Pulmonary Hypertension

Classical Transient Receptor Potential 1 and 6 Contribute to Hypoxic Pulmonary Hypertension Through Differential Regulation of Pulmonary Vascular Functions

Yang Xia, Xiao-Ru Yang, Zhenzhen Fu, Omkar Paudel, Joel Abramowitz, Lutz Birnbaumer, James S.K. Sham

Abstract—Hypoxic pulmonary hypertension is characterized by increased vascular tone, altered vasoreactivity, and vascular remodeling, which are associated with alterations in Ca²⁺ homeostasis in pulmonary arterial smooth muscle cells. We have previously shown that classical transient receptor potential 1 and 6 (TRPC1 and TRPC6) are upregulated in pulmonary arteries (PAs) of chronic hypoxic rats, but it is unclear whether these channels are essential for the development of pulmonary hypertension. Here we found that pulmonary hypertension was suppressed in TRPC1 and TRPC6 knockout (Trpc1⁻/⁻ and Trpc6⁻/⁻) mice compared with wild-type after exposure to 10% O₂ for 1 and 3 weeks. Muscularization of pulmonary microvessels was inhibited, but rarefaction was unaltered in hypoxic Trpc1⁻/⁻ and Trpc6⁻/⁻ mice. Small PAs of normoxic wild-type mice exhibited vasomotor tone, which was significantly enhanced by chronic hypoxia. Similar vasomotor tone was found in normoxic Trpc1⁻/⁻ PAs, but the hypoxia-induced enhancement was blunted. In contrast, there was minimal vascular tone in normoxic Trpc6⁻/⁻ PAs, but the hypoxia-enhanced tone was preserved. Chronic hypoxia caused significant increase in serotonin-induced vasoconstriction; the augmented vasoreactivity was attenuated in Trpc1⁻/⁻ and eliminated in Trpc6⁻/⁻ PAs. Moreover, the effects of 3-week hypoxia on pulmonary arterial pressure, right ventricular hypertrophy, and muscularization of microvessels were further suppressed in TRPC1-TRPC6 double-knockout mice. Our results, therefore, provide clear evidence that TRPC1 and TRPC6 participate differentially in various pathophysiological processes, and that the presence of TRPC1 and TRPC6 is essential for the full development of hypoxic pulmonary hypertension in the mouse model. (Hypertension. 2014;63:173-180.)

Key Words: hypotension, pulmonary □ hypoxia □ TRPC1 channel □ TRPC6 channel □ vascular smooth muscle

A cute exposure to alveolar hypoxia triggers reversible hypoxic pulmonary vasoconstriction (HPV), whereas prolonged exposure, as occurring in high-altitude inhabitants or in patients with respiratory diseases, causes pulmonary hypertension (PH). PH is characterized by potentiating vascular tone, altered reactivity to agonists, and profound vascular remodeling, eventually leading to right heart (RV) hypertrophy and failure.1 Although the mechanism of pathogenesis is complex, the intrinsic changes in Ca²⁺ homeostasis in pulmonary arterial smooth muscle cells (PASMCs) are the major determinants contributing to PASMC proliferation and vasoconstriction in chronic hypoxic pulmonary hypertension (CHPH).2 Cytosolic Ca²⁺ concentration is regulated by intracellular Ca²⁺ release and extracellular Ca²⁺ influx, which is gated by voltage-dependent Ca²⁺ channels and voltage-independent nonselective cation channels. There is growing evidence supporting the pivotal role of multiple nonselective cation channels in acute3 and prolonged hypoxic responses.4–6

