Endothelin-1 mediates hypoxia-induced inhibition of voltage-gated K⁺ channel expression in pulmonary arterial myocytes


Division of Pulmonary and Critical Care Medicine and Vascular Biology Program, Institute for Cell Engineering; and Departments of Medicine, Pediatrics, Oncology, and Radiation Oncology and McKusick-Nathans Institute of Genetic Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland

Submitted 8 March 2007; accepted in final form 28 November 2007

Hypoxia-induced inhibition of voltage-gated K⁺ (Kv) channels may contribute to the development of pulmonary hypertension by increasing intracellular calcium concentration ([Ca²⁺]i). The peptide endothelin-1 (ET-1) has been implicated in the development of pulmonary hypertension and acutely decreases Kv channel activity. ET-1 also activates several transcription factors, although whether ET-1 alters Kv channel expression is unclear. The hypoxic induction of ET-1 is regulated by the transcription factor hypoxia-inducible factor-1 (HIF-1), which we demonstrated to regulate hypoxia-induced decreases in Kv channel activity. In this study, we tested the hypothesis that HIF-1-dependent increases in ET-1 lead to decreased Kv channel expression and subsequent elevation in [Ca²⁺]i. Resting [Ca²⁺]i and Kv channel expression were measured in cells exposed to control (18% O₂, 5% CO₂) and hypoxic (4% O₂, 5% CO₂) conditions. Hypoxia caused a decrease in expression of Kv1.5 and Kv2.1 and a significant increase in resting [Ca²⁺]i. The increase in [Ca²⁺]i was reduced by nifedipine, an inhibitor of voltage-dependent calcium channels, and removal of extracellular calcium. Treatment with BQ-123, an ET-1 receptor inhibitor, prevented the hypoxia-induced decrease in Kv channel expression and blunted the hypoxia-induced increase in [Ca²⁺]i in PASMCs, whereas ET-1 mimicked the effects of hypoxia. Both hypoxia and overexpression of HIF-1 under normoxic conditions increased ET-1 expression. These results suggest that the inhibition of Kv channel expression and rise in [Ca²⁺]i during chronic hypoxia may be the result of HIF-1-dependent induction of ET-1.

hypoxygen-inducible factor-1; pulmonary hypertension

In patients with chronic lung disease, prolonged hypoxia causes contraction and proliferation of pulmonary arterial smooth muscle cells (PASMCs), leading to the development of pulmonary hypertension and increased mortality due to eventual right heart failure. Despite extensive study, the cellular mechanisms responsible for the pathogenesis of hypoxic pulmonary hypertension remain poorly understood, although increasing data suggest that an elevation in PASMC intracellular Ca²⁺ concentration ([Ca²⁺]i) may be involved (2, 31, 41, 57). Acutely, hypoxia causes a rapid and reversible increase in [Ca²⁺]i that can be blocked by inhibitors of voltage-dependent or store-operated Ca²⁺ channels (6, 11, 23, 33, 36, 38, 52, 56, 59). With prolonged hypoxia, [Ca²⁺]i remains elevated even after return to normoxic conditions (2, 31, 41, 57). This increase in [Ca²⁺]i requires Ca²⁺ influx and contributes to both contraction and proliferation of PASMCs, characteristic changes that occur during chronic hypoxia (CH) (25, 26, 28). In PASMCs, [Ca²⁺]i is regulated to a large extent by membrane potential (Em), with depolarization activating voltage-dependent Ca²⁺ channels (VDCC) and Ca²⁺ influx. Em in PASMCs is controlled primarily by the activity of voltage-gated K⁺ (Kv) channels, with Kv channel inhibition causing increased [Ca²⁺]i (1, 67). Kv channels are composed of both pore-forming α-subunits and regulatory β-subunits. The α-subunits are classified into several subfamilies, of which Kv1.2, Kv1.5, Kv2.1/9.3, and Kv3.1 are expressed in PASMCs and exhibit oxygen sensitivity (1, 5, 10, 31, 54, 58, 68). Exposure to hypoxia, either in vivo or in vitro for several hours to a few days, resulted in decreased Kv channel activity, which was directly correlated with downregulation of gene transcription and reduced protein expression of Kv1.2, Kv1.5, and Kv2.1 (1, 10, 31, 54). Kv1.5 and Kv2.1 were also downregulated in pulmonary arteries from chronically hypoxic rats (34, 58). Moreover, Kv2.1 has been shown to regulate basal Em (1), whereas gene transfer of Kv1.5 attenuated hypoxic pulmonary hypertension (32). Thus, these two channel subtypes exhibit O₂-dependent expression and play significant roles in PASMC function.

