Upregulation of osmo-mechanosensitive TRPV4 channel facilitates chronic hypoxia-induced myogenic tone and pulmonary hypertension

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Upregulation of osmo-mechanosensitive TRPV4 channel facilitates chronic hypoxia-induced myogenic tone and pulmonary hypertension. Am J Physiol Lung Cell Mol Physiol 302: L555–L568, 2012. First published December 29, 2011; doi:10.1152/ajplung.00005.2011.—Chronic hypoxia causes pulmonary hypertension with vascular remodeling, increase in vascular tone, and altered reactivity to agonists. These changes involve alterations in multiple Ca2+ pathways in pulmonary arterial smooth muscle cells (PASMCs). We have previously shown that vanilloid (TRPV)- and melastatin-related transient receptor potential (TRPM) channels are expressed in pulmonary arteries (PAs). Here we found that TRPV4 was the only member of the TRPV and TRPM subfamilies upregulated in PAs of chronic hypoxic rats. The increase in TRPV4 expression occurred within 1 day of hypoxia exposure, indicative of an early hypoxic response. TRPV4 expression in PASMCs were found to be mechanosensitive. Osmo-mechanical stress imposed by hypotonic solution activated Ca2+ transients; they were inhibited by TRPV4 specific short interfering RNA, the TRPV blocker ruthenium red, and the cytochrome P450 epoxyxygenase inhibitor N-(methylsulphonyl)-2-(2-propynyl)benzenehexanamide. Consistent with TRPV4 upregulation, the Ca2+ response induced by the TRPV4 agonist 4α-phorbol 12,13-didecanoate and hypotonicity was potentiated in hypoxic PASMCs. Moreover, a significant myogenic tone, sensitive to ruthenium red, was observed in pressurized endothelium denuded small PAs of hypoxic but not normoxic rats. The elevated basal intracellular Ca2+ concentration in hypoxic PASMCs was also reduced by ruthenium red. In extension of these results, the development of pulmonary hypertension, right heart hypertrophy, and vascular remodeling was significantly delayed and suppressed in hypoxic trpv4−/− mice. These results suggest the novel concept that TRPV4 serves as a signal transduction mechanism that promotes pulmonary hypertension via facilitated Ca2+ influx, subsequently enhanced myogenic tone and vascular remodeling.

Moreover, substantial evidence indicates that chronic hypoxia causes intrinsic changes in ionic balance and Ca2+ homeostasis in pulmonary arterial smooth muscle cells (PASMCs), leading to membrane depolarization, elevation of resting intracellular Ca2+ concentration ([Ca2+]i), and changes in electrophysiological and Ca2+ responses to vasoconstrictors and vasodilators (26, 41, 49–51, 53). These functional changes of PASMCs involve alterations in multiple Ca2+ pathways (45, 52), and they are crucial for the development of pulmonary hypertension.

The transient receptor potential (TRP) gene superfamily, which consists of three major subfamilies of canonical (TRPC), melastatin-related (TRPM), and vanilloid-related (TRPV) channels, and four lesser subfamilies, is known to encode a large repertoire of nonselective cation channels (33). To date, ≥10 TRP channels have been identified in vascular smooth muscle cells (VSMCs) and implicated in various vascular functions (2). In search for Ca2+ pathways participating in the development of chronic hypoxic pulmonary hypertension, we (26) have previously characterized TRPC channels in rat intralobar PASMCs and provided the first evidence that chronic hypoxia upregulates the store-operated TRPC1 and the receptor-operated TRPC6 expression in rat pulmonary arteries (PAs). Moreover, the enhanced store-operated Ca2+ entry was found to be responsible for the increase in resting [Ca2+]i, and basal tone of PAs of hypoxic pulmonary hypertensive rats. A subsequent study by others (60) showed that the upregulation of TRPC1 and TRPC6 is the direct effect of hypoxia on PASMCs and requires the full expression of hypoxia inducible factor (HIF-1α). In addition, idiopathic pulmonary arterial hypertension has been shown to be associated with the overexpression of TRPC6 and TRPC3 in PASMCs, and inhibition of TRPC6 expression markedly attenuated proliferation of PASMCs from these patients (68). Interestingly, a functional single-nucleotide polymorphism in the TRPC6 gene promoter region was identified in patients with idiopathic pulmonary arterial hypertension, and was shown to enhance NF-κB-mediated promoter activity and stimulated TRPC6 expression in PASMCs (69). These studies revealed a critical involvement of TRPC channels in pulmonary hypertension.

We have further extended our efforts to identify TRPV and TRPM channels in rat PAs (65). Multiple channel transcripts including TRPV1, TRPV2, TRPV4, TRPM2, TRPM3, TRPM4, TRPM7, and TRPM8 were detected, with TRPV4 and TRPM8 being the most abundantly expressed TRPV and TRPM subtypes, respectively. Moreover, these channels constitute functional Ca2+ entry pathways in PASMC, inasmuch as the TRPV4 agonist 4α-phorbol 12,13-didecanoate (4α-PDD) and the TRPM8 agonist menthol elicited a significant increase in

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endothelium was removed by gently rubbing the luminal surface with matically isolated and transiently cultured as previously described in accordance with the guidelines and approved by the Johns Hopkins translation may contribute to the enhanced vascular tone and vas-pulmonary arterial pressure (Ppa) in hypoxia, and its upregu-
serves as a signal transduction mechanism for the elevated hypoxic pulmonary hypertension. Our results suggest that functions in PASMCs and their roles in the pathogenesis of chronic hypoxia. We further investigated their physiological study, we compared the expression of TRPV and TRPM functions and pathophysiological roles of TRPV and TRPM channels in PASMCs have not been investigated. In the present study, we found that the TRPV4 is the only channel upregulated by PASMCs and their roles in the pathogenesis of hypoxic pulmonary hypertension. Our results suggest that TRPV4 are osmo-mechanosensitive channels, which may serve as a signal transduction mechanism for the elevated pulmonary arterial pressure (Ppa) in hypoxia, and its upregu-
lation may contribute to the enhanced vascular tone and vas-
cular remodeling in hypoxic pulmonary hypertension.

