Chronic hypoxia selectively enhances L- and T-type voltage-dependent Ca\(^{2+}\) channel activity in pulmonary artery by upregulating Ca\(_{\text{v}1.2}\) and Ca\(_{\text{v}3.2}\)

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Chronic hypoxia selectively enhances L- and T-type voltage-dependent Ca\(^{2+}\) channel activity in pulmonary artery by upregulating Ca\(_{\text{v}1.2}\) and Ca\(_{\text{v}3.2}\). Hypoxia-induced pulmonary hypertension (HPH) is characterized by sustained pulmonary vasoconstriction and vascular remodeling, both of which are mediated by pulmonary artery smooth muscle cell (PASMC) contraction and proliferation, respectively. An increase in cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) is a major trigger for pulmonary vasoconstriction and an important stimulus for cell proliferation in PASMCs. Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCC) is an important pathway for the regulation of [Ca\(^{2+}\)]\(_{\text{cyt}}\). The potential role for L- and T-type VDCC in the development of HPH is still unclear. Using a hypoxic-induced pulmonary hypertension mouse model, we undertook this study to identify if VDCC in pulmonary artery (PA) are functionally upregulated and determine which type of VDCC are altered in HPH. Mice subjected to chronic hypoxia developed pulmonary hypertension within 4 wk, and high-K\(^+\)- and U-46619-induced contraction of PA was greater in chronic hypoxic mice than that in normoxic control mice. Additionally, we demonstrate that high-K\(^+\)- and U-46619-induced Ca\(^{2+}\) influx in PASMC is significantly increased in the hypoxic group. The VDCC activator, Bay K8864, induced greater contraction of the PA of hypoxic mice than in that of normoxic mice in isometric force measurements. L-type and T-type VDCC blockers significantly attenuated absolute contraction of the PA in hypoxic mice. Chronic hypoxia did not increase high-K\(^+\)- and U-46619-induced contraction of mesenteric artery (MA). Compared with MA, PA displayed higher expression of calcium channel voltage-dependent L-type \(\alpha_{\text{1c}}\)-subunit (Ca\(_{\text{v}1.2}\)) and T-type \(\alpha_{\text{1I}}\)-subunit (Ca\(_{\text{v}3.2}\)) upon exposure to chronic hypoxia. In conclusion, both L-type and T-type VDCC were functionally upregulated in PA, but not MA, in HPH mice, which could result from selectively increased expression of Ca\(_{\text{v}1.2}\) and Ca\(_{\text{v}3.2}\).

In hypoxic pulmonary hypertension (HPH), pulmonary hypertension associated with hypoxia belongs to the third group in the classification of pulmonary hypertension according to the proceedings of the Fourth World Symposium on Pulmonary Hypertension at Dana Point 2008 (17). Over time, HPH causes right ventricle hypertrophy, right ventricular failure, and death (1, 37). Pulmonary hypertension related to chronic obstructive pulmonary disease (COPD) is one of the most common forms of HPH and is significantly associated with increased mortality (8, 36). Currently, there is no specific therapy for pulmonary hypertension associated with COPD, which provides motivation for researchers to understand the pathogenic mechanisms of HPH.

Sustained pulmonary vasoconstriction and vascular remodeling are the predominant features of HPH, both of which are respectively mediated by pulmonary artery smooth muscle cell (PASMC) contraction and proliferation (27). A rise of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{\text{cyt}}\)) is a major trigger for pulmonary vasoconstriction and an important stimulus for PASMC proliferation. Chronic hypoxia increases the resting [Ca\(^{2+}\)]\(_{\text{cyt}}\) and alters the electromechanical coupling in PASMC, and normoxic recovery reverses the alterations in the electrophysiological properties of PASMC and causes normalization of pulmonary arterial pressure (5, 6).

In PASMC, [Ca\(^{2+}\)]\(_{\text{cyt}}\) is regulated by two pathways: voltage-dependent Ca\(^{2+}\) influx and voltage-independent Ca\(^{2+}\) influx. The latter includes Ca\(^{2+}\) entry through receptor-operated Ca\(^{2+}\) channels (ROC) and store-operated Ca\(^{2+}\) channels (SOC). Lin et al. showed that both ROC and SOC of PASMC were upregulated by chronic hypoxia and contributed to the enhanced vascular tone in HPH (29). In PASMC when the membrane is depolarized, Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels (VDCC) contributes to another important pathway to regulate [Ca\(^{2+}\)]\(_{\text{cyt}}\).