Transient receptor potential (TRP) proteins encode a large repertoire of nonselective cation channels in vascular smooth muscle cells.7 We have previously identified the TRP channels of classical/canonical (TRPC)-, melastatin-, and vanilloid-related subfamilies in rat PASMCs.4,8 Functional studies show that TRPC1 and TRPC6 mediate store-operated and receptor-operated Ca²⁺ entry, respectively.9 Most importantly, chronic hypoxia (CH) upregulates the expression of TRPC1 and TRPC6, as well as the associated store-operated and receptor-operated Ca²⁺ entry in rat distal pulmonary arteries (PAs).4 A subsequent study confirmed the upregulation of TRPC1 and TRPC6 expression in the murine model of CPH and suggested that the process requires the full expression of hypoxia-inducible factor-1α.10 Abnormalities in TRPC
expression have also been identified in various types of PH. For example, PASMC of idiopathic pulmonary arterial hypertension patients excessively expresses TRPC3 and TRPC6, resulting in augmented store-operated Ca\(^{2+}\) entry and proliferation.\(^{10}\) In monocrotaline-induced PH, increased TRPC1 expression and store-operated Ca\(^{2+}\) entry contribute to the enhanced vasoconstriction to endothelin-1.\(^{11}\) Additionally, treatment of experimental PH with sildenafil and sodium tanshinone IIA sulfonate suppresses TRPC1 and TRPC6 expression.\(^{12,13}\) All of the information hints that TRPC1 and TRPC6 are critically involved in CHPH, but leaves open the question of whether the altered expression and functions of these TRPC channels are essential for the development of the disease. The present study was undertaken to address these issues by using TRPC1- (Trpc1\(^{-/-}\)), TRPC6- (Trpc6\(^{-/-}\)), and TRPC1-TRPC6- (Trpc1\(^{-/-},\)Trpc6\(^{-/-}\)) null mice to examine how TRPC1 and TRPC6 channels affect vasomotor tone, agonist-induced vasoconstriction, and pulmonary vascular remodeling, and whether genetic deletion of these two cation channels could prevent the animals from developing CHPH.

Materials and Methods
Models of CHPH Trpc1\(^{-/-}\) and Trpc6\(^{-/-}\) mice (1:1; 129Sv:C57BL/6J background) were initially provided by the NIEHS Comparative Medicine Branch.\(^{14,15}\) Corresponding wild-type (WT) mice of the same background were used as control. Subsequent generations of colonies and Trpc1\(^{-/-}\) and Trpc6\(^{-/-}\) mice were maintained at Johns Hopkins University. Age-matched, male WT and knockout mice (10–12 weeks old) were placed in a hypoxic chamber and exposed to 10% O\(_2\) for 1 or 3 weeks to induce hypoxic PH.\(^{16}\) All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Johns Hopkins Animal Care and Use Committee (see online-only Data Supplement for details).

Results

TRPC Expression in Trpc1\(^{-/-}\), Trpc6\(^{-/-}\), and WT Mice
Expression of TRPC subtypes in endothelium-denuded PAs of Trpc1\(^{-/-}\), Trpc6\(^{-/-}\), and WT mice was compared by real-time RT-PCR. In WT PAs, the mRNA level of TRPC1 was the highest, followed by TRPC6 and then TRPC3, whereas TRPC4, 5, and 7 expressions were minimal. Similar TRPC mRNA expression profiles were found in Trpc1\(^{-/-}\) and Trpc6\(^{-/-}\) PAs, except they were devoid of TRPC1 and TRPC6 mRNA, respectively (Figure 1A and 1B). Similar to previous observations in CH rats and mice,\(^4,9\) TRPC1 and TRPC6 protein levels were significantly increased by 246±51% (n=6) and 103±38% (n=6), respectively, in PAs of WT mice exposed to 3 weeks of 10% O\(_2\) (Figure 1C and 1D). Comparable increases in TRPC1 (167±31%; n=3) and TRPC6 proteins (110±33%; n=6) were observed in PAs of CH Trpc6\(^{-/-}\) and Trpc1\(^{-/-}\) mice, respectively (Figure 1E and 1F). These results suggest that there is no compensatory shift in TRPC expression, and the hypoxic regulation of TRPC expression is unaltered in PAs of the knockout mice.

Deletion of TRPC1 or TPRC6 Suppresses CHPH
Trpc1\(^{-/-}\), Trpc6\(^{-/-}\), and WT mice were exposed to 10% O\(_2\), for 0, 1, and 3 weeks. Right ventricular systolic pressure (RVSP) and mean pulmonary arterial pressure (MPAP) were identical in the 3 types of mice under normoxic condition. Elevation of RVSP, MPAP, and RV mass index (RVMI) were evident in WT mice after 1 week of hypoxia exposure and progressed to higher levels after 3 weeks (Figure 1). Compared with WT, the increase in RVSP, MPAP, and RVMI were significantly less in Trpc1\(^{-/-}\) mice after 1 week...
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*P<0.05, normoxia vs hypoxia for each genotype; +P<0.05 vs WT under different treatments; #P=0.056 vs WT.

