Hypoxia Inhibits Gene Expression of Voltage-gated K⁺ Channel α Subunits in Pulmonary Artery Smooth Muscle Cells

Jian Wang,* Magdalena Juhaszova,† Lewis J. Rubin,*‡ and Xiao-Jian Yuan*‡

*Department of Medicine, Division of Pulmonary and Critical Care Medicine; †Department of Physiology, University of Maryland School of Medicine, Baltimore, Maryland 21201

Abstract

Activity of voltage-gated K⁺ channels (Kᵥ) in pulmonary arterial smooth muscle cells (PASMC) is pivotal in controlling membrane potential, cytoplasmic free Ca²⁺ concentration ([Ca²⁺]ₜₒᵣᵢ₃), and pulmonary vasomotor tone. Acute hypoxia selectively inhibits Kᵥ channels, depolarizes PASMC, raises [Ca²⁺]ₜₒᵣᵢ₃, and causes pulmonary vasoconstriction and vascular remodeling. Prolonged hypoxia (24–60 h) decreased significantly the mRNA levels of Kᵥ channel α subunits, Kᵥ1.2 and Kᵥ1.5. Consistently, the protein levels of Kᵥ1.2 and Kᵥ1.5 were also decreased significantly by hypoxia (48–72 h). Nevertheless, hypoxia affected negligibly the mRNA levels of Kᵥ channel β subunits (Kᵥβ1, Kᵥβ2, and Kᵥβ3). The native Kᵥ channels are composed of pore-forming α and auxiliary β subunits. Assembly of Kᵥ β subunits with α subunits confers rapid inactivation on the slowly or non-inactivating delayed rectifier Kᵥ channels. Kᵥ β subunits also function as an open-channel blocker of Kᵥ channels. Thus, the diminished transcription and expression of Kᵥ α subunits may reduce the number of Kᵥ channels and decrease Kᵥ currents. Unchanged transcription of Kᵥ β subunits may increase the fraction of the Kᵥ channel α subunits that are associated with β subunits and further reduce the total Kᵥ currents. These data demonstrate a novel mechanism by which chronic hypoxia may cause pulmonary vasoconstriction and hypertension. (J. Clin. Invest. 1997. 100: 2347–2353.) Key words: Kᵥ1.2 • Kᵥ1.5 • α subunits • β subunits • reverse transcription-PCR • Western blotting

Introduction

In pulmonary arterial smooth muscle cells (PASMC), activity of voltage-gated K⁺ (Kᵥ) channels is an important determinant in controlling resting membrane potential (Eₘₑₚ) (1–3) which, in turn, regulates cytosolic free calcium concentration ([Ca²⁺]ₜₒᵣᵢ₃) because of the voltage dependence of sarcolemmal Ca²⁺ channels (1, 4). Elevation of [Ca²⁺]ₜₒᵣᵢ₃ in PASMC is a major trigger for pulmonary vasoconstriction (5) and vascular smooth muscle cell proliferation (leading to vascular remodeling) (6, 7).

Acute hypoxia (< 3 min) inhibits Kᵥ channels in PASMC (3, 8–10). The resultant decrease in Kᵥ currents (Iᵥ(Kᵥ)) depolarizes the myocytes, increases [Ca²⁺]ₜₒᵣᵢ₃, and causes pulmonary vasoconstriction (11–14). Chronic exposure to hypoxia (1–20 d) causes a steady increase in pulmonary arterial pressure that is significant by day 2 and maximized by day 20 (15). An early work by McMurtry et al. (16) indicates that the pressor response to acute hypoxia in lungs from chronically hypoxic rats is decreased significantly, while the response to vasoconstrictor agonists (angiotensin II, prostaglandin E₂, and norepinephrine) is augmented. They suggest that this reduced pressor responsiveness may result from abnormalities in the mechanism that couples acute hypoxia with contraction of the pulmonary vascular smooth muscle (16). Recently, reduced Iᵥ(Kᵥ) and associated membrane depolarization have been observed in PASMC isolated from chronically hypoxic rats (17, 18). These data imply that chronic hypoxia may interact directly with the coupling mechanism (e.g., the Kᵥ channels) by which acute hypoxia causes pulmonary vasoconstriction.