The mechanisms regulating Kv channel expression in PASMCs are not well understood. Endothelin-1 (ET-1), a potent endothelium-derived vasoconstrictor abundant in the pulmonary vasculature, has been implicated in the development of pulmonary hypertension. In the lung, ET-1 gene expression is increased by hypoxia (7, 21), and ET-1 receptor inhibitors attenuate hypoxia-induced increases in pulmonary arterial pressure (4, 21, 29), indicating that ET-1 plays a key role in the pathogenesis of hypoxic pulmonary hypertension (4, 13). We and others have previously demonstrated that acute application of ET-1 decreased Kv current activity in normoxic PASMCs (35, 42, 44). Whether ET-1 also alters Kv channel expression is unclear, but not unlikely, given that ET-1 can activate transcription factors that may be involved in the regulation of Kv channel expression (63, 66).
Hypoxic induction of ET-1 occurs primarily via activation of gene transcription by hypoxia-inducible factor-1 (HIF-1), a heterodimer consisting of HIF-1α and HIF-1β subunits, with HIF-1α conferring sensitivity and specificity for hypoxic induction. Homozygous knockout of the Hif1a locus, which encodes HIF-1α, was embryonic lethal, but studies comparing mice with one null allele at the Hif1a locus with their wild-type litter mates (Hif1a+/−) demonstrated a critical role for HIF-1 in the development of hypoxic pulmonary hypertension (Lippi, 2007). In a subsequent study, Kv channel activity was reduced in PASMCs from Hif1a−/− mice exposed to CH, whereas Kv currents in PASMCs from Hif1a+/− mice were unaltered (Lippi, 2008). Recent evidence indicates that HIF-1 regulates induction of Ca2+−activated K+ channels in tumors (Perez-Soler, 2004) and that abnormally elevated HIF-1α expression in the Fawn-hooded rat was associated with reduced Kv channel expression in pulmonary vascular smooth muscle (Cook, 2006).

Given that Kv1.5 and Kv2.1 were consistently reduced by hypoxia (Lippi, 2009), and that these channels play an important role in regulating PASMC function (1, 32), we chose these genes as targets to further explore the hypoxic regulation of Kv channels. We hypothesized that a hypoxia-induced increase in ET-1 levels, mediated by HIF-1, leads to decreased Kv channel expression and elevation of [Ca2+], in PASMCs and tested this hypothesis using gain-of-function and loss-of-function models.

**METHODS**

**Hypoxic exposure in vivo.** All procedures were approved by the Animal Care and Use Committee of The Johns Hopkins University School of Medicine. Male adult (250–350 g) Wistar rats or littermate Hif1a+/− and Hif1a−/− mice (>8 wk old) were exposed to normoxia or hypoxia (10% O2) for 21 days as previously described (Cook, 2009, 2011). The chamber was continuously flushed with a mixture of room air and 100% N2 to maintain 10 ± 0.5% O2 and low CO2 concentrations (<0.5%). Chamber O2 concentration was continuously monitored (PRO-OX; RCI Hudson, Anaheim, CA). Animals were exposed to room air for 10 min twice a week to clean the cages and replenish food and water supplies.

**Isolation of intrapulmonary arteries and culture of PASMCs.** Animals were anesthetized with pentobarbital sodium (130 mg/kg ip), and the heart and lungs were removed en bloc. Intrapulmonary arteries (200–600 μm outer diameter for rats; 200–400 μm outer diameter for mice) were dissected free from connective tissue in ice-cold HBSS containing (in mM): 130 NaCl, 5 KCl, 1.2 MgCl2, 10 HEPES, and 10 glucose with pH adjusted to 7.2 with 5 M NaOH. The arteries were opened and the lumen gently scraped to remove endothelial cells. The arteries were cut into 1–2 mm segments and placed in Ham’s F-12 medium with L-glutamine (Mediatech) supplemented with 4% FCS, 1% streptomycin, and 1% penicillin for 24–48 h. For preliminary experiments using a handheld oxygen monitor (PRO-OX; RCI Hudson, Anaheim, CA). Animals were exposed to a chamber was continuously flushed with a mixture of room air and 4% O2–5% CO2. The chamber and the nonhypoxic controls (18% O2–5% CO2) were placed in an incubator at 37°C for 60 h. The chamber was flushed before beginning exposure and again at 48 h. Initial experiments were performed using a handheld oxygen monitor (model 5577, Hudson RCI) to assure that the chamber was able to sustain the desired level of hypoxia for a minimum of 48 h.