Figure 3. Morphological analysis of pulmonary vascular remodeling. A, Proportion of non- (<25%), partially (25%–75%), and completely (>75%) muscularized resistance pulmonary vessels (<100 μm). B, Quantification of pulmonary vascular densities in lungs of normoxic and hypoxic Trpc1−/−, Trpc6−/−, and wild-type (WT) mice. There were 4 to 6 mice in each group. *P<0.05; **P<0.01; ***P<0.001 vs WT or as specified.

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and 3 weeks of hypoxia. The changes in RVSP and MPAP were suppressed, and RV hypertrophy was virtually absent in Trpc6−/− mice after 1 week of hypoxia. However, PH and RVMI of Trpc6−/− mice progressed in subsequent weeks, with marginal reduction in MPAP (P=0.056) and RVMI (P=0.116) after 3 weeks. Polycythemia developed in CH WT, Trpc6−/−, and Trpc1−/− mice, with hematocrit of Trpc6−/− mice slightly higher than that of WT. Heart rate and mean systemic arterial
pressure were similar in normoxic and CH WT and knock-out mice (Figure 2F and 2G). These results, for the first time, show that TRPC1 and TRPC6 are required for the full development of CHPH.

**TRPC1 and TRPC6 Contribute to CH-Induced Pulmonary Vascular Remodeling**

Morphological analysis showed that the percent distributions of nonmuscularized, partially muscularized, and completely muscularized small vessels (<100 μm) were similar in normoxic WT, Trpc1−/−, and Trpc6−/− mice (Figure 3A). Significant increase in partially (normoxia, 14.9±3.0%; CH, 30.1±5.2%; P<0.01) and completely muscularized vessels (normoxia, 4.3±0.9%; CH, 11.9±2.2%; P<0.01) was observed in CH WT mice. In contrast, there was no significant reduction in the nonmuscular vessels or increase in the partially muscularized vessels, except a marginal increase in the completely muscularized vessels of CH Trpc1−/− and Trpc6−/− mice. Furthermore, vessel density was significantly reduced in CH WT mice, and similar reduction was observed in CH Trpc1−/− and Trpc6−/− mice (Figure 3B). These results clearly indicate that TRPC1 and TRPC6 are important contributing factors to CH-induced neomuscularization of small PAs, but have little influence on rarefraction of small PAs in CHPH.

**TRPC1 and TRPC6 Contribute Differentially to Pulmonary Vascular Tone**

We devised a strategy to estimate vascular tone at different preloads by measuring the active and passive wall tension of size-matched (ID ≈ 200 μm) PA rings using a wire myograph. Increasing vessel width from the resting position (zero tension) in 50-μm steps was associated with progressive increase in wall tension (Figure 4A and Figure S1 in the online-only Data Supplement). Active tension at each point was determined by subtracting total wall tension by passive tension measured after complete relaxation by Ca2+ removal and addition of papaverine (10 μmol/L; Figure 4B). The vessel width versus tension relation shows significant active tone in normoxic WT PAs, and it increased with vessel width in a quasilinear manner (Figure 4C). Active tension was significantly enhanced in PAs of CH WT mice and abolished by Ca2+ removal. Stretch-activated spontaneous contractions were observed occasionally in CH WT PAs (Figure S2). Compared with normoxic WT PAs, vascular

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**Figure 4.** Pulmonary vascular tone in Trpc1−/−, Trpc6−/−, and wild-type (WT) pulmonary arteries (PAs). A, Representative tracings of wall tension generated by stepwise increase (50 μm) in vessel width in PAs of normoxic (top) and chronic hypoxic (CH; bottom) mice in Ca2+-containing, Ca2+-free, and Ca2+-free plus papaverine solutions. B, Wall tension vs vessel width relations generated from small PAs isolated from normoxic and hypoxic Trpc1−/− (nor: n=22; hyp: n=21), Trpc6−/− (nor: n=20; hyp: n=20), and WT mice (nor: n=33; hyp: n=38) in Ca2+-containing (black), Ca2+-free (red), or Ca2+-free plus 10 μmol/L papaverine solution (blue). C, Active tone of normoxic and CH WT PAs, calculated by the difference between wall tensions obtained in Ca2+-containing and Ca2+-free plus papaverine solution. D and E, Active tone of PAs from mice of different genotypes after normoxic and chronic hypoxic exposure, respectively; **P<0.01 vs WT. F, Active tone of PAs of normoxic and hypoxic mice at Δ width of 250 μm (left) and 350 μm (right). **P<0.01 and ***P<0.001 vs normoxia; + P<0.05 vs WT.
tone was similar in Trpc1−/− PAs at the lower vessel width, but it leveled off when vessel width was further increased (Figure 4D). In contrast, the active tone of Trpc6−/− PAs was significantly lower throughout the whole range of vessel widths tested (P<0.001). Moreover, vascular tone was suppressed in both CH Trpc1−/− and Trpc6−/− PAs (Figure 4E). At Δ width of 250 and 350 μm, where wall tensions were equivalent to transmural pressures of ≈15 and 25 mm Hg (see Methods in the online-only Data Supplement), CH caused a significant increase in vascular tone in PAs of WT and Trpc6−/− mice (Figure 4F).