Kᵥ1.2 and Kᵥ1.5 are two Shaker-like Kᵥ channel α subunits cloned recently from smooth muscle cells (19, 20). Adda et al. (21) identified Kᵥ1.2 and Kᵥ1.5 in human airway smooth muscle cells. In rat PASMC, we have found recently that, in addition to expressing Kᵥ1.2 and Kᵥ1.5, the cells also express three Kᵥ channel β subunits (Kᵥβ1, Kᵥβ2, and Kᵥβ3) (22). Electrophysiological studies on the expressed Kᵥ1.2 and Kᵥ1.5 channels indicate that these channels are slowly or non-inactivating delayed rectifier Kᵥ channels and are sensitive to the Kᵥ channel blocker, 4-aminopyridine (19, 20, 23, 24). Activity of the 4-aminopyridine–sensitive Kᵥ channels in PASMC plays a critical role in regulating Eₘₑₚ and [Ca²⁺]ₜₒᵣᵢ₃ (1–4) and in initiating hypoxia-mediated membrane depolarization and vasoconstriction (3, 8–10, 17).

Kᵥ β subunits can bind specifically to the Shaker-like Kᵥ α subunits (Kᵥ1 subfamily) (25, 26) through a highly conserved region in the amino-terminal domains (amino-terminal A and B box) of α subunits (27). Association of Kᵥ channel β subunits with Kᵥ1.2 or Kᵥ1.5 alters profoundly the biophysical properties of the channels (26, 28, 29). Coexpression of Kᵥβ1 with Kᵥ1.2 or Kᵥ1.5 not only confers rapid inactivation on these slowly or non-inactivating delayed rectifier channels (26,
In this study, the effects of prolonged hypoxia (1–3 d) on the mRNA and protein levels of Kv channel α subunits (Kv1.2 and Kv1.5) and β subunits (Kvβ1, Kvβ2, and Kvβ3) were determined to test the hypothesis that transcriptional regulation of K+ channels by chronic hypoxia plays an important role in the development of pulmonary hypertension.

**Methods**

**Cell culture and treatment with hypoxia.** Primary cultured PASMC were obtained from rat intrapulmonary arteries (third or fourth division) and branches of the main pulmonary artery (second division). The methods used to dissociate the cells and to prepare the cultures were described previously (1). The cells, grown on 10-cm petri dishes, were fed twice a week with 10% fetal bovine DME (containing 5.5 mM glucose) and incubated in a humidified atmosphere containing 5% CO2, 28, 29, but also inhibits the activity of K+ channels as an open-channel blocker (30).

**Reverse transcription-PCR (RT-PCR).** Total RNA was prepared from the primary cultured PASMC by the acid guanidinium thiocyanate-phenol-chloroform extraction method (31). Isolated total RNA was dissolved in diethyl pyrocarbonate water at 1 μg/μl, and stored at −70°C. Reverse transcription (RT) was performed using the First-Strand cDNA Synthesis kit (Pharmacia Biotech, Piscataway, NJ). 3′-Actin Sense 5′-GCCTGGAGACTCTGCCTGAGTTCAGGGATG-3′ (J04731)* Antisense 5′-AGGACTATAGATCCTAAGGC-3′ (X76724)* Antisense 5′-ATAGCCTGGTGCCTGAGGAA-3′ (X76723)* Antisense 5′-AATGCTGTCGATCTCGTGGA-3′ (X76722)* Antisense 5′-CACGGTGGAAGGATATGCTC-3′ (X76721)* Antisense 5′-GACTCATCGTACTCCGTCT-3′