VDCC are organized into six subtypes by their functional characteristics and further divided into groups based on their sensitivity to membrane depolarization (high voltage: L, P, Q, R, and N type and low voltage: T type). L-, T- and P/Q-type channels have been identified in vascular smooth muscle cells (18, 44); however, expression of P- and Q-type channels has not been investigated in the pulmonary vasculature. L-type VDCC are activated at more depolarized potentials and display slower inactivation and faster deactivation times compared with T-type VDCC. The L-type VDCC antagonists nifedipine or verapamil can prevent hypoxic pulmonary vasoconstriction.
VDCC are altered compared with VDCC from the mesenteric artery (PA) upregulated by chronic hypoxia and determine which type of VDCC in the PA are functionally important for causing pulmonary hypertension (HPH). These data imply that Ca\(^{2+}\) influx through L-type VDCC is one of the important [Ca\(^{2+}\)]\(_{cyt}\) regulatory pathways in HPH (6, 12, 13, 23, 24, 47). T-type VDCC have been identified in human PASMC and are required for cell cycle progression and proliferation (44). Currently, the potential role for T-type VDCC in the development of HPH is still not understood.

Hypoxia affects all systems of the body, and not only causes pulmonary vasoconstriction and HPH, but also induces systemic vasodilation (28, 32). Previous data have shown that acute hypoxia reduces K\(^{+}\) currents in PASMC but not in mesenteric arterial smooth muscle cells (MASMC) and that chronic hypoxia downregulates expression of voltage-gated K\(^{+}\) (K\(_V\)) channels in PASMC but not in MASMC (40, 63). Suppression of K\(_V\) channel expression reduces K\(_V\) current, causes membrane depolarization, which activates VDCC, and increases Ca\(^{2+}\) influx, ultimately leading to a rise in [Ca\(^{2+}\)]\(_{cyt}\) in PASMC (40, 63). In MASMC, hypoxia negligibly affects K\(_V\) channel expression, but increases ATP-sensitive K\(^{+}\) current, and induces hyperpolarization (40, 63, 64). Based on these different responses to hypoxia, comparative research on pulmonary and systemic circulation could be helpful in exploring the mechanisms of HPH to facilitate the development of treatments for HPH. In this regard, we used the HPH mouse model to examine whether VDCC in the PA are functionally upregulated by chronic hypoxia and determine which type of VDCC are altered compared with VDCC from the mesenteric artery (MA).

**MATERIALS AND METHODS**

**HPH mouse model.** HPH was induced by exposure of male mice (8 wk old C57BL/6) to chronic hypoxia (10\% O\(_2\)) in a normobaric ventilated chamber (46). Briefly, adult-age-matched male mice were kept in hypoxic conditions for 4 wk to develop pulmonary hypertension. All studies were approved by the University of Illinois at Chicago Institutional Animal Care and Use Committee and were performed according to the guidelines of the University of Illinois at Chicago that comply with national and international regulations.

**Hemodynamic and right ventricular hypertrophy.** Pulmonary hemodynamic and right ventricular hypertrophy measurements were done as described (46). Briefly, right ventricular systolic pressure (RVSP) was measured by a catheter (Millar, Houston, TX) inserted in the right ventricle (RV) via the external right jugular vein. To determine RV hypertrophy, the RV was separated from the left ventricle (LV) and septum (S). The Fulton index or the ratio of RV weight to LV + S weight [RV/(LV + S)] was determined and calculated as a measurement for RV hypertrophy.

**Histology and pulmonary vascular morphometry.** Before removal, the lungs were perfused with 10 ml of saline through the RV. The lung tissue or vessels were then fixed in 10\% formalin and paraffin embedded. Sections (6 \(\mu\)m thick) were cut, and hematoxylin and eosin (H&E) staining was performed to analyze the thickness of pulmonary artery (PA).

**Isolation of PA and mesentery artery.** After anesthesia using a procedure approved by the Institutional Animal Care and Use Committee, the lungs and mesentery were quickly removed from the mouse, washed with cold saline (to remove blood from the lung tissue), and placed in a dissection plate. Under a stereomicroscope, the right and left branches of the intrapulmonary arteries (250–400 \(\mu\)m diameter) and MA (250–400 \(\mu\)m diameter) were isolated from the mice. Adipose and connective tissues were carefully removed with fine forceps and ophthalmological scissors, and the remaining arteries were cut into 2–3-mm-long rings.

