Partial HIF-1α deficiency impairs pulmonary arterial myocyte electrophysiological responses to hypoxia

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Prolonged exposure to decreased alveolar O2 tension, as occurs with many pulmonary diseases, leads to sustained pulmonary vasoconstriction, vascular remodeling, and pulmonary hypertension. Development of pulmonary hypertension is associated with eventual right heart failure and increased morbidity and mortality. Morphological changes associated with chronic hypoxia include pulmonary arterial smooth muscle cell (PASMC) hypertrophy and hyperplasia, muscularization of precapillary arterioles, and increased deposition of extracellular matrix components (11, 17, 18). Functional changes that occur in the pulmonary vasculature as a consequence of chronic hypoxia include membrane depolarization (23, 25) and altered vasoreactivity in response to numerous agonists (4, 5, 9, 10, 15, 19, 27).

Membrane potential plays a vital role in regulating vascular caliber and the proliferative state of PASMCs through control of cytosolic Ca2+ concentration ([Ca2+]i). In PASMCs, 4-aminopyridine (4-AP), but not charybdotoxin (ChTX), causes membrane depolarization and increased [Ca2+]i (2, 22, 31), indicating that resting membrane potential is regulated predominantly by a specific subtype of voltage-gated K+ (KV) channel that is 4-AP sensitive and ChTX insensitive. Furthermore, in vivo exposure to chronic hypoxia attenuates KV channel currents (23, 24), causes membrane depolarization (23, 25), and elevates basal [Ca2+]i (21) in PASMCs.

Hypoxia-inducible factor-1 (HIF-1) is a transcription factor composed of HIF-1α and HIF-1β subunits. Although HIF-1β is constitutively expressed in the lung, expression of HIF-1α is tightly regulated by O2 tension (29). HIF-1α has been shown to activate the transcription of genes encoding several factors important in the development of hypoxic pulmonary hypertension including endothelin-1, erythropoietin, inducible nitric oxide synthase, and vascular endothelial growth factor (7, 13, 20).

Mouse embryonic stem cells carrying a null allele at the Hif1a locus encoding HIF-1α were generated, and mice with complete [Hif1a(−/−)] or partial [Hif1a(+/−)] HIF-1α deficiency were analyzed (8). Hif1a(−/−) embryos died by gestational day 10.5, whereas Hif1a(+/−) mice were viable and indistinguishable from their wild-type [Hif1a(+/+)] littermates (8). Hif1a(+/−) mice exposed to 10% O2 for 3 wk exhibited right heart hypertrophy, elevated pulmonary arterial pressure, polycythemia, and extension of muscle into previously nonmuscular pulmonary arterioles (30). Development of pulmonary hypertension, polycythemia, and vascular remodeling was blunted in Hif1a(+/−) mice exposed to chronic hypoxia (30), indicating that HIF-1α plays an important role in the development of...
hypoxygen pulmonary hypertension. The mechanisms underlying contraction and proliferation of pulmonary vascular smooth muscle during the development of pulmonary hypertension are complex and poorly understood, however, and much is yet to be determined regarding the role of HIF-1α in mediating the pathogenesis of hypoxygen pulmonary hypertension. Moreover, although partial deficiency for HIF-1α was associated with impaired hypoxygen-induced pulmonary vascular remodeling (30), it was unclear whether this was due to altered PASMC electrophysiology, hyperplasia, and/or hypertrophy.

Given that the development of vasoconstriction and vascular remodeling in response to chronic hypoxygen are likely to be influenced by membrane potential, we hypothesized that partial deficiency for HIF-1α would limit the changes in PASMC electrophysiology induced by chronic hypoxygen. To test this hypothesis, we used whole cell patch-clamp techniques to determine the effect of chronic hypoxygen on cell capacitance, membrane potential, and K⁺ currents in PASMCs isolated from Hif1a(+/−) and Hif1a(+/+) mice.

METHODS

All procedures were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. Mice were generated on a C57B6 × 129 genetic background as previously described (8). Hif1a(+/−) and mice with one null allele at the Hif1a locus ([Hif1a(+/−)]) were mated, and offspring genotype was determined by PCR (8). Male Hif1a(+/+) and Hif1a(+/−) mice (8 wk old) were placed in a hypoxygen chamber and exposed to either normoxia or normobaric hypoxygen for 21 days. The chamber was continuously flushed with either room air or a mixture of room air and N₂ (10 ± 0.5% O₂) to maintain low CO₂ concentrations (<0.5%). Chamber O₂ and CO₂ concentrations were continuously monitored (OM-11 O₂ analyzer and LB-2 CO₂ analyzer, Sensormedics, Anaheim, CA). The mice were exposed to room temperature and no food was provided throughout the experiment. All experiments were conducted at room temperature (22–25°C) under normoxic conditions.

