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# Subacute hypoxia suppresses Kv3.4 channel expression and whole-cell $K^+$ currents through endogenous 15-hydroxyeicosatetraenoic acid in pulmonary arterial smooth muscle cells

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**Abstract**

We have previously reported that subacute hypoxia activates lung 15-lipoxygenase (15-LOX), which catalyzes arachidonic acid to produce 15-HETE, leading to constriction of neonatal rabbit pulmonary arteries. Subacute hypoxia suppresses Kv3.4 channel expression and results in an inhibition of whole-cell $K^+$ currents ($I_K$). Although the Kv channel inhibition is likely to be mediated through 15-HETE, direct evidence is still lacking. To reveal the role of the 15-LOX/15-HETE pathway in the hypoxia-induced down-regulation of Kv3.4 channel expression and inhibition of $I_K$, we performed studies using 15-LOX blockers, whole-cell patch-clamp, semi-quantitative PCR, ELISA and Western blot analysis. We found that Kv3.4 channel expression at the mRNA and protein levels was greatly up-regulated in pulmonary arterial smooth muscle cells after blockade of 15-LOX by CDC or NDGA. The 15-LOX blockade also partially restored $I_K$. In comparison, 15-HETE had a stronger effect than 12-HETE on the expression of Kv3.4 channels. 5-HETE had no noticeable effect on Kv3.4 channel expression. These data indicate that the 15-LOX pathway via its metabolite, 15-HETE, seems to play a role in the down-regulation of Kv3.4 expression and $I_K$ inhibition after subacute hypoxia.
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*Keywords:* Kv3.4; 15-lipoxygenase; 15-hydroxyeicosatetraenoic acid; Subacute hypoxia; Pulmonary arterial smooth muscle cells

## 1. Introduction

Subacute exposure to moderate hypoxia produces pulmonary vasoconstriction, which is essential for the ventilation perfusion balance in the pulmonary circulation. The subacute hypoxic response is unique to the pulmonary circulation, the mechanisms of which are still unclear. Previous studies have suggested that $K^+$ channels in pulmonary arterial smooth muscle cells (PASMCs) are inhibited by subacute hypoxia, leading to depolarization, an increase in $[Ca^{2+}]_i$ and constriction of pulmonary arteries (Post et al, 1992; Weir and Archer, 1995; Yuan, 1995; Archer et al, 1996; Osipenko et al, 1997). These $K^+$ channels are voltage-gated and sensitive to 4-aminopyridine (4-AP) (Yuan, 1995; Post et al., 1995; Archer et al., 1996). However, how the $K^+$ channels are inhibited after subacute hypoxia remains elusive. Both direct and indirect effects have been proposed for the channel inhibition. In the carotid body and pulmonary neuroepithelium, similar $K^+$ channels are inhibited by hypoxia directly, through molecular $O_2$ (Ganfornina and Lopez-Barneo, 1992). There is evidence suggesting that lipoxygenases (LOX) and their products play a role (Zhang et al, 2005) in the process.

We have previously found that subacute hypoxia up-regulates the activity of 15-lipoxygenase (15-LOX) in PASMCs (Zhang et al, 2005). The conversion of arachidonic acid to 15-hydroxyeicosatetrienoic acid (15-HETE), a product of 15-LOX, is enhanced in microsomal and cytosolic fractions of pulmonary

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arteries from subacute hypoxic neonatal rabbits (Zhang et al, 2005). Exogenous 15-HETE causes robust and concentration-dependent contractions of pulmonary arterial rings after subacute hypoxia, which could be blocked by inhibition of endogenous 15-HETE production with lipoxygenase inhibitor (Zhang et al, 2005). Furthermore, blockade of Kv channels by 4-AP inhibits the 15-HETE-induced constriction of pulmonary arterial rings in hypoxic rabbits and rats (Weissmann et al, 1998; Pfister et al, 1998; Zhu et al, 2003; Han et al, 2004). 15-HETE decreases Kv currents ($I_{K)}$) in freshly dispersed rabbit and rat PASMCs (Han et al, 2004) and raises $[Ca^{2+}]_i$ level in PASMCs (Zhang et al, 2005). We also have evidence that 15-HETE down-regulates Kv3.4 channel expression and inhibits $I_K$ (Li et al, 2006a,b). Although these studies indirectly suggest that subacute hypoxia suppresses Kv channels via a 15-HETE-mediated mechanism, direct evidence remains unavailable.

In the present study, the expression of Kv3.4 channels at mRNA and protein levels and functional $I_K$ were studied in PASMCs after blockade of 15-LOX. Our results suggest that subacute hypoxia down-regulates Kv3.4 channel expression and suppresses $I_K$ through endogenous 15-HETE. 15-HETE was found to be more potent than 5-HETE and 12-HETE in mediating hypoxia-induced down-regulation of Kv3.4 channel expression. These results fill a gap in defining how subacute hypoxia inhibits Kv channels leading to membrane depolarization and an increase in $[Ca^{2+}]_i$ level in PASMCs, and demonstrate the link between 15-LOX, 15-HETE formation, and pulmonary vasoconstriction after subacute hypoxia.

## 2. Materials and methods

### 2.1. Reagents and instruments

15-HETE, 5-HETE, 12-HETE, nitro-L-argininemethyl ester (L-NAME), cinnamyl 3, 4-dihydroxy-[alpha]-cyanocinnamate (CDC), nordihydro-guiairetic acid (NDGA) and 15(S)-HETE EIA Kit were all purchased from Cayman Chemical Company (Michigan, USA). Anti-potassium channel Kv3.4 antibody was purchased from Sigma (USA). RT-PCR kit was purchased from Invitrogen (California, USA). All other reagents were from common commercial sources.

