TRPV4 Is Required for Hypoxic Pulmonary Vasoconstriction

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ABSTRACT

Background: Hypoxic pulmonary vasoconstriction (HPV) is critically important in regionally heterogeneous lung diseases by directing blood toward better-oxygenated lung units, yet the molecular mechanism of HPV remains unknown. Transient receptor potential (TRP) channels are a large cation channel family that has been implicated in HPV, specifically in the pulmonary artery smooth muscle cell (PASMC) Ca^{2+} and contractile response to hypoxia. In this study, the authors probed the role of the TRP family member, TRPV4, in HPV.

Methods: HPV was assessed by using isolated perfused mouse lungs or by intravital microscopy to directly visualize pulmonary arterioles in mice. *In vitro* experiments were performed in primary human PASMC.

Results: The hypoxia-induced pulmonary artery pressure increase seen in wild-type mice (5.6 ± 0.6 mmHg; mean ± SEM) was attenuated both by inhibition of TRPV4 (2.8 ± 0.5 mmHg), or in lungs from TRPV4-deficient mice (*Trpv4*$^{-/-}$) (3.4 ± 0.5 mmHg; n = 7 each). Functionally, *Trpv4*$^{-/-}$ mice displayed an exaggerated hypoxemia after regional airway occlusion (p_aO_2 71% of baseline ± 2 *vs.* 85 ± 2%; n = 5). Direct visualization of pulmonary arterioles by intravital microscopy revealed a 66% reduction in HPV in *Trpv4*$^{-/-}$ mice. In human PASMC, inhibition of TRPV4 blocked the hypoxia-induced Ca^{2+} influx and myosin light chain phosphorylation. TRPV4 may form a heteromeric channel with TRPC6 as the two channels coimmunoprecipitate from PASMC and as there is no additive effect of TRPC and TRPV4 inhibition on Ca^{2+} influx in response to the agonist, 11,12-epoxyeicosatrienoic acid.

Conclusion: TRPV4 plays a critical role in HPV, potentially *via* cooperation with TRPC6. **(Anesthesiology 2015; 122:1338-48)**

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HYPOXIC pulmonary vasoconstriction (HPV) optimizes gas exchange by redirecting blood flow from poorly aerated to well-ventilated areas of the lung.[1] Many molecular aspects of this process have been elucidated to date, including the recent identification that the hypoxic signal is initially sensed at the level of the capillary endothelium and propagated upstream to resistance arterioles as a conducted response.[2] The ultimate effector of HPV is the pulmonary artery smooth muscle cell (PASMC). Hypoxia-induced PASMC contraction—and therefore vasoconstriction—stems from membrane depolarization and Ca^{2+} influx from several sources including voltage-operated Ca^{2+} channels,[3] store-operated Ca^{2+} channels,[4] and sarcoplasmic reticulum Ca^{2+} release channels.[5]

An important class of Ca^{2+} channel in lung vascular biology is the transient receptor potential (TRP) channel family.[1,6] TRP channels are polymodal cation channels that are expressed in cellular membranes as homotetramers or heterotetramers and have been implicated as sensors of various cellular functions including mechanosensation and thermosensation.[6,7] Specifically, the classical channel, TRPC6, has been implicated in the intact HPV response: TRPC6 is expressed in human PASMC, and inhibition of TRPC6 diminished the PASMC Ca^{2+} response to hypoxia.[8–10] TRPC6 is activated by key mediators of HPV, such as epoxyeicosatrienoic acids (EETs),[11,12] and

What We Already Know about This Topic

- Hypoxic pulmonary vasoconstriction is an intrinsic response of the pulmonary vasculature that is critical to perfusion–ventilation matching
- Vasoconstriction effected by pulmonary artery smooth muscle cells is mediated by calcium-activated contraction, but the route of calcium entry is unclear

What This Article Tells Us That Is New

- A role for the calcium-permeant transient receptor potential channel TRPV4 in hypoxic pulmonary vasoconstriction was demonstrated in mouse models *in vivo* and *in vitro* both pharmacologically and in Trpv4$^{-/-}$ mice
- TRPV4 is critical for hypoxia-induced pulmonary vasoconstriction in contrast to its vasodilatory roles in renal and mesenteric arteries

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lungs isolated from *Trpc6*$^{-/-}$ mice have an attenuated HPV response.[13]

Vanilloid subfamily (TRPV) channels are highly abundant in endothelial and vascular smooth muscle cells and in perivascular neural tissue.[14] TRPV channels are differentially expressed in different vascular beds, indicating their specific roles in different organs. For example, nanomolar concentrations of the TRPV1 agonist capsaicin cause endothelial-dependent relaxation of mouse mesenteric, yet not renal arteries, whereas TRPV4 stimulation causes endothelial-dependent vasodilation in both vascular beds.[15] Stimulation of TRPV4 channels in arterial smooth muscle cells of the systemic circulation likewise promotes vasodilation *via* activation of large-conductance Ca^{2+}-activated K^+ channels, resulting in membrane hyperpolarization.[16,17] Conversely, activation of TRPV4 in PASMC by serotonin results in vasoconstriction.[18] Similar to TRPC6, TRPV4 is highly expressed in PASMC[19] and activated by EETs.[20] Our group and others have demonstrated a role for endothelial TRPV4 in the disruption of vascular permeability and edema formation due to increased hydrostatic pressure or ventilator pressure.[21–23] Based on these findings, an oral TRPV4 inhibitor has been developed that decreases cardiogenic lung edema in mice and is the current focus of further clinical testing.[24] There is similar interest in pharmacological targeting of TRPV channels for therapeutic benefit in asthma, acute lung injury, and other relevant lung diseases.[25,26]

In addition to its general vasoactive role, TRPV4 has been shown to be up-regulated in chronic hypoxia in rat pulmonary arteries and was responsible for the increased myogenic tone in pulmonary vessels of hypoxic rats.[27] Although these data demonstrate a clear link between TRPV4 and the PASMC response to agonists or chronic hypoxia, a potential role for TRPV4 in acute HPV has not been addressed so far. In this study, we hypothesized that TRPV4 would be required for an intact HPV response and that loss of TRPV4 function would result in a blunted PASMC Ca^{2+} response to hypoxia.

Materials and Methods

Reagents

HC-067047 and SKF-96365 were purchased from Sigma (Canada). Hank's buffered salt solution (HBSS), 15-μm diameter yellow-green fluorescent microspheres, and Fura-2-acetoxymethyl ester (AM) were purchased from Invitrogen (USA). Anti-caveolin-1 antibody was purchased from BD Biosciences (Canada), and rabbit anti-TRPV4 antiserum was a generous gift from Dr. Stefan Heller (Stanford University, Stanford, California).[28] Antimyosin light chain (MLC) and anti-phospho-MLC were purchased from Cell Signaling Technology (USA).