**Isometric tension measurements.** Two tungsten hooks (125 \(\mu\)m diameter) were inserted through the lumen of the rings. One hook was mounted on the bottom of a perfusion chamber, and the other was connected to an isometric force transducer (Harvard Apparatus, Holliston, MA). Isometric tension was continuously monitored and acquired using DATAQ software (DATAQ Instruments, Akron, OH).

**Mouse PASMC isolation.** PASMC were isolated from mouse lungs as described previously, using a modification of the Marshall et al. (33) method. A mixture of 5 ml of medium 199 (M199) growth medium containing 5 g/l low-melting-point agarose type VII (Sigma, St. Louis, MO), 5 g/l iron beans (diameter <10 \(\mu\)M; Sigma), and antibiotics (penicillin and streptomycin) was slowly injected over a period of 60 s through the RV, thereby perfusing the PA. M199 growth medium (1 ml) containing 5 g/l agarose type VII was injected in airways through the trachea. The lungs were plunged in cold PBS to denude the agarose to gel. Because of the rapidly solidifying nature of the agarose and the size of the iron particles, the likelihood of traversing the capillary space is minimized. All the lobes were then isolated and finely minced in a petri dish. The tissue was further disrupted by passing through a 16-gauge followed by an 18-gauge needle approximately five times. The suspension was then mixed in M199 growth medium containing 80 U/ml type IV collagenase (Sigma) and incubated at 37\(^\circ\)C for 90 min. With the use of a magnetic column (Invitrogen), the arteries containing the iron beads were collected on the side. The supernatant was aspirated, and, following three washes, the arteries were suspended in 5 ml M199 containing 20\% FBS. Aliquots of the suspension were transferred to T25 culture flasks. Cells from the hypoxic group were incubated at 3\% O\(_2\), whereas cells from the normoxic control were cultured in air. Smooth muscle cell purity was determined by immunostaining with smooth muscle specific actin antibody.

**[Ca\(^{2+}\)]\(_{cyt}\) measurement.** Cells were cultured on 25-mm cover slips (Fisher Scientific, Waltham, MA) and were then placed in a recording chamber on the stage of an inverted fluorescent microscope (Eclipse Ti-E; Nikon, Tokyo, Japan) equipped with an objective lens (S Plan Fluor 20×/0.45 ELWD; Nikon) and an EM-CCD camera (Evolve; Photometrics, Tucson, AZ). [Ca\(^{2+}\)]\(_{cyt}\) was monitored using a membrane-permeable Ca\(^{2+}\)-sensitive fluorescent indicator, fura 2-acetoxyethyl ester (fura 2-AM; Invitrogen-Molecular Probes, Eugene, OR), and imaged with NIS Elements 3.2 software (Nikon). These cells were incubated in HEPS-buffered solution containing 4 \(\mu\)M fura 2-AM for 60 min at room temperature (25\(^\circ\)C). The loaded cells were then washed with HEPS-buffered solution for 10 min to remove

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excess extracellular indicator. Fura 2 was excited with 340- and 380-nm wavelengths (D340×2 and D380×2 filters, respectively; Chroma Technology, Bellows Falls, VT) by a xenon arc lamp (Lambda LS; Sutter Instrument, Novato, CA) and an optical filter changer (Lambda 10-B). Emission of fura 2 was collected through a dichroic mirror (400DCLP) and a wide band emission filter (D510/80m). [Ca^{2+}]_{ext}, within a region of interest (5 × 5 μm), which was placed at the peripheral region of each cell, was measured as the ratio of fluorescence intensities (F_{405}/F_{500}) every 2 s. The recording chamber was continuously perfused at a flow rate of 2 ml/min using a mini-pump (model 3385; Control, Friendswood, TX). [Ca^{2+}]_{ext}, measurements were carried out at 32°C using an automatic temperature controller (TC-344B; Warner Instruments, Hamden, CT), since increased fura 2 compartmentalization in organelles and cell dye leakage have been reported to occur at physiological temperature (45).