**Table 1. Characteristics of Primers and Conditions of RT-PCR**

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer</th>
<th>Sequence</th>
<th>Location</th>
<th>Fragment size</th>
<th>Number of cycles</th>
<th>Total [RNA] [μl]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kv1.2</td>
<td>Sense</td>
<td>5′-TACATGGGAGATACAGGAGG-3′</td>
<td>1953–1971</td>
<td>295</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>(J04731)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kv1.5</td>
<td>Antisense</td>
<td>5′-ATATCTCTGTGTCTAAAAATCA-3′</td>
<td>2228–2247</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(M27158)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kvβ1</td>
<td>Sense</td>
<td>5′-GGTGTAAGCAGGAGTGGCAGGCTAAGG-3′</td>
<td>1536–1565</td>
<td>1111</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>(X70662)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kvβ2</td>
<td>Antisense</td>
<td>5′-CTCGAGAAAGCACCGTACAG-3′</td>
<td>2617–2646</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(X76724)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kvβ3</td>
<td>Antisense</td>
<td>5′-AGGACTATAGATCCTAAGGC-3′</td>
<td>1521–1540</td>
<td>150</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>(X76723)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Sense</td>
<td>5′-GACTCATCGTACTCCGTCT-3′</td>
<td>2731–2750</td>
<td>244</td>
<td>25</td>
<td>3</td>
</tr>
<tr>
<td>(J00691)*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*GenBank accession numbers for the sequences used in designing the primers.

2848 Wang et al.
Proteins solubilized in SDS buffer were separated by SDS-PAGE. The 10% gels were calibrated with prestained protein molecular weight markers (Bio-Rad Laboratories, Richmond, CA). Proteins were then transferred to the Hybond-C extra nitrocellulose membrane (Amerham Corp., Arlington Heights, IL) as described (32). The efficiency of the transfer was verified by Ponceau-S staining. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20. The blots were then incubated with the affinity-purified polyclonal antibodies specific for K$_V$1.2 (1:300, Alomone Labs, Jerusalem, Israel), K$_V$1.5 (1:1000; Upstate Biotechnology Inc., Lake Placid, NY), and α-actin (1:1000; Boehringer Mannheim Biochemicals). The membranes were washed three times for 5 min each and incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG for 1 h, and an enhanced chemiluminescence detection system (ECL; Amersham Corp.) was used for detection of the bound antibody.

**Statistical analysis.** The composite data are expressed as means±SE. Statistical analyses were performed using paired Student’s t test. Differences were considered to be significant when $P<0.05$. 

**Results**

The quantity of PCR products for β-actin and K$_V$1.2 correlated linearly with the change of cycle numbers between 23 and 28 cycles, while 3.0 μg total RNA and 3.0 μl cDNA were used in RT-PCR (Fig. 1 A). With 25 cycles and 3.0 μg total RNA used for amplifying the messages in PCR, the change in cDNA level of β-actin and K$_V$1.3 between 1.5 and 5.0 μl correlated linearly with the amount of the PCR products (Fig. 1 B). When 3.0 μl cDNA and 25 cycles were used in PCR, the change in total RNA levels between 1.5 and 5.0 μg also correlated linearly with the quantity of the PCR products of β-actin, K$_V$1.2, and K$_V$1.3 (Fig. 1 C). These results indicate that the experimental protocol for RT-PCR used in this study (3 μg total RNA for RT, 3 μl cDNA and 25 cycles for PCR) was appropriate to quantify the mRNA levels of K$_V$ channels.

**Effects of hypoxia on mRNA levels of K$_V$ channel α subunits.** Total RNA was extracted from primary cultured rat PASMC incubated under normoxic (5% CO$_2$ in air, PO$_2$ = 130–140 Torr) and hypoxic (3% O$_2$/5% CO$_2$ in N$_2$, PO$_2$ = 25–35 Torr) conditions, respectively. After RT, the same amount of first-strand cDNA from each of the normoxic and hypoxic cells was used in PCR consisting of the specific primers for K$_V$ channels and β-actin. The gene transcription (mRNA levels) of K$_V$ channel α subunits (K$_V$1.2 and K$_V$1.5) and β subunits (K$_V$β1, K$_V$β2 and K$_V$β3) were examined, and the β-actin mRNA level was used as control.

