Notch Activation of Ca^{2+} Signaling in the Development of Hypoxic Pulmonary Vasoconstriction and Pulmonary Hypertension

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Abstract

Hypoxic pulmonary vasoconstriction (HPV) is an important physiological response that optimizes the ventilation/perfusion ratio. Chronic hypoxia causes vascular remodeling, which is central to the pathogenesis of hypoxia-induced pulmonary hypertension (HPH). We have previously shown that Notch3 is up-regulated in HPH and that activation of Notch signaling enhances store-operated Ca^{2+} entry (SOCE), an important mechanism that contributes to pulmonary arterial smooth muscle cell (PASMC) proliferation and contraction. Here, we investigate the role of Notch signaling in HPV and hypoxia-induced enhancement of SOCE. We examined SOCE in human PASMCs exposed to hypoxia and pulmonary arterial pressure in mice using the isolated perfused/ventilated lung method. Wild-type and canonical transient receptor potential (TRPC) $6^{-/-}$ mice were exposed to chronic hypoxia to induce HPH. Inhibition of Notch signaling with a γ -secretase inhibitor attenuates hypoxia-enhanced

SOCE in PASMCs and hypoxia-induced increase in pulmonary arterial pressure. Our results demonstrate that hypoxia activates Notch signaling and up-regulates TRPC6 channels. Additionally, treatment with a Notch ligand can mimic hypoxic responses. Finally, inhibition of TRPC6, either pharmacologically or genetically, attenuates HPV, hypoxia-enhanced SOCE, and the development of HPH. These results demonstrate that hypoxia-induced activation of Notch signaling mediates HPV and the development of HPH via functional activation and up-regulation of TRPC6 channels. Understanding the molecular mechanisms that regulate cytosolic free Ca^{2+} concentration and PASMC proliferation is critical to elucidation of the pathogenesis of HPH. Targeting Notch regulation of TRPC6 will be beneficial in the development of novel therapies for pulmonary hypertension associated with hypoxia.

Keywords: pulmonary artery; notch; hypoxia; TRPC6; store-operated Ca^{2+} entry

Hypoxic pulmonary vasoconstriction (HPV) is a compensatory physiological mechanism that is important for maintaining efficient gas exchange by optimizing ventilation-perfusion matching

via diverting blood from poorly ventilated areas of the lung to the regions that are better ventilated (1, 2). However, persistent hypoxia causes hypoxia-induced pulmonary hypertension (HPH) by

inducing sustained pulmonary vasoconstriction and pulmonary vascular medial hypertrophy. Clinically, HPH occurs in patients with chronic obstructive pulmonary disease and obstructive sleep

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Clinical Relevance

Our previous studies have demonstrated that Notch is up-regulated in patients with pulmonary hypertension and in animals with experimental pulmonary hypertension. In this study, we demonstrate that acute hypoxia-induced pulmonary vasoconstriction and chronic hypoxia-induced pulmonary hypertension are mediated by Notch-induced enhancement of store-operated Ca^{2+} entry in pulmonary arterial smooth muscle cells. This is the first report to show that Notch signaling activates Ca^{2+} signaling and that the Notch-mediated activation of Ca^{2+} signaling involves two mechanisms: acute functional activation and chronic up-regulation of Ca^{2+} -permeable channels (e.g., canonical transient receptor potential [TRPC] 6). Targeting Notch regulation of TRPC6 will be beneficial in the development of novel therapies for pulmonary hypertension associated with hypoxia.

apnea and is a risk factor for right heart failure (3, 4). The pathogenesis of HPH