### 2.2. Animals

All animal experiments complied fully with the Institutional Animal Care and Use Committee of Harbin Medical University. Wistar rats weighing 125–250 g were used in the study. The animals were housed in the Animal Research Center of Harbin Medical University, at a controlled ambient temperature of 22–24 °C with 50% relative humidity and a 12-h light–dark cycle. During experiments, the rats were anesthetized with 4% halothane. The chest was opened, the heart and lungs were removed *en bloc*, and pulmonary arteries were dissected under a stereo-microscope.

### 2.3. Dissociation and culture of PASMCs

Primary cultures of PASMCs were prepared from rat pulmonary arteries (Yuan et al, 1993). The isolated distal arterial rings were incubated in Hanks' balanced salt solution containing 1.5 mg/ml of collagenase for 20 min (Wang et al, 2005). After incubation, a thin layer of the adventitia was carefully stripped off with fine forceps and the endothelium was removed by gently scratching the intimal surface with a surgical blade. The smooth muscle was then digested with 1.0 mg/ml of collagenase and 0.5 mg/ml of elastase (Sigma) for 60 min at 37 °C. The cells were plated onto 25×25 mm cover slides (for patch-clamp experiments) or 10-cm petri dishes (for molecular biological experiments) in 20% fetal bovine serum (FBS)-containing DMEM and cultured in a humidified incubator with 5% $CO_2$ for 3–5 days at 37 °C. Before each experiment, the cells were incubated in 0.3% FBS-DMEM for 12 to 24 h to stop cell growth.

The purity of PASMCs in the primary cultures was confirmed by specific monoclonal antibody raised against smooth muscle α-actin. The total number of the primary cultured cells was estimated with membrane-permeable 4',6'-diamidino-2-phenylindole (DAPI, 5 μM; Molecular Probes) for nucleic acid stain. All the DAPI-stained cells were cross-reacted with SMC α-actin antibodies, indicating that the cells were pure PASMCs.

### 2.4. Experiment and groups

Quiescent (growth-arrested) PASMCs cultured in 0.3% FBS-DMEM were divided into ten groups. Group 1 (normoxia) was maintained in an incubator containing 5% $CO_2$ and 95% $O_2$. Group 2 (subacute hypoxia) was incubated with a gas mixture composed of 3% $O_2$, 5% $CO_2$, and 92% $N_2$ for 24 h. Groups 3 and 4 were treated with 1 μM 15-HETE under subacute hypoxic and normoxic conditions as positive controls. Groups 5 and 6 were subjected to inhibition of endogenous 15-HETE, in which the cells were treated with 15-LOX blocker, 5 μM CDC or 50 μM NDGA, respectively, followed by subacute hypoxia. Groups 7 and 8 were exposed to exogenous 15-HETE and subacute hypoxia, in which the cells were treated with 1 μM 15-HETE under subacute hypoxic conditions after blockade of endogenous 15-HETE with CDC and NDGA. Groups 9 and 10 were designed to examine the effects of 5-HETE and 12-HETE in subacute hypoxia. The PASMCs were incubated under either normoxic or subacute hypoxic conditions for 24 h before each experiment. For cells cultured under normoxic conditions, $pO_2$ in the culture medium was 140±5 mmHg and was retained at this level until the cells were harvested for measurement of Kv3.4 channel expression or studies of $I_K$. There were no significant changes in pH values in culture medium or in cell morphology during the 24-h incubation period in the hypoxic incubator.

### 2.5. Western blot procedures

Primary cultured PASMCs were gently washed twice in cold PBS, scraped into 0.3 ml lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/ml of phenylmethylsulfonyl fluoride, and 30 μl/ml of aprotinin), and incubated for 30 min on ice. The lysates were then sonicated and centrifuged at 16,099 *g* for 10 min, and the insoluble fraction was discarded. The protein concentrations in the supernatant were determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL,

USA) with bovine serum albumin (BSA) as standard. Fifty micrograms of protein were mixed and boiled in SDS-PAGE sample buffer for 5 min. The proteins fractionated by 10% SDS-PAGE were then transferred to nitrocellulose membranes by electroblotting in a Mini Trans-Blot cell transfer apparatus (Bio-Rad) under conditions recommended by the manufacturer. After incubation overnight at 4 °C in a blocking buffer (0.1% Tween 20 in PBS) containing 5% nonfat dry milk powder, the membranes were incubated with affinity-purified rabbit polyclonal antibodies specific for Kv3.4 (Sigma). The monoclonal antibody specific for smooth muscle β-actin (Sigma) was used as an internal control. The membranes were then washed and incubated with anti-rabbit horseradish peroxidase-conjugated IgG for 120 min at room temperature. The bound antibodies were detected with an enhanced chemiluminescence detection system (Amersham, USA).

### 2.6 RT-PCR

Sequences of rat Kv3.4 cDNA were obtained from the GenBank™ data base (Accession # X62841). Accordingly, sense primer, 5'-TGTGCGTGATGTGGTTCA-3', and antisense primer, 5'-GAATTCGCTCAGCATA ATAGAT-3', were designed for a 356-bp fragment. For β-actin control, sense primer, 5'-CCGTAAAGACC TCTATGCC AACA-3', and antisense primer, 5'-CGGACTCATCGTACTCCTGCT-3', were used to obtain a 500-bp PCR fragment.

Total RNA prepared from primary cultured PASMCs by the acid guanidinium thiocyanate-phenol-chloroform extraction method was reverse transcribed with the Superscript First-strand cDNA Synthesis kit (Invitrogen). The fidelity and specificity of the sense and antisense oligonucleotides were examined with the BLAST program. The PCR products were amplified in a DNA thermal cycler, followed by electrophoresis through a 1% agarose gel. The amplified cDNA bands were then visualized with GelStar staining. To quantify the PCR products, an invariant mRNA of β-actin was used as an internal control. The OD values for the channel signals, measured by a Kodak electrophoresis documentation system, were normalized to the OD values for the β-actin signals; the ratios are expressed as arbitrary units for quantitative comparison (Wang et al, 1997).