Animals

All mice were housed in accordance with national guidelines, and all animal experiments were approved by the Animal Care Committee at St. Michael's Hospital (Toronto, Ontario, Canada). Male C57/Bl6 mice were obtained from Charles River Laboratories (Canada). Male mice deficient in TRPV4 (*Trpv4*$^{-/-}$) bred on a background of C57/Bl6 were from Dr. W. Liedtke[29] and were maintained on site. For experiments, mice were anesthetized with a single intraperitoneal injection of ketamine and xylazine, unless otherwise indicated.

Isolated Perfused Lung

Mice were anesthetized as described in Intravital Microscopy section and ventilated *via* a tracheostomy with a tidal volume of 10 ml/kg and at a rate of 90 breaths/min. The mice were then sacrificed by exsanguination. The pulmonary artery and left atrium were cannulated, and the lungs were perfused with 20% fetal bovine serum in HBSS containing Ca^{2+} and Mg^{2+} at 37°C, with a flow rate of 50 ml min^{-1} kg^{-1} as previously reported.[2] For normoxic treatment, the ventilating gas contained 21% O_2, 5% CO_2, and 74% N_2. Hypoxic gas was 1% O_2 in 5% CO_2, and 94% N_2. After stabilization of the pulmonary artery pressure (PAP) on normoxic gas, ventilation was switched to hypoxia for 5 min. The peak PAP during this 5-min period was recorded. Ventilation was then returned to normoxia, and the PAP was allowed to return to baseline.

Intravital Microscopy

Mice were anesthetized with an intraperitoneal injection of fentanyl, midazolam, and meditomidine. After tracheostomy, they were ventilated with room air at 100 breaths/min. A window into the right chest was surgically opened as described earlier.[30] An intrapleural catheter was inserted, the chest was resealed with a polyvinylidine membrane, and negative intrapleural pressure was reestablished at −3 mmHg. Mice were placed on a custom-made heated, motorized stage under an upright microscope (Axiotechvario 100HD; Zeiss, Germany). After baseline image acquisition (TillVision; Till Photonics, Germany) of subpleural pulmonary arterioles (PAs), ventilation was shifted to a fraction of inspired oxygen (F_{IO_2}) of 0.11. Vessels were imaged again after 10 min of hypoxic ventilation, and changes in vessel diameter in response to hypoxia were calculated from the acquired images.

Intratracheal Saline Instillation

The effect of ventilation/perfusion (V/Q) mismatch *in vivo* was assessed in C57/Bl6 or *Trpv4*$^{-/-}$ mice as previously described.[2] In brief, the left carotid artery was cannulated with a heparinized catheter. An arterial blood gas sample was measured to obtain a baseline reading of the arterial partial pressure of oxygen (p_aO_2). A small amount of normal saline (1 μl/g) was instilled intratracheally using a 25-gauge needle inserted into the tracheal tube (time 0). After a further 2 min of ventilation, a second arterial blood gas sample was analyzed.

One-lung Ventilation

As described earlier,[2] anesthetized mice were ventilated, and their right internal jugular vein was cannulated. One-lung

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ventilation was established by advancing the tracheal tube into the left mainstem bronchus. After 5 min, a 150 μl bolus of 15-μm fluorescent microspheres was delivered to the right atrium *via* the internal jugular vein. Mice were then sacrificed by exsanguination. Each lung was harvested and weighed and then dissolved overnight in 4 N KCl. Microspheres were recovered by filtration and then were dissolved in Cellsolve acetate (Sigma). Fluorescence emission in each lung sample was assessed using a standard plate reader, and fluorescence was normalized per milligram of lung weight. A small section of kidney tissue was used as a negative control to ensure complete microsphere capture in the pulmonary circulation during the first passage (data not shown).

Intracellular Ca^{2+} Measurements

Human primary PASMC were purchased (Lonza, Switzerland) and cultured in normoxic conditions according to the manufacturer's instructions. Cells were routinely used between passages 4 and 9. For real-time fluorescence imaging, cells were plated on 25-mm square coverglasses in six well plates. Cells were loaded with Fura 2-AM as per the manufacturer's directions. Coverglasses were mounted inside a perfusion chamber heated to 37°C and were perfused with HBSS containing Ca^{2+} and Mg^{2+}. Hypoxia was induced by bubbling HBSS with 1% O_2 in a hypoxic chamber for 45 min before experiments, and oxygen content in the perfusate was verified using a clinical blood gas analyzer. Hypoxic buffer routinely had a pO_2 less than 35 mmHg. Fura-2 imaging was performed as described previously,[2] and 340/380 Fura-2 ratios were converted to Ca^{2+} concentration based on appropriate calibration parameters.[31] Confocal experiments using Fluo-4 (Invitrogen) were performed similarly. Data were collected using a Leica GET SCOPE INFO and MetaMorph (Molecular Devices, USA) and are expressed as the peak change in Fluo-4 intensity after treatment, relative to baseline.

Fractionation of Caveolae

Primary human PASMC were maintained as mentioned in the section Intracellular Ca^{2+} Measurements. PASMC were exposed to hypoxia for 15 min in a hypoxic chamber, or left in room air, before lysis. Lysates were homogenized and then applied to sucrose gradients (40% to 5%) and centrifuged for 18 h at 60,000*g*. Seven equal fractions were collected, and equal amounts of protein were resolved on a 10% polyacrylamide gel. Membranes were immunoblotted for TRPV4 and caveolin-1 to identify caveolae. Data were quantified and expressed as the percent of TRPV4 signal in cav-1-containing fractions relative to total TRPV4.

Coimmunoprecipitation

Mice were anesthetized and ventilated as in the isolated perfused lung experiments. After 3 min of normoxic or hypoxic ventilation (the time at which the maximal PA pressure was recorded in other experiments), samples of whole lung were extracted and snap frozen in liquid nitrogen. Whole-lung lysates were collected by homogenization of these samples in ice-cold lysis buffer. Equal amounts of precleared whole-lung lysate were immunoprecipitated with anti-TRPV4 antibody, which was captured on Protein A/G beads (GE Healthcare, Canada). After washing the beads, captured protein was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were probed with anti-TRPV4 or anti-TRPC6 (Alomone Labs, Israel). Supernatants from beads as well as samples incubated with beads alone (*i.e.*, in the absence of immunoprecipitating TRPV4 antibody) also were run as negative controls.

Statistical Analysis

Data are shown as mean ± SEM and as scatter plots for four or less samples. Statistical analyses were performed using GraphPad Prism (GraphPad Software, USA). Pairwise comparisons were carried out using the Mann–Whitney U Test. Multiple comparisons used Kruskall–Wallace testing and Dunn multiple comparison test. A *P* value less than 0.05 was considered significant. Animals for experimentation were randomly selected from a given cage by the experimenter. As most data were collected in real time, the investigator was not blinded to the experimental conditions. Sample sizes were based on previous experience, and no formal power calculation was carried out before the study. There were no missing data in any experiments.