**Measurement of [Ca2+]i.** [Ca2+]i was measured in rat PASMCs incubated with 5 μM fura-2 AM for 60 min at 37°C. Cells were placed in a laminar flow cell chamber perfused with physiological saline solution containing (in mM): 118.3 NaCl, 4.7 KCl, 1.2 MgSO4, 25 NaHCO3, 1.1 glucose, and 1.2 KH2PO4, gassed with 16% O2–5% CO2, and washed for 15 min at 37°C to remove extracellular dye and allow complete deesterification of cytosolic dye. Ratiometric measurement of fluorescence from the dyes was performed in a workstation based on a Nikon TES 100 Eclipse inverted microscope with epifluorescence attachments. The colлимated light beam from a xenon arc lamp was filtered by interference filters at 340 and 380 nm and focused onto the PASMCs under examination via a ×20 fluorescence objective (Super Fluor 20, Nikon). Light emitted from cells was returned through the objective and detected by a cooled charge-coupled device imaging camera. An electronic shutter was used to minimize photobleaching of dye. Protocols were executed and data collected online with InCyte software (Intracellular Imaging, Cincinnati, OH).

**RT-PCR.** Total RNA was isolated from PASMC endothelium-denuded pulmonary arteries via TRIzol extraction, dissolved in distilled pyrocatechol water, and stored at −80°C until use. Reverse transcription was performed using the First-Strand cDNA Synthesis kit (Invitrogen). RNA (4 μg) was reverse transcribed using random hexamers and incubated for 5 min at 90°C. The specific primers used for PCR were designed from sequences corresponding to the rat and mouse genes of interest (Table 1). PCR was performed using 3 μl of the first-strand cDNA mixture, and products were amplified by denaturing at 94°C for 1 min, annealing for 1.5 min at 60°C, and extending at 72°C for 2 min, for a total of 30 cycles. This was followed by a final extension at 72°C, for 10 min, to ensure complete product extension. The PCR products were electrophoresed through a 1% agarose gel and visualized by ethidium bromide staining.

**Immunoblot assays.** PASMCs were washed with PBS and scraped in cold lysis buffer containing (in mM): 25 HEPES, 1 DTT, and protease inhibitor cocktail (Complete tablets, Boehringer). Protein concentrations were determined by Bradford assay. For each sample, 10 μg of total protein was used and separated on 10% SDS-PAGE gels and transferred to nitrocellulose membranes. Membranes were blocked in 5% nonfat milk for 2 h and probed with primary antibodies to Kv1.5 or Kv2.1, as described previously (Cook, 2009). Membranes were then stripped and re-probed with a-actin. Densitometry was performed using NIH ImageJ software, and values obtained for the protein of interest were normalized to a-actin in the same lane to control for loading.

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

<table>
<thead>
<tr>
<th>Standard Names</th>
<th>Predicted Size, bp</th>
<th>Sense/Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat Kv1.5</td>
<td>267</td>
<td>5′-AGTTCCCGAGGGAGGACATGA3′-3′</td>
</tr>
<tr>
<td>(NM_012972)</td>
<td></td>
<td>5′-GCTTGCGTGTTCCTCTTCTGAG3′-3′</td>
</tr>
<tr>
<td>Rat Kv2.1</td>
<td>269</td>
<td>5′-GACACGATCGACATCTTCAAGG3′-3′</td>
</tr>
<tr>
<td>(NM_013186)</td>
<td></td>
<td>5′-CTAATTTGTCGACGACCCGCA3′-3′</td>
</tr>
<tr>
<td>Mouse Kv1.5</td>
<td>176</td>
<td>5′-CGCGAGGAGGACACAGTAAG3′-3′</td>
</tr>
<tr>
<td>(BC021787)</td>
<td></td>
<td>5′-GACGACGAGGACAGAAGTCG3′-3′</td>
</tr>
<tr>
<td>Mouse Kv2.1</td>
<td>189</td>
<td>5′-GACGAGATGACAGAAAAAAGG3′-3′</td>
</tr>
<tr>
<td>(BC0361501)</td>
<td></td>
<td>5′-AATGGTGGAGAGGACAATG3′-3′</td>
</tr>
<tr>
<td>b-actin</td>
<td>244</td>
<td>5′-GACATGCGAGGCTTGGGCT3′-3′</td>
</tr>
<tr>
<td>(NM_031144)</td>
<td></td>
<td>5′-GACATGCGAGGCTTGGGCT3′-3′</td>
</tr>
<tr>
<td>ET-1</td>
<td>231</td>
<td>5′-GGAGATGTTGACGACGATGAG3′-3′</td>
</tr>
<tr>
<td>(NM_012548)</td>
<td></td>
<td>5′-GAGGATGTTGACGACGATGAG3′-3′</td>
</tr>
<tr>
<td>VE-Cadherin</td>
<td>85</td>
<td>5′-GTTGCTGACTTCTGGGTTGAG3′-3′</td>
</tr>
<tr>
<td>(NM_001795)</td>
<td></td>
<td>5′-CTTGAGAGGATATGAGGAGT3′-3′</td>
</tr>
</tbody>
</table>