TRPC1 and TRPC6 Contribute to Pulmonary Vasoreactivity in CH PAs

5-HT elicited concentration-dependent contraction in endothelium-denuded PAs of normoxic WT and knockout mice, with similar maximum response (Eₘₐₓ) and sensitivity (−log EC₅₀) except a slightly lower Eₘₐₓ in PAs of Trpc6−/− mice (Figure 5A–5C). Consistent with previous reports, the maximal response elicited by 5-HT in CH WT PAs was augmented (normoxia: 117.8±2.08%; n=12; hypoxia: 146.8±4.4%; n=12; P<0.001) without significant change in EC₅₀ (Figure 5B). In contrast, the CH-induced enhancement of Eₘₐₓ was completely eliminated (normoxia: 110.9±1.0%; n=17; CH: 112.9±2.0%; n=12; P=0.594), and the sensitivity for 5-HT was slightly reduced in CH Trpc6−/− PAs. The 5-HT–induced maximal response was also significantly suppressed in CH Trpc1−/− PAs compared with WT. These data suggest that TRPC6 is critically involved in 5-HT–induced contractile responses in PA under CH, and TRPC1 contributes in part to the enhanced vasoreactivity to 5-HT in CH.

TRPC1-TRPC6 Double-Knockout Further Suppressed CHPH

Because TRPC1 and TRPC6 regulate different vascular functions, we further examined the effects of TRPC1 and TRPC6 double-deletion on CHPH. RVSP, MPAP, and RVMI were all significantly lower in normoxic Trpc1−/−Trpc6−/− mice compared with WT, whereas heart rate and mean systemic arterial pressures were the same (Figure 6A–6E). The double-knockout mice developed less severe PH compared with WT mice, as indicated by significantly lower RVSP (WT: 26.2±0.5; Trpc1−/−/Trpc6−/−: 21.3±0.6; P<0.001), MPAP (WT: 18.2±0.4; Trpc1−/−/Trpc6−/−: 14.6±0.4; P<0.001), and RVMI (WT: 0.33±0.01; Trpc1−/−/Trpc6−/−: 0.28±0.01; P<0.001). RVSP and MPAP of CH double-knockout mice were also significantly lower than those of CH Trpc1−/− or Trpc6−/− mice (Figure S3A and S3B). Morphological analysis found that the neomuscularization of small PAs was virtually absent in CH Trpc1−/−Trpc6−/− mice (Figure 6F). However, significant reduction of vessel density was still observed in lungs of CH Trpc1−/−Trpc6−/− mice (Figure 6G). In conjunction with the results observed in Trpc1−/− and Trpc6−/− mice, these data support the notion that TRPC1 and TRPC6 are major contributing factors in the pathogenic processes of CHPH.

Discussion

In the present study, we used genetic mouse models to test the hypothesis that TRPC1 and TRPC6 are crucial for CHPH development, and to examine their roles in pulmonary vascular functions. Trpc1−/− and Trpc6−/− mice are suitable for these purposes because the expressions of TRPC subtypes in PA are...
unaltered, consistent with previous reports in systemic arteries of \textit{Trpc1}^{−/−} mice\textsuperscript{14} and in PAs of \textit{Trpc6}^{−/−} mice\textsuperscript{1} (but see also Dietrich et al\textsuperscript{15}), and their regulation by CH is similar to that in rats and WT mice.\textsuperscript{4,9} Our results show that TRPC1 and TRPC6 participate differentially in the three salient features of PH: elevated pulmonary vasomotor tone, altered vascular reactivity, and vascular remodeling. Ablation of TRPC1 or TRPC6 has minimal effect on pulmonary circulation under normoxic conditions, but mitigates PH and RV hypertrophy induced by CH.