**Real-time RT-PCR analysis.** The total RNA from dissected pulmonary arteries of either normoxic control mice or hypoxic mice was purified using Trizol reagent (Invitrogen, Grand Island, NY) and quantified with NanoDrop 2000c (Thermo Scientific, Waltham, MA). Equal amounts of RNA were reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Indianapolis, IN) and gene-specific primers for calcium channel voltage-dependent L-type channel (Cav1.2, forward: 5′-TGAGCAACCTTGTGGCATCCTTG-3′; reverse: 5′-AGAGATGTCGTAATGCGAA-3′), Cav2.1 (forward: 5′-CAAAGA-3′; reverse: 5′-GGG TGT AGT-3′), Cav3.1 (forward: 5′-AGA ATG TCA GCT TCT-3′; reverse: 5′-CAT GCC GGC CAT GAC AAT GAA GAA-3′), Cav3.2 (forward: 5′-GCT GAA CAC CAA CGA TGC CAA AGA-3′), calcium channel voltage-dependent L-type channel α1C-subunit (Ca v1.2, forward: 5′-TGAGCAACCTTGTGGCATCCTTG-3′; reverse: 5′-ACGGGTCTGCA-TCTCATCGAAGTT-3′), Ca,2.1 (forward: 5′-TGTTGATCTCAATGCGAA-3′; reverse: 5′-AGACAAGTGCACGACC-3′), Cav2.2 (forward: 5′-AGCGGAGCTTAACTCAGT-3′; reverse: 5′-CATGCCGATCCTCCTGCTCGAAGA-3′), Cav3.1 (forward: 5′-CACTCTCATCGAAGTT-3′; reverse: 5′-CATGCCGATCCTCCTGCTCGAAGA-3′), Cav3.2 (forward: 5′-CACTCTCATCGAAGTT-3′; reverse: 5′-CATGCCGATCCTCCTGCTCGAAGA-3′), and 18S rRNA (forward: 5′-GGG TGT AGT-3′; reverse: 5′-CATGCCGATCCTCCTGCTCGAAGA-3′). RNA quantities were normalized using 18S rRNA as an internal standard and analyzed by the Bio-Rad CFX Manager Software. The change in ΔΔCt with respect to control was calculated by making normoxic values equal to one and adjusting corresponding hypoxic values proportionally.

**Immunohistochemistry.** For immunohistochemistry, sections of isolated PA and MA were incubated with the following primary antibodies: rabbit anti-Ca,1.2 (Sigma) and rabbit anti-Ca,3.2 (Santa Cruz). Alexa Fluor 488-labeled chicken anti-rabbit IgG (Molecular Probes) was used as secondary antibody.

**Western blotting.** Protein samples were prepared by homogenizing mouse lung tissue using a glass Dounce tissue grinder, followed by brief sonication, in 1× RIPA lysis buffer (Millipore) containing 2% n-dodecyl-β-D-maltoside (Thermo Scientific) and protease inhibitor cocktail (Roche). Insoluble tissue was removed by centrifugation (5,000 g, for 5 min). Protein concentrations were determined by the BCA Protein Assay Kit (Pierce). Proteins were loaded equally (30 μg) into separate wells for 4–20% SDS gradient gels (Mini-PROTEAN TGX; Bio-Rad), separated at 120 V for 2 h, and transferred onto polyvinylidene fluoride membranes. Membranes were then stained with Ponceau S and cut in half at 75-kDa standard marker, with the top used for Ca,1.2 or Ca,3.2 analysis (∼250 kDa) and the bottom for β-actin loading controls (∼44 kDa). Primary antibodies used for analysis included: monoclonal mouse anti-Ca,1.2 (Millipore) and anti-Ca,3.2 at 1:200 (Alomone); monoclonal β-actin at 1:1,000 (Santa Cruz Biotechnology). Intensity of Western blot bands was determined with ImageJ software.

**RESULTS**

**Chronic hypoxia induces pulmonary hypertension in mice.** To investigate the role of VDCC in the development of HPH, we initially begin by examining the development of HPH in the mouse model. Mice exposed to sustained hypoxia (10% O2) developed pulmonary hypertension within 4 wk as reflected by a significant increase in RVSP: 34.256 ± 1.413 mmHg in hypoxic mice vs. 22.354 ± 1.522 mmHg in normoxic mice (Fig. 1A). Lung tissue sections with H&E staining showed that the wall thickness of the vessels was greater in HPH mice than in the normoxic group (Fig. 1B). Additionally, a significant increase in the Fulton index [RV/(LV + S)] was found in the hypoxic group (0.225 ± 0.0113) compared with that in the normoxic control (0.334 ± 0.0344, Fig. 1C). These results demonstrate that chronic hypoxia induces pulmonary hypertension in mice.