### 2.7. Patch-clamp methods

A drop of the cultured rat PASMCs was placed in a chamber on the stage of an inverted microscope. Experiments were performed at 24–25 °C. The external solution contained (in mmol/L): NaCl 135.0, KCl 4.7, $MgCl_2$ 1.0, $CaCl_2$ 2.0, HEPES 10.0 and glucose 10.0 at pH 7.4. The composition of the pipette solution was KCl 125.0, $MgCl_2$ 4.0, $Na_2ATP$ 5.0, ethylene glycol-bis (beta-aminoethyl ether)-N, N, N',N'-tetraacetic acid (EGTA) 10.0 and HEPES 10.0 at pH 7.2. Outward currents were generated by progressive voltage steps (10 mV increments, 400 ms duration) from a constant holding potential of −40 mV to 60 mV. Hypoxia was achieved by bubbling the reservoir that fed the perfusion chamber with 100% $N_2$, obtaining a final $pO_2$ level in the perfusion chamber of ~15 mmHg (Stefan et al, 2005). The peak current amplitude was determined in each cell as the average of 3 to 5 trials. The component of $I_K$ sensitive to 1 mM 4-AP was calculated and considered to be the Kv current. A set of 10 mV hyperpolarizing currents was used to subtract the leak currents. Currents are presented as a function of membrane potentials in standard current-voltage relationships by using the pClamp 6.0 software (Axon Instruments, Inc).

### 2.8. Measurement of 15-HETE levels

To estimate the amount of 15(S)-HETE, cells were grown for 24 h under hypoxic conditions with CDC and NDGA. At the end of the incubation period, cells and medium were collected to determine the protein concentrations in the supernatant by the Bradford method with BSA as standard. Endogenous 15-HETE was measured by using the 15(S)-HETE EIA Kit (Catalog No. 534721, Cayman). The results were analyzed with the data reduction software (Cayman).

### 2.9. Statistical analysis

All values are expressed as means ± S.E.M. The significance of mean values among the experimental groups was calculated by using two-tailed analyses of variance (ANOVA) followed by Dunnett's test. Differences were considered to be significant if $P \leq 0.05$.

## 3. Results

### 3.1. Effects of subacute hypoxia and exogenous 15-HETE on Kv3.4 channel expression in PASMCs

If the hypoxia-induced down-regulation of Kv3.4 channel expression is mediated through 15-HETE, 15-HETE alone should suppress Kv3.4 channel expression. To test this

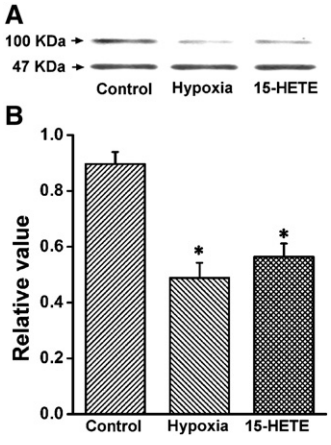


Fig. 1. Effects of 15-HETE on Kv3.4 channel expression in cultured rat PASMCs. A: Western blot analysis of Kv3.4 channels in PASMCs cultured under normoxia, hypoxia or with 1 μm/l 15-HETE. B: Summarized data showing the protein levels of Kv3.4 channels in PASMCs cultured under normoxia, hypoxia or with 1 μm/l 15-HETE. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$.

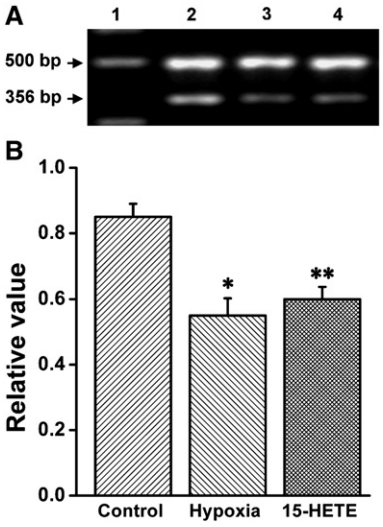


Fig. 2. Effects of 15-HETE on the expression of Kv3.4 channel mRNA in cultured rat PASMCs. A: RT-PCR analysis of Kv3.4 channels in PASMCs cultured under normoxia, hypoxia or with 1 μm/l 15-HETE. B: Summarized data showing the mRNA levels of Kv3.4 channels in PASMCs cultured under normoxia, hypoxia or with 1 μm/l 15-HETE. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$.

hypothesis, Western blot analysis and RT-PCR techniques were used to examine the effect of exogenous 15-HETE on Kv3.4 channel expression in PASMCs. The results show that subacute hypoxia produced a significant inhibition of Kv3.4 channel expression at the protein and mRNA levels in comparison with that of the normoxic control group (Figs. 1 and 2). Similar results were observed with exogenous 15-HETE under normoxic conditions.

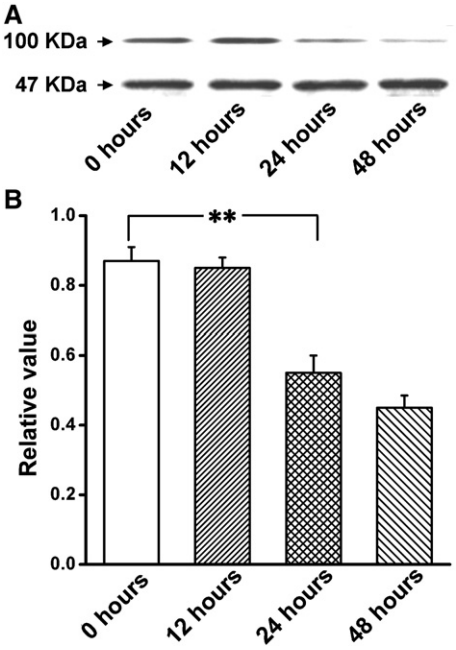


Fig. 3. Time course of the expression of Kv3.4 channel protein in cultured rat PASMCs exposed to hypoxia. A: Western blot of Kv3.4 channel expression in PASMCs. B: Densitometric analysis of the Western blot. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$.