ET-1, endothelin-1.
Adenovirus infection. PASMCs were cultured to 80% confluence in Ham’s F-12 media. After 24 h in serum-free media. PASMCs were infected with replication-defective recombinant adenoviruses encoding either β-galactosidase (AdLacZ) or a constitutively active form of HIF-1α (AdCA.5), which contains mutations that inhibit degradation of the protein under nonhypoxic conditions (16) as previously described (57). PASMCs were inoculated with 50 plaque-forming units per cell and incubated for 48 h at 37°C under nonhypoxic conditions.

Measurement of ET-1. ET-1 levels in cell media were determined via a commercial ELISA (Assay Designs). Media was collected immediately following challenge and stored at −80°C until use. ET-1 concentrations are expressed in pg/ml and were calculated from a standard curve performed with each experiment using ET-1 peptide standard provided by the manufacturer.

Statistical analysis. Comparisons between groups were performed using Student’s t-test and ANOVA with Holm-Sidak post hoc test as appropriate. Ratios of protein of interest to α-actin were subjected to arcsin transformation before analysis. All experiments were performed at least three times using cells isolated from at least three different animals.

RESULTS

Effect of CH on K\textsubscript{\text{V}} channel expression in Hif1a\textsuperscript{+/+} and Hif1a\textsuperscript{+/−} mice. We previously observed a decrease in K\textsubscript{\text{V}} channel activity in PASMCs isolated from chronically hypoxic Hif1a\textsuperscript{+/−} mice (40) that was associated with an increase in resting [Ca\textsuperscript{2+}], (57). To determine whether the hypoxia-induced decrease in K\textsubscript{\text{V}} channel activity was associated with a reduction in K\textsubscript{\text{V}} channel expression, we measured K\textsubscript{\text{V}}1.5 and K\textsubscript{\text{V}}2.1 mRNA levels using RT-PCR. We found that exposure to CH decreased expression of both channels in endothelium-denuded pulmonary arteries isolated from Hif1a\textsuperscript{+/+} mice (Fig. 1). This reduction in K\textsubscript{\text{V}} channel expression was consistent with our findings in the chronically hypoxic rat model (58). We found that partial deficiency of HIF-1α had no effect on K\textsubscript{\text{V}}1.5 or K\textsubscript{\text{V}}2.1 gene expression under normoxic conditions; however, in contrast to the reduction in expression observed in Hif1a\textsuperscript{+/+} mice, there was no significant difference between K\textsubscript{\text{V}}1.5 and K\textsubscript{\text{V}}2.1 expression in pulmonary arteries isolated from normoxic and chronically hypoxic Hif1a\textsuperscript{+/−} mice. These results are in accordance with our previous findings that chronic hypoxia had no effect on K\textsubscript{\text{V}} currents and resting [Ca\textsuperscript{2+}]\textsubscript{i} in PASMCs from Hif1a\textsuperscript{+/−} mice (40, 57).

Effect of HIF-1 overexpression on K\textsubscript{\text{V}} channel expression. To confirm a role for HIF-1 in regulating K\textsubscript{\text{V}} channel gene expression, we employed a gain-of-function model using an adenoviral construct that contained a constitutively active form of HIF-1α (AdCA.5) and results in an increase in HIF-1α protein and HIF-1 DNA binding activity (16). In PASMCs isolated from normoxic rats and cultured under control conditions, overexpression of HIF-1 with the AdCA.5 virus resulted in a decrease in both K\textsubscript{\text{V}}1.5 and K\textsubscript{\text{V}}2.1 RNA and protein expression compared with PASMCs transfected with a control adenovirus containing LacZ (AdLacZ) (Fig. 2). The adenovirus constructs contained a green fluorescent protein (GFP)-tag that allowed us to visually confirm transfection efficiency; however, since GFP interferes with fura-2 measurements, we were unable to determine whether overexpression of HIF-1 also increased basal [Ca\textsuperscript{2+}]\textsubscript{i}.