Results

To probe for a potential role for TRPV4 in HPV, we first used the isolated perfused mouse lung model. In lungs isolated from wild-type C57/Bl6 mice, upon shifting ventilating gas from a normoxic (21% O_2) to a hypoxic (1% O_2) mixture, we observed a rapid increase in PAP (fig. 1). The maximal increase in PAP during a 5-min period of hypoxic ventilation was 5.6 ± 0.6 mmHg. When the specific TRPV4 inhibitor, HC-067047[32], was added to the perfusate at a concentration of 20 μM, the hypoxic response was severely attenuated, by approximately 50%. This result indicates that TRPV4 is involved in the HPV response in isolated mouse lungs. To further assess this effect, we tested lungs isolated from mice lacking functional TRPV4 expression (*Trpv4*$^{-/-}$).[29] Lungs from *Trpv4*$^{-/-}$ mice also exhibited a diminished HPV response, with a similar magnitude as that seen with pharmacological inhibition of TRPV4. These results indicate that TRPV4 is a required component of the normal HPV response. Basal perfusion pressures during normoxia did not differ between groups (10 ± 0.6 mmHg), indicating that the different reponses to hypoxia were not the result of different baseline offsets. Because TRPC6 has previously been shown to be involved in HPV,[13] we tested for a possible compensatory change in TRPC6 expression in our *Trpv4*$^{-/-}$ mice. Western blots of whole-lung lysates from *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice revealed no significant difference in TRPC6 protein expression in mice lacking TRPV4 compared with

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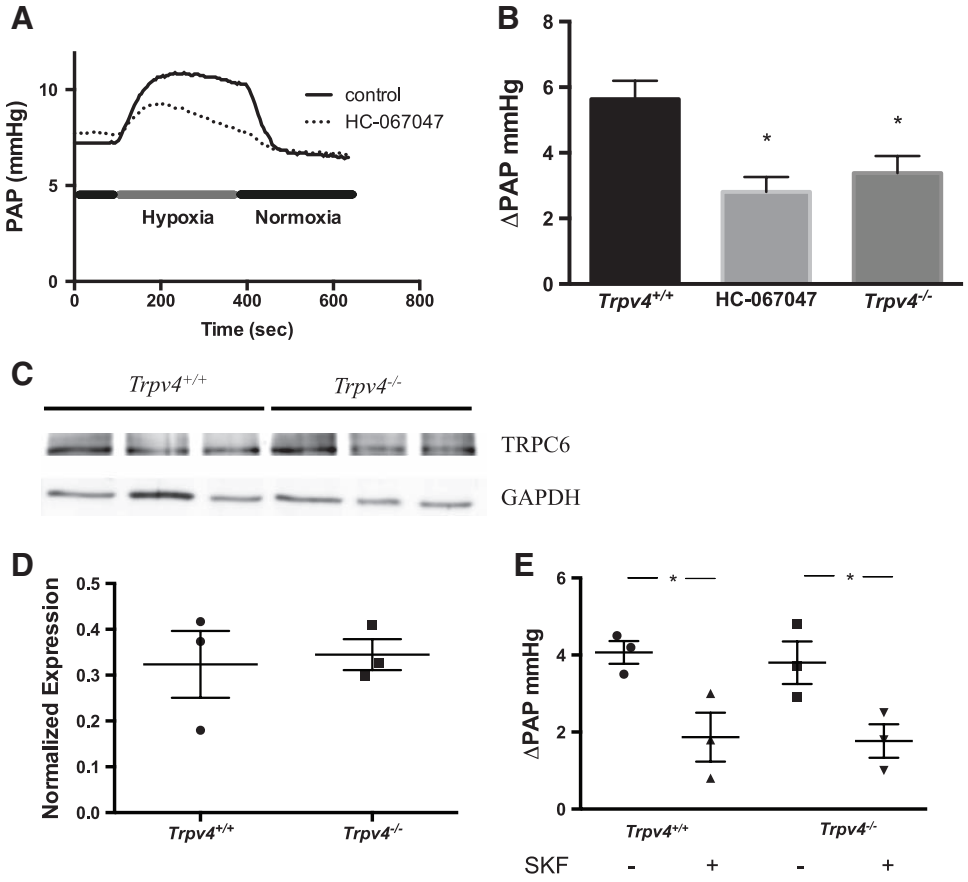

Fig. 1. TRPV4 is required for hypoxic pulmonary vasoconstriction (HPV) in isolated perfused mouse lungs. Lungs were isolated from *Trpv4*$^{+/+}$ or *Trpv4*$^{-/-}$ mice as described in Materials and Methods section. For treatments with the TRPV4 inhibitor, HC-067047, inhibitor was added at 20 μM to the perfusate for 15 min before hypoxia and continued throughout. (*A*) Representative tracings of pulmonary artery pressure (PAP) in isolated perfused wild-type mouse lungs under constant perfusion rate during normoxia (21% O_2) or hypoxia (1% O_2). Note the diminished increase in PAP during hypoxia in wild-type lungs perfused with TRPV4 inhibitor. (*B*) Group data (n = 7 lungs per group) showing the HPV response as the maximal change in PAP during a 5-min period of hypoxic ventilation. Note that both pharmacological inhibition of TRPV4 and genetic deletion of TRPV4 attenuate the HPV response. *$P < 0.05$ *versus* control. (*C*) Western blots of whole-lung lysates taken from *Trpv4*$^{+/+}$ or *Trpv4*$^{-/-}$ mice to probe for TRPC6 protein. (*D*) Group data of the Western blots shown in *C*. There was no significant difference in TRPC6 expression, normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression, between wild-type and *Trpv4*$^{-/-}$ mice (n = 3 mice per group). (*E*) The functional response to TRPC6 activation with hyperforin (10 μM) is unchanged in *Trpv4*$^{-/-}$ compared with *Trpv4*$^{+/+}$ mice. In isolated perfused mouse lungs, TRPC6 activation caused the same increase in PAP (ΔPAP) in *Trpv4*$^{+/+}$ or *Trpv4*$^{-/-}$ mice (n = 3). In both strains, the response to hyperforin was attenuated by the TRPC inhibitor, SKF-96365 (SKF, 30 μM). *$P > 0.05$, n = 3 each.

controls (fig. 1C). Similarly, in a functional assay in the isolated perfused mouse lung, we observed no difference in pulmonary vasoconstriction in response to the TRPC6 channel agonist, hyperforin,[33] in *Trpv4*$^{+/+}$ and *Trpv4*$^{-/-}$ mice, with an increase in PAP of 4.0 ± 0.3 mmHg in wild-type *versus* 3.8 ± 0.6 mmHg in *Trpv4*$^{-/-}$ mice ($P = 0.9$) (fig. 1E). In both cases, this increase could be largely attenuated by treatment with the TRPC inhibitor, SKF-96365 (SKF). Together, these data demonstrate that the loss of TRPV4 function is not compensated for by modulating TRPC6 expression or function *in vivo*.