Effect of chronic hypoxygen on whole cell K⁺ currents. To identify the K⁺ currents present in mouse PASMCs, membrane currents were activated by depolarizing pulses of 800 ms from a holding potential of −60 mV to test potentials ranging from −50 to +40 mV in +10-mV step increments. Current measurements were made under control conditions 3–4 min after the cells were treated with 100 nM ChTX to inhibit Ca²⁺-activated K⁺ (Kᵥ) channels and 3–4 min after the subsequent addition of 10 mM 4-AP to inhibit Kᵥ channels. In subsequent experiments, Kᵥ currents were isolated by pretreating cells with ChTX (100 nM) and with [Ca²⁺]i buffered with 5 mM 4-AP to inhibit 4-AP-sensitive K⁺ channels (22). The effect of chronic hypoxygen on Kᵥ currents was determined by comparison of current-voltage (I-V) relationships of peak Kᵥ current density measured in cells from normoxic and hypoxygen Hif1a(+/−) and Hif1a(+/+) animals.

Effect of chronic hypoxygen on membrane potential. Membrane potential was measured in current-clamp mode with I = 0. Membrane potential was measured for 2 min, and cells with unstable membrane potentials were discarded. Membrane potential for each cell was determined by calculating the standard deviation in membrane potential for the 2-min recording period.

Drugs and Chemicals

ChTX was obtained from American Peptides (Sunnyvale, CA). All other chemicals were obtained from Sigma (St. Louis, MO). A stock solution of ChTX (10⁻⁴ M) was made up in a physiological level (0.5%). Chamber O₂ and CO₂ concentrations were continuously measured with an internal solution containing (in mM) 35 KCl, 90 potassium gluconate, 10 NaCl, and 10 HEPES, with the pH adjusted to 7.2 with 5 M KOH. GTP (0.5 mM) was added to provide substrate for signal transduction pathways. MgATP (5 mM) was included to inhibit ATP-activated K⁺ currents and provide substrate for energy-dependent processes. Because [Ca²⁺], may affect K⁺ channel activity (16), in some experiments, 10 mM 1,2-bis(o-aminophenoxethyl)ethane-N,N,N',N'-tetraacetic acid (BAPTA) and 3 mM Ca²⁺ were added to buffer the [Ca²⁺], to a physiological level (∼75 nM). Whole cell currents were recorded with an Axopatch 200A amplifier (Axon Instruments) in voltage-clamp mode; membrane potential was recorded in current-clamp mode. Pipette potential and capacitance and access resistance were electronically compensated for. Voltage-clamp protocols were applied with the use of pCLAMP software (Axon Instruments). Data were filtered at 5 kHz, digitized with a Digidata 1200 analog-to-digital converter (Axon Instruments), and analyzed with pCLAMP software. Cell capacitance was calculated from the area under the capacitative current elicited by a 10-mV hyperpolarizing pulse from a holding potential of −70 mV. Whole cell current was normalized to cell capacitance and is expressed as picoamperes per picofarad (pA/pF).

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in distilled water, divided into aliquots, and kept frozen at −20°C until used. 4-AP was made up daily as a stock solution (10⁻¹ M) in PSS, and the pH was adjusted to 7.4 with HCl. On the day of experiment, stock solutions were diluted as needed with PSS to appropriate concentrations.

Data Analysis
Amplitudes of currents are expressed as current density obtained by normalizing peak current with cell capacitance. To examine the inactivation kinetics of Kv currents, current was separated into inactivating and noninactivating components. The time course of inactivation of Kv current at +30 mV was fit with the exponential equation (22) \( I(t) = A_0 + A_1 e^{-t/\tau_1} \), where \( I(t) \) is the current at time \( t \), \( A_0 \) is the steady-state current, \( A_1 \) is the amplitude of the exponential, and \( \tau_1 \) is the time constant.

Statistical significance was determined with Student’s t-test (paired or unpaired as applicable). A value of \( P < 0.05 \) was accepted as significant. Data are expressed as means ± SE; \( n \) is the number of cells tested.