MATERIALS AND METHODS

Rats and mouse models of chronic hypoxia-induced pulmonary hypertension. Male Wistar rats (150 to 250 g), TRPV4 null (trpv4−/−) mice, and age-matched wild-type mice (C57BL/6J, 8–10 wk old) were used as the experimental animals. The generation of trpv4−/− mice has been previously described (25). They were placed in a hypoxic chamber and exposed to either normoxia or normobaric 10% O2 for 4 wk to induce hypoxic pulmonary hypertension as described previously (26). Animals were anesthetized with sodium pentobarbital (50 mg/kg ip). Pulmonary hypertension was evaluated in both models by measuring right ventricular (RV) systolic pressure (RVSP) and RV hypertrophy. RV pressure was measured with a Mikro-Tip pressure catheter (SPR-671; Millar Instruments) approached through cannula-
lation of the right jugular vein. Mixed venous blood of mice was collected from the jugular vein or punctured vena cava with a heparinized micro-hematocrit capillary tube, and hematocrit was mea-
sured after centrifugation with a Damon IEC MB Centrifuge. Heart and lung were then removed after exsanguination. RV was separated from the left ventricle and septum (LV + S). The mass ratio of RV/(LV + S) was determined. All animal procedures were performed in accordance with the guidelines and approved by the Johns Hopkins Animal Care and Use Committee.

Isolation and transient culture of PASMCs. PASMCs were enzymatically isolated and transiently cultured as previously described (65). In brief, intralobar PAs (300–800 μm) were isolated, and the endothelium was removed by gently rubbing the luminal surface with a cotton swab. Deendothelialized PAs were allowed to recover for 30 min in cold (4°C) HBSS, followed by 20 min in reduced-Ca2+ (20 μM) HBSS at room temperature. The tissue was digested at 37°C for 20 min in 20 μM Ca2+ HBSS containing collagenase (type I; 1,750 U/ml), papain (9.5 U/ml), BSA (2 mg/ml), and dithiothreitol (1 mM) and then washed with Ca2+-free HBSS to stop digestion. PASMCs were dispersed gently by trituration with a small-bore pipette in Ca2+-free HBSS at room temperature. Cells were then placed on 25-mm glass coverslips. PASMCs from chronic hypoxic and normoxic animals were cultured transiently (16–24 h) in Ham’s F-12 medium (with l-glutamine) supplemented with 0.5% FCS, 100 U/ml streptomycin, and 0.1 mg/ml penicillin inside a modular incubator chamber (Billups-Rothenberg) under 4% O2-5% CO2 and 21% O2-5% CO2, respectively, before use. The 4% O2 was used for providing a hypoxic environment for the transient culture of PASMCs of chronic hypoxic rats. It has been tested that transient culture of PASMCs for 24 h under this condition did not alter the expression of TRPV4 and TRPC1 channel proteins, as well as 4α-PPD and thapsigargin-induced store-operated Ca2+ entry.

RNA preparation and quantitative real-time RT-PCR. Deendothe-
ialized PASs frozen in liquid nitrogen were mechanically homoge-
nized. Total RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). Genomic DNA contamination was removed by TURBO DNA-free DNase (Ambion, Austin, TX). An aliquot of total RNA (0.5 μg) was used for first-strand cDNA synthesis using random hexamer primers and Superscript III RNase H− reverse transcrip-
tase (Invitrogen, Carlsbad, CA) according to the manufactur-
er’s protocol. Quantitative real-time RT-PCR was used to quantify the changes in the expression of TRPV and TRPM subtypes. PCR reactions were set up with iQ SYBR Green PCR Supermix (Bio-Rad, Hercules, CA), using 1 μl of cDNA as the template in each 20-μl reaction mixture. Gene-specific real-time PCR primers for TRPV and TRPM subtypes were described previously (65). PCR protocol, consisting of an initial step at 95°C for 5 min, followed by 40 cycles at 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min, was performed using the iQ5 Multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). Standard curves were generated from serial dilutions of purified PCR products of known copy number. Data were normal-
ized with the amount of 18S rRNA in individual samples to correct for sample variability. Conventional RT-PCR for TRPV4 and TRPV2 was also performed in some experiments using primers and protocols described previously (65).

Western blotting. PASs frozen in liquid nitrogen were crushed and homogenized using a mortar and pestle and resuspended in ice-cold lysis buffer containing 50 mM Tris Cl (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 0.1% SDS, 0.5% NP-40, and protease inhibitor cocktail (Roche, Mannheim, Germany). The homogenate was cen-
trifuged at 4°C with 1,000 g for 5 min, the supernatant was collected, and the protein concentration was estimated using the BCA assay. Protein samples of 20 μg were resolved in an 8% SDS-PAGE gel and electrotransfered onto a PVDF membrane (Millipore, Bedford, MA). The membrane was blocked with 5% (wt/vol) nonfat dry milk in PBS containing 0.02% Tween 20 (PBST) for 1 h at room temperature, and followed by incubation at 4°C overnight with a specific primary antibody. The primary antibodies were polyclonal rabbit anti-TRPV2 (1:1,000 dilution) and anti-TRPM8 (1:500 dilution) from Abcam (Cambridge, MA) and anti-TRPV1 (1:500 dilution) from Alomone (Jerusalem, Israel). β-Actin was used as loading control. After being washed, the membrane was incubated with peroxidase-conjugated goat-anti-rabbit secondary antibody (Bio-Rad, Hercules, CA) at 1:3,000 dilution at room temperature for an hour. After wash, protein bands were detected with enhanced chemiluminescence (Pierce, Rockford, IL) and imaged by a Gel Logic 200 image system (Kodak, New Haven, CT).