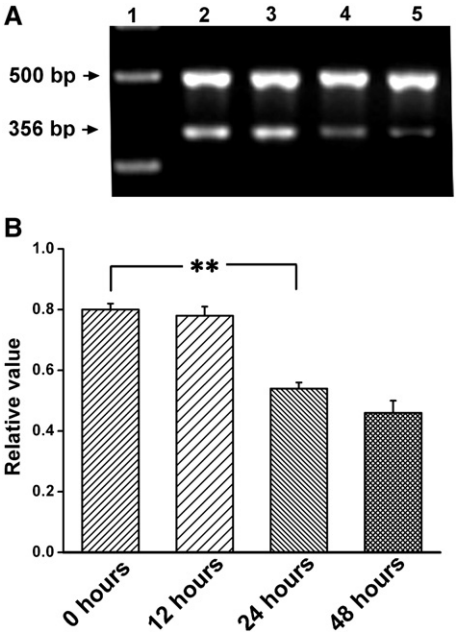


Fig. 4. Time course of the expression of Kv3.4 channel mRNA in cultured rat PASMCs exposed to hypoxia. A: RT-PCR of Kv3.4 channel mRNA expression in PASMCs. B: Densitometric analysis of the RT-PCR. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$.

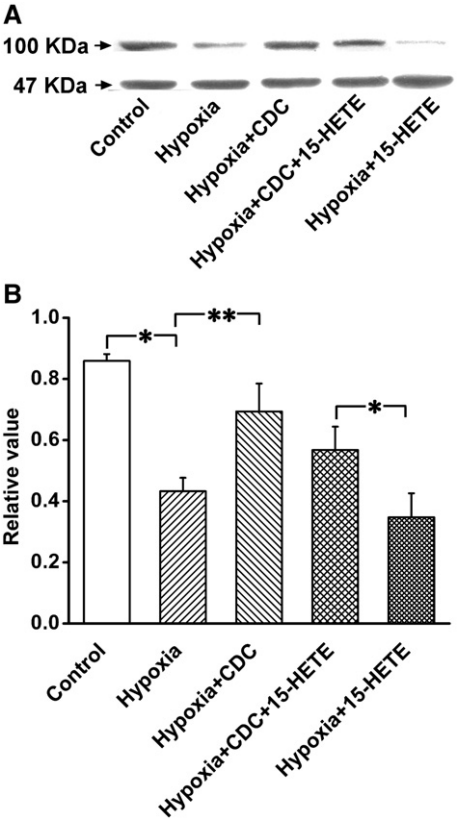


Fig. 5. Hypoxia-induced down-regulation of Kv3.4 channel expression in cultured rat PASMCs was partly restored by 15-LOX inhibitor CDC (5 μM) under subacute hypoxia. A: Western blot of Kv3.4 channel expression in PASMCs. B: Densitometric analysis of the Western blot. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$.

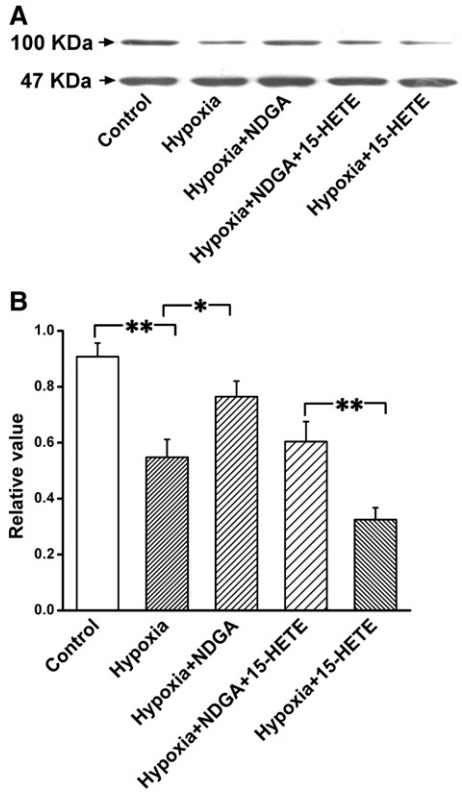


Fig. 6. Hypoxia-induced down-regulation of Kv3.4 channel expression in cultured rat PASMCs was partly restored by 15-LOX inhibitor NGDA (50 μM) under subacute hypoxia. A: Western blot of Kv3.4 channel expression in PASMCs. B: Densitometric analysis of the Western blot. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$.

### 3.2. Time-dependent changes in the expression of Kv3.4 channels after subacute hypoxia

The time-dependent changes in the expression of Kv3.4 channel protein and mRNA were studied by using Western blot and RT-PCR analysis in rat PASMCs cultured under subacute hypoxic conditions. We found that the expression of Kv3.4 channel protein decreased after 24 h of exposure to hypoxia (Fig. 3). No significant change in expression was found within 12 h of exposure to hypoxia. Similarly, the mRNA levels of Kv3.4 were also decreased after 24 h of exposure to moderate hypoxia (Fig. 4).