RESULTS
Effect of Chronic Hypoxia on Right Ventricular Hypertrophy and Hematocrit

The development of pulmonary hypertension was assessed by measuring RV hypertrophy in hypoxic mice. RV free wall and LV+S weights were similar in normoxic Hif1a(+/+) and Hif1a(−/−) mice (Table 1). In Hif1a(+/+) mice exposed to chronic hypoxia, RV wall weight was significantly increased, resulting in an increase in the RV-to-LV+S ratio, which was due entirely to the increase in RV weight because LV+S weight was not different between groups. The hypoxia-induced increase in RV weight was attenuated in Hif1a(+/−) mice, resulting in a lower RV-to-LV+S ratio in hypoxic Hif1a(+/−) than in Hif1a(+/+) mice. Exposure to hypoxia increased hematocrit in chronically hypoxic Hif1a(+/+) mice. Hif1a(+/+) mice still developed polycythemia during exposure to chronic hypoxia, although the increase in hematocrit was blunted compared with that observed in Hif1a(+/+) hypoxic mice. These results verify the development of pulmonary hypertension in our model and are consistent with those previously reported (30).

Effect of Chronic Hypoxia on Cell Capacitance

Cell capacitance was measured in single freshly isolated PASMCs under conditions of whole cell patch clamp. Average cell capacitance in PASMCs from normoxic Hif1a(+/+) and Hif1a(−/−) mice was 14.4 ± 2.2 nS (\( n = 8 \)) and 12.8 ± 3.2 (\( n = 7 \)) pA, respectively (Fig. 1). These values are similar to the value previously reported in rat PASMCs by our laboratory (22). Average cell capacitance increased significantly in PASMCs from Hif1a(+/+) mice exposed to chronic hypoxia, reaching a value of 23.6 ± 1.2 pA (\( n = 5 \)). Measurement of capacitance in PASMCs from Hif1a(+/−) mice exposed to chronic hypoxia showed no significant change in cell capacitance from values measured in normoxic mice (13.2 ± 0.8 pA; \( n = 8 \)), indicating that partial HIF-1α deficiency prevented the development of hypoxia-induced PASMC hypertrophy.

Identification of \( K^+ \) Currents in Mouse PASMCs
Application of step depolarizations from a holding potential of −60 mV to test potentials positive to −30 mV elicited outward whole cell currents. The whole cell \( K^+ \) current in PASMCs from normoxic animals could be separated into two main components (Fig. 2). One component was noisy, large, slowly activating at test potentials positive to +10 mV, and nonactivating. This current was not sensitive to 4-AP but could be inhibited by 100 nM ChTX. These characteristics are

<table>
<thead>
<tr>
<th>Hif1α genotype</th>
<th>RV Weight, g</th>
<th>LV+S Weight, g</th>
<th>RV/LV+S</th>
<th>Hct, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic Hif1a(+/+)</td>
<td>0.0264 ± 0.0011</td>
<td>0.1059 ± 0.005</td>
<td>0.249 ± 0.003</td>
<td>44.5 ± 1.6</td>
</tr>
<tr>
<td>Normoxic Hif1a(−/−)</td>
<td>0.0249 ± 0.0015</td>
<td>0.1020 ± 0.004</td>
<td>0.244 ± 0.004</td>
<td>42.5 ± 2.8</td>
</tr>
<tr>
<td>Hypoxic Hif1a(+/+)</td>
<td>0.0326 ± 0.0009</td>
<td>0.1038 ± 0.003</td>
<td>0.316 ± 0.017</td>
<td>65.7 ± 2.1</td>
</tr>
<tr>
<td>Hypoxic Hif1a(−/−)</td>
<td>0.0291 ± 0.0006</td>
<td>0.1061 ± 0.003</td>
<td>0.291 ± 0.006</td>
<td>61.0 ± 1.1</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n = 4 \) animals. CH, chronic hypoxia; RV, right ventricle; LV+S, left ventricle plus septum; RV/LV+S, ratio of RV weight to LV+S weight; Hct, hematocrit; Hif1α (+/+), wild-type; Hif1α(−/−), having one null allele at the Hif1α locus encoding hypoxia-inducible factor (HIF)-1α. *Significant difference from normoxic value; †Significant difference from hypoxic Hif1α(+/+) value.
quent experiments, Kv currents were studied in the presence of 100 nM ChTX to inhibit KCa currents. In PASMCs, resting membrane potential was measured in whole cell patch clamp in current-clamp mode. In PASMCs from normoxic mice, the average resting membrane potential was 1.16 ± 1.1 µA/pF at +30 mV; n = 6. This reflected a 54.2% reduction in peak Kv current density with exposure to chronic hypoxia and was similar to the magnitude of Kv current reduction previously reported in PASMCs from chronically hypoxic rats (23, 24). The Kv current in PASMCs from chronically hypoxic Hif1α(+/-) mice did not appear to exhibit time-dependent inactivation, and thus Kv current inactivation could only be fit to an exponential in one of five cells. Partial deficiency for HIF-1α appeared to prevent the chronic hypoxia-induced reduction in Kv current because peak Kv current density measured in PASMCs from Hif1α(+/-) mice exposed to chronic hypoxia was not different from that measured in PASMCs from normoxic mice (5.1 ± 1.16 µA/pF at +30 mV; n = 6). Furthermore, there was no significant change in Kv current inactivation kinetics in PASMCs from Hif1α(+/-) mice exposed to chronic hypoxia (Table 2).