Measurement of intracellular [Ca2+]i. [Ca2+]i, was monitored using the membrane-permeable Ca2+-sensitive fluorescent dye fluo 3-AM. PASMCs were loaded with 5–10 μM fluo 3-AM (dissolved in DMSO with 20% pluronic acid) for 45 min at room temperature (∼23°C) in normal Tyrode solution containing the following (in mM): 137 NaCl, 5.4 KCl, 2 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose, pH 7.4 (adjusted with NaOH). Cells were then washed and rested for 15–30 min to allow for complete deesterification of cytosolic dye.
Fluo-3 was excited at 488 nm, and emission light at >515 nm was detected using a Nikon Diaphot microscope equipped with a micro-fluorometer. Protocols were executed and data collected online with a Digidata analog-to-digital interface and a pClamp software package (Axon Instruments, Foster City, CA). [Ca\(^{2+}\)] was calibrated using the equation \( [\text{Ca}^{2+}] = K_0 (F - F_b)(F_{\text{max}} - F) \), where \( F_b \) was background fluorescence and \( F_{\text{max}} \) was the maximum fluorescence determined in situ in cell superfused with 10 \( \mu \)M 4-B-Bromo A-23187. For the hypotonicity experiments, PASMCs were first equilibrated in modified Tyrode solutions with half of NaCl replaced with equi-osmol of mannitol. Hypotonicity-induced response was initiated by removal of mannitol from the external solution. All the experiments were conducted under normoxic condition at room temperature.

**Mn\(^{2+}\) quenching of fura-2.** Rate of Ca\(^{2+}\) entry was quantified by quenching of fura-2 with Mn\(^{2+}\). PASMCs were loaded with fura-2 AM as described above. Fura-2 was excited at the isosbestic point (360 nm), and emission light was recorded at >510 nm. PASMCs were then bathed in a Ca\(^{2+}\)-free (with 0.1 mM EGTA) nifedipine (1 \( \mu \)M) containing Tyrode solution. After a stable baseline fluorescence was attained, 4\( \alpha \)-PDD was given to PASMCs for 15 min before 500 \( \mu \)M Mn\(^{2+}\) was applied through a multibarrel pipette positioned <50 \( \mu \)m from PASMCs. The rates of quenching of fura-2 fluorescence in PASMCs with/without treatments were determined.

**Short interfering RNA knockdown of TRPV4.** PASMCs were isolated and seeded onto coverslips in 12-well cell culture plate and cultured for ~24 h to 80% confluence. Short interfering (si)RNA against TRPV4 (5’-CGUCAAAACCGCUAGUAUUA-3’ or scrambled control oligonucleotide (5’-UUCCUGAAGCUAGGUCAU-3’) synthesized by Dharmacon (Lafayette, CO) was transfected into PASMCs using Geneporter2 transfection reagent (Genlantis, San Diego, CA) according to the manufacturer’s instructions. Then, 3.5 \( \mu \)g of siRNA were mixed with 25 \( \mu \)l of DNA dilution B solution and 5 \( \mu \)l of Geneporter2 transfection reagent was diluted into 20 \( \mu \)l of serum-free Ham’s F-12 medium. The two solutions were then combined and incubated for 10 min at room temperature to allow complex formation. PASMCs were washed twice, and 50 \( \mu \)l of siRNA/Geneporter2 complexes were added to each well together with 650 \( \mu \)l of fresh Ham’s F-12 medium without serum or antibiotics. The cells were incubated with the complexes for 4–6 h (37°C, 5% CO\(_2\)), and 700 \( \mu \)l of Ham’s F-12 containing 2% FBS were added for further incubation for 12 h. The medium was then changed, and the cells were cultured in growth medium for 36 h before use for experiments.

**Analysis of vasomotor tone in pressurized microvessels.** Small branches of PAs (100–200 \( \mu \)m internal diameter) were dissected in ice-cold Krebs-Ringer bicarbonate solution containing the following (in mmol/l): 118.3 NaCl, 4.7 KCl, 1.2 MgSO\(_4\), 1.2 KH\(_2\)PO\(_4\), 2.5 CaCl\(_2\), 25.0 NaHCO\(_3\), and 11.1 glucose and transferred to a vessel chamber containing the same solution. The proximal end of the artery was cannulated with a tapered glass pipette, secured, and gently flushed to remove any blood from the lumen. The arterial lumen was rubbed with a strand of moose mane to disrupt the endothelium before the distal end of the artery was cannulated. The artery was stretched longitudinally to approximate its in situ length and pressurized to 15 mmHg with a servo-controlled peristaltic pump (Living Systems Instrumentation, Burlington, VT). Any arteries with apparent leaks were discarded. The chamber was superfused with Krebs-Ringer solution at 37°C, pH 7.4 (gassed with 16% O\(_2\)-5% CO\(_2\)), and placed on the stage of an inverted microscope (X20, Nikon TS-100) connected to a video camera (Sony, CCTV camera) and a video monitor. The internal diameter of the vessel was determined continuously by a video dimension analyzer (Living Systems) and recorded using a BIO-PAC (Santa Barbara, CA) data-acquisition system. The effective-
ness of endothelial disruption was verified by the lack of a vasodilatory response to acetylcholine (1 μM) in arteries constricted with U46619 (10 –100 nM). Arteries were exposed to a series of 10-mmHg pressure steps (3 min each) beginning at 5 mmHg and ending at 55 mmHg to examine myogenic tone. Paired experiments were conducted in the absence or presence of ruthenium red (3 μM). To determine the passive diameter at each pressure step, a pressure-diameter curve was recorded after incubation (30 min) of the arteries with Ca²⁺-free control solution (containing 3 mM EGTA). Pressure-diameter curves were also obtained after incubation (15 min) of the arteries with Ca²⁺-free control solution containing 10 μM papaverine. Myogenic tone was calculated as the percent difference in internal diameter observed for Ca²⁺-containing vs. Ca²⁺-free Krebs plus papaverine at each pressure for all groups.