K\textsubscript{\text{V}} channel expression and [Ca\textsuperscript{2+}]\textsubscript{i} in PASMCs exposed to hypoxia ex vivo. We further explored the mechanism(s) by which hypoxia regulated K\textsubscript{\text{V}} channel expression by exposing PASMCs to hypoxia ex vivo, which removes the influence of pressure and circulating factors. We found that exposure to 4% O\textsubscript{2} for 60 h caused a reduction in both K\textsubscript{\text{V}}1.5 and K\textsubscript{\text{V}}2.1 gene and protein expression (Fig. 2, C and D). These findings are consistent with previous results in cultured PASMCs (1, 31, 54) and chronically hypoxic animals (10, 34, 58). We next tested whether downregulation of K\textsubscript{\text{V}} channel expression in this model was associated with activation of VDCC and elevated resting [Ca\textsuperscript{2+}]\textsubscript{i}. We found that exposure to hypoxia ex vivo resulted in a significant increase in basal [Ca\textsuperscript{2+}]\textsubscript{i} from 120.3 ± 5.6 nM in cells cultured under control conditions (n = 190 cells) to 191.0 ± 9.5 nM in PASMCs cultured under hypoxic conditions (n = 141 cells; Fig. 3C). The magnitude of the increase in basal [Ca\textsuperscript{2+}]\textsubscript{i} was similar to that observed in PASMCs isolated from chronically hypoxic animals. Removal of extracellular Ca\textsuperscript{2+}, by perfusing the cells with Ca\textsuperscript{2+}-free Krebs solution containing 1 mM EGTA to chelate any residual Ca\textsuperscript{2+} (Fig. 3A), or blockade of VDCC with nifedipine (1 μM; Fig. 3B), had no effect on basal [Ca\textsuperscript{2+}]\textsubscript{i} in control PASMCs, but significantly reduced [Ca\textsuperscript{2+}]\textsubscript{i} in PASMCs cultured under hypoxic conditions.

Role of ET-1 in mediating hypoxia-induced changes in [Ca\textsuperscript{2+}]\textsubscript{i} and K\textsubscript{\text{V}} channel expression. ET-1 receptor inhibitors attenuated the development of hypoxic pulmonary vasocon-
stricture and hypoxic pulmonary hypertension and blunted the reduction in $K^+$ channel activity observed in PASMCs cultured for several weeks under hypoxic conditions (4, 21, 29, 30, 49). We found that pretreatment with BQ-123 (10 $\mu$M), an ET$_A$ receptor inhibitor, beginning 1 h before hypoxia, completely prevented the decrease in $K_V$ channel gene and protein expression in PASMCs exposed to hypoxia ex vivo (Fig. 4). On average, pretreatment with BQ-788 (10$^{-8}$ M), an ET$_B$ receptor inhibitor, also prevented the hypoxia-induced reduction in $K_V$ channel gene and protein expression, although the results were more variable than those observed with BQ-123, and in a few experiments reduction of RNA or protein was still observed, as can be seen in Fig. 4. We next tested whether BQ-123 and BQ-788 pretreatment could also attenuate the hypoxia-induced increase in $[Ca^{2+}]_i$. Treatment with BQ-123 or BQ-788 for 60 h under control conditions had no effect on basal $[Ca^{2+}]_i$ (120.3 $\pm$ 5.6 nM for control conditions; $n$ = 190 cells vs. 116.9 $\pm$ 5.4 nM for BQ-123-treated cells; $n$ = 72 cells and 110.4 $\pm$ 4.6 nM for BQ-788 treated cells; $n$ = 108 cells, Fig. 5). The hypoxia-induced increase in $[Ca^{2+}]_i$, observed in untreated cells was significantly attenuated in the presence of BQ-123 (191.0 $\pm$ 9.5 nM for untreated; $n$ = 141 cells vs. 135.6 $\pm$ 6.4 nM for BQ-123 treated; $n$ = 84 cells) or BQ-788 (129.1 $\pm$ nM; $n$ = 118 cells).