The mRNA levels of K$_V$1.2 and K$_V$1.5 were decreased significantly by exposure to hypoxia in a time-dependent manner (Fig. 2, A and B). The inhibition of K$_V$1.2 and K$_V$1.5 mRNA appeared to start at 24 h (the shortest time tested) and continued to 60 h (the longest time tested) of hypoxia (Fig. 2, A and B, right).

**Effects of hypoxia on mRNA levels of K$_V$ channel β subunits.** In contrast to the inhibitory effect on K$_V$1.2 and K$_V$1.5, hypoxia affected negligibly the mRNA levels of K$_V$β1, K$_V$β2, and K$_V$β3 (Fig. 3). Actually, the mRNA level of K$_V$β1 was increased slightly during hypoxia (Fig. 3 A), although no significance was observed. These results suggest that the effect of hypoxia on K$_V$ α subunits (K$_V$1.2 and K$_V$1.5) differs from the effect on β subunits in rat PASMC. Hypoxia inhibits gene transcription of K$_V$ α subunits (K$_V$1.2 and K$_V$1.5), but had no effect on gene transcription of K$_V$ β subunits (K$_V$β1, K$_V$β2, and K$_V$β3).

**Effects of hypoxia on protein levels of K$_V$ channel α subunits.** To confirm that hypoxia-induced inhibition of K$_V$ channel α subunits (K$_V$1.2 and K$_V$1.5) transcription leads to decreased production of the channel proteins, immunoblotting was used to compare protein levels of the channels in PASMC incubated under normoxia and hypoxia. Consistent with the inhibitory effects on transcription of K$_V$1.2 and K$_V$1.5, hypoxia (48–72 h) reduced significantly the amounts of K$_V$1.2 and K$_V$1.5 channel proteins, while the protein level of α-actin was not changed significantly (Fig. 4).

**Discussion**

**Molecular characteristics of K$_V$ channel α and β subunits.** The K$_V$ channel is composed of four membrane-bound, pore-forming α subunits and four auxiliary, hydrophilic β subunits (24, 26, 28, 29). There are at least six subfamilies of K$_V$ channel α subunit genes that encode 18 K$_V$ channels: K$_V$1.1–1.7 (Shaker), K$_V$2.1–2.2 (Shab), K$_V$3.1–3.4 (Shaw), K$_V$4.1–4.3 (Shal), K$_V$5.1, and K$_V$6.1 (24). K$_V$ channel β subunits were cloned recently from brain (K$_V$β1.1–1.3, K$_V$β2, and K$_V$β3) and heart (K$_V$β1.1)
(25, 28, 29, 33). Association of Kv channel β with α subunits confers the fast A-type inactivation on the slowly or non-inactivating delayed rectifier Kv channels (e.g., Kv1.2 and Kv1.5) (25, 28). Association of Kv1.2 and Kv1.5 subunits with Kv2.1 and Kv2.2 subunits in brain Kv1 channels is restrictive to Kv1 family members; for example, Kvβ1 and Kvβ2 can be associated with Kv1.1, Kv1.2, Kv1.4, and Kv1.5, but not with Kv2.1 and Kv4.1 (25, 26). In addition to changing kinetic properties of Kv α subunits, Kv β subunits can also

![Diagram A](image1.png)

![Diagram B](image2.png)

![Diagram C](image3.png)

Figure 2. Effect of hypoxia on mRNA levels of Kv1 channel α subunits (Kv1.2 and Kv1.5) in PASMC. PCR-amplified products are displayed in agarose gels for Kv1.2 (295 bp, A), Kv1.5 (1,111 bp, B), and β-actin (244 bp, A and B), when the first-strand cDNAs, synthesized from total RNA extracted from PASMC incubated in normoxia (Nor) and hypoxia for 24 (H24), 36 (H36), 48 (H48), and 60 (H60) h, were amplified using the specific sense and antisense primers for β-actin and Kv1 channel α subunits (Kv1.2 and Kv1.5; see Table I). M, Marker. Right panels. Data that were normalized to the amount of β-actin are expressed as means ± SEM (experiments were repeated three to four times independently). ***P < 0.001 vs. normoxic controls (open bars).