**Pulmonary vascular morphometry.** Mice were exsanguinated after deep anesthesia with pentobarbital sodium (130 mg/kg ip). The trachea was cannulated and the lungs were inflated to 20 mmHg by injection of 0.5% UltraPure low melting point agarose PBS solution (gelling temperature of 24–28°C; Invitrogen). The inflated lungs were cooled on ice for 10 min, cut into smaller pieces, and fixed with 4% formaldehyde at 4°C. The fixed lungs were embedded in paraffin, sectioned into 5-μm slices at levels parallel to, but away from, the

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**Fig. 2.** Ca²⁺ response induced by hypotonic solution on intracellular Ca²⁺ concentration ([Ca²⁺]ᵢ) in rat pulmonary arterial smooth muscle cells (PASMCs). Effects of Ca²⁺-free solution (A) and the TRPV channel antagonist ruthenium red (RuR; 3 μM; B) on the Ca²⁺ transients induced by hypotonic solution on [Ca²⁺]ᵢ in rat PASMCs. C: effect of N-(methylsulfonyl)-2-(2-propynyloxy)-benzenethanamine (MS-PPOH; 30 μM) on Ca²⁺ influx induced by hypotonic solution. D: mean percent change in hypotonic solution-induced Ca²⁺ response after removal of Ca²⁺ (n = 15 cells) and application of RuR (n = 11 cells). E: average change in [Ca²⁺]ᵢ, (Δ[Ca²⁺]ᵢ), induced by hypotonic solution in the presence (n = 17) or absence (n = 16) of MS-PPOH. Experiments were conducted in the presence of 1 μM nifedipine. *P < 0.05, significant difference from control.

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**Fig. 3.** Short interfering (si)RNA knockdown of TRPV4 mRNA and protein in PASMCs. A: conventional (top) and real-time (bottom) RT-PCR analysis of TRPV4 and TRPV2 mRNA expression in PASMCs transfected with siRNA against TRPV4 or control scrambled sequence (n = 8). B: Western blot of TRPV4 protein in PASMCs transfected siRNA against TRPV4 or a control sequence (n = 12). *P < 0.05, significant difference from control.
plane of the hilum to avoid the large vessels. The lung sections were stained with hematoxylin and eosin for gross morphology or with a rabbit polyclonal antibody against smooth muscle α-actin (AB15267; Abcam, Cambridge, MA) to identify muscularized pulmonary arteries. Lung sections were examined under an Olympus BX51 microscope, and images were taken with an Olympus Q-color 5 digital camera and the Qcapture software (Qimaging, Surrey, Canada) and analyzed with the SimplePCI software (Compix, Cranberry, PA).

Vessels of internal diameter <100 μm were classified into nonmuscularized, partially muscularized (25–75%), and completely muscularized (>75%) vessels. Approximately 300 vessels were scored in multiple lung sections from three to four animals in each group. Percent wall thickness (%WT) was quantified for completely muscularized, partially muscularized (25–75%), and completely muscularized (>75%) vessels. Approximately 300 vessels were scored in multiple lung sections from three to four animals in each group. Percent wall thickness (%WT) was quantified for completely muscularized vessels.

Statistical analysis. All data are expressed as means ± SE. The numbers of animals, tissues, preparations, and cells are specified in the text. Statistical significance (P < 0.05) of the changes was assessed by paired or unpaired Student’s t-tests, the nonparametric Mann-Whitney U-tests, or by one- or two-way ANOVA with Tukey’s range test for post hoc analysis, wherever applicable.

RESULTS

Chronic hypoxia upregulates TRPV4 expression in PASMCs. Pulmonary hypertension developed in rats after exposure to 10% O2 for 4 wk, as indicated by a significant increase in RVSP.

![Image](http://ajplung.physiology.org/)
Ca2+-/H9251-PDD (0.3 μM) implicated as mechanosensitive channels in other vascular n.

To examine whether TRPV4 indeed mediates the hypotonicity-induced Ca2+ entry in PASMCs, TRPV4 expression was inhibited specifically using siRNA (Fig. 3, A and B). Transfection of normoxic PASMCs with siRNA against TRPV4 resulted in a 75% reduction in TRPV4 mRNA and 56% reduction in protein expression, compared with PASMC transfected with a nonsilencing random sequence. Expression of the closely related TRPV2 mRNA was unaltered, confirming that the siRNA-mediated gene knockdown was specific. Moreover, there was no change in mRNA levels of TRPM4 (scrambled sequence: 0.50 ± 0.04, n = 8; siRNA: 0.47 ± 0.03, n = 8), TRPC1 (scrambled sequence: 0.12 ± 0.01, n = 8; siRNA: 0.13 ± 0.01, n = 8), and TRPC6 (scrambled sequence: 0.07 ± 0.02, n = 8; siRNA: 0.07 ± 0.01, n = 8), which have been implicated as mechanosensitive channels in other vascular smooth muscle cells.