**Effect of Chronic Hypoxia on Membrane Potential**

Resting membrane potential was measured in PASMCs with whole cell patch clamp in current-clamp mode. In PASMCs from normoxic mice, the average resting membrane potential was $-38.0 \pm 2.6$ mV in Hif1α(+/-) animals (n = 8) and $-36.9 \pm 2.4$ mV in Hif1α(+/-) animals (n = 8; Fig. 4). This is consistent with values previously reported in rat and human PASMCs (22, 31). Exposure to chronic hypoxia caused marked depolarization in PASMCs from Hif1α(+/-) mice, with an average resting membrane potential of $-11.5 \pm 2.6$ mV measured in these cells (n = 9). The hypoxia-induced depolarization was significantly attenuated but not completely prevented in PASMCs

Table 2. Effect of partial deficiency of HIF-1α and CH on Kv current amplitudes and time constant of inactivation

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>$A_0$</th>
<th>$A_1$</th>
<th>$\tau_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxic Hif1α(+/-)</td>
<td>7</td>
<td>42.5 ± 17.3</td>
<td>18.9 ± 6.6</td>
<td>36.4 ± 19.7</td>
</tr>
<tr>
<td>Normoxic Hif1α(+-)</td>
<td>8</td>
<td>86.0 ± 47.4</td>
<td>11.7 ± 3.1</td>
<td>36.7 ± 11.6</td>
</tr>
<tr>
<td>Hypoxic Hif1α(+-)</td>
<td>6</td>
<td>77.1 ± 35.4</td>
<td>22.4 ± 5.9</td>
<td>27.6 ± 12.6</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of cells. $A_0$, amplitude of nonactivating component; $A_1$, amplitude of inactivating component; $\tau_1$, time constant of inactivating component. Voltage-gated K+ (Kv) current in pulmonary arterial smooth muscle cells (PASMCs) isolated from hypoxic Hif1α(+/-) mice did not exhibit time-dependent inactivation.
A large body of data indicates that alterations in PASMC structure and function occur during the development of pulmonary hypertension. We demonstrated that PASMC size, as reflected in the measurement of cell capacitance, was increased in cells from chronically hypoxic Hif1a(+/-) mice, a response that was completely absent in Hif1a(+/+) mice. Although the molecular mechanisms underlying PASMC hypertrophy and proliferation are not clear, our data indicate that normal levels of HIF-1α are required for hypoxia-induced hypertrophy of pulmonary vascular smooth muscle. The absence of PASMC hypertrophy in hypoxic Hif1a(+/-) mice provides a basis for the attenuation of vascular remodeling that has been reported previously (30).

Exposure to chronic hypoxia resulted in a reduction in whole cell Kv current density in PASMCs from Hif1a(+/-) mice, similar to results previously described in rat models (23, 24). Reduction of Kv current density is also a feature of human pulmonary hypertension (32). Because Kv current was normalized to cell capacitance, the reduction in Kv current density in these cells could be due to the fact that PASMC size is increased in chronically hypoxic Hif1a(+/-) mice. For the observed reduction in Kv current density (54% at +30 mV), PASMC capacitance would have to increase by >100%. However, PASMC capacitance increased 63% in response to chronic hypoxia, suggesting that PASMC hypertrophy alone cannot account for the entire decrease in Kv current. Moreover, the Kv current inactivation kinetics are markedly different in PASMCs from chronically hypoxic Hif1a(+/-) and Hif1a(+/-) mice, providing further evidence that the reduction in Kv current density is not simply due to an increase in cell size. These data suggest that at least a portion of the decrease in current density is attribut-