**High-K⁺- and U-46619-induced contraction of PA is enhanced in HPH mice.** Pulmonary hypertension is due in part to sustained pulmonary vasoconstriction and vascular remodel-
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ing. To investigate the effect of hypoxia on pulmonary vasoconstriction, we measured the contraction of PA from normoxic and hypoxic mice in response to high K\(^+\) and U-46619. Mounted PA rings were tested for endothelial function by preconstricting vessels with phenylephrine (PE, 100 nM), followed by a vasodilation challenge with acetylcholine (ACh, 10 μM). Assessment of intact endothelial layer function in mouse PA vessels has been previously reported by our group (25).

ACh treatment of endothelium-denuded PA preparations produced a slight relaxation in active tension (0.1768 ± 0.0654 g) that was not significantly different from PE-induced active tension (0.2050 ± 0.0819 g) or washout (0.2295 ± 0.0936 g). These data indicate the endothelial layer was functionally disrupted (Fig. 2).

As we have previously shown (57), both high-K\(^+\) solution and U-46619, a thromboxane A\(_2\) analog, induced vasoconstriction in pulmonary arteries from normoxic mice (Fig. 3, A and B, left). The vasoconstriction observed in response to high-K\(^+\) solution (10–120 mM K\(^+\)) and U-46619 (0.3–300 nM) was concentration dependent (Fig. 3, A–C). However, the vasoconstriction observed in pulmonary arteries of HPH mice in response to high-K\(^+\) and U-46619 was significantly greater than in normoxic mice (Fig. 3, A–C, right). These data indicate that high-K\(^+\) - and U-46619-induced vasoconstriction in HPH mice is significantly enhanced compared with normoxic mice.

**Ca\(^{2+}\) influx through VDCC is increased in the PA of HPH mice.** Increased [Ca\(^{2+}\)]\(_{cyt}\) is a major trigger for pulmonary vasoconstriction, and Ca\(^{2+}\) influx through VDCC is an important mechanism for the increased [Ca\(^{2+}\)]\(_{cyt}\). To determine the effect of hypoxia on Ca\(^{2+}\) influx through VDCC, we measured [Ca\(^{2+}\)]\(_{cyt}\) in PASMC from normoxic and hypoxic mice. Raising the extracellular K\(^+\) concentration causes membrane depolarization, which subsequently increases open probability of VDCC and promotes Ca\(^{2+}\) influx (51, 57). In primary PASMC isolated from normoxic mice, application of a high-K\(^+\) (40 mM) solution had a small effect on the [Ca\(^{2+}\)]\(_{cyt}\) (Fig. 4, A, left, and C, left). However, in primary PASMC from hypoxic mice, high K\(^+\) significantly increased the [Ca\(^{2+}\)]\(_{cyt}\) (Fig. 4A, right, and C, left). Similarly, U-46619 had minimal effect on the [Ca\(^{2+}\)]\(_{cyt}\) in normoxic PASMC (Fig. 4, B, left, and C, right), but U-46619 significantly increased the [Ca\(^{2+}\)]\(_{cyt}\) in primary PASMC isolated from hypoxic mice (Fig. 4, B, right, and C, right). These data suggest that the activity of VDCC is enhanced in hypoxic mice, leading to significantly increased [Ca\(^{2+}\)]\(_{cyt}\).

**Activation of VDCC in HPH mice enhances pulmonary vasoconstriction.** Increased [Ca\(^{2+}\)]\(_{cyt}\) in PASMC can lead to contraction. Given that [Ca\(^{2+}\)]\(_{cyt}\) and VDCC activity is enhanced in PASMC from hypoxic mice, we investigated the effect of activation of VDCC on PA contraction. Bay K8864 selectively activates VDCC and induces vascular contraction. Using isometric force measurements, we show that treatment of normoxic PA rings with increasing concentrations of Bay K8864 results in vasoconstriction (Fig. 5, A, left, and B). Treatment of hypoxic PA rings with Bay K8864 results in a significantly stronger contraction than that of normoxic PA rings (Fig. 5, A, right, and B). These data further demonstrate the enhanced activity of VDCC can contribute to HPH in mice.