To examine whether TRPV4 indeed mediates the hypotonicity-induced Ca2+ entry in PASMCs, TRPV4 expression was inhibited specifically using siRNA (Fig. 3, A and B). Transfection of normoxic PASMCs with siRNA against TRPV4 resulted in a 75% reduction in TRPV4 mRNA and 56% reduction in protein expression, compared with PASMC transfected with a nonsilencing random sequence. Expression of the closely related TRPV2 mRNA was unaltered, confirming that the siRNA-mediated gene knockdown was specific. Moreover, there was no change in mRNA levels of TRPM4 (scrambled sequence: 0.50 ± 0.04, n = 8; siRNA: 0.47 ± 0.03, n = 8), TRPC1 (scrambled sequence: 0.12 ± 0.01, n = 8; siRNA: 0.13 ± 0.01, n = 8), and TRPC6 (scrambled sequence: 0.07 ± 0.02, n = 8; siRNA: 0.07 ± 0.01, n = 8), which have been implicated as mechanosensitive channels in other vascular smooth muscle cells.

Knockdown of TRPV4 function was verified by examining cation entry elicited by the TRPV4 agonist 4α-PDD (0.3 μM).

The maximum rate of Mn2+-quenching of fura-2 fluorescence, an index of nonselective cation entry, was significantly attenuated in PASMCs transfected with TRPV4 siRNA (0.77 ± 0.17% s⁻¹, n = 13), compared with the control sequence (1.33 ± 0.19% s⁻¹, n = 13; P < 0.05; Fig. 4, A and B). 4α-PDD-mediated Ca2+ entry was significantly reduced in PASMCs transfected with TRPV4 siRNA (control: 266.58 ± 64.25 nM, n = 13; TRPV4 siRNA: 122.53 ± 36.71 nM, n = 16; P < 0.05; Fig. 4, C and D). Furthermore, Ca2+ entry elicited by hypotonic solution was greatly reduced in TRPV4 siRNA transfected PASMCs (TRPV4 siRNA: 18.65 ± 6.81 nM, n = 14; control: 80.16 ± 25.48 nM, n = 14; P < 0.05; Fig. 4, E and F). These results for the first time show that TRPV4 channels operate as osmo-mechanosensitive Ca2+ channels in rat PASMCs.

Chronic hypoxia enhances TRPV4-mediated Ca2+ entry in PASMCs. The effect of chronic hypoxia on the activity of TRPV4 in PASMCs was evaluated by measuring Ca2+ entry induced by 4α-PDD (0.3 μM; Fig. 5, A and B). Ca2+ entry was augmented significantly in PASMCs isolated from 4 wk chronic hypoxic rats [normoxic PASMCs: 224.14 ± 37.88 nM (n = 30); hypoxic PASMCs: 528.69 ± 124.70 nM, n = 25; P < 0.01]. There was no significant Ca2+ response when vehicle solution (DMSO 1:1,000) was applied instead of 4α-PDD (normoxic cells: −3.9 ± 3.6 nM, n = 8; hypoxic cells: 0.2 ± 0.9 nM, n = 12). The maximal rate of quenching induced by 4α-PDD was doubled in the chronic hypoxic PASMCs (normoxic PASMCs: 0.72 ± 0.14% s⁻¹, n = 8; hypoxic PASMCs: 1.81 ± 0.46% s⁻¹, n = 8; P < 0.01; Fig. 5, C and D). Similarly, Ca2+ transients activated by hypotonic solution were significantly larger in chronic hypoxic PASMCs, with a large transient Ca2+ release followed by a sustained elevation of [Ca2+]i due to Ca2+ influx (Fig. 6, A and B). The initial Ca2+ release recorded in Ca2+-free solution was 22.8 ± 4.51 (n = 25) nM normoxic and 90.76 ± 17.91 nM (n = 22; P < 0.01) in hypoxic PASMCs. The Ca2+ entry signal re-
The enhanced Ca^2+ entry via osmo-mechanosensitive TRPV4 channels in hypoxic PASMCs might facilitate vasoconstriction in response to elevated Ppa. This possibility was investigated in isolated endothelium-denuded pressurized small PAs (internal diameter <200 μm) of normoxic and chronic hypoxic rats. The effectiveness of endothelial disruption was verified by the lack of a vasodilatory response to acetylcholine (1 μM) in arteries constructed with U46619 (10–100 nM). Elevation of transmural pressure from 5 to 55 mmHg (in 10-mmHg steps) caused a monotonic increase in the steady-state diameter of microvessels of normoxic rats. The pressure-diameter relation was unaffected by the removal of extracellular Ca^2+ (in the presence of 3 mM EGTA) or by the addition of papaverine (10 μM) in Ca^2+-free solution to induce maximal vasodilatation (Fig. 7A), indicating that there was no active myogenic tone in normoxic PAs. In contrast, significant myogenic tone developed in small PAs of chronic hypoxic rats. These arteries dilated significantly in Ca^2+-free solution with or without papaverine (repeated-measures ANOVA, P < 0.001), and the differences in the pressure-diameter relation were readily noticeable at intraluminal pressure between 25–55 mmHg (Fig. 7B). Myogenic tone was further analyzed by calculating the percentage difference in internal diameter recorded after readmission of extracellular Ca^2+ which had been shown to normalize the elevated [Ca^2+]i elicited by hypotonic solution in normoxic (n = 22) and hypoxic PASMCs (before ruthenium red: 104.3 ± 260.7 nM; after ruthenium red: 260.7 ± 26.1 nM, P < 0.001; Fig. 8, A and B). Moreover, ruthenium red (3 μM) had no significant effect on the basal [Ca^2+]i of normoxic (control: 294.2 ± 34.9 nM, n = 18; ruthenium red: 260.7 ± 26.1 nM, n = 18; Fig. 8, A and B). However, ruthenium red (3 μM), which had no significant effect on the basal [Ca^2+]i, of hypoxic PASMCs (before ruthenium red: 104.3 ± 9.5 nM; after RuR: 96.7 ± 6.5 nM, n = 9), caused a significant reduction in the elevated basal [Ca^2+]i of the hypoxic PASMCs (before ruthenium red: 134.0 ± 6.5 nM; after ruthenium red: 96.7 ± 6.5 nM, n = 9), which had been shown to normalize the elevated [Ca^2+]i in hypoxic PASMCs (26), also blocked the 4α-PDD induced Ca^2+ influx (control: 476.2 ± 64.7 nM, n = 11; La^3+; 52.0 ± 5.7, n = 11; P < 0.001; Fig. 8, E and F). Since TRPV4 is the
only TRPV channel upregulated by chronic hypoxia, our results suggest that it is likely to be responsible for the enhanced myogenic tone and contributes to the elevated basal [Ca^{2+}]i in chronic hypoxic PAs.