Although these whole-organ studies demonstrated a functional role for TRPV4 in HPV, we next aimed to verify this effect *in vivo*. To this end, we used intravital microscopy to visualize the HPV response in real time in mice. Anesthetized mice were ventilated through a tracheostomy, and a thoracic window was prepared as described in Materials and Methods section. Imaging of medium-sized PAs of 20 to 50 μm in diameter, which we have shown previously to yield a robust diameter response to hypoxia, allowed us to visualize HPV directly (fig. 2). In control C57Bl/6 mice (*Trpv4*$^{+/+}$), hypoxic ventilation resulted in an approximately 10% decrease in arteriolar diameter. In contrast, *Trpv4*$^{-/-}$ mice had two thirds less vasoconstriction in response to hypoxia than wild-type controls. These data indicate that TRPV4 plays a critical role in HPV not only in isolated lungs but also *in vivo*.

Although these data clearly indicate that TRPV4 is required for HPV in both the intact lung and the whole animal, the functional relevance of this channel had not yet been established. To assess the physiological effect of TRPV4 in HPV, we

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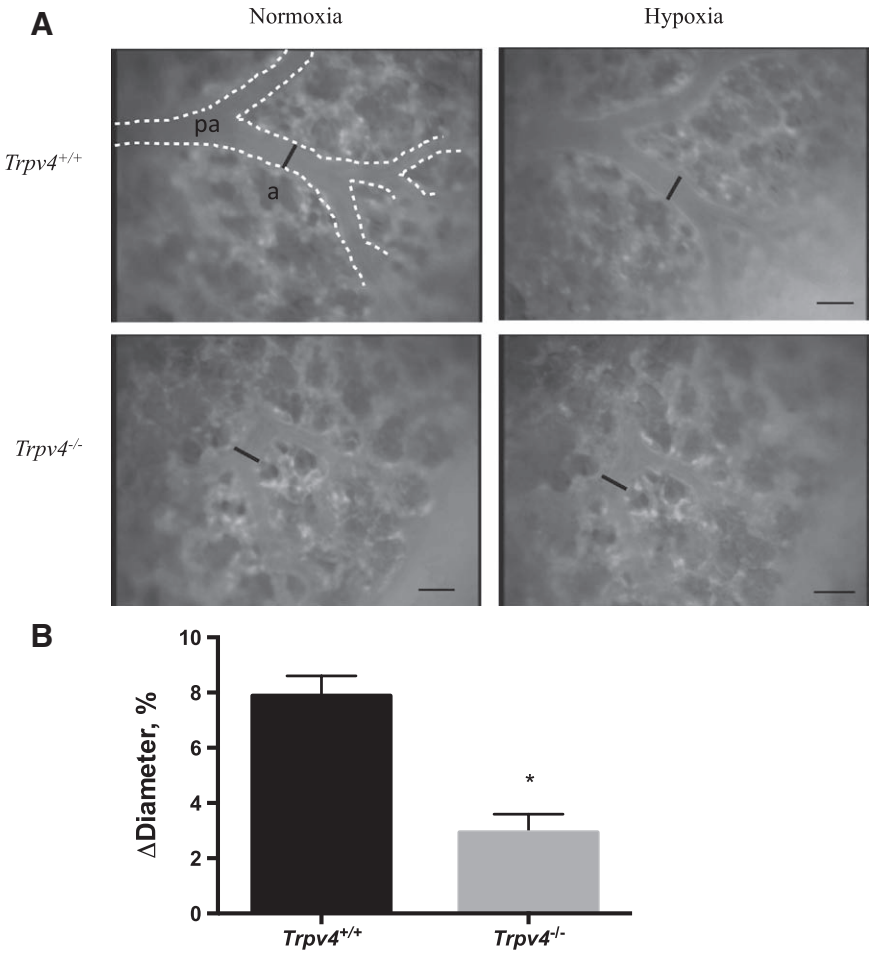

Fig. 2. Intravital microscopy reveals deficient hypoxic pulmonary vasoconstriction in *Trpv4*$^{-/-}$ mice. Mice were anesthetized and ventilated and prepared for intravital microscopy as described in Materials and Methods section. (*A*) Representative images taken from real-time imaging of subpleural pulmonary arterioles (pa) during normoxic or hypoxic ventilation. *Dotted lines* in the first image outline a branching pa. a = an alveolar sac. *Black lines* are drawn across representative arterioles to indicate the point of vessel diameter measurement. *Scale bar* = 50 μm. (*B*) Group data (>25 arterioles from three mice per group) showing the maximal change in vessel diameter during hypoxic ventilation compared with normoxia expressed as percent change in vessel diameter. Note the marked attenuation of hypoxic pulmonary vasoconstriction as directly visualized by intravital microscopy in *Trpv4*$^{-/-}$ mice compared with wild-type controls. *P > 0.0001 *versus* control.

used an *in vivo* model of induced ventilation-perfusion (V/Q) mismatch. In this system, the carotid artery of anesthetized, ventilated mice is cannulated to facilitate arterial blood gas analysis. After a baseline measurement, a small volume (25 μl) of 0.9% saline solution is instilled intratracheally. These saline droplets then occlude small airways in both lungs, inducing V/Q mismatch.[2,13] A further arterial blood gas sample is then analyzed 2 min later to assess the degree of hypoxemia in the animal. In a control animal, an appropriate HPV response should be invoked to minimize hypoxemia in response to the induced V/Q mismatch. However, if TRPV4 is of functional importance in HPV, a *Trpv4*$^{-/-}$ mouse should demonstrate worsened hypoxemia after saline instillation. This is, in fact, the case (fig. 3A). In control mice, 2 min after V/Q mismatch induction, p_aO_2 decreased to 85% of baseline. In mice lacking functional TRPV4 expression, however, p_aO_2 dropped to 71% of baseline, indicating a failure to compensate for V/Q mismatch *via* HPV. These data demonstrate that functional loss of TRPV4 attenuates an animal's ability to invoke HPV in response to V/Q mismatch.