### 3.3. Alleviation of the hypoxia-induced Kv3.4 channel down-regulation with 15-LOX blockade

To elucidate whether 15-LOX plays a role in the hypoxia-induced inhibition of Kv3.4 channel expression, we suppressed 15-LOX activity by using 15-LOX blockers. Inhibition of endogenous 15-HETE with 5 μM CDC relieved the inhibition of Kv3.4 channel down-regulation caused by subacute hypoxia ($P<0.01$ with CDC, $n=4$; in comparison with hypoxia alone). Similar results were obtained with another 15-LOX inhibitor NDGA (50 μM) ($P<0.05$, $n=4$, Fig. 5).

If the alleviation of hypoxia-induced Kv3.4 channel down-regulation is mediated through 15-HETE, the effect should be diminished or blocked with addition of exogenous 15-HETE. To test this possibility, 1 μM 15-HETE was added to the cultured PASMC, and the expression of Kv3.4 channel was examined with or without 15-LOX blockers. The alleviation of hypoxia-induced Kv3.4 channel inhibition by 5 μM CDC or 50 μM NDGA, respectively, was significantly attenuated in the presence of exogenous 15-HETE (Figs. 5 and 6). Without inhibition of endogenous 15-HETE formation, subacute hypoxia produced an even greater inhibitions of the expression of Kv3.4 channel in the presence of exogenous 15-HETE (Figs. 5 and 6), suggesting that both endogenous and exogenous 15-HETEs have inhibitory effects on Kv3.4 channel expression.

### 3.4. The effect of 15-LOX blockade on the expression of Kv3.4 channel mRNA

Subacute hypoxia significantly inhibited the expression of Kv3.4 channels mRNA in cultured rat PASMCs compared with the effect of normoxia ($P<0.05$, $n=4$). Similar to protein expression, the expression of Kv3.4 channel mRNA was partly restored after inhibition of endogenous 15-HETE by CDC or

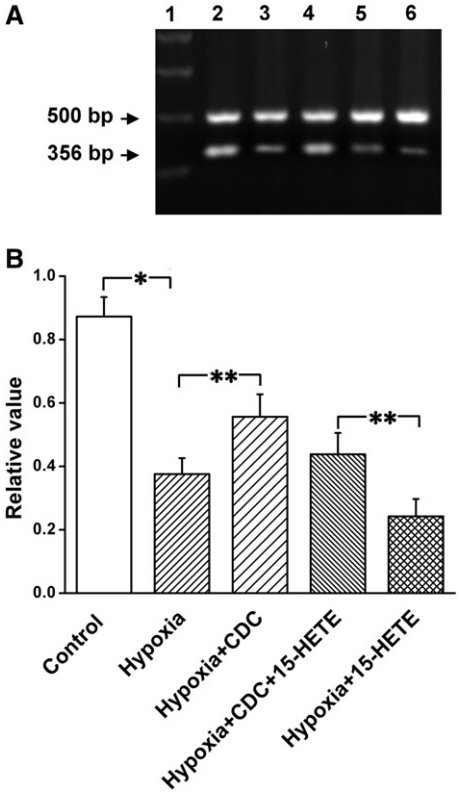


Fig. 7. Hypoxia-induced down-regulation of Kv3.4 channel mRNA expression in cultured rat PASMCs was partly reversed by 15-LOX inhibitor CDC (5 μM) under subacute hypoxia. A: The PCR-amplified products displayed in agarose gels stained with ethidium bromide for Kv3.4 (356 bp) and β-actin (500 bp) transcripts. Lane 1, marker; Lane 2, control; Lane 3, subacute hypoxia; Lane 4, subacute hypoxia+CDC; Lane 5, subacute hypoxia+CDC+15-HETE; Lane 6, subacute hypoxia+15-HETE. B: Summarized data normalized to the ratio of Kv3.4 and β-actin in PASMCs ($n=4$, $^{**}P<0.01$, $^{*}P<0.05$).

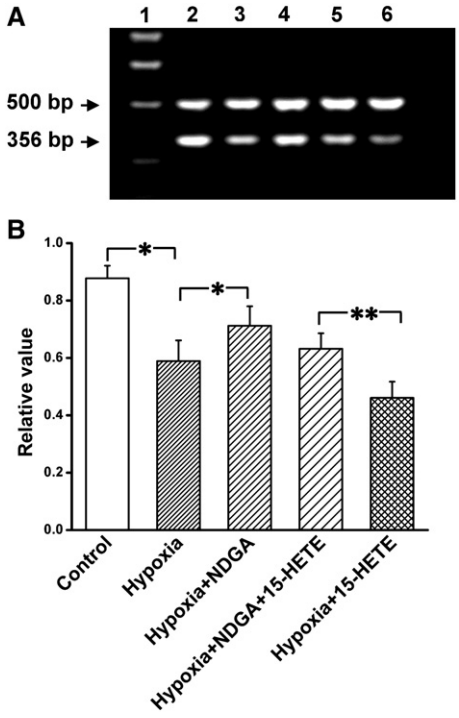


Fig. 8. Hypoxia-induced down-regulation of Kv3.4 channel mRNA expression in cultured rat PASMCs was partly reversed by 15-LOX inhibitor NGDA (50 μM) under subacute hypoxic conditions. A: The PCR-amplified products displayed in agarose gels stained with ethidium bromide for Kv3.4 (356 bp) and β-actin (500 bp) transcripts. Lane 1, marker; Lane 2, control; Lane 3, subacute hypoxia; Lane 4, subacute hypoxia+NDGA; Lane 5, subacute hypoxia+NDGA+15-HETE; Lane 6, subacute hypoxia+15-HETE. B: Summarized data normalized to the ratio of Kv3.4 and β-actin in PASMCs ($n=4$, $^{**}P<0.01$, $^{*}P<0.05$).

