Chronic hypoxia inhibits K\textsubscript{v} channel gene expression in rat distal pulmonary artery

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Wang, Jian, Letitia Weigand, Wenqian Wang, J. T. Sylvester, and Larissa A. Shimoda. Chronic hypoxia inhibits K\textsubscript{v} channel gene expression in rat distal pulmonary artery. Am J Physiol Lung Cell Mol Physiol 288:L1049–L1058, 2005. First published January 21, 2005; doi:10.1152/ajplung.00379.2004.—In pulmonary arterial smooth muscle cells (PASMCs), voltage-gated K\textsuperscript{+} (K\textsubscript{v}) channels play an important role in regulating membrane potential, cytoplasmic free Ca\textsuperscript{2+} concentration, and pulmonary vasomotor tone. Previous studies demonstrated that exposure of rats to chronic hypoxia decreased K\textsubscript{v} channel function in PASMCs from distal pulmonary arteries (dPAs). To determine whether this decrease in function was due to decreased expression of K\textsubscript{v} channel proteins and which K\textsubscript{v}, proteins might be involved, we analyzed K\textsubscript{v} channel gene expression in intact, endothelium-denuded dPAs obtained from rats exposed to 10% O\textsubscript{2} for 3 wk. K\textsubscript{v.1}, K\textsubscript{v.2}, K\textsubscript{v.4}, K\textsubscript{v.5}, K\textsubscript{v.6}, K\textsubscript{v.1.6}, K\textsubscript{v.2.1}, K\textsubscript{v.3.1}, K\textsubscript{v.4.3}, and K\textsubscript{v.9.3} channel \(\alpha\)-subunits and K\textsubscript{v.1}, K\textsubscript{v.2}, and K\textsubscript{v.3} \(\beta\)-subunits were expressed in dPAs but did not alter gene or protein expression of these channels in aorta. Furthermore, chronic hypoxia did not alter the mRNA levels of \(\beta\)-subunits in dPAs. These results suggest that diminished transcription of K\textsubscript{v} \(\alpha\)-subunits may reduce the number of functional K\textsubscript{v} channels in dPAs during prolonged hypoxia, causing the decreased K\textsubscript{v} current previously observed in PASMCs and leading to pulmonary artery vasconstriction.

In pulmonary arterial smooth muscle cells (PASMCs), the activity of voltage-gated K\textsuperscript{+} (K\textsubscript{v}) channels plays an important role in regulating resting membrane potential (2, 29, 37), which, in turn, modulates the intracellular calcium concentration ([Ca\textsuperscript{2+}]) because of Ca\textsuperscript{2+} influx through sarcolemmal voltage-dependent Ca\textsuperscript{2+} channels. Intracellular Ca\textsuperscript{2+} levels play a key role in regulating smooth muscle function, and, in PASMCs, a rise in [Ca\textsuperscript{2+}], is required for both the hypoxic pulmonary vasoconstriction (7, 14, 23, 24) and smooth muscle growth and proliferation (12, 32) characteristic of hypoxic pulmonary hypertension.