To directly assess the degree of V/Q mismatch, we next measured the distribution of blood flow between both lungs during one-lung ventilation. To this end, mice were ventilated *via* tracheostomy, and the endotracheal tube was advanced into the left mainstem bronchus to allow for ventilation of the left lung only. After 5 min of one-lung ventilation, fluorescent microspheres of 15 μm diameter were introduced into the pulmonary circulation *via* the right internal jugular vein. Microsphere fluorescence in each lung, therefore, yields a quantitative measure of blood flow to that organ. In a control animal, one would expect one-lung ventilation to result in the redistribution of blood flow to the ventilated side. However, if HPV is dysfunctional, this redistribution should be attenuated. Our results were consistent

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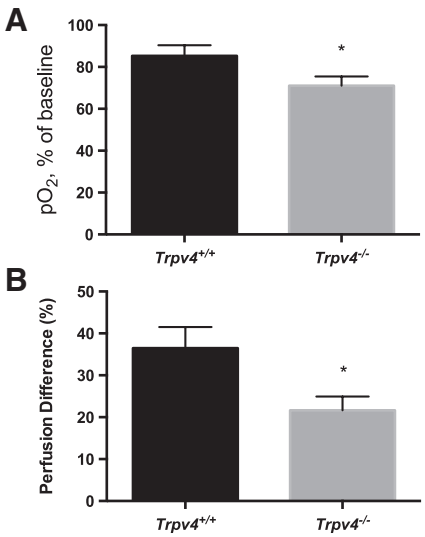

Fig. 3. Loss of TRPV4 function causes a deficiency in ventilation/perfusion (V/Q) matching and arterial oxygenation. (*A*) Control (*Trpv4*$^{+/+}$) or *Trpv4*$^{-/-}$ mice were ventilated as described in Materials and Methods section. Normal saline 25 μl was instilled intratracheally, and mice were ventilated for further 2 min. A blood sample was taken from the internal carotid artery for blood gas analysis. Data indicate the p_aO_2 at this point as a percentage of baseline p_aO_2 before saline administration. Note the worsened degree of hypoxemia in *Trpv4*$^{-/-}$ mice compared with controls. Data are mean ± SEM for five mice per condition. *$P < 0.001$ (*B*) Control (*Trpv4*$^{+/+}$) or *Trpv4*$^{-/-}$ mice were anesthetized and endobronchially intubated to selectively ventilate the left lung. After 5 min, a bolus of fluorescent microspheres was delivered to the right atrium *via* the jugular vein. Mice were sacrificed, and each lung was dissolved in strong alkali. Dissolved tissue was filtered, and fluorescence assessed using a plate reader. Data are presented as the percent difference in perfusion between the ventilated and nonventilated lungs, normalized to lung tissue weight, and demonstrate a failure of pulmonary blood flow redistribution in *Trpv4*$^{-/-}$ mice compared with wild-type controls. Data are mean ± SEM for four mice per condition; *$P < 0.001$.

with this notion (fig. 3B); after one-lung ventilation, control animals had a 36% difference in pulmonary blood flow between the ventilated and nonventilated lungs. In contrast, *Trpv4*$^{-/-}$ mice demonstrated only a 22% blood flow discrepancy between lungs, indicating a failure to redistribute pulmonary blood flow appropriately in response to regional alveolar hypoxia during one-lung ventilation.

TRPV4 forms a channel for bivalent cations and is known to be highly permeable to Ca^{2+}.[34] Therefore, we next directly measured the role of TRPV4 for the PASMC Ca^{2+} response to hypoxia. Primary human PASMC were loaded with the ratiometric Ca^{2+} indicator, Fura 2-AM, and were imaged during perfusion with normoxic or hypoxic buffer solutions. As expected, perfusion with hypoxic buffer produced a significant increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (fig. 4). To study the role of TRPV4 in the observed hypoxia-induced $[Ca^{2+}]_i$ response, we perfused PASMC with 20 μM HC-067047 for 10 min before hypoxia and continued perfusion with the inhibitor throughout the hypoxic period. When TRPV4 activity was inhibited, we observed a significant attenuation of the maximal $[Ca^{2+}]_i$ increase during hypoxia. These data suggest that the characteristic $[Ca^{2+}]_i$ response to hypoxia is mediated, in large part, by TRPV4. Intracellular Ca^{2+} is a critical factor in several signaling events during HPV, most importantly, the activation of MLC kinase and the subsequent phosphorylation of MLC. This is a critical regulatory step in HPV because MLC phosphorylation is required for cross-bridge formation and contraction of the PASMC and, therefore, vasoconstriction.[1] Acute exposure of PASMC to hypoxia resulted in robust phosphorylation of MLC. However, pretreatment with the TRPV4 inhibitor, HC-067047, attenuated this effect (fig. 4, D and E). This finding suggests that Ca^{2+} influx through TRPV4 is involved in MLC kinase activation and subsequent phosphorylation of MLC as a necessary precondition for PASMC contraction and vasoconstriction.

TRPV4 has several known biological agonists,[26] including the arachidonic acid metabolites of the EET family.[35] Interestingly, EET species, such as 11,12-EET, are produced in the lung during acute hypoxia and can activate TRPC6 in PASMC as part of the HPV response.[36] Thus, we hypothesized that increased abundance of EET may similarly activate TRPV4 in PASMC. To test this hypothesis, we imaged PASMC loaded with the confocal Ca^{2+} indicator, Fluo-4. Stimulation with 3 nMol 11,12-EET caused a robust increase in $[Ca^{2+}]_i$ in these cells, which could be partially blocked by TRPV4 inhibition (fig. 5A).

Epoxyeicosatrienoic acids can also activate TRPC6, which has previously been shown to be critically involved in HPV in that *Trpc6*$^{-/-}$ mice largely lack the lung vasoconstrictive response to hypoxia.[13] We therefore tested next whether TRPV4 and TRPC6 contribute independently to the PASMC Ca^{2+} response to EETs or whether their roles may be functionally linked. Notably, TRPC6 and TRPV4 share a series of similarities that make a potential functional link particularly interesting. First, both are activated by EETs and inhibited by cyclic guanosine monophosphate.[21,37] Second, in sensory neurons, TRPV4 and TRPC6 have been shown to have a cooperative function in nociception.[38] Indeed, different TRP channel subunits can associate to form heteromeric mature channels,[39] thus providing a structural correlate to potential functional interdependencies. To test the role of TRPC6 in EET-stimulated Ca^{2+} flux in our system, we treated PASMC with the TRPC inhibitor SKF (30 μM) before stimulating the cells with 11,12-EET. SKF attenuated the 11,12-EET-stimulated increase in $[Ca^{2+}]_i$ to a similar degree as seen with TRPV4 inhibition. Yet, a combination of both TRPC and TRPV4 inhibitors showed no additive effect, suggesting functional cooperation between TRPC6 and TRPV4 in the PASMC Ca^{2+} response. These data are consistent with, but not limited to, a model whereby TRPC6 and TRPV4 form a heteromeric channel in the PASMC membrane.