NDGA (Figs. 7 and 8, $P<0.01$ with CDC and $P<0.05$ with NDGA, vs hypoxia, respectively, $n=4$). The effects of CDC and NDGA were significantly attenuated in the presence of exogenous 15-HETE during subacute hypoxia. The inhibitors increased the expression of Kv3.4 channel mRNA in comparison with the effect of exogenous 15-HETE if endogenous 15-HETE formation was not inhibited under subacute hypoxic conditions (Figs. 7 and 8, $P<0.01$ with CDC or NDGA, vs exogenous 15-HETE alone, respectively, $n=4$). These data imply that the down-regulation of Kv3.4 channel mRNA as a result of subacute hypoxia is mediated through 15-HETE.

### 3.5. The 15-LOX blockade also partly restored the whole-cell $K^+$ currents

Subacute hypoxia significantly inhibited $I_K$ (Fig. 9B and H, $P<0.01$, $n=4$). However, after inhibition of endogenous 15-HETE formation by CDC or NDGA, $I_K$ was nearly completely restored in comparison with the effect of subacute hypoxia without CDC or NDGA (Fig. 9C and E, $P<0.05$, $n=4$). Furthermore, pretreatment of PASMCs with 5 μM CDC or 50 μM NDGA markedly augmented the $I_K$ in response to exogenous 15-HETE (Fig. 9G, D and F), suggesting that the 15-LOX/15-HETE signaling pathway contributes to $I_K$ inhibition after subacute hypoxia.

### 3.6. Effects of other HETEs isoforms

The specificity of 15-HETE in causing the down-regulation of Kv3.4 channel expression in cultured rat PASMCs was examined by using both exogenous 5-HETE and 12-HETE. Under subacute hypoxic conditions, 12-HETE inhibited the

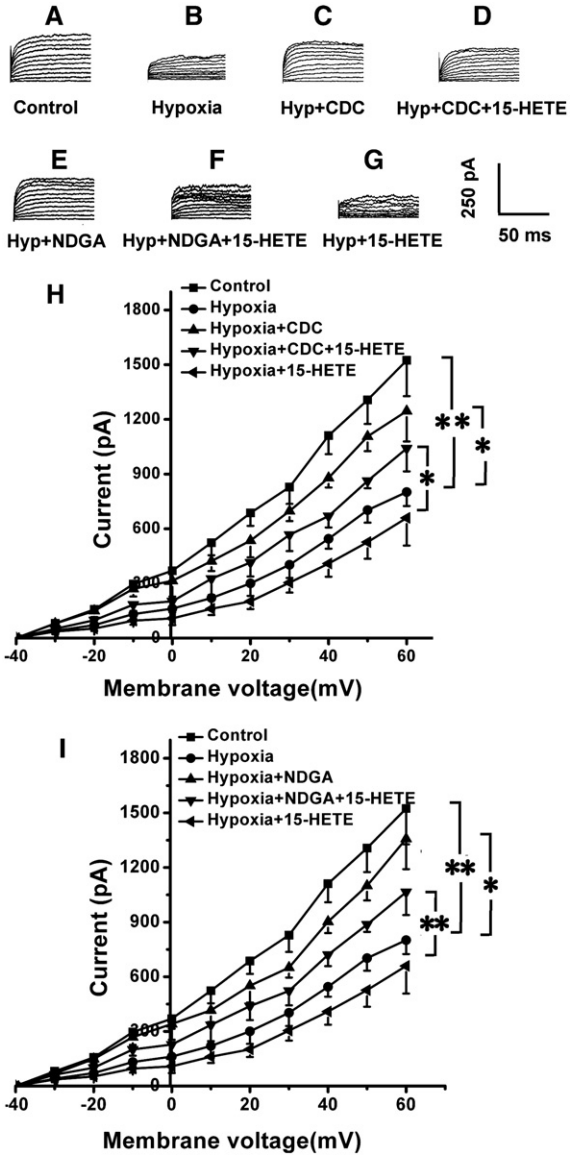


Fig. 9. The effect of endogenous 15-HETE on $I_K$ in cultured rat PASMCs under subacute hypoxia. A: Currents were elicited by depolarizing the cells to a series of test potentials ranging from −40 to +60 mV in 10 mV increments. B: Currents were inhibited by subacute hypoxia. C: Currents were restored by 5 μM CDC under subacute hypoxia. D: Currents were partly inhibited by 1 μM exogenous 15-HETE while endogenous formation was blocked by 5 μM CDC under subacute hypoxia. E: Currents were restored by 50 μM NDGA under subacute hypoxia. F: Currents were partly inhibited by 1 μM exogenous 15-HETE while endogenous formation was blocked by 50 μM NDGA under subacute hypoxic conditions. G: Currents were inhibited by exogenous 15-HETE under subacute hypoxic conditions. H: $K^+$ current (pA)-membrane voltage (mV) relationship in control, subacute hypoxia, subacute hypoxia+CDC, subacute hypoxia+CDC+15-HETE, and subacute hypoxia+15-HETE ($n=4$, $^{**}P<0.01$, $^{*}P<0.05$). I: $K^+$ current (pA)-membrane voltage (mV) relationship in control, subacute hypoxia, subacute hypoxia+NDGA, subacute hypoxia+NDGA+15-HETE, and subacute hypoxia+15-HETE ($n=4$, $^{**}P<0.01$, $^{*}P<0.05$).

