs transfected with AdCA5 (6.92 ± 0.02; n = 76 cells; Fig. 6, A and B). In addition, Na⁺/H⁺ exchange activity was significantly greater in PASMCs transfected with AdCA5 (0.177 ± 0.01; n = 76 cells) than in PASMCs transfected with AdLacZ (0.068 ± 0.06; n = 77 cells; Fig. 6C).

DISCUSSION

Previous studies demonstrated that the development of hypoxic pulmonary hypertension was associated with alterations in PASMC pH homeostasis, including an alkaline shift in basal pH due to increased expression and activity of NHE1 (39). However, the mechanism responsible for these changes was not known. In the current study, we demonstrated that the changes in pH homeostasis observed in PASMCs isolated from chronically hypoxic animals were due to a direct effect of hypoxia and used loss-of-function and gain-of-function models to demonstrate a critical role for HIF-1 in regulating these responses.

The factors controlling transcriptional regulation of NHE1 are poorly understood. The promoter region contains putative binding sites for a variety of transcription factors, including HIF-1. We previously demonstrated that numerous pulmonary responses to hypoxia are regulated by this transcription factor, including elevated RV pressure (an estimate of pulmonary arterial pressure), decreased K⁺ channel activity, PASMC hypertrophy, alterations in Ca<sup>2+</sup> homeostasis, and pulmonary vascular remodeling (42, 48, 50). In this study, we used mice with partial deficiency for HIF-1α to evaluate the role of HIF-1 in the changes in pH homeostasis induced by CH. Consistent with our previous results in C57BL/6 mice, an alkaline shift in pH<sub>i</sub> increased Na⁺/H⁺ exchange activity, and augmented NHE1 expression were observed in pulmonary vascular smooth muscle isolated from chronically hypoxic wild-type mice. These alterations were absent in Hif1a<sup>−/−</sup> mice, indicating that full HIF-1 activity was required for the alterations in pH homeostasis observed in response to chronic hypoxia in vivo.
In the intact animal, exposure to hypoxia results in increased pulmonary arterial pressure and alterations in circulating factors, which may influence the pulmonary vascular changes observed in this model (6, 9, 19). Some of the circulating factors, such as endothelin-1, are regulated by HIF-1 (13). Moreover, as we previously reported (50), measurement of right ventricular hypertrophy verified that development of pulmonary hypertension in response to CH was blunted in mice partially deficient for HIF-1. Thus it was impossible to determine whether the lack of changes in NHE1 expression, Na\(^+\)/H\(^+\) exchange activity, and pHi in chronically hypoxic mice was a direct consequence of reduced HIF-1-dependent NHE1 gene transcription, simply a result of decreased pulmonary arterial pressure, or a difference in the circulating factors to which the PASMCs were exposed in vivo.

We sought to address this issue in two ways. First, to explore whether the changes in PASMC pH homeostasis were due to a direct effect of hypoxia on PASMCs, an indirect effect mediated by pressure, or hypoxia-induced factors secreted by endothelial cells, we used an ex vivo model to assess the ability of hypoxia to alter pHi, Na\(^+\)/H\(^+\) exchange activity, and NHE1 expression in pulmonary arteries from chronically hypoxic mice. Hypoxia had no effect on NHE1 expression in mice partially deficient for hypoxia-inducible factor 1. β-Actin or α-actin expression was similar in all groups. Results shown are similar to those obtained in 3 separate experiments. Right: bar graphs show mean fold induction in NHE1 mRNA and protein expression normalized to β-actin or α-actin expression in chronically hypoxic mice and normoxic and chronically hypoxic mice (n = 3 samples consisting of cells isolated from 3 mice each).

Fig. 4. Effect of chronic hypoxia on NHE1 expression in pulmonary arteries from Hif1a\(^{+/−}\) and Hif1a\(^{+/+}\) mice. Left: representative gels illustrating increased mRNA (A) and protein (B) expression of NHE1 in endothelium-denuded pulmonary arteries from chronically hypoxic Hif1a\(^{+/−}\) mice. Hypoxia had no effect on NHE1 expression in mice partially deficient for hypoxia-inducible factor 1. β-Actin or α-actin expression was similar in all groups. Results shown are similar to those obtained in 3 separate experiments. Right: bar graphs show mean fold induction in NHE1 mRNA and protein expression normalized to β-actin or α-actin expression in chronically hypoxic Hif1a\(^{+/−}\) mice and normoxic and chronically hypoxic Hif1a\(^{+/+}\) mice (n = 3 samples consisting of cells isolated from 3 mice each).

Fig. 5. Effect of overexpressing HIF-1α under nonhypoxic conditions on NHE1 expression. Left: representative gel images demonstrating NHE1 mRNA (A) and protein (B) expression in PASMCs transfected with an adenovirus encoding a constitutively active form of HIF-1α (AdCA5) or a control adenovirus encoding β-galactosidase (AdLacZ). Right: bar graphs showing average fold induction in NHE1 expression normalized to β-actin (n = 3 experiments for each group).
We next used a gain-of-function model to determine the effect of increased HIF-1 expression in the absence of hypoxia. Overexpression of HIF-1α resulted in an increase in NHE1 gene and protein expression that was similar to that observed in PASMCs exposed to hypoxia ex vivo and in pulmonary arteries isolated from chronically hypoxic animals. In vivo, in the absence of increased pulmonary arterial pressure or circulating factors, caused changes in pH and Na⁺/H⁺ exchange activity that were qualitatively and quantitatively similar to those observed in cells isolated from animals exposed to hypoxia in vivo. We also found that NHE1 gene and protein expression were significantly increased in these cells to levels similar to those measured in endothelium-denuded pulmonary arteries from chronically hypoxic animals. Interestingly, in rat PASMCs, NHE1 protein was present as a double band. Other investigators have observed similar patterns of protein expression, due to the presence of several glycosylation sites (29, 51). It is likely that the faster migrating band corresponds to newly synthesized, core-glycosylated NHE1, whereas the slower migrating band corresponds to mature (terminally glycosylated) NHE1 (4). The data from PASMCs exposed to hypoxia ex vivo provide confirmation that hypoxia induces PASMC-autono-
mous changes in pH homeostasis and NHE1 expression that are similar to those observed during development of hypoxic pulmonary hypertension.

In summary, we used in vivo loss-of-function and ex vivo gain-of-function models to demonstrate that HIF-1 plays a crucial role in controlling NHE1 expression, Na⁺/H⁺ exchange activity, and alkalization of PASMCs in response to hypoxia. Although the current study was restricted to PASMCs, HIF-1-dependent hypoxia-induced changes in pH homeostasis may also regulate proliferation and apoptosis in other cells types. For example, exposure of cardiac myocytes to hypoxia induces apoptosis that can be attenuated by NHE inhibitors (44), and inhibition of Na⁺/H⁺ exchange in tumor cells decreases cell proliferation (11). Further study will also be required to determine whether HIF-1-dependent hypoxic induction of NHE1 is restricted to PASMCs or occurs in other pulmonary vascular cell types (e.g., endothelial cells and fibroblasts) that proliferate in response to chronic hypoxia.

ACKNOWLEDGMENTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-67919, HL-73859, and HL-55338 and by American Heart Association Scientist Development Grants AHA0930255N and AHA0430037N.

AJP-Lung Cell Mol Physiol • VOL 291 • NOVEMBER 2006 • www.ajplung.org
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