To further probe this model, we performed coimmunoprecipitation experiments in whole-lung lysates collected during

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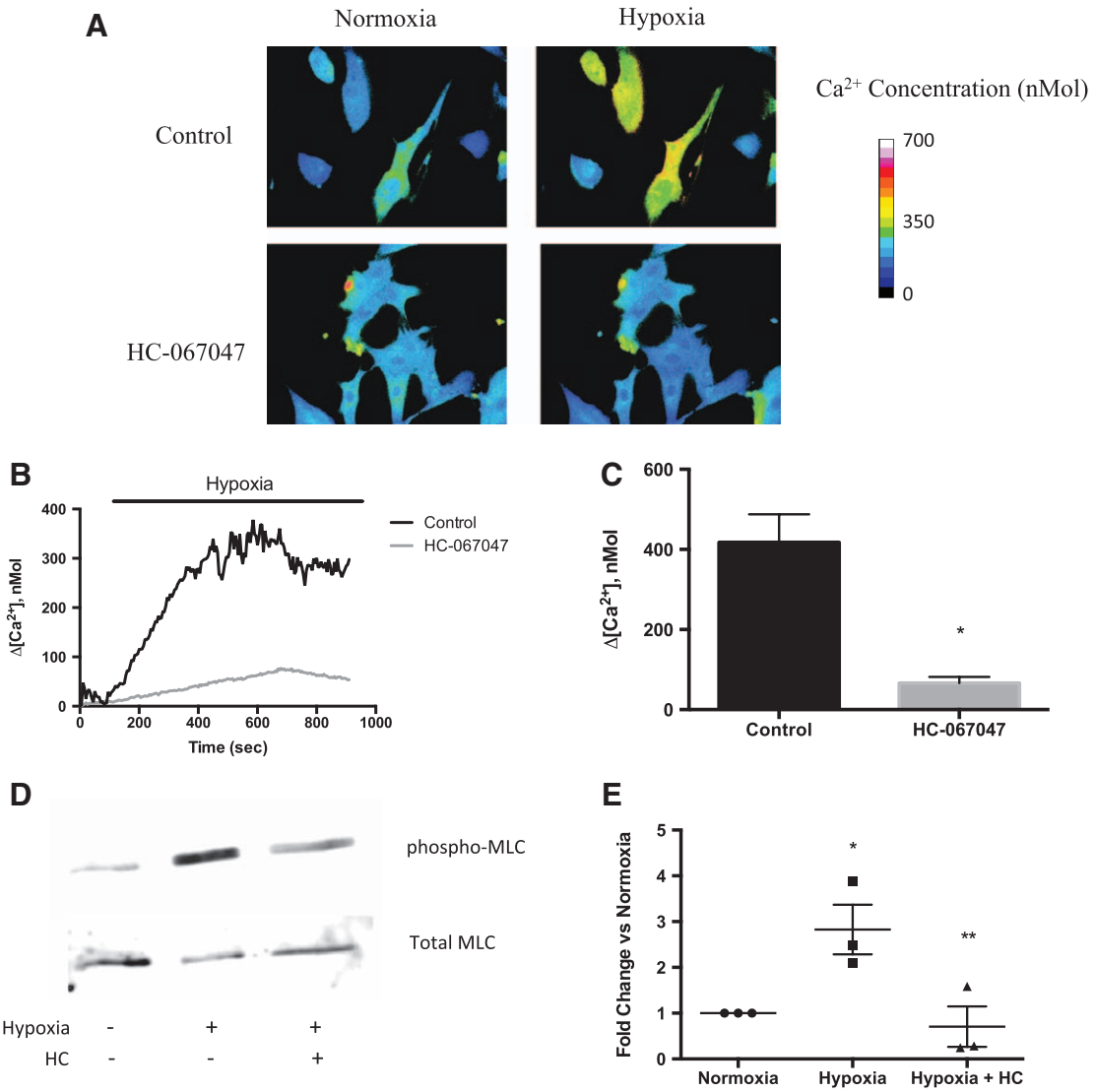

Fig. 4. TRPV4 inhibition blocks the $[Ca^{2+}]_i$ response to hypoxia in pulmonary artery smooth muscle cells (PASMCs). PASMCs were loaded with the ratiometric calcium indicator, Fura-2. Cells grown on coverslips were perfused with Hank's buffered salt solution (HBSS) plus 2 mM Ca^{2+}. Fluorescence images were recorded at wavelengths of 340 and 380 nm every 5 s. As indicated, perfusate was switched to Ca^{2+}-containing HBSS that had been bubbled with 1% O_2 to induce hypoxia. For experiments involving the TRPV4 inhibitor, HC-067047, cells were perfused with HC-067047 (20 μM) for 15 min in HBSS + Ca^{2+} before imaging. Further treatments were then in the presence of HC-067047. (*A*) Representative images show Fura-2-loaded PASMC color coded for Ca^{2+} concentration (nMol). Note the attenuation of the PASMC $[Ca^{2+}]_i$ increase during hypoxia when TRPV4 is inhibited. (*B*) Representative recordings of the PASMC $[Ca^{2+}]_i$ response to hypoxia in the presence or absence of the TRPV4 inhibitor, HC-067047. (*C*) Group data depicting the maximal change in cytosolic Ca^{2+} concentration ($\Delta[Ca^{2+}]$) in response to hypoxia. Baseline Ca^{2+} concentration in all cells was 200 ± 20 nMol. For cells treated with the inhibitor, if no peak was present, Ca^{2+} concentration was determined at the time point when the peak occurred in control cells. Data are mean ± SEM for 15–20 cells from three independent experiments. **P* < 0.001. (*D*) Hypoxia results in increased phosphorylation of myosin light chain (phospho-MLC) in a TRPV4-depended manner. PASMC were exposed to 1% O_2 (hypoxia) for 5 min in the presence or absence of HC-067047 (HC, 20 μM). (*E*) Group data show TRPV4-dependent phosphorylation of MLC during hypoxia. **P* < 0.05 *versus* normoxia; ***P* < 0.05 *versus* hypoxia; n = 3.

normoxic or hypoxic ventilation (fig. 5B). During both normoxia and hypoxia, TRPC6 protein could be detected after immunoprecipitation of TRPV4. Omission of TRPV4 antibody resulted in loss of the TRPC6 signal, demonstrating the specificity of TRPC6 binding to TRPV4. These data demonstrate that TRPV4 and TRPC6 form a structural complex in the mouse lung both at normoxia and hypoxia.