Depolarization (31) and reduced K\textsubscript{v} channel activity (28, 30) observed in PASMCs following exposure to chronic hypoxia suggest alterations in K\textsuperscript{+} channel regulation or expression. Because these effects are observed for several hours after return to normoxic conditions and most adaptations to chronic hypoxia are accomplished by changes in gene expression, it is possible that the observed decrease in K\textsubscript{v} channel activity is mediated by transcriptional regulation. Few studies have sought to determine the effect of prolonged hypoxia on K\textsuperscript{+} channel expression. In PASMCs from normoxic animals cultured under hypoxic conditions for 72 h, mRNAs encoding K\textsubscript{v.1.1}, K\textsubscript{v.1.2}, K\textsubscript{v.1.5}, K\textsubscript{v.2.1}, K\textsubscript{v.4.3}, and K\textsubscript{v.9.3} channel \(\alpha\)-subunits decreased (9–19, 33), suggesting that hypoxia could repress K\textsuperscript{+} channel expression. However, the effect of short-term in vitro exposure to hypoxia in cultured cells may not accurately reflect the effects of chronic hypoxia on K\textsuperscript{+} channel expression in the intact animal, where the duration of exposure and level of hypoxia or changes in pulmonary arterial pressure and circulating factors may alter the response. In intact animals, the protein expression of K\textsubscript{v.2.1} and K\textsubscript{v.1.5} was shown to decrease in pulmonary arteries isolated from rats exposed to 2–3 wk of hypoxia (16, 20). The decrease in K\textsubscript{v.1.5} protein levels was found to be associated with a reduction in mRNA expression; however, the effect on K\textsubscript{v.2.1} gene expression was not examined. Although several other K\textsubscript{v} channel subtypes are expressed in distal pulmonary arteries (dPAs) (2, 6, 33), a detailed analysis of K\textsubscript{v} channel gene expression in intact dPAs from chronically hypoxic rats has been not performed. Thus, in this study, we determined whether exposure of rats to 10% O\textsubscript{2} for 3 wk altered dPA expression of regulatory \(\beta\)-subunits (K\textsubscript{v.1.1–1.3}) and several pore-forming \(\alpha\)-subunits of K\textsubscript{v} channel families known to exist in vascular smooth muscle, including Shaker (K\textsubscript{v.1.1–1.6}), Shab (K\textsubscript{v.2.1}), Shaw (K\textsubscript{v.3.1}), and Shal (K\textsubscript{v.4.3}) as well as the \(\alpha\)-subunit that assembles with Shab family members to form functional heteromeric channels.

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Fig. 1. Messenger RNAs for Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv3.1, Kv4.3, and Kv9.3 were expressed in rat distal pulmonary arteries (PA) and aorta (A and B, n = 3 experiments, each on pooled tissue from 3 different animals). Messenger RNA for Kv1.3 was not expressed in these tissues but was readily detected in rat brain (C, n = 3 experiments, each on pooled tissue from 3 different animals).

Table 1. Kv primer sequence for RT-PCR

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To determine whether the effects of chronic hypoxia on Kv channel expression were specific for pulmonary arteries, results obtained in pulmonary arteries were compared with those obtained in aorta.

**METHODS**

**Chronically hypoxic rats.** All procedures were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine. As previously described (28), male Wistar rats (250–300 g) were placed in a Plexiglas chamber and exposed to normobaric hypoxia for 21 days. The chamber was continuously flushed with a mixture of room air and N\textsubscript{2} (10 ± 0.5% O\textsubscript{2}). Chamber O\textsubscript{2} concentration was continuously monitored (PRO-OX 110 oxygen analyzer; BioSpherix, Redfield, NY). The rats were exposed to room air for 10 min twice a week to clean the cages and replenish food and water supplies. Normoxic controls were kept in room air.

**RT-PCR.** Total RNA was prepared from deendothelialized fourth-generation or smaller dPA (200–600 \(\mu\)m outer diameter) and aorta (tissue from 3 rats was pooled per sample) by TRIzol (GIBCO BRL) extraction. Isolated total RNA was dissolved in diethyl pyrocarbonate water and stored at \(-80°C\) until use. Reverse transcription was performed using the First-Strand cDNA Synthesis Kit (Pharmacia Biotech, Austin, TX) as previously described (34). Three micrograms of the total RNA was reverse transcribed using random hexamers [pd(N\textsubscript{6}) primer]. The reaction mixture was incubated for 1 h at 37°C and heated at 90°C for 5 min to inactivate the reverse transcriptase.

PCR was performed using a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA), as previously described (34). Three microliters of the first-strand cDNA reaction mixture was used in a 50-\(\mu\)l PCR reaction consisting of 200 \(\mu\)M each primer, 10 mM Tris·HCl, pH 8.3, 50 mM KCl, 2 mM MgCl\textsubscript{2}, 200 \(\mu\)M each dNTP, and 1 unit of Taq DNA polymerase. The sense and antisense PCR oligonucleotide primers (Table 1) chosen to amplify the cDNA were specifically designed from coding regions of various Kv channels as described previously (9, 19, 33).