We established a strategy for studying vasomotor tone at different muscle length in murine small PA, and much novel information has been revealed. In contrast to the lack of basal vasomotor tone in normoxic rat microvessels,\textsuperscript{5,17} murine PA exhibits a small component of active tone. This active tone is myogenic and Ca\textsuperscript{2+}-dependent, judging by the increase in magnitude with mechanical stretch and by its complete inhibition after Ca\textsuperscript{2+} removal. The vascular tone was enhanced after 3-week CH, similar to the de novo appearance of myogenic tone in PAs of CH rats.\textsuperscript{5,17} Interestingly, the basal tone in normoxic PAs and the CH-enhanced vasomotor tone are apparently two separate components mediated by different mechanisms. Deletion of TRPC6 suppressed the basal tone in normoxic PAs, but did not interrupt the CH-induced elevation in vasomotor tone. The reduction in vasomotor tone of \textit{Trpc6}^{−/−} PAs is consistent with reports showing that TRPC6 is mechanosensitive and mediates myogenic response,\textsuperscript{18,19} but it is in contrast to the enhanced myogenic tone in \textit{Trpc6}^{−/−} cerebral arteries where compensatory upregulation of TRPC3 is evident.\textsuperscript{15} Compared with TRPC6, deletion of TRPC1 has little effect on the vascular tone of normoxic PAs at the lower vessel width, but eliminated the increase in active tone at the higher levels of mechanical stretch. This is consistent with findings that TRPC1 is mechanosensitive in nonvascular cells,\textsuperscript{20} but it does not play a significant role in myogenic tone under normal physiological conditions. More importantly, the disappearance of enhanced tone in CH \textit{Trpc1}^{−/−} PA suggests that TRPC1 is recruited to facilitate the enhanced vascular tone under pathological conditions of CHPH. This is in concordance with previous reports suggesting that TRPC1 upregulation is responsible for the elevated basal tone in CH rat PAs and resting Ca\textsuperscript{2+} concentration of hypoxic PASMCs.\textsuperscript{4,9,12}

A wealth of data has been accumulated suggesting alterations of vasoreactivity of CH rat PAs in response to agonists. 5-HT elicited enhanced contractile response in PAs of our CH WT mice, after normalization with maximal KCl-induced contraction to account for changes in other nonreceptor-dependent mechanisms. This is consistent with previous reports on CH rats and mice.\textsuperscript{6,21} Moreover, the CH-enhanced 5-HT response was noticeably suppressed in \textit{Trpc1}^{−/−} PAs and virtually abolished in \textit{Trpc6}^{−/−} PAs, suggesting that TRPC1 plays a contributing role whereas TRPC6 is required for the enhanced response. The clear participation of TRPC6 and TRPC1 in 5-HT–induced contraction in hypoxic but not normoxic PA could be related to the upregulation of the TRPC channels, and it may also reflect changes in the signaling mechanism. 5-HT–induced pulmonary vasoconstriction is mediated primarily by 5-HT\textsubscript{1A}, and to a lesser extent by 5-HT\textsubscript{1B} receptor in normoxic PAs.\textsuperscript{21,22} 5-HT\textsubscript{1A} and 5-HT\textsubscript{1B} expressions are upregulated and the contribution of 5-HT\textsubscript{1B} to pulmonary vasoconstriction is augmented in CH PAs.\textsuperscript{21,23} It will be
interesting for future studies to investigate whether 5-HT\textsubscript{1A} or 5-HT\textsubscript{2A} receptors are preferentially coupled to the upregulated TRPC6 channels in CH PAs.

TRPC1 and TRPC6 both play a significant role in neomuscularization of small PAs, which was largely suppressed in CH Trpc1\textsuperscript{-/-} and Trpc6\textsuperscript{-/-} mice. This is consistent with the well-recognized roles of TRPC1 and TRPC6 in PASMC proliferation.\textsuperscript{10,24} Lessening of muscularization may reduce PA vasmotor tone and reactively and hence attenuates PH in CH Trpc1\textsuperscript{-/-} and Trpc6\textsuperscript{-/-} mice. It is noteworthy that CH caused a 30% to 40% reduction in the density of pulmonary microvessels, which could lead to an increase in parallel resistance of pulmonary circulation and elevate PAP. Pulmonary vascular rarefaction is well documented in CH rats and mice and is related to alterations in VEGF and other signaling pathways.\textsuperscript{25,26} This process, however, is independent of TRPC1 and TRPC6 because deletion of either or both channels did not reverse the vascular regression.