Figure 3. Effect of hypoxia on mRNA levels of Kv1 channel β subunits (Kvβ1, Kvβ2, and Kvβ3) in PASMC. PCR-amplified products are displayed in agarose gels for Kvβ1 (150 bp, A), Kvβ2 (141 bp, B), Kvβ3 (178 bp, C), and β-actin (244 bp, A, B, and C), when the first-strand cDNAs, synthesized from total RNA extracted from PASMC incubated in normoxia (Nor) and hypoxia for 24 (H24), 36 (H36), 48 (H48), and 60 (H60) h, were amplified using the specific sense and antisense primers for β-actin and Kv1 channel β subunits (Kvβ1, Kvβ2, and Kvβ3; see Table I). M, Marker. Right panels. Data that were normalized to the amount of β-actin are expressed as means ± SEM (experiments were repeated three to four times independently).
block Kv channels and reduce K_v currents as an open-channel blocker (30). The regulatory interaction between α and β subunits and formation of heteromultimeric channels by different membrane-bound α and hydrophilic β subunits contribute significantly to the diversity of native Kv channels and their physiological properties (28, 29).

The time required for reaching a new steady state level of channel activity is dependent on the half-life (t_{1/2}) of the channel protein and mRNA. Na⁺ and Ca²⁺ channels are relatively very stable (t_{1/2} = 1–2 d). The endogenous Kv channels, however, turn over very rapidly, e.g., t_{1/2} for Kv1.5 channel protein and mRNA is 4 and 0.5 h, respectively (34). The very short half-life of Kv channels also suggests that the cells undergo rapid exchange of both channel proteins and mRNA under physiological conditions.

**Inhibitory effect of hypoxia on gene transcription of Kv channel α subunits.** Chronic hypoxia increases significantly pulmonary arterial pressure by eliciting pulmonary vasoconstriction and vasoconstruction (15). Pulmonary arterial pressure in animals placed in a hypobaric hypoxic chamber starts to rise at 24 h and is elevated significantly by 48 h of hypoxia. The pressor response is maximized at day 20 of hypoxia, whereas right ventricular hypertrophy occurs after 5 d of hypoxia (15). Thus, in this study, we examined the effect of 24–72 h of hypoxia on gene transcription and expression of Kv channels in PASMC. The data obtained from this study show that: (a) hypoxia (24–60 h) inhibited gene transcription of PASMC Kv α subunits (Kv1.2 and Kv1.5), (b) hypoxia (24–60 h) affected negligibly gene transcription of Kv β subunits (Kvβ1, Kvβ2, and Kvβ3) (the Kvβ1 mRNA level was increased slightly), and (c) hypoxia (48–72 h) reduced significantly expression of Kv1.2 and Kv1.5 channel proteins. The results suggest that prolonged hypoxia (~ 72 h) can alter activity of Kv channels by selectively inhibiting gene transcription of Kv channel α subunits.

Amplitude of single-channel K⁺ current is positively proportional to the channel conductance and the electrochemical driving force. Whole-cell K⁺ currents (I_{K}) are determined by the following equation:

\[ I_K = g_K \times N \times P_{\text{open}} \times (E_m - E_K) , \]

where g_K is the single-channel conductance, N is the total number of K⁺ channels, P_{\text{open}} is the steady state open probability of K⁺ channels, E_m is the membrane potential (−40 to −55 mV in PASMC), and E_K is the K⁺ equilibrium potential (about −85 mV). Transcriptional inhibition of Kv α subunits (Kv1.2 and Kv1.5) during hypoxia would lead to a decrease in the Kv channel gene products, thereby reducing the number of K_v channels. Unchanged transcription of Kv β subunits during hypoxia may increase the fraction of the Kv channel α subunits that are associated with β subunits (e.g., Kv1.5–Kvβ1). Decreased number of Kv channels, along with the blockade effect of Kv β subunits on Kv channel activity (30), would lead to reduction of I_{K}, which has been described in PASMC isolated from chronically hypoxic rats (17, 18). The consequent increase in [Ca²⁺]_{cyt} (due to Ca²⁺ influx through voltage-gated Ca²⁺ channels and Ca²⁺-induced Ca²⁺ release from intracellular stores) may play an important role in the development of pulmonary vasoconstriction and vascular remodeling (5–7).