Fig. 2. Messenger RNA levels of Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv1.6, and Kv2.1 in rat distal PA and aorta (Ao) isolated from normoxic and chronically hypoxic rats. Top: results from individual experiments illustrating the effect of chronic hypoxia on Kv channel and \(\beta\)-actin expression. Bottom: bar graphs represent mean Kv channel expression (normalized to \(\beta\)-actin). Exposure to chronic hypoxia decreased mRNA levels of Kv1.1, Kv1.2, Kv1.5, Kv1.6, and Kv2.1. N, normoxia; CH, chronic hypoxia. *Significant difference (\(P < 0.05\)) from normoxic value (\(n = 3\) experiments, each on pooled tissue from 3 different animals).
System were normalized to the OD values in the β-actin signals; the ratios are expressed as arbitrary units for quantitative comparison.

Protein isolation and measurement by Western blotting. Deendothelialized dPA and aorta were washed with PBS and homogenized using an Ultra-Turrax T-25 at 20,000 rpm in 0.5 ml of HEPES buffer containing 10 mM HEPES, 1 mM DTT, and 1 tablet of protease inhibitor (Complete tablets; Boehringer Mannheim Biochemicals, Indianapolis, IN). Protein concentrations were determined by BCA protein assay, using BSA as a standard. For each sample, 20 μg of total protein were separated by SDS-PAGE on 10% gels. Proteins were then transferred to a Hybond-C extra nitrocellulose membrane (Bio-Rad, Hercules, CA), and the efficiency of the transfer was verified by Ponceau S staining. Membranes were blocked with 5% nonfat dry milk and probed with the affinity-purified polyclonal antibodies specific for various Kv channels (Alomone Laboratories, Jerusalem, Israel and Upstate Biotechnology, Charlottesville, VA), followed by incubation with anti-rabbit or anti-mouse horseradish peroxidase-conjugated IgG for 1 h. Enhanced chemiluminescence (ECL; Amersham, Piscataway, NJ) was used for detection of the bound antibody. To determine the total amount of protein in each lane, membranes were also probed with, and all protein concentrations normalized to, β-actin (Sigma, St. Louis, MO).

Statistical analysis. Data are expressed as means ± SE; n is the number of experiments performed, as indicated in RESULTS and in the figure legends. Statistical comparisons were performed using Student’s t-test. Differences were considered to be significant when P < 0.05.

RESULTS

Effects of chronic hypoxia on mRNA expression of Kv channel α-subunits in rat dPA and aorta. Consistent with previous reports in cultured PASMCs (33), we found that mRNAs for Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv3.1, Kv4.3, and Kv9.3 were expressed in rat dPAs (Fig. 1A). Similarly, aorta also expressed mRNAs for Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv3.1, Kv4.3, and Kv9.3 (Fig. 1B). Messenger RNA for Kv1.3 was not expressed in these tissues but was readily detected in rat brain (Fig. 1C), indicating functional primers and PCR conditions for this gene. Exposure to chronic hypoxia significantly reduced the mRNA levels of Kv1.1 (−66.1 ± 5.9%), Kv1.2 (−42.5 ± 3.4%), Kv1.5 (−55.5 ± 2.8%), Kv1.6 (−34.3 ± 3.4%), Kv2.1 (−54.8 ± 6.3%), Kv4.3 (−39.2 ± 6.5%), and Kv9.3 (−37.1 ± 2.6%) in dPAs (Figs. 2 and 3); however, chronic hypoxia had no consistent effect on the mRNA level of Kv1.4 (−0.2 ± 23.6%) and Kv3.1 (3.6 ± 1.1%) (Figs. 2 and 3). The inhibitory effects of hypoxia on Kv channel α-subunits appear to be selective for pulmonary arteries, as hypoxia had no significant effect on expression of these channels in the aortas from the same animals (range: −2.1 ± 4.2 to +2.5 ± 4.5%).