The contributions of TRPC1 and TRPC6 to CHPH are different at various stages of the disease. For example, PH and RV hypertrophy was greatly suppressed in Trpc6\textsuperscript{-/-} mice exposed for 1-week hypoxia, but the suppression was diminished after 3 weeks. The early suppression of PH in Trpc6\textsuperscript{-/-} mice may suggest the TRPC6-dependent vasoreactivity is a major factor in the early development of PH. But it is more likely related to the important role of TRPC6 in HPV. It has been shown that acute hypoxia activates TRPC6 in PASMCs through diacylglycerol accumulation, and HPV is completely abolished in Trpc6\textsuperscript{-/-} mice.\textsuperscript{2,7} Because HPV occurs immediately after exposure to hypoxia and is blunted within a week after prolonged exposure to hypoxia,\textsuperscript{28,29} the impact of TRPC6-mediated HPV on HPV is most prominent in the first week of CH and subsides thereafter as PH progresses. This is congruent with the observations in CH Trpc6\textsuperscript{-/-} mice. Whereas, TRPC1 is engaged in the development of the intrinsic vasoconstrictor/myogenic tone, which continues to affect PAP throughout CH exposure. Hence, PH was consistently suppressed in Trpc1\textsuperscript{-/-} mice after 1 and 3 weeks of CH.

TRPC1 and TRPC6 double-deletion experiments provided further insights into the combined influence of these TRPC channels in pulmonary vasculatures. The hypotension of pulmonary circulation observed in normal Trpc1\textsuperscript{+/+}Trpc6\textsuperscript{+/+} mice demonstrates the importance of TRPC1 and TRPC6 in the regulation of pulmonary vascular tone, contrasting to the systemic circulation where MAP was unaltered. The dramatic attenuation of CH-induced increase in RVSP, mean PA pressure, and RVMI in Trpc1\textsuperscript{+/+}Trpc6\textsuperscript{+/+} mice, as opposed to the somewhat limited effects of single gene deletion, further suggests that the combined actions of the two channels have significantly larger influence than TRPC1 or TRPC6 alone. Targeting both (or multiple) TRPC channels simultaneously, therefore, could be an effective approach for the treatment of PH. It has to be mentioned, however, that there are other TRPC1/TRPC6-independent mechanisms, such as vascular rarefaction, that still remain effective, causing the residual elevation of PAP in CH Trpc1\textsuperscript{+/+}Trpc6\textsuperscript{+/+} animals.

Perspectives

This study showed that TRPC1 and TRPC6 are crucial for the regulation of vasoconstrictor tone, vasoreactivity, and neomuscularization of pulmonary vasculatures. These vascular functions contribute at different stages of CHPH, and their participations are essential for the full manifestation of CHPH in the murine model. In view of their multifaceted contributions to PH, manipulation of TRPC functions may offer a promising therapeutic strategy for hypoxia-related PH.

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Disclosures

None.

References


Novelty and Significance

What Is New?

• To our knowledge, this is the first study using genetic models to examine the roles of TRPC1 and TRPC6 in pulmonary vascular functions during chronic hypoxic pulmonary hypertension (CHPH).
• We identified, for the first time, that TRPC1 and TRPC6 differentially regulate pulmonary vasomotor tone, vasoreactivity, and neomuscularization; their presence is essential for the full manifestation of CPHH.

What Is Relevant?

• Our results clearly indicate that TRPC1 and TRPC6 play crucial roles in CPHH. Manipulation of TRPC functions may offer a novel therapeutic strategy for hypoxia-related PH.

Summary

Our knockout mice models proved that TRPC1 and TRPC6 are major contributing factors for the development of CPHH.
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TRPC1 and TRPC6 Contribute to Hypoxic Pulmonary Hypertension through Differential Regulation of Pulmonary Vascular Functions

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Running title: Roles of TRPC1 and TRPC6 in pulmonary hypertension

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MATERIALS AND METHODS

Mouse model of chronic hypoxia-induced pulmonary hypertension. Trpc1−/− and Trpc6−/− mice (1:1, 129Sv:C57BL/6J background) generated as reported earlier were originally obtained from NIEHS 1,2. Corresponding wild type (WT) mice of the same background were used as control. Subsequent generations of the colonies and Trpc1−/− Trpc6−/− double knockout mice were bled and maintained in the animal facilities of JHU under a protocol approved by the Johns Hopkins Animal Care and Use Committee. Age-matched knockout and WT mice (10–12 wk old) were placed in a hypoxic chamber and exposed to normobaric 10% O2 for 1 or 3 weeks to induce hypoxic pulmonary hypertension as described previously 3. Male mice were used in the study to avoid variations related to hormone and sex differences. Normoxic mice were housed in room air as controls.