TRPV4 gene deletion reduces chronic hypoxia-induced pulmonary hypertension. As a test of the concept that TRPV4 contributes to the development of chronic hypoxic pulmonary hypertension, TRPV4 null (trpv4−/−) mice and age-matched wild-type mice were exposed to 10% O2 for 0, 1, 2, and 4 wk. Increase in RVSP and RV/(LV+S) mass ratio were clearly evident in the wild-type mice after 1 wk of hypoxia exposure, and progressed to higher levels in the second and the fourth week (Fig. 9, B and C). These changes were virtually undetectable in trpv4−/− mice after 1 wk of hypoxia, and the increases in RVSP and RV/(LV+S) in the subsequent weeks were significantly attenuated compared with the wild-type controls. Polycythemia was developed to the similar extent in the trpv4−/− and wild-type mice at all the time-points examined (Fig. 9D). These results show for the first time that the trpv4 gene contributes the development of hypoxic pulmonary hypertension.

Morphological analysis was performed to compare vascular remodeling in wild-type and trpv4−/− mice exposed to 4 wk of hypoxia. Smooth muscle was clearly observed in small PAs and precapillary alveolar arterioles in wild-type hypoxic lungs, as a hallmark of hypoxic pulmonary hypertension, in contrast to the thin medial layer in small arteries and the absence of smooth muscle in arterioles of normoxic lungs (Fig. 10, A and B). Frequency distributions of resistance PAs (inner diameter < 100 μm) show a dramatic increase in the muscularized small PA (30 μm) in hypoxic lungs (Fig. 10, C and D). Median diameter of muscularized vessels was shifted from a median of 41.3 μm in normoxic lungs to 21.2 μm in hypoxic lungs (P = 0.001). Muscularization was observed, but to a less extent, in small PAs of chronic hypoxic trpv4−/− mice. Median diameter of muscularized PAs in hypoxic trpv4−/− lungs was 24.9 μm, which is significantly different from the hypoxic wild-type lung (Mann-Whitney rank sum test, P = 0.001). Moreover, the density of the completely muscularized vessels was lower, and the thickness of medial layers (expressed in %diameter or %area) was significantly decreased in the hypoxic trpv4−/− lungs (Fig. 10, E and F). Even though there is a possibility...
that residual vascular tone in the lungs of trpv4−/− mice before fixation may affect the percent wall thickness and area, our results on the density of muscularized vessels clearly suggest that the trpv4 gene contributes to the hypertrophy and/or hyperplasia of smooth muscle in small PAs of hypoxic animals. Nevertheless, the significant increase in muscularization of PA in hypoxic trpv4−/− lungs indicates that other mechanisms in addition to TRPV4 are also involved.

**DISCUSSION**

The purpose of the present study is to identify the vanilloid- and melastatin-related TRP channels that are regulated by chronic hypoxia, as an endeavor to discover alternative Ca2+ pathways contributing to hypoxic pulmonary hypertension. Our survey on the 14 known TRPV and TRPM isoforms found that TRPV4 was the only channel upregulated in PA of rats after four wk of hypoxia exposure. This specific upregulation of TRPV4 mRNA expression was an early response to hypoxia occurring within the first day of challenge and persisted throughout the development of pulmonary hypertension. Functional experiments performed in PASMCs showed that TRPV4 could be activated by osmomechanical stress, and its activity was significantly enhanced by chronic hypoxia. The upregulation of TRPV4 in PASMCs was associated with the appearance of a pressure-induced myogenic constrictor response, which was abolished by ruthenium red, in small PAs of chronic hypoxic rats. The elevated basal [Ca2+]i in hypoxic PASMCs was also reduced by ruthenium red. Moreover, deletion of the trpv4 gene delayed and suppressed the development of pulmonary hypertension and right heart hypertrophy, as well as reduced the muscularization of resistance PAs in hypoxic trpv4−/− mice. These results for the first time provide direct evidence at the molecular, cellular, organ, and animal levels, indicating that the expression and function of TRPV4 channels in PAs are regulated by chronic hypoxia, and they are required for the full development of hypoxic pulmonary hypertension.

Our previous study (26) showed that chronic hypoxia enhances the expression of store-operated TRPC1 and receptor-operated TRPC6 channels in rat PAs. The enhanced store-operated Ca2+ entry is thought to be responsible for the increase of basal [Ca2+]i in PASMCs and resting tone in PA of chronic hypoxic rats (26, 28, 60). The present study has placed TRPV4 as the third member of the TRP superfamily regulated by chronic hypoxia. The early transcriptional regulation of TRPV4 by hypoxia suggests that it could be a target gene of hypoxia-dependent transcription pathways, such as HIF-1. This notion is concordant with previous studies showing hypoxia-induced structural and physiological changes in pulmonary vasculatures, including membrane depolarization, increase in resting [Ca2+]i, reduction in Kv currents, and upregulation of Na+/H+ exchange which all require the full expression of HIF-1α (48, 49, 67), and HIF-1α regulates the increase in expression of TRPC1 and TRPC6 in hypoxic PASMCs (60). Alternatively, TRPV4 expression could be regulated by other oxygen-sensitive or Ca2+-dependent transcription factors such as early growth-response gene, NF-κB, and nuclear factor of
activated T cells (4, 5, 64), which are known to be activated by hypoxia.