Fig. 3. Messenger RNA levels of Kv3.1, Kv4.3, and Kv9.3 in rat distal PA and aorta. Left: PCR results from individual experiments. Right: bar graphs represent mean Kv subunit expression (normalized to β-actin). Exposure to chronic hypoxia decreased mRNA subunit expression of Kβ subunits. *Significant difference from normoxic value (n = 3 experiments from 3 different samples, P < 0.05).
Effects of chronic hypoxia on mRNA expression of Kv channel β-subunits in rat dPA. Three Kv β-subunits were identified in rat dPAs and aorta: Kv1.1, Kv1.2, and Kv1.3 (Fig. 4). Exposure to chronic hypoxia did not significantly alter the mRNA levels of any of the Kv β-subunits examined in rat dPAs (Kv1.1 = +5.7 ± 0.6%, Kv1.2 = +2.9 ± 0.2%, and Kv1.3 = −0.4 ± 5.0%) or aorta (Kv1.1 = +4.0 ± 0.8%, Kv1.2 = −3.5 ± 0.6%, and Kv1.3 = +3.4 ± 0.3%) (Fig. 4).

Differential effects of chronic hypoxia on protein expression of Kv channel α-subunits in rat dPA and aorta. Immunoblot analysis was used to confirm the protein expression of Kv α-subunits identified by RT-PCR in rat dPAs and aorta. In both pulmonary arteries and aorta, protein expression of Kv1.1, Kv1.2, Kv1.4, Kv1.5, Kv1.6, Kv2.1, Kv3.1, and Kv4.3 was observed (Figs. 5–8). Similar to results obtained with RT-PCR, protein expression of Kv1.3 was not observed in either dPA or aorta (data not shown). We could not test for Kv9.3 protein because an antibody was not available. Exposure to chronic hypoxia significantly reduced the protein levels of Kv1.1 (−52.8 ± 4.9%), Kv1.5 (−49.6 ± 6.6%), Kv1.6 (−39.2 ± 3.5%), Kv2.1 (−61.2 ± 9.7%), and Kv4.3 (−45.6 ± 7.9%) (Figs. 5, 6, and 8), whereas the protein expression of Kv1.2, Kv1.4, and Kv3.1 was not altered by chronic hypoxia (−8.9 ± 4.2, +5.1 ± 7.4, and +0.32 ± 2.9%, respectively) (Figs. 6 and 8). In contrast to the hypoxia-induced reduction in Kv channel protein levels observed in dPAs, exposure to chronic hypoxia had no effect on the expression of any of the Kv channel α-subunits in the aorta (Figs. 5–8).

DISCUSSION

We (26, 28) and others (2, 3, 9, 19, 20, 30) previously demonstrated that exposure to prolonged hypoxia decreased Kv channel activity in PASMCs. This decrease was associated with depolarization and increased [Ca2+]i in PASMCs and enhanced vasoreactivity in pulmonary arteries (9, 19, 20, 26–28, 30, 31). Based on results obtained in PASMCs cultured under hypoxic conditions (19, 33) and the effects of prolonged hypoxia on Kv1.2, Kv1.5, and Kv2.1 channel expression in intact animals (2, 9, 20), we hypothesized that a change in the expression of multiple Kv channel α- (pore-forming) or β- (regulatory) subunits could account for the reduction in Kv channel activity observed in our rat model of hypoxic pulmonary hypertension. In the present study, we found that exposure...
to chronic hypoxia decreased gene and protein expression of several $K_v$ channel subtypes in dPAs of rat. The effect of hypoxia was specific for pulmonary arteries, as similar decreases in $K_v$ channel expression were not observed in aortas isolated from the same animals. Furthermore, the inhibitory effect of hypoxia was restricted to the $K_v$ channel $\alpha$-subunits, as hypoxia negligibly affected the expression of the cytoplasmic regulatory $\beta$-subunits. Our results also provide a comprehensive exploration of the effects of chronic hypoxia on $K_v$ channel expression in intact rats and indicate that the reduction of PASMC $K_v$ channel activity previously described in this model (28, 30) is due to the pulmonary-specific downregulation of multiple $K_v$ channel $\alpha$-subunits in conjunction with maintained $\beta$-subunit expression.