Hemodynamic measurements and Right Ventricular Hypertrophy Determination. Animals were anesthetized with sodium pentobarbital (50 mg/kg i.p.), trachea was intubated and ventilated (0.02 ml/g body weight, 130 cycles/min) with a Harvard Apparatus Inspira ASV Animal Ventilator. After thoracotomy, right ventricular (RV) systolic pressure (RVSP) and pulmonary arterial pressure (PAP) were measured using a Mikro-tip pressure catheter (SPR-1000; Millar Instruments, Houston, TX) approached through direct puncture of RV, followed by advancing the catheter into the main PA. Arterial blood was collected from left ventricle with a heparinized needle, and hematocrit was measured after centrifugation with a Damon IEC MB Centrifuge. Heart and lung were then harvested after exsanguination. RV was separated from the left ventricle and septum (LV+S). Both portions were blot dry and weighed and the mass ratio of RV/(LV+S) was determined. In some animals, mean arterial pressure (MAP) was measured through carotid artery before thoracotomy. All animal procedures pertaining to housing conditions and animal handling were approved by the Johns Hopkins Animal Care and Use Committee.

RT-PCR and quantitative real-time PCR. The total RNA was extracted from de-endothelialized PAs, and first-stand cDNA was synthesized as described before 4. Primer sequences specific for mouse TRPC subfamily and 18S were designed based on published sequences in GenBank to obtain predicted PCR products with 100-150 bases and listed on Table 1. Real-time PCR reactions were carried out with SYBR Green PCR Master Mix on an IQ5 Real-time PCR detection system. Confirmation with single peak in the melting curve as well as a single band of expected size in the agarose gel showed the specificity of PCR. Standard curves were generated and the absolute copy numbers of each gene were determined. The TRPCs mRNA levels were expressed as their representative copy number relative to that of 18S for each sample measured in the same run.

Western bolt. Endothelium-denuded PAs from 3 mice were harvested and pooled together, considering as one sample. The tissues were then homogenized 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 left on ice for 30 min, allowing complete cell lysis. After centrifuging at 4°C at 10,000 rpm
for 10 min, the supernatant was collected and quantified by a Nanodrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). 10 μg protein was resolved on SDS-PAGE (8%), followed by transferring onto polyvinylidene difluoride membranes, immunobloting with rabbit polyclonal antibodies against TRPC1 (ab75322, Abcam, Cambridge, MA), TRPC6 (ACC-017, Alomone Laboratories, Jerusalem, Israel) and β-Actin (13E5, Cell signaling, Danvers, MA) at 1:500, 1:1000 and 1:10,000 dilution, respectively. The membranes were then probed with horseradish peroxidase-conjugated goat anti-rabbit IgG (172-1019, Bio-Rad, Hercules, CA). Bound antibodies were visualized by Pierce ECL (Thermo Scientific). The intensity of interested bands was quantified using ImageJ software.