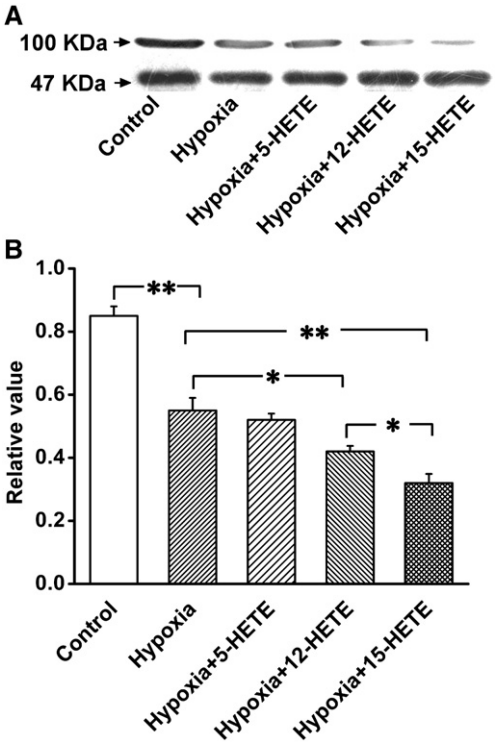


Fig. 10. Influence of exogenous 5-HETE, 12-HETE and 15-HETE on the expression of Kv3.4 channels in cultured rat PASMCs under subacute hypoxia. A: Western blot of the expression of Kv3.4 channels in PASMCs. B: Densitometric analysis of the Western blot. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$.

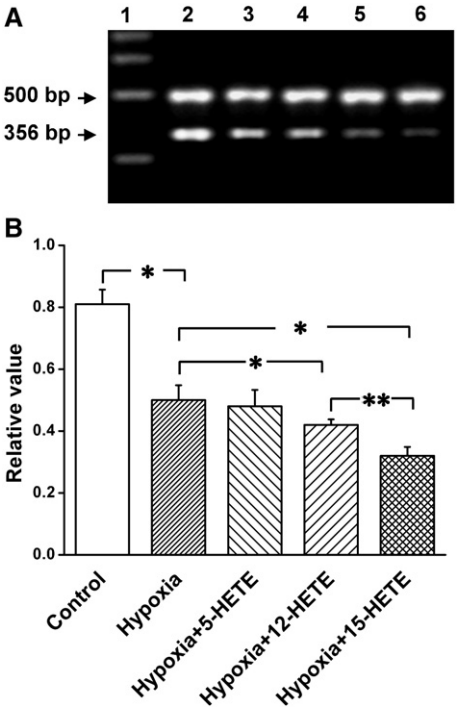


Fig. 11. Influence of exogenous 5-HETE, 12-HETE and 15-HETE on the expression of Kv3.4 channel mRNA in cultured rat PASMCs under subacute hypoxia. A: RT-PCR of Kv3.4 channel mRNA expression in PASMCs. B: Densitometric analysis of the RT-PCR. $n=4$, $^{**}P<0.01$, $^{*}P<0.05$.

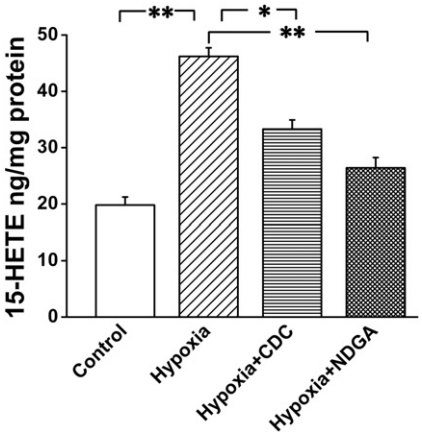


Fig. 12. Endogenous 15-HETE levels detected by 15(S)-HETE ELLISA Kit in PASMCs after hypoxia and with CDC or NDGA. 15-HETE was detected in passage 3 pulmonary arterial vascular smooth muscle cells grown under normoxic or hypoxic conditions and with CDC or NDGA. $n=2$, $^{**}P<0.01$, $^{*}P<0.05$.

Kv3.4 channel expression in cultured PASMCs but 5-HETE had no noticeable effect. 12-HETE was found to be less potent than 15-HETE in causing inhibition of the Kv3.4 channel, as further decreases in the channel expression occurred in the 15-HETE treated cells (Figs. 10 and 11). These data suggest that 15-HETE is more potent than 5-HETE in bring about hypoxia-induced down-regulation of Kv3.4 expression.

### *3.7. Measurements of 15-HETE levels after hypoxia with or without 15-LOX blockers*

If 15-HETE is critical, its concentration should change with hypoxia. Therefore, we measured endogenous 15-HETE levels in PASMCs, using the 15(S)-HETE ELISA Kit. Our results showed that subacute hypoxia produced a marked increase in 15-HETE level in PASMCs (Fig. 12, $P<0.01$, $P<0.05$, $n=2$ vs normoxic group, respectively). The production of endogenous 15-HETE after subacute hypoxia was significantly attenuated with CDC or NDGA. These results are consistent with other observations in the present study.

## 4. Discussion

We have recently reported that hypoxia enhances 15-LOX protein expression and 15-HETE production (Zhu et al, 2003), and that 15-HETE augments pulmonary arterial tone through $K^+$ channels in vascular smooth muscle cells (Han et al, 2004). We have further proved that Kv1.5, Kv2.1, and Kv3.4 channels contribute to the 15-HETE-induced pulmonary vasoconstriction (Han et al, 2004; Li et al, 2006a,b). In the present study, we found that the 15-LOX pathway via its metabolite, 15-HETE, plays a role in the hypoxia-induced down-regulation of Kv3.4 channel expression and inhibition of $I_K$.

The hypoxia-induced down-regulation of Kv3.4 channel expression mediated through 15-HETE is a novel finding in PASMCs. Although both subacute hypoxia and exogenous 15-HETE inhibit Kv3.4 channel expression and $I_K$, no direct

evidence was available to fill the gap in our knowledge of how hypoxia induces membrane depolarization and Kv channel inhibition. If the subacute hypoxia induced inhibition of Kv3.4 channel expression is dependent on 15-HETE, blockade of endogenous 15-HETE production with LOX inhibitors should be able to prevent down-regulation of Kv3.4 channel expression. Supporting this scenario, our results showed that the expression of Kv3.4 channel protein and mRNA in PASMCs was markedly increased after pretreatment with 15-LOX blockers. These results thus provide direct evidence that subacute hypoxia down-regulates Kv3.4 expression at both RNA and protein levels via endogenous 15-HETE.