To gain insights into the mechanism by which TRPV4 may become activated during HPV, we probed for the subcellular localization of TRPV4 in PASMC during hypoxia. Previous work has demonstrated the importance of caveolae in lung vascular biology.[40] Caveolae are plasma membrane microdomains that form nuclei for complex signaling networks between proteins and lipids. Key mediators of endothelial

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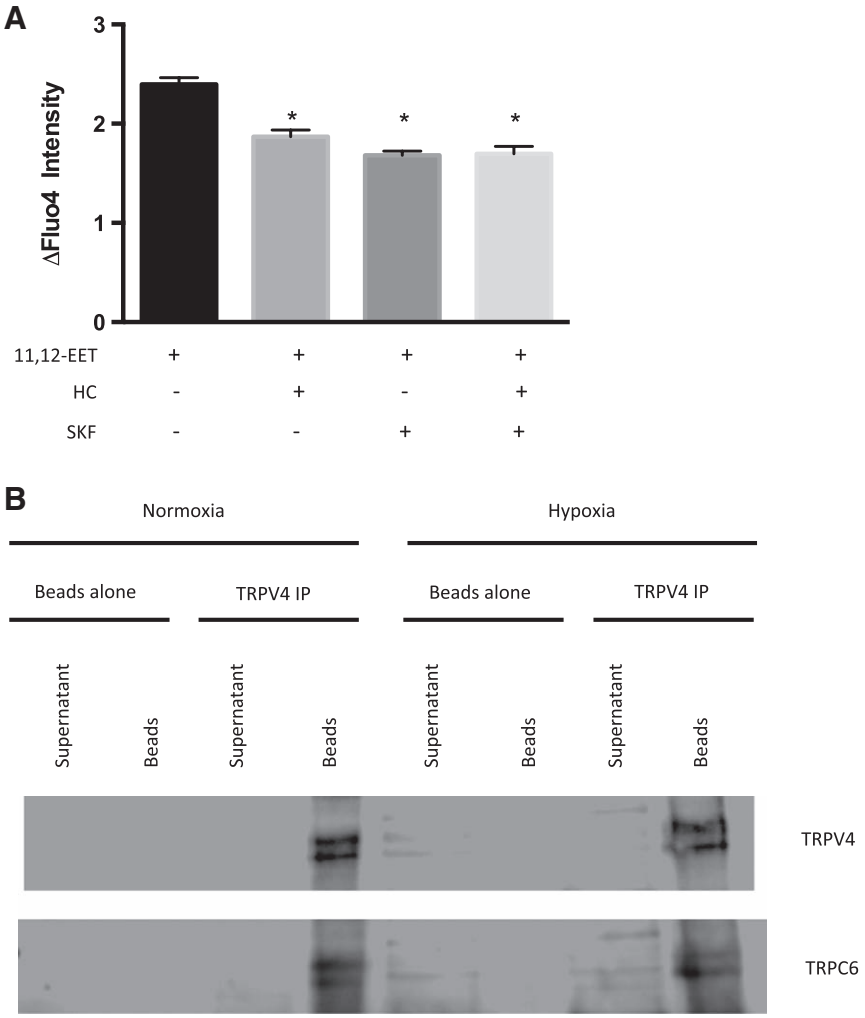

Fig. 5. The effect of epoxyeicosatrienoic acids (EETs) on pulmonary artery smooth muscle cell Ca^{2+} entry *via* TRPV4 and TRPC6. (*A*) Fluo-4-loaded pulmonary artery smooth muscle cell were treated with vehicle or the indicated inhibitors, HC-067047, 20 μM (HC) and/or SKF-96365, 30 μM (SKF) for 15 min, followed by stimulation with 11,12-EET (3 μMol). Data indicate the peak increase in Fluo-4 signal intensity, relative to baseline fluorescence. Data are mean ± SEM for 30–50 cells per condition, taken from three independent experiments. **P* < 0.0001 *versus* 11,12-EET alone. (*B*) TRPV4 forms protein–protein complexes with TRPC6. Wild-type mice were subjected to normoxic or hypoxic ventilation, and whole-lung lysates were collected. Immunoprecipitation (IP) was performed using an anti-TRPV4 antibody. Captured proteins were electrophoresed and subsequently probed for TRPV4 or TRPC6. In each condition, samples were run in parallel in the absence of immunoprecipitating antibody (beads alone) to control for nonspecific binding to beads. After collection of protein-bound beads, both the supernatants and bead samples were run on gels. Note that TRPC6 coimmunoprecipitates with TRPV4 in both normoxia and hypoxia. No protein is seen in negative control lanes (beads alone).

and vascular smooth muscle biology, for example, endothelial nitric oxide synthase, are found in caveolae and require this localization for normal function. Association with caveolae can also be dynamic and may be a consequence of stimulation. TRPV4 has previously been found within caveolae in human umbilical vein endothelial cells.[41] In addition, TRPC6 has previously been shown to translocate to caveolae during hypoxia in PASMC.[42] The proposed interaction and functional cooperation between TRPV4 and TRPC6 would therefore suggest that TRPV4 in PASMC may similarly translocate to caveolae in response to hypoxia. Using sucrose gradient ultracentrifugation to purify caveolae, we found a significant redistribution of TRPV4 to caveolin-1-containing cellular fractions in response to 15 min of hypoxia (fig. 6). There was an approximately two-fold increase in the amount of TRPV4 associated with caveolae during hypoxia compared with controls. This finding sets up an intriguing possible scenario, in which TRPV4 activation and signaling is modulated during hypoxia by cooperation with TRPC6 and affiliation with the unique protein and lipid environment of caveolae.

Discussion

Our current studies have established a critical role for TRPV4 in the pulmonary vascular response to hypoxia. Using *in vitro*, *in vivo*, and whole-organ approaches, we have demonstrated that TRPV4 is required for a normal HPV response and that

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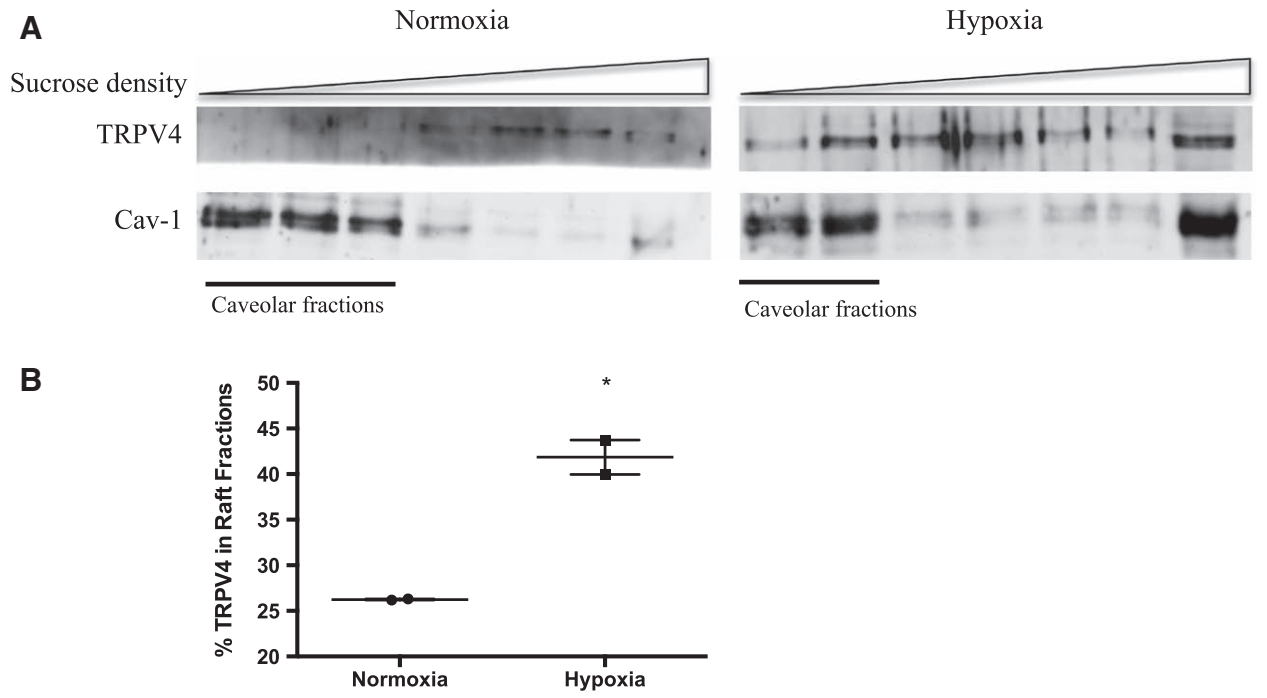