To determine whether exogenous ET-1 could mimic the effects of hypoxia, PASMCs were treated with ET-1 ($10^{-10}$ to $10^{-8}$ M) for 48 h under nonhypoxic conditions. At all concentrations, ET-1 caused a reduction in both $K_V$1.5 and $K_V$2.1 gene and protein expression (Fig. 6). Prolonged exposure to $10^{-8}$ M ET-1 also increased basal $[Ca^{2+}]_i$, from 113.6 $\pm$ 8.3 nM ($n$ = 84 cells) in control PASMCs to 176.5 $\pm$ 8.8 nM ($n$ = 80 cells) in PASMCs treated with ET-1. In these experiments, the media was changed (ET-1 removed) before the 1-h fura-2 loading period to eliminate acute effects of ET-1 on Ca$^{2+}$ mobilization. Pretreatment with BQ-123 ($10^{-5}$ M) prevented the ET-1-induced decrease in $K_V$1.5 and $K_V$2.1 gene and protein expression (Fig. 7, A and B). Similarly, BQ-123 blocked the increase in basal $[Ca^{2+}]_i$, in response to prolonged ET-1 exposure.

**ET-1 expression in PASMCs.** The fact that BQ-123 or BQ-788 was able to alter the effect of hypoxia in cultured PASMCs was intriguing since the primary source for ET-1 synthesis is thought to be endothelial cells (12, 19). Analysis of ET-1 levels in the media of PASMCs exposed to hypoxia showed a significant increase compared with PASMCs cultured for the same duration under control conditions (Fig. 8A). Control PASMCs exhibited substantial expression of preproendothelin-1 (ppET-1), the gene that encodes the inactive precursor of ET-1 (Fig. 8B). Control PASMCs exhibited significant expression of ppET-1, whereas exposure to hypoxia ex vivo (4% O$_2$, 60 h) increased ppET-1 expression. To determine whether our PASMC cultures were contaminated with endothelial cells, the presence of an endothelial cell-specific gene product, VE-cadherin, was determined in our samples. VE-cadherin was readily detected in rat microvascular endothelial cells; however, the same PCR conditions and primers failed to detect significant levels of VE-cadherin in PASMCs (Fig. 8C). Since hypoxic induction of ET-1 in endothelial cells has been shown to be regulated by HIF-1, we tested whether this was also the case in PASMCs. Similar to the effect of hypoxia, ppET-1 expression was greater in PASMCs in which HIF-1

---

Fig. 2. Effect of HIF-1a gain-of-function on $K_V$ expression. Analysis of $K_V$1.5 and $K_V$2.1 mRNA (A) and protein (B) expression in pulmonary arterial smooth muscle cells (PASMCs) transfected with an adenovirus encoding a constitutively active form of HIF-1a (AdCA.5) or a control adenovirus encoding $\beta$-galactosidase (AdLacZ). Bar graphs show $K_V$1.5 and $K_V$2.1 mRNA and protein expression normalized to $\beta$-actin (means $\pm$ SE; $n$ = 3 experiments each; *$P < 0.05$). C: Representative images showing PCR products for $K_V$1.5, $K_V$2.1, and $\beta$-actin in PASMCs cultured under control conditions (C, 18% O$_2$ for 60 h) or exposed to hypoxia (H, 4% O$_2$ for 60 h). Bar graphs represent means $\pm$ SE data from 3 experiments. D: Representative images illustrating the effect of hypoxia on $K_V$1.5 and $K_V$2.1 protein expression. Bar graphs represent means $\pm$ SE data from 3 experiments.
was overexpressed by infection with AdCA.5 compared with PASMCs infected with AdLacZ.

DISCUSSION

Although the effect of CH on Kᵥ channel activity and expression in PASMCs has been known for some time (1, 10, 31, 40, 43, 58), the mechanisms by which hypoxia modulated Kᵥ channel expression remained unclear. In this study, we found that the hypoxia-induced reduction in Kᵥ channel expression was mediated by a process requiring activation of HIF-1 and induction of ET-1. The effects of hypoxia, HIF-1, and ET-1 on Kᵥ channel expression correlated with changes in basal [Ca²⁺]ᵢ, suggesting that modulation of Kᵥ channel expression may affect Ca²⁺ homeostasis, and, as a result, PASMC contraction and growth.