The novel observation of TRPV4 upregulation in hypoxic PAs prompted us to investigate its physiological functions in pulmonary vasculature and its pathophysiological roles in pulmonary hypertension. TRPV4 has a widespread expression in many cell types and is best recognized as an osmo-mechanosensitive channel (24). It can be activated multimodally by hypotonicity, mechanical, and thermal (warmth) stimuli, as well as by naturally occurring and synthetic chemical compounds such as the arachidonic acid metabolites epoxyeicosatrienoic acids (EETs) and the phorbol ester derivative 4α-PDD (9, 59, 61). It has been established in certain cell types that osmo-mechanical stimuli can activate phospholipase A2 to generate arachidonic acid and its downstream cytochrome P450 epoxygenase metabolites 5,6-EET and 8–9-EET to activate TRPV4 channels (58, 59, 61).

Our present study clearly suggests that TRPV4 can operate as an osmo-mechanosensitive cation channel in PASMCs. It is based on a series of evidence that cell swelling induced by hypotonic solution, a common surrogate for mechanical stretch, activated a significant Ca\(^{2+}\) transient in PASMCs. The Ca\(^{2+}\) response was dependent on extracellular Ca\(^{2+}\) and blocked by the TRPV antagonist ruthenium red and the cytochrome P450 epoxygenase inhibitor MS-PPOH. Specific knockdown of TRPV4 with siRNA attenuated both the Ca\(^{2+}\) response induced by 4α-PDD and hypotonic solution. Mechanosensitive cation channels have been recorded in adult rabbit PASMCs (39, 40). The density of these channels is significantly higher, and the threshold for activation is lower in PAs than in systemic arteries, suggesting adaptive tuning for the low pressure in pulmonary circulation (40). The preferential expression of mechanosensitive channels in rabbit PA is consistent with our previous (65) finding that TRPV4 is abundantly expressed in rat PASM with a transcript level significantly higher than aorta.

The prominent expression of the mechanosensitive TRPV4 in PASMCs and their immediate upregulation after hypoxia exposure suggest that mechanical stimulus, an underappreciated factor in pulmonary hypertension, may play a significant role in the pathogenesis of chronic hypoxic pulmonary hypertension. In systemic circulation, an increase in intravascular pressure is known to activate mechanosensitive channels to generate myogenic tone (10, 54, 63), and mechanical stretch of VSMCs can stimulate cell proliferation and vascular remodeling (6, 18, 43, 47). In pulmonary circulation, Ppa and vascular resistance are inherently low, and vascular tone is minimal or nonexistent. However, hypoxia exposure initiates hypoxic pulmonary vasoconstriction and subsequent development of significant vascular tone (3, 26, 38, 50). The elevated Ppa may exert sufficient mechanical stretch to activate the upregulated TRPV4 channels to generate myogenic tone. This is evident in the present study of the pressurized deendothelized small PAs of chronic hypoxic rats. The myogenic tone, which was ruthenium red sensitive, increased with transmural pressure to a maximum of ~10% reduction in diameter at 35 mmHg. Since the activation of myogenic tone (15–35 mmHg) occurs well within the dynamic range of Ppa of hypoxic animals, and 10% reduction in vessel diameter can account
for significant elevation in vascular resistance (Poiseuille’s law: resistance \( \propto 1/r^4 \)), the hypoxia-induced myogenic tone could contribute in part to hypoxic pulmonary hypertension. Several other TRP channels including TRPC1, TRPC6, TRPM4, and TRPV2 have been implicated as mechanosensitive cation channels in VSMCs (10, 31, 35, 54, 63). However, TRPV4 is the only mechanosensitive TRP channel that is upregulated by hypoxia and blocked by ruthenium red. Hence, it is most likely the Ca\(^{2+}\) influx pathway responsible for the ruthenium red sensitive myogenic tone observed in the chronic hypoxic PAs.

It is noteworthy that ruthenium red reduces the elevated basal [Ca\(^{2+}\)] in PASMCs of chronic hypoxic rats. Our previous study (26) showed that La\(^{3+}\), at a low concentration (10 \( \mu \text{M} \)) that blocks store-operated Ca\(^{2+}\) entry, normalized the elevated basal [Ca\(^{2+}\)] in PASMCs of chronic hypoxic rats. Since ruthenium red does not inhibit store-operated Ca\(^{2+}\) entry (Fig. 8, A and B) and La\(^{3+}\) can inhibit 4\( \alpha \)-PDD induced Ca\(^{2+}\) entry (Fig. 8, E and F), it is likely that the facilitation of Ca\(^{2+}\) influx via TRPV4 contributes at least in part to the elevated basal [Ca\(^{2+}\)] of these cells. However, it has been shown that siRNA knockdown of TRPC1 and TRPC6 reduced store-operated Ca\(^{2+}\) entry partially reduced the elevated [Ca\(^{2+}\)] in the hypoxic PASMCs (28). Hence, the relative contributions and interactions of TRPV4 channels, TRPC channels, and store-operated Ca\(^{2+}\) entry in the regulation of basal [Ca\(^{2+}\)], of hypoxic PASMCs require further investigations.