Development of pulmonary hypertension during chronic hypoxia results from a reduction in vascular caliber due to both sustained constriction and remodeling. Pulmonary vasoconstriction and smooth muscle cell hypertrophy and hyperplasia are associated with elevated resting [Ca^{2+}]_i levels, a consequence of reduced Kv channel activity and depolarization. Using a murine model of hypoxic pulmonary hypertension, we found that partial deficiency for HIF-1α results in a significant attenuation of RV hypertrophy and polycythemia, results consistent with previous observations by our laboratory (30). We also found that hypoxia induced PASMC hypertrophy, reduction of Kv current, and depolarization in Hif1a(+/-) mice, effects that were reduced or absent in Hif1a(+/-) mice. These results delineate a critical role for HIF-1α in the pathophysiological response of PASMCs to hypoxia.
able to a reduction in Kv channel activity. The effect of chronic hypoxia on K_v current density observed in Hif1a (+/+) mice was absent in Hif1a (+/-) mice. Although one reason for the lack of reduction in K_v current density may be the prevention of PASMC hypertrophy in hypoxic Hif1a (+/-) mice, maintenance of normal K_v channel activity is also likely to contribute to the preservation of K_v current density, as supported by the fact that K_v current inactivation kinetics were not altered in these cells. These findings indicate that full expression of HIF-1α is required for the hypoxia-induced decrease in K_v current.

It is unclear whether reduced K_v channel activity in response to chronic hypoxia is due to a change in PASMC phenotype, altered regulation of the channels, decreased K_v channel expression, or a combination of these factors. Regulatory pathways involved in the inhibition of K^+ channels include association with β-subunits or phosphorylation by protein kinase C (PKC) (1, 3, 12, 14). Hypoxia alters PKC isoyme expression and subcellular distribution (6, 28). Exposure of cultured PASMCs to hypoxia decreases mRNA and protein expression of K_v α-subunits, whereas K_v β-subunit expression is unaffected (26). If these changes in protein expression occur in vivo, then the hypoxia-induced reduction in K_v current density could be explained by a decrease in K_v channel number in concert with increased association with inhibitory β-subunits.

Under normal conditions, the major regulator of resting membrane potential in PASMCs appears to be the K_v channel because inhibition of these channels causes membrane depolarization, activation of voltage-gated Ca^{2+} channels, and increased [Ca^{2+}]_i (22, 31). The observed reduction in K_v current density in PASMCs from Hif1a (+/+) mice after exposure to chronic hypoxia is consistent with the observed hypoxia-induced depolarization in these animals. The magnitude of depolarization in Hif1a (+/+) mice is similar to that previously reported in the rat and in humans (23, 24). Depolarization was significantly attenuated, although not completely prevented, in PASMCs from chronically hypoxic Hif1a (+/-) mice. This finding, coupled with the demonstration that the hypoxia-induced reduction in K_v current was completely eliminated in these mice, suggests that depolarization in response to chronic hypoxia is due to more than simply a change in K^+ channel conductance. Other ion channels can participate in the regulation of resting membrane potential, and a decrease in Cl^- conductance and/or an increase in Na^+ or Ca^{2+} conductance could also result in depolarization. Nonetheless, our data provide compelling evidence that partial deficiency of HIF-1α blunts hypoxia-induced changes in PASMC electrophysiology.

In summary, we have demonstrated that exposure to chronic hypoxia increased cell capacitance, reduced K_v current density, and depolarized PASMCs and that these effects were reduced or absent in PASMCs from mice partially deficient for HIF-1α. No other gene product has been shown to have such a profound effect on pulmonary vascular and, specifically, PASMC responses to chronic hypoxia. These striking results indicate that hypoxic induction of HIF-1α is required for both PASMC depolariization and hypertrophy, although whether these represent primary or secondary responses to chronic hypoxia is not clear. PASMC hypertrophy contributes to vascular remodeling through medial thickening of arterioles, whereas reduction of K_v current and depolarization may play a causal role in the regulation of cell function through control of [Ca^{2+}]_i, which is associated with cell contraction, proliferation, and gene expression. Our data indicate that HIF-1α plays a pivotal role in mediating both the vasoconstriction and vascular remodeling observed during the pathogenesis of hypoxic pulmonary hypertension, which contributes significantly to morbidity and mortality in patients with chronic lung disease.