**Isometric tension measurement of intralobar PAs**  
Upon removal, the heart and lungs were immediately immersed in oxygenated modified Krebs solution containing (in mM) 118 NaCl, 4.7 KCl, 0.57 MgSO₄, 1.18 KH₂PO₄, 25 NaHCO₃, 10 dextrose, and 1.25 CaCl₂. Intralobar pulmonary arteries (~200 μm OD) were isolated, free of connective tissue, and then endothelium was denuded by gently rubbing the lumen with a moose mane. The arteries were cut into 1.8 mm rings and mounted on wire-myograph system (Model 6200; Danish Myo Technology, Aarhus, Denmark). The Krebs solution was continuously gassed by 16% O₂ plus 5% CO₂ and isometric tension was online recorded in LabChart 7 (PowerLab; ADInstruments, Colorado Springs, CO). For vasoreactivity experiments, basal tension was determined by normalization module in LabChart 7 with the target pressure setting at 15 mmHg or 25 mmHg for normoxic and hypoxic PAs, respectively. After 60 min equilibrium, the rings were exposed to 60 mM KCl for 3 times and to phenylephrine (PE, 1 μM) followed by ACh (10 μM) for verification of complete disruption of endothelium. PAs with >20% Ach-induced relaxation were discarded. The active tensions induced by cumulative-concentrations of agonist were normalized to the maximum-tension generated by 60 mM of KCl. For the vascular tone experiments, the resting initial width of the vessel was determined by increasing the distance between the two wires of the myograph until they touched the vessel wall, as evident by a sudden increase in tension reading. Vessel-width was then increased in 50 μm steps from 0 to 600 μm once every three minutes to generate a length-tension relation in 1.5 mM Ca²⁺ containing modified Krebs solution. The same procedure was repeated in the same PA ring in Ca²⁺ free and Ca²⁺ free +10 μM papaverine Krebs solutions, with a recovery period of 30 min under zero-tension between each trial. Active tension at each point was calculated by subtracting the total wall-tension recorded under Ca²⁺ containing condition with the passive wall-tension measured after the vessel was completely relaxed in Ca²⁺ free +10 μM papaverine solutions. The conversion of wall-tension (Tᵢ) to transmural pressure (Pᵢ) at a given vessel-width was calculated based on the Laplace law, Pᵢ = Tᵢ / rᵢ = Tᵢ / (ICᵢ / 2π), where rᵢ and ICᵢ are the radius and inner circumference of the vessel and ICᵢ equals to the resting circumference (IC₀) plus 2 x Δ-width.

**Morphology of Pulmonary Vascular Structure**  
Mice were exsanguinated after deep anesthesia with pentobarbital sodium. 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 30 min, cut into 4-5 pieces, and then fixed in 4% formaldehyde at 4°C overnight. Formalin fixed lungs
were embedded in paraffin, sectioned into 5 μm slices and stained with smooth muscle α-actin to identify muscularized pulmonary arteries, as described before. An Olympus BX51 microscope equipped with an Olympus Q-color 5 digital camera was used to examine the sections under a 20x objective. Three images from each of the four lung sections were captured from each animal with the Qcapture software (Qimaging, Surrey, Canada). Since it is impossible to distinguish arterioles from venules at this anatomical level, all vessels of internal diameter <100 μm were taken into account and were classified into nonmuscularized (<25%), partially muscularized (25–75%), and completely muscularized (> 75%) vessels. The scoring area covered levels from upper lobe to lower lobe and the data are expressed as percent distributions of different types of vessels for the evaluation of pulmonary vascular remodeling. Vessel density refers to total number of vessels in each microscopic field.

Data Analysis All data are expressed as means±SE. N specified in the text refers to numbers of animals or preparations. Concentration-response curves were fitted with the three parameter logistic model: R = Emax/(1+(|A|/EC50)^b) using the SigmaPlot software, where R is the normalized developed tension, Emax is the maximal response, |A| is the concentration of the agonist, EC50 is the effective concentration for 50% response, and b is the slope factor. Statistical significance (P <0.05) of the changes was estimated in Sigma plot by paired or unpaired Student’s t-tests, or by one or two-way ANOVA with Bonferroni post hoc test, wherever applicable.

Chemicals and drugs PE, 5-HT, papaverine and other chemicals were purchased from Sigma Chemical (St. Louis, MO). Stock solutions were prepared in distill water 1000 times the concentration used in the experiments.
REFERENCES


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Figure S1. Representative original tracings of vessel-width and wall-tension experiments in PAs of normoxic and hypoxic WT (A, B) and Trpc1−/− (C, D) and Trpc6−/− (E, F) mice. Stepwise increase (50 μm) of vessel-width was applied to PAs after equilibrated at resting length in the presence of 1.25 mM Ca2+. The protocol was repeated after Ca2+ removal and after further addition of 10 μM papaverine. Arrow indicates application of stepwise increase in vessel-width.
Figure S2. A representative tracing showing the appearance of spontaneous oscillatory contraction in a CH WT PA at the higher levels of mechanical stretch. The inset shows oscillations at an expanded scale. Arrow indicates application of stepwise increase in vessel-width.
Figure S3. Comparison of right ventricular systolic pressure (A), mean pulmonary arterial pressure (B), and the right heart mass index (RV/(LV+S), C) between WT, Trpc1−/−, Trpc6−/− and Trpc1−/−Trpc6−/− mice. The data are summarized from Figure 2 and 6 of the manuscript. *, **, and *** indicate P<0.05, 0.01, and 0.001 compared to WT. # indicates P<0.05 compared to Trpc1−/−Trpc6−/− mice.