We previously demonstrated that Kᵥ currents were reduced in PASMCs from chronically hypoxic Hif1a⁺/⁺ mice, but not Hif1a⁻/⁻, mice (40). Results from the current study suggest that this finding is likely due to reduced Kᵥ channel expression in chronically hypoxic Hif1a⁺/⁺ mice. That Kᵥ1.5 and Kᵥ2.1 expression was not decreased in chronically hypoxic Hif1a⁻/⁻ mice clearly demonstrated that hypoxic induction of HIF-1 was
required for downregulation of Kv channels, perhaps via direct regulation of gene expression by HIF-1. However, the possibility also existed that this finding was simply related to the decreased pulmonary arterial pressure measured in the chronically hypoxic Hif1a−/− mice (65). To further explore this possibility, we used an ex vivo model of hypoxia, which eliminated influences from changes in mechanical forces or other external influences. Downregulation of Kv channels was observed in PASMCs cultured under hypoxic conditions, consistent with previous reports (31, 54), confirming that the mechanism by which hypoxia reduced Kv channel expression was intrinsic to these cells. We next addressed whether induction of HIF-1 alone, in the absence of hypoxia, was sufficient to cause Kv channel gene repression and decreased protein expression. Overexpression of HIF-1α resulted in a downregulation of Kv1.5 and Kv2.1 expression that was qualitatively similar to that observed in response to hypoxia. This finding suggested that 1) induction of HIF-1 is sufficient to cause repression of Kv channel expression and 2) the effects of HIF-1 on Kv channel expression were not secondary to effects on pulmonary arterial pressure or circulating factors.

Very little is known regarding regulation of Kv channel expression. Although our results demonstrated a role for HIF-1 in regulating Kv channel expression, it was not clear that HIF-1 was directly repressing gene transcription. Indeed, there is evidence to suggest that this was unlikely to be the case. For example, the downregulation in gene expression was not readily observed until greater than 48 h of hypoxic exposure (data not shown). Since HIF-1 levels increase within minutes of a decrease in oxygen tension (14, 53), and the half-life of Kv channel mRNA is relatively short (50), direct gene repression by HIF-1 during hypoxia should be apparent at earlier time points. Thus, we searched for HIF-1-inducible products that might mediate Kv channel repression. ET-1, a peptide known...
to be involved in the pathogenesis of hypoxic pulmonary hypertension and whose expression is regulated by HIF-1 (4, 12), activates c-jun, which has been demonstrated repress Kv channel expression (63, 66). Thus, ET-1 provided a possible link between HIF-1 and Kv channel expression. This link was supported by recently published observations showing that Fawn-hooded rats, which develop idiopathic pulmonary hypertension and exhibit elevated ET-1 levels (46), had increased HIF-1, and decreased Kv channel, expression (3). In the current study, the role of ET-1 was tested by treating cells with an ETA or ETB receptor antagonist during hypoxic exposure. That ET receptor blockade prevented both the hypoxia-induced downregulation of Kv1.5 and Kv2.1 expression, as well as the increase in basal [Ca$^{2+}$)], suggests that hypoxic induction of ET-1 was required for these responses. Although both ETA and ETB receptors have been found to be expressed on pulmonary vascular smooth muscle, most studies find that separate ET receptor subtypes are coupled to separate pathways, both of which are required for the response. Further experiments will be required to determine whether this is indeed the case. We also found that an increase in ET-1 levels under nonhypoxic conditions was sufficient to mimic the effects of hypoxia on Kv channel expression and basal [Ca$^{2+}$]). As expected, similar to our results with hypoxia, the effects of ET-1 were prevented when ETA receptors were blocked.

It is widely held that the main source for ET-1 is the vascular endothelium (12, 18). However, we observed an effect of hypoxia in cultured PASMCs that could be blocked by ET-1 receptor antagonists, suggesting either contamination of the cultures with endothelial cells, or that our PASMCs are also a source for ET-1. The latter possibility is not unreasonable, since PASMCs have been shown to secrete ET-1 under a variety of conditions (24, 62, 64). We have performed extensive staining on our cell isolations and have shown that our cultures are greater than 95% PASMCs (55). Moreover, we were unable to detect VE-cadherin gene expression in our cultures using protocols that produced positive results in rat microvascular endothelial cells, suggesting minimal endothelial cell contamination. In contrast, in situ staining demonstrated that the genes encoding the precursor for ET-1, ppET-1, and the endothelin converting enzyme, are present in rat pulmonary arterial smooth muscle, and protein levels of both increased with hypoxic exposure (27). We found low levels of ppET-1 gene expression in PASMCs cultured under control conditions and substantial ET-1 in the media from these cells, suggesting that ET-1 can be produced in cultured PASMCs.}

![Fig. 8. A: bar graph illustrating means ± SE values for ET-1 measured in media collected from PASMCs under control and hypoxic conditions. B: representative image showing PCR products for preproendothelin-1 (ppET-1) and β-actin in rat PASMCs exposed to control (18% O2) or hypoxic (4% O2) conditions. Bar graphs are means ± SE for 3 experiments in cells from 3 animals. C: representative PCR images demonstrating that amplicons correlating the VE-cadherin (VE) were detected in rat pulmonary microvascular endothelial cells (RMVECs) but not PASMCs cultured under control or hypoxic conditions. D: representative images and bar graphs of average data showing ppET-1 and β-actin expression in PASMCs transplanted with AdCa.5 or AdLac-Z. *P < 0.05.]