$K_v$ channels are hetero- or homomeric tetramers composed of the pore-forming $\alpha$-subunits and the cytoplasmic regulatory $\beta$-subunits (11). Using RT-PCR and immunoblot analyses, investigators have identified a number of $K_v$ channel $\alpha$- and $\beta$-subunits expressed in pulmonary vascular smooth muscle (5, 38). We found that endothelium-denuded rat dPAs expressed $K_v$1.1, $K_v$1.2, $K_v$1.4, $K_v$1.5, $K_v$1.6, $K_v$2.1, $K_v$3.1, $K_v$4.3, and $K_v$9.3. This agrees with previously published reports in rat cultured PASMCs and freshly isolated pulmonary arteries (9, 19, 33, 38), although some studies find $K_v$1.3 and not $K_v$1.4 (3). The reason for this discrepancy is not clear, although given that those results were solely from immunoblot, perhaps antibody strength or specificity could contribute. Our finding of $K_v$1.4 mRNA was confirmed by immunoblot, and the lack of $K_v$1.3 mRNA expression was not due to primer or PCR errors, as $K_v$1.3 in brain was readily detected.

Of the $K_v$ channel subtypes expressed in pulmonary vascular smooth muscle, we found that the expressions of $K_v$1.1, $K_v$1.2, $K_v$1.5, $K_v$1.6, $K_v$2.1, $K_v$3.1, $K_v$4.3, and $K_v$9.3 were significantly depressed in dPAs from chronically hypoxic rats. These dPAs were denuded of endothelium by a process we have previously demonstrated to render these vessels unresponsive to endothelium-dependent vasodilators (27), indicating disruption of the endothelium. Logically, the downregulation of $K_v$ channel mRNA expression in rat pulmonary arterial smooth muscle during chronic hypoxia would be reflected as a decrease in protein expression. Indeed, this is the case for $K_v$1.1, $K_v$1.5, $K_v$1.6, $K_v$2.1, and $K_v$4.3, the protein expressions of which were

![immunoblots demonstrate protein expression for $K_v$1.1, $K_v$1.5, and $\alpha$-actin in distal PA and aorta from normoxic (Nor) and chronically hypoxic (Hyp) rats. Bottom: bar graphs represent mean protein expression of $K_v$1.1 and $K_v$1.5 (normalized to $\alpha$-actin). Both $K_v$1.1 and $K_v$1.5 protein levels decreased in distal PA from chronically hypoxic rats. Chronic hypoxia did not alter protein expression of $K_v$1.1 and $K_v$1.5 channels in aorta. *Significant difference ($P < 0.05$) from normoxic value ($n$ = 3–4 experiments, each on pooled tissue from 3–4 different animals).]
all decreased in dPAs isolated from chronically hypoxic rats. However, Kv1.2 presented a different scenario, in which protein did not decrease with reduced mRNA expression. This phenomenon is not uncommon as mRNA levels do not always reflect the exact amount of encoded protein. This being the case, protein expression data are more closely correlated with changes in the numbers of functional Kv channels. Unfortunately, we were unable to determine protein expression for Kv9.3 because an antibody was not available.

It would appear that the effect of chronic hypoxia on Kv channel expression is vessel specific. In contrast to the marked reduction in Kv channel mRNA and protein levels observed in dPAs, aortas from the same animals showed no alteration in either mRNA or protein expression of Kv channels. This finding is consistent with maintained expression of Kv channel expression in cultured mesenteric smooth muscle cells subjected to short-term hypoxia (19). The lack of effect of chronic hypoxia on Kv expression in aorta suggests that this response is not generalized and may be restricted to the pulmonary vasculature.

The decrease in Kv channel expression likely explains the reduced Kv channel currents observed in PASMC isolated from chronically hypoxic rats (28, 30). It is interesting that Kv1.4 expression was not altered by exposure to chronic hypoxia. Kv1.4 is typically characterized as a rapidly inactivating, or transient, current (13, 21). Examination of the inactivation kinetics of the whole cell Kv current in normoxic and chronically hypoxic PASMCs demonstrated a faster inactivation following exposure to chronic hypoxia with a greater proportion of the transient, rapidly inactivating current (28). The present data suggest that a decrease in the number of channels conducting non- or slowly inactivating currents, in concert with maintained expression of channels conducting rapidly inactivating currents, could contribute to the faster inactivation kinetics previously observed in PASMCs from chronically hypoxic rats (28, 30).