**L-type and T-type VDCC blockers significantly attenuate contraction of the PA in HPH mice.** To determine which VDCC are involved in the enhanced contraction of the PA in HPH mice, we examined the high-K\(^+\)-and U-46619-induced vasoconstriction of PA rings after treatment with VDCC blockers. Nifedipine, mibebradil, and o-agonists are L-type, T-type, and P/Q-type VDCC blockers, respectively. In PA rings from normoxic mice, both nifedipine and mibebradil caused dose-dependent inhibition of high-K\(^+\) (40K)-induced PA contraction (Fig. 6, Aa, left, and Ab). Nifedipine and mibebradil in-
systemic arteries (15), and chronic hypoxia induces pulmonary hypertension but not systemic hypertension (28). We therefore investigated the high-K⁺- and U-46619-induced vasoconstriction of MA isolated from normoxic and hypoxic mice. Treatment with high K⁺ (e.g., 40 mM K⁺) induced contraction of MA rings with no significant difference between normoxic and hypoxic mice (Fig. 8, A and B). Additionally, U-46619 dose-dependently induced similar vasoconstriction in MA from normoxic and hypoxic mice (Fig. 8, A and C). These results demonstrate that hypoxia does not enhance vasoconstriction in MA (Fig. 8), and hypoxia selectively enhances vasoconstriction in PA (Figs. 3 and 5–7).

We also investigated the activity of VDCC in MA from normoxic and hypoxic mice. Treatment with Bay K8644 (0.1–10 μM) did not induce contraction of MA rings isolated either from normoxic or hypoxic mice (Fig. 8, D and E). These data indicate that VDCC activity is not enhanced in the MA of hypoxic mice. Together, these results show that pulmonary vasoconstriction due to Ca²⁺ influx through nifedipine- and mibefradil-sensitive VDCC is enhanced in PA but not MA of HPH mice.

Chronic hypoxia upregulates expression of Ca³.1 and Ca³.2 in the PA. To determine whether hypoxia altered the expression of VDCC, we examined the mRNA expression of L-type (Cav1.2), T-type (Cav3.1 and Cav3.2), and P/Q-type (Cav2.1) VDCC by real-time RT-PCR. In the PA, exposure to chronic hypoxia resulted in increased expression of Cav1.2, Cav3.1, and Cav3.2 compared with normoxic controls (Fig. 9Ac). In the MA, chronic hypoxia resulted in increased expression of Cav3.1 (Fig. 9Ab). The increase in expression of Cav3.1 after hypoxia is similar in PA and MA (Fig. 9Ac). However, the increase in expression of Cav1.2 and Cav3.2 after hypoxia is only seen in the PA (Fig. 9Ac).

In addition to the upregulated mRNA expression, immunohisto-
chemistry showed increased expression of Cav1.2 and Cav3.2 in PA from hypoxic mice compared with MA in which there was no significant change due to hypoxia (Fig. 9B). Western blot analysis of mouse lungs from normoxic and hypoxic animals indicates that hypoxia upregulates expression of Cav1.2 by ~10-fold, whereas protein expression of Cav3.2 increases only slightly, by ~40% with hypoxia (Fig. 9C). These results demonstrate that hypoxia selectively upregulates protein expression of L-type (Cav1.2) and T-type (Cav3.2) VDCC in the mouse PA and that hypoxia up-regulates protein expression of another subtype of T-type VDCC, Cav3.1, in both PA and MA.

DISCUSSION

In the present study, our findings demonstrate that HPH mice have 1) increased high-K⁺- and U-46619-induced contraction of the PA, 2) increased high-K⁺- and U-46619-induced Ca²⁺ influx in primary PASMC, and 3) enhanced pulmonary vasorestriction due to Ca²⁺ influx through VDCC compared with normoxic mice. In addition, the data from this study show that 1) L-type and T-type, but not P/Q-type, channels contribute to the enhanced high-K⁺- and U-46619-induced pulmonary vasoconstriction and 2) Cav1.2 and Cav3.2 are significantly upregulated in PA (but not in MA) from hypoxic mice compared with normoxic mice. We also show that the hypoxia-mediated changes are specific to the PA by demonstrating that there is no difference in high-K⁺- and U-46619-induced vasorestriction or VDCC function and expression in MA from normoxic and hypoxic mice. Together, these data suggest that chronic hypoxia functionally enhances pulmonary vasorestriction associated with Ca²⁺ influx through L-type and T-type VDCC by selectively upregulating Cav1.2 and Cav3.2 channels in PASMC. To determine if chronic hypoxia affected VDCC in the systemic circulation, we challenged the MA with high K⁺ and U-46619 but did not find significant difference between normoxic and hypoxic mice, demonstrating the effects of hypoxia on VDCC are specific to the pulmonary vasculature and not the systemic (or mesenteric) vasculature.