![Fig. 9. Schematic illustrating proposed mechanisms involved in chronic hypoxia-induced modulation of Kv channel expression and consequent changes in PASMC function. Solid black lines represent established pathways; dashed gray lines represent possible pathways requiring further investigation. NCX, Na$^{+}$/Ca$^{2+}$ exchange; VDCC, voltage-dependent Ca$^{2+}$ channel; P$_{pe}$, pulmonary arterial pressure; KBR, KB-R7943; BPD, bepridil; I$_{Kv}$, Kv current.]

by 10.220.33.2 on November 26, 2017 http://ajplung.physiology.org/ Downloaded from
expression. Induction of ET-1 via HIF-1 has been demonstrated in human umbilical vein endothelial cells (12), and our data confirm that this is also the case in the pulmonary vasculature. Moreover, these findings are consistent with the possibility that PASMCs can produce ET-1 and suggest that in vivo, both endothelial cells and PASMCs may contribute to local elevated ET-1 levels during hypoxia. Indeed, this possibility is supported by data showing that reductions in K_v channel expression in response to in vivo hypoxia occur more rapidly than with ex vivo hypoxia (10), where ET-1 levels are likely to be lower due to culture conditions (i.e., dilution in the media) and the absence of endothelial cells.

Downregulation of K_v channel expression leads to depolarization in PASMCs. Since \( E_m \) is a major factor controlling \([Ca^{2+}]_i\), in these cells, the decrease in K_v channel gene expression is likely to influence cell function through modulation of \([Ca^{2+}]_i\). Indeed, cells exposed to hypoxia or isolated from chronically hypoxic animals exhibited elevated resting \([Ca^{2+}]_i\), which required Ca^{2+} influx from extracellular sources. Interventions that prevented the reduction in K_v channel expression, including partial deficiency of HIF-1 and treatment with BQ-123 or BQ-788, also prevented the increase in \([Ca^{2+}]_i\), whereas ET-1 mimicked the hypoxia-induced downregulation of K_v channel expression and the increase in \([Ca^{2+}]_i\). Unfortunately, due to the GFP-tag, we could not measure \([Ca^{2+}]_i\), in adenoviral infected cells. The exact mechanisms by which depolarization modulates \([Ca^{2+}]_i\), is still being explored. In cells exposed to acute (minutes to hours) or prolonged (several days) hypoxia ex vivo, the increase in \([Ca^{2+}]_i\) could be reversed by inhibitors of VDCC. However, in PASMCs from chronically hypoxic animals, blockade of these channels had little effect on \([Ca^{2+}]_i\), or tone (41). Rather, it appears that other \( E_m \)-dependent pathways (i.e., reverse mode Na^+/Ca^{2+} exchange) may participate. Indeed, consistent with this possibility, we have found that both a general Na^+/Ca^{2+} exchange inhibitor and an inhibitor specific for reverse mode Na^+/Ca^{2+} exchange (Ca^{2+}-entry mode), caused a decrease in basal \([Ca^{2+}]_i\), in PASMCs isolated from chronically hypoxic, but not normoxic, rats (45). While these data provide a possible functional link between K_v channel expression and cell function during CH (Fig. 9), further experiments will be required to determine the exact pathways involved.

In summary, our results indicate that during prolonged hypoxia, the inhibition of PASMC K_v channel expression may result from increased ET-1 levels secondary to induction of HIF-1. We have previously established that HIF-1 mediated development of hypoxic pulmonary hypertension via control of K_v channel activity, \( E_m \), PASMC hypertrophy, and vascular remodeling (40, 65), although the exact mechanisms involved in these responses were unresolved. In contrast, the role of ET-1 in the pathogenesis of pulmonary hypertension was presumed to be due largely to its contractile (60) or mitogenic (9) effects. The data from the current study provide an additional mechanism by which HIF-1 and ET-1 mediate the development of hypoxic pulmonary hypertension, through control of K_v channel expression.

REFERENCES


GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-51912, HL-67919, HL-67191, and HL-55338 and American Heart Association Grant AHA-0430037N.


46. Stelzer TJ, O'Brien RF, Yanagisawa M, Sakurai T, Sato K, Webb S,本文内容已被删除，无法下载。