Because 15-HETE and subacute hypoxia had similar effect on the expression of Kv3.4 channels, we presumed that exogenous 15-HETE would decrease the expression of Kv3.4 in cultured PASMCs pretreated with lipoxygenase inhibitors under subacute hypoxic conditions, and that the expression of Kv3.4 channels would be higher than that in PASMCs treated only with 15-HETE under subacute hypoxic conditions without lipoxygenase inhibitors. We found that the expression of Kv3.4 channels in PASMCs pretreated with CDC or NDGA was greatly increased compared with that of PASMCs without blockade of 15-LOX in response to exogenous 15-HETE. These data provide further evidence that blocking endogenous 15-HETE formation decreased the inhibiting effect of subacute hypoxia on Kv3.4 channel expression.

At least four different major families of $K^+$ channels have been identified in single PASMCs. (i) Kv channels, (ii) large conductance $Ca^{2+}$-activated $K^+$ channels ($BK_{Ca}$), (iii) ATP-sensitive $K^+$ channels ($K_{ATP}$), and (iv) inward rectifier $K^+$ channels ($K_{IR}$). The $K^+$ currents found in the present study were voltage-gated and sensitive to 4-AP, indicating that they belong to the Kv channel family (Post et al., 1995; Archer et al., 1996). It is known that several members of the Kv channel family are sensitive to hypoxia, including Kv1.2 (Conforti et al., 2000), Kv1.5 (Archer et al., 2001), Kv2.1 (Patel et al., 1997), Kv3.1b (Osipenko et al., 2000), Kv3.3 (Wang et al., 1996), and Kv 4.2 (Perez-Garcia et al., 1999). Our previous studies have shown that 15-HETE suppresses the expression of Kv1.5, Kv2.1 and Kv3.4 channels, and inhibits $I_K$ in PASMCs. Similar results were observed for PASMCs maintained under subacute hypoxic conditions (Li et al, 2006a,b). In the present study, we focused our attention on the role of 15-HETE in inhibiting $K^+$ channels in PASMCs under subacute hypoxic conditions. This study did not reveal how many subtypes of Kv channels were involved in the 15-HETE-mediated signal transduction pathway under subacute hypoxic conditions. However, our patch-clamp studies showed that whole-cell $K^+$ currents were higher in the presence of 15-LOX blockers, suggesting that subacute hypoxia may inhibit the $K^+$ current through an endogenous 15-HETE-mediated mechanism, leading to pulmonary vasoconstriction. Furthermore, exogenous 15-HETE further reduced whole-cell $K^+$ currents in PASMCs preincubated with CDC or NDGA after hypoxia. As whole-cell $K^+$ currents are recorded from multiple $K^+$ channels, it is hard to distinguish which types of $K^+$ channels contribute to the PASMC membrane depolarization induced by endogenous 15-HETE. Even so, the increased transmembrane $K^+$ currents through these channels were confirmed in the presence of 15-LOX blockers under subacute hypoxic conditions. Therefore, it is very likely that 15-HETE is the key player linking hypoxia with Kv channel inhibition.

The LOXs comprise a family of non-heme iron-containing dioxygenases that catalyze, in a stereospecific manner, the oxygenation of the 5-, 12-, or 15-carbon atoms of arachidonic acid. There is a previous report that 5-LOX is involved in the regulation of lung vascular tone and in the development of chronic pulmonary hypertension in hypoxic rodent models (Burhop et al., 1988). 12-LOX and its product, 12(S)-HETE, are important intermediates in hypoxia-induced pulmonary arterial smooth muscle proliferation and may participate in hypoxia-induced pulmonary hypertension (Preston et al., 2005). In the present study, we found that CDC and NDGA only partially restored the hypoxia-induced decrease in Kv channel expression. We also studied whether other HETEs affect the Kv3.4 channel. Our results showed that both 12-HETE and 15-HETE but not 5-HETE inhibited the expression of Kv3.4 channel in cultured PASMCs under subacute hypoxic conditions. Since the 5-LO pathway has been shown to participate in the development of hypoxia-induced pulmonary hypertension (Voelkel et al., 1996), it is possible that 5-HETE induces pulmonary vascular constriction through other Kv channels. In comparison, 15-HETE had a stronger effect than 12-HETE on the expression of Kv3.4 channels. Thus it is likely that an array of LOXs is activated following hypoxia, with each affecting different target molecules, while the 15-LOX/15-HETE pathway may play a more important role in the down-regulation of the expression of Kv3.4 channels induced by subacute hypoxia.

In conclusion, we have demonstrated that blocking the formation of the endogenous 15-HETE increased Kv3.4 expression in PASMCs under subacute hypoxic conditions at both protein and mRNA levels. Inhibition of endogenous 15-HETE up-regulates the expression of Kv3.4 channels down-regulated by exogenous 15-HETE in PASMCs under conditions of subacute hypoxia. Likewise, patch-clamp studies support the role of 15-HETE in blocking Kv channels in PASMCs during subacute hypoxia. 15-HETE had a stronger effect than 12-HETE on the expression of Kv3.4 channels in cultured PASMCs. All these data suggest that the decreased Kv3.4 channel expression, induced by subacute hypoxia, is regulated by 15-HETE. These studies are the first effort to establish the molecular and ionic mechanisms of subacute hypoxia in inhibiting Kv channels and provide insight into the potential contribution of 15-HETE to the regulation of $K^+$ channel activity.

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