HPV is an important mechanism for maintaining a proper ventilation-to-perfusion ratio by diverting blood away from poorly ventilated areas to areas of the lung with relatively high ventilation. Persistent hypoxia or exposure to chronic hypoxia, however, causes sustained HPV and pulmonary arterial medial hypertrophy leading to increased pulmonary vascular resistance and pulmonary arterial pressure. Studies have demonstrated that acute hypoxia causes pulmonary vasoconstriction.

Fig. 6. L- and T-type VDCC blockers inhibit high-K⁺-induced active tension in PA rings from hypoxic mice to a greater extent than in PA rings from normoxic mice. A–C: representative tracings (a) and summarized data expressed as absolute decrease (b) and percent decrease (c) to 40K-mediated active tension in the presence of various concentrations of nifedipine (0.03–3 μM; A), mibefradil (0.03–3 μM; B), and ω-agatoxin (0.1–30 nM; C) in PA rings isolated from Nor (left, and open circles, n = 4) and Hyp (right, and solid circles, n = 4) mice. *P < 0.05 and **P < 0.01 vs. Nor. The inhibitory effects of nifedipine, a L-type VDCC blocker, and mibefradil, a T-type VDCC blocker, on 40K-induced vasoconstriction is greater in PA rings from hypoxic mice than in PA rings from normoxic mice.
nifedipine, an L-type VDCC blocker, on U-46619-induced vasoconstriction is greater in PA rings from hypoxic mice than in PA rings from normoxic mice. 

motes Ca\(^{2+}\) membrane depolarization, increases open probability of VDCC, produced contraction after exposure to chronic hypoxia (9, 23). In our study, we have demonstrated that hypoxia leads to an increase in [Ca\(^{2+}\)]\(_{cyt}\) in mouse PASMC potentially because of enhanced VDCC activity. However, it should be mentioned that Ca\(^{2+}\) measurements were taken at subphysiological temperature. Although fura 2 is a widely used fluorescent probe for examining dynamic changes of intracellular Ca\(^{2+}\) in live cells, there are several limitations, namely organelle compartmentalization and dye leakage from loaded cells that prevent accurate Ca\(^{2+}\) measurements at 37°C (45). One method used for circumventing these potential artifacts is performing experiments at lower temperatures. Whether these data reflect actual Ca\(^{2+}\) responses to hypoxia at physiological temperature is unknown and will require further study.

In addition to enhanced high-K\(^{+}\)-induced PA contraction, in the present study, we provide evidence of enhanced agonist-induced contraction in HPH mice. We demonstrate that PA contraction induced by the thromboxane A\(_2\) analog U-46619 was increased in HPH mice. Thromboxane A\(_2\), an arachidonic acid metabolite, is an endothelium-derived constricting factor...
that binds specifically to Gq/11 protein-coupled receptors (e.g., TP receptor) and causes increased [Ca\(^{2+}\)]\(_{cyt}\) and sensitization of the contractile proteins to Ca\(^{2+}\) in PASMC (38, 48, 51, 60). Inhibition of thromboxane synthase by furegrelate sodium attenuates the development of HPH in neonatal piglet (22). Additionally, thromboxane A\(_2\) inhibits Kv channels (along with other K\(^+\) channels), which causes membrane depolarization and opens VDCC, resulting in pulmonary vasoconstriction (10). The thromboxane A\(_2\)-mediated membrane depolarization is also associated with its activating effect on nonselective cation channels (60). In rat PASMC, U-46619 induces a nonselective cation current that is sensitive to ruthenium red, a blocker of transient potential vanilloid-related channels. The results of our study demonstrate that enhanced VDCC expression and function also contribute to the enhanced agonist-induced contraction in HPH mice.