**Possible mechanisms involved in hypoxia-induced inhibition of Kv channel transcription.** Reduced O₂ tension has a significant influence on gene regulation in a variety of tissues and cells. The cell signaling pathways by which hypoxia regul-
lates gene transcription and translation appear to be very complex (35, 36). In pulmonary vascular endothelial cells, hypoxia inhibits expression of nitric oxide synthase (eNOS) by suppressing the transcriptional rate of the eNOS gene and decreasing the half-life (t1/2) of the eNOS mRNA (37). In PASMC, hypoxia also decreases mRNA transcripts of ornithine decarboxylase and S-adenosylmethionine decarboxylase (38). Distinct action of various oxygen radicals generated by hemoproteins (e.g., cytochrome, or NADPH oxidoreductase) as a function of changes in O2 tension and cellular redox state may be involved in the down- or upregulation of gene transcription (35, 36, 39–41). The main molecular pathways appear to be the modification of O2-regulated transcriptional factors that subsequently turn on or off the target genes (36). It has been demonstrated recently that the hypoxia-inducible factor (HIF-1) plays an important role in inducing gene transcription of O2-dependent proteins, such as erythropoietin (41, 42). Chronic exposure to hypoxia (~3 d) increases HIF-1 DNA-binding activity in isolated rat lungs (43) and in cultured bovine PASMC (44). Whether HIF-1 is related to induction of an intermediate mediator that downregulates gene transcription of K+ channels during hypoxia has not yet been elucidated.

The precise cellular and molecular mechanisms through which hypoxia inhibits mRNA levels of K1,1.2 and K1,1.5 (e.g., whether it is due to decreased transcriptional rate or changes in mRNA stability) are unknown. It may be related to the cellular redox state, hemoprotein (e.g., cytochrome, or NADPH oxidoreductase) activity, and reactive oxygen intermediates (35–37, 39–42, 45). Hypoxia alters cellular redox status and inhibits the heme-containing proteins (1, 10, 39–42). Thus, it is reasonable to speculate that the hypoxia-induced inhibition of heme- and/or metal-containing enzymes may serve as an intermediate to downregulate gene transcription of K+ channels (39–42).

Summary and conclusions. The results from this study demonstrate that prolonged hypoxia downregulates gene transcription and expression of K1 channel α subunits (K1,1.2 and K1,1.5), but affects negligibly transcription of K0 channel β subunits (K0β1, K0β2, and K0β3) in rat PASMC. The consequent decrease in the number of K0 channels would lead to decreased K0 currents, due to a reduction in current availability (17), and depolarized membrane potential, which have been observed in PASMC isolated from chronically hypoxic animals (17, 18). The hypoxia-mediated transcriptional regulation of K0 channel genes in PASMC may play a causal role in the development of HPV and pulmonary hypertension during chronic hypoxia.

Acknowledgments

We thank A.M. Aldinger for technical assistance and Dr. S. Sigrid for providing primer sequences.

This work was supported by grants from the National Institutes of Health (HL54043 to X.-J. Yuan, and HL02659 to L.J. Rubin) and by the American Heart Association–Maryland Affiliate, Inc. (to X.-J. Yuan). X.-J. Yuan is a Parker B. Francis Fellow in Pulmonary Research and a recipient of the Giles F. Filley Memorial Award and the Research Career Enhancement Award from the American Physiological Society.

References

Hypoxia Inhibits Voltage-gated K⁺ Channel Gene Expression