Fig. 6. Hypoxia induces TRPV4 translocation into caveolae. Pulmonary artery smooth muscle cells were kept in room air (normoxia) or 1% O_2 (hypoxia) for 15 min and then lysed. Lysates were subjected to sucrose density gradient ultracentrifugation, and seven fractions were collected from the resulting gradients. Each fraction was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis, and membranes were subsequently immunoblotted for TRPV4 and caveolin-1, as a marker for caveolae. (*A*) Representative Western blot images. Sucrose gradient fractions are shown in increasing density. The *top row* depicts TRPV4 immunoblots in normoxia and hypoxia, as indicated. *Bottom panel* shows caveolin-1 (cav-1) as a marker of caveolae. Note the leftward movement of TRPV4 during hypoxia into cav-1-containing fractions. *Black bar* indicates the fractions considered to be caveolae for subsequent analysis. (*B*) Densitometric analysis was performed, and the fraction of TRPV4 signal in the caveolin-1-containing fractions was expressed relative to total TRPV4 signal in all fractions. Data shown are ± SEM for n = 2 experiments. *P = 0.01.

the loss of TRPV4 function is associated with deficient oxygenation in animals challenged with V/Q mismatch. In addition, we have shown that during one-lung ventilation—a necessary step for a variety of cardiothoracic procedures—TRPV4 is required for maximal redistribution of pulmonary blood flow to the ventilated lung. Finally, we have determined that TRPV4 is required for the hypoxia-triggered $[Ca^{2+}]_i$ increase and MLC phosphorylation in isolated human PASMC, that TRPV4 can form protein–protein complexes in these cells with TRPC6, and that hypoxia results in the redistribution of TRPV4 to plasma membrane microdomains.

The polymodal cation channel TRPV4 is well positioned to play a pivotal role in HPV. It is highly expressed in PASMC[19] and in pulmonary endothelial cells,[43] both of which are required for HPV.[1,2] Furthermore, TRPV4 is activated by EETs,[20] the levels of which are increased in the lung during acute hypoxia, whereas depletion of EETs has been shown to diminish the HPV response.[36] In the systemic vasculature, TRPV4 has previously been shown to be involved in the regulation of vasodilatory responses: mesenteric vessels express TRPV4, and such vessels isolated from *Trpv4*$^{-/-}$ mice have a diminished vasodilatory response to acetylcholine.[16] In vessel myographs, TRPV4 agonists relax large conducting renal arteries, mesenteric arteries, and vasa recta and vascular dilation in response to TRPV4 agonists in isolated perfused kidneys is abrogated in *Trpv4*$^{-/-}$ mice.[15] In contrast to this documented role of TRPV4 in vascular dilation, we demonstrate here a key role of TRPV4 as an important prerequisite in pulmonary vasoconstriction. Importantly, the fact that given stimuli such as hypoxia, serotonin, or EETs cause opposite effects with respect to vasomotor tone between the pulmonary and systemic circulations is well documented,[44] and the divergent effects of TRPV4 on lung and systemic vascular tone may provide a possible mechanistic explanation for some of these divergences. Given the broad expression pattern of TRPV4, a tissue-specific knockout mouse would be an ideal animal model for studying the role of TRPV4 in individual tissues. However, to our knowledge, such an animal model has not been constructed at this time.

In line with the demonstrated role of TRPV4 as a bivalent cation channel, our findings suggest that the functional role of TRPV4 in HPV relates to its ability to mediate the characteristic $[Ca^{2+}]_i$ increase in PASMC in response to hypoxia that has previously been shown to be critical for hypoxia-induced PASMC contraction.[1] The mechanisms by which hypoxia causes activation of TRPV4 remain to be elucidated but may involve formation of EET, which have previously been shown to activate TRPV4,[22] and to mediate HPV,[36] and/or activation of src kinase which similarly has been shown to activate

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TRPV4 *via* phosphorylation of its tyrosine residues[45] and also contributes to the lung vascular response to hypoxia.[46] In addition, we show here that TRPV4 activation during hypoxia is associated with its translocation to caveolae. This finding complements a series of striking similarities between two different members of the TRP family in the regulation of HPV, namely TRPV4 and TRPC6. Similar to TRPV4, TRPC6 has previously been shown to contribute critically to HPV and to mediate the PASMC $[Ca^{2+}]_i$ response to hypoxia.[13] In addition, TRPC6 is—similar to TRPV4—activated by EETs[47] and translocates to caveolae in response to hypoxia.[36] This analogy between the function and regulation of TRPV4 and TRPC6 hence bears the question that whether both channels may in fact be functionally linked in the context of HPV. Indeed, functional cooperation between both channels was recently reported in the context of nociceptor sensitization, in that antisense to TRPC6 reversed the mechanical hyperalgesia induced by the TRPV4-selective agonist 4αPDD.[38] Interestingly, TRP channels are composed of four subunits that commonly assemble to homomeric but occasionally heteromeric pore-forming channels, which may explain cooperativity between different TRP channels.[6,48] TRPV4 has been shown to form a heteromer with TRPC1, and this heteromerization increases the surface expression and calcium conductance of TRPV4[49] and allows TRPV4 to function as a store-operated calcium channel, essentially bestowing a function associated with TRPC proteins upon TRPV4. By immunoprecipitation analyses in PASMCs, we show here that TRPV4 can similarly engage in protein–protein interactions with TRPC6, which may form the molecular basis for the observed parallel roles of both channels in HPV, and for their nonadditive effects in the PASMC $[Ca^{2+}]_i$ response to EET. Our finding that the interaction between TRPV4 and TRPC6 in PASMC remained unaltered during hypoxia further suggests that both channels form heteromers already at baseline in PASMC and become recruited to caveolae (and presumably, activated) conjointly upon exposure to hypoxia.

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Competing Interests

The authors declare no competing interests.

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