In PASMC, L- and T-type VDCC are the main pathways for voltage-mediated Ca\(^{2+}\) influx involved in excitation-contraction coupling and proliferation (16, 26, 50). In the present study, we show that L-type (Ca\(_{1.2}\)) and T-type (Ca\(_{3.2}\)) channels are specifically upregulated in the PA (but not in the MA) of hypoxic mice, suggesting a role for these channels in the pathogenesis of HPH. Inhibition of L- and T-type channels with nifedipine and mibebradil, respectively, significantly attenuated the enhanced high-K\(^+\)- and U-46619-induced vasoconstriction to a greater extent in hypoxic mice than in normoxic mice. Paradoxically, hypoxic tissues experience a decrease in sensitivity to nifedipine as indicated by a rightward shift in the dose-response curve, whereas sensitivity to mibebradil remains unchanged compared with normoxic preparations (Figs. 6 and 7). Although the diminished effect of nifedipine on HPH mice was unexpected, it was not surprising, since we have also shown that Ca\(_{1.2}\) expression is significantly increased in hypoxic lung mouse tissue (Fig. 8). Therefore, the diminished effect of nifedipine on L-type VDCC is most likely due to enhanced expression and activity of VDCC in the chronically hypoxic pulmonary vasculature.

Recently, we have shown that the dihydropyridine Ca\(^{2+}\) channel blockers, including nifedipine, can activate the Ca\(^{2+}\)-sensing receptor, which we have also shown is upregulated in PASMC isolated from idiopathic PAH patients (59), leading to enhanced extracellular Ca\(^{2+}\)-induced Ca\(^{2+}\) influx. Clinical use of nifedipine has also proven to be problematic. Inhibition of VDCC with nifedipine or other VDCC blockers significantly attenuates smooth muscle cell proliferation cultured in media containing growth factors and serum (30, 49, 55), which would indicate that Ca\(^{2+}\) influx through L-type and T-type VDCC is required for smooth muscle cell proliferation. Silenced expression of T-type VDCC in human PASMC was also shown to significantly attenuate proliferation (44), providing further evidence that T-type channels are essential in controlling prolif-
currents over L-type Ca\(^{2+}\) currents (~10–30 more) \(20, 34\); however, in cultured rat spinal motoneurons, mibebradil was equally effective at blocking L-type Ca\(^{2+}\) currents \(52\). Additionally, a metabolized form of mibebradil has been reported to preferentially block L-type over T-type Ca\(^{2+}\) currents in insulin-secreting cells \(56\). To our knowledge, the overlapping pharmacology of these two inhibitors has not been extensively studied in vascular myocytes; however, we must always be cognizant of the limitations these pharmacological tools bring. Therefore, the use of more highly specific inhibitors and knockout mouse studies will be required to elucidate the role of L- and T-type Ca\(^{2+}\) channels in the vasculature, particularly pertaining to their contribution in the development of PAH. The molecular mechanism involved in hypoxia-mediated selective upregulation of Ca\(_{\text{1.2}}\) and Ca\(_{\text{3.2}}\) in PASMC and PA is unclear. The results from this study demonstrate that chronic hypoxia significantly upregulated the mRNA expression level of Ca\(_{\text{1.2}}\) and Ca\(_{\text{3.2}}\) only in the PA but not in the MA. Hypoxia also upregulates Ca\(_{\text{3.1}}\), a T-type VDCC found in neurons and cardiac tissue; however, the augmenting effect occurred to the same extent in the PA and MA. These data indicate that the augmenting effect of chronic hypoxia on the mRNA expression level of Ca\(_{\text{1.2}}\) and Ca\(_{\text{3.2}}\) is specific to only PA, but the augmenting effect of hypoxia on the Ca\(_{\text{3.2}}\) expression level seems to be a nonspecific phenomenon that occurs in both the PA and MA. The data from immunohistochemistry experiments confirmed that chronic hypoxia selectively upregulated the protein expression of Ca\(_{\text{1.2}}\) and Ca\(_{\text{3.2}}\) in the PA but not the MA.

In conclusion, our study demonstrates that chronic hypoxia enhances the high-K\(^+\)- and U-46619-induced contraction in the PA, but not in the MA, because of selective functional upregulation of L-type (Ca\(_{\text{1.2}}\)) and T-type (Ca\(_{\text{3.2}}\)) VDCC in the PA. These studies provide insight into the role of Ca\(^{2+}\)-dependent tone in the development of HPH.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise are declared by the authors.

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