Another possibility accounting for faster inactivation of Kv currents in PASMCs isolated from chronically hypoxic rats is the lack of effect of chronic hypoxia on Kvβ1, Kvβ2, and Kvβ3 expression. Characterization of cloned Kv channels indicates...
that the native Shaker-related Kᵥ channels are most likely heteromultimers formed by both α- and β-subunits, with each β-subunit associating with an α-subunit (18). The Kᵥ channel β-subunits bind to the cytoplasmic side of the α-subunits (35), conferring rapid inactivation onto the non- or slowly inactivating Kᵥ channel α-subunits (e.g., Kᵥ1.2, Kᵥ1.4, and Kᵥ1.5) (22) and blocking the α-subunits as an open channel blocker (8). Thus, maintained Kᵥ β-subunit expression, coupled with reduced Kᵥ α-subunit expression, might allow for more interaction between Kᵥ α- and β-subunits and faster current inactivation. It is impossible to delineate at this point which of the two possibilities, maintained expression of Kᵥ1.4 or greater interaction between Kᵥ α- and β-subunits, is responsible for the change in current inactivation kinetics previously observed.

Little is known about the factors regulating Kᵥ channel gene transcription. The mechanism by which Kᵥ channels are downregulated by chronic hypoxia is unknown but is likely to involve modulation of a transcription factor. For example, hypoxia-inducible factor-1 (HIF-1) has been implicated in the regulation of an increasing number of genes, including vascular endothelial factor, endothelin-1, and nitric oxide synthase-2 (25). Indeed, the reduction in Kᵥ current magnitude observed in PASMCs isolated from chronically hypoxic mice was absent in mice partially deficient for HIF-1 (26), indicating a crucial role of this transcription factor in modulation of Kᵥ channel expression by hypoxia. Unfortunately, these data do not allow determination of whether HIF-1 directly mediates Kᵥ channel expression in PASMCs or influences expression via an intermediate factor. For example, in addition to HIF-1, accumulating evidence suggests that the c-fos and c-jun family of genes can also be induced by hypoxia (4) and may participate in regulation of Kᵥ channel gene expression. Data suggest that overexpression of c-jun significantly decreases whole cell Kᵥ current (36), making c-jun a suitable candidate for the transcriptional downregulation of Kᵥ channel expression during chronic hypoxia. Moreover, the c-jun transcription factor functionally cooperates with HIF-1, potentiating its transcriptional activity (1). Regardless of the exact mechanism of downregulation, decreased transcription of Kᵥ channels is likely to be key in the pathogenesis of chronic hypoxic pulmonary hypertension because the consequent reduction

Fig. 7. Exposure to chronic hypoxia had no effect on Kᵥ1.2 and Kᵥ1.4 protein levels in distal PA and aorta from normoxic and chronically hypoxic rats. Top: immunoblots demonstrate protein expression for Kᵥ1.2, Kᵥ1.4, and α-actin. Bottom: bar graphs represent mean protein expression of Kᵥ1.2 and Kᵥ1.4 (normalized to α-actin, n = 3–4 experiments, each on pooled tissue from 3–4 different animals).
in outward K+ current contributes to a sequence of events that ultimately leads to reduced vascular caliber and elevated pulmonary arterial pressure.

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Fig. 8. Top: immunoblots demonstrate protein expression for Kv 3.1, Kv 4.3, and α-actin in distal PA and aorta from normoxic and chronically hypoxic rats. Bottom: bar graphs represent mean protein expression of Kv 3.1 and Kv 4.3 (normalized to α-actin). Kv 4.3 protein levels decreased in PA, but not in aorta, from chronically hypoxic rats. *Significant difference (P < 0.05) from normoxic value (n = 3–4 experiments, each on pooled tissue from 3–4 different animals). Chronic hypoxia did not alter protein expression of Kv 3.1 channels in PA or aorta.