In addition to myogenic tone and basal [Ca\(^{2+}\)], the increased expression of TRPV4 in PAs may contribute to hypoxic pulmonary hypertension through other potential mechanisms. EETs, the endogenous activators of TRPV4 channels, are derived from cytochrome P450 epoxygenases. Cytochrome P450 epoxygenases are highly expressed in pulmonary endothelial cells, PASMCs, as well as airway epithelial and SMCs (19). A previous study (42) showed that exposure of mice to hypoxia upregulates P450 epoxygenases and increase the production of EETs (8,9-EET, 11,12-EET, and 13,14-EET) in the lungs. Since all EET regioisomers, including the putative systemic EDHF 11,12-EET, have been reported to cause vasoconstriction in pressurized rabbit PAs (72), the enhanced EET production in hypoxic lung cells may facilitate pulmonary vasoconstriction through paracrine activation of TRPV4 in PAs. Moreover, serotonin is capable of activating a TRPV4 like non-selective cation channels in rat PASMCs (8) presumably through receptor-mediated phospholipase A2 activation and EET production (8, 14). Since the serotonin signaling pathway is intricately linked to hypoxic pulmonary hypertension (11, 21, 29), the upregulation of TRPV4 in hypoxic PAs may contribute to the serotonin-dependent mechanisms. It is also recognized that chronic hypoxia enhances basal vascular tone and reactivity to agonists in part through Rho/Rho kinase signaling pathway (3, 12, 36, 62). The enhanced myofilament Ca\(^{2+}\) sensitivity mediated by Rho kinase, and the increased Ca\(^{2+}\) influx via TRPV4 channels may function synergistically to promote pulmonary vascular reactivity in hypoxic PAs.

Fig. 10. Morphological analysis of pulmonary vascular remodeling in wild-type and trpv4\(^{-/-}\) mice exposed to 4-wk hypoxia. A and B: lung sections of normoxic and hypoxic wild-type mice immunostained with smooth muscle \( \alpha \)-actin antibody (brown). Arrows indicate small peri-alveolar vessels. C and D: Size distributions of muscularized vessels in lungs of normoxic and hypoxic wild-type and trpv4\(^{-/-}\) mice. Box plots show the median and range of the diameter of muscularized vessels. E: density of partially (partially M; 25–75% actin positive) and completely muscularized (completely M; >75% actin positive) arteries in normoxic and hypoxic lungs. F: %medial thickness and %cross-sectional area of completely muscularized vessels. Both the density and the thickness of the completely muscularized vessels were significantly less in hypoxic trpv4\(^{-/-}\) mice. More than 300 vessels were analyzed from 4 mice in each group.
The notion that TRPV4 channels are critically involved in the pathogenesis of hypoxic pulmonary hypertension is strongly supported by our results from trpv4<sup>−/−</sup> mice. The complete absence of pulmonary hypertension after 1 wk of hypoxia; the significant suppression of subsequent increase in Ppa and right heart hypertrophy; and the reduction in the density and medial thickening of muscularized small PAs in hypoxic trpv4<sup>−/−</sup> mice indicate that TRPV4 participates in both the early development and the maintenance of hypoxic pulmonary hypertension. These effects are unrelated to a generalized impairment of the HIF-1 pathway because the levels of polycythemia in the wild-type and trpv4<sup>−/−</sup> mice were similar throughout the period of hypoxia exposure. Since vascular remodeling is mild in the early stage of hypoxic pulmonary hypertension in mice, the dramatic differences between the wild-type and trpv4<sup>−/−</sup> mice in the first week of hypoxia are likely related to the TRPV4-dependent vascular tone. Moreover, the reduction in muscularization of perialveolar arterioles and medial thickening of distal PAs in the 4 wk hypoxic trpv4<sup>−/−</sup> mice suggests that TRPV4 may participate in PASMC hyperplasia and/or hypertrophy during vascular remodeling. It has been established that TRPC1 and TRPC6 upregulation are associated with mitogen/growth factor-induced PASMC proliferation, and knockdown of these channels inhibits PASMCs proliferation (13, 56, 70). The TRPV4-dependent muscularization of resistance PAs may provide an additional mechanism to further strengthen the vasoconstriction mediated by Ca<sup>2+</sup> influx through TRPV4 channels and contribute to the progression of pulmonary hypertension.

It is recognized that systemic deletion of TRPV4 in trpv4<sup>−/−</sup> mice may affect other pulmonary cells that are involved in hypoxic pulmonary hypertension. For example, endothelial TRPV4 are critical for shear-stress-induced vasodilatation, nitric oxide, and EDHF production (16, 22, 46), as well as the increase in alveolar barrier permeability in high vascular pressure- and ventilator-induced lung injury (1, 15, 20, 66). These processes are not functioning normally in trpv4<sup>−/−</sup> mice. Since the development of hypoxic pulmonary hypertension depends on the complex interplays between PASMCs and other pulmonary cells, the specific contributions of TRPV4 of PASMCs and other lung cells require future investigations perhaps in tissue-targeted knockout animals.

In summary, we have identified the osmo-mechanosensitive TRPV4 channel in PASMCs as a novel Ca<sup>2+</sup> entry pathway upregulated by chronic hypoxia. It contributes to the enhanced myogenic response and vascular response in hypoxic PAs and is required for the full development of hypoxic pulmonary hypertension. These results lay out an attractive concept that TRPV4 acts as a signal transducer for the elevated Ppa and other stimulators. Its upregulation during hypoxia exposure provides a feed-forward mechanism to facilitate Ca<sup>2+</sup> influx to enhance myogenic tone and vascular remodeling (see schematics in Fig. 11). Together with the upregulated store-operated TRPC6 and receptor-operated TRPC6 that contribute to the increased resting [Ca<sup>2+</sup>]<sup>+</sup>, and agonist induced responses, respectively (26, 28), TRPV4 served as another multifaceted Ca<sup>2+</sup> entry pathway participating in the exacerbation of hypoxic pulmonary hypertension. In view of its significant contribution to pulmonary hypertension, TRPV4 channel can be considered as a potential therapeutic target for the treatment of this dreadful disease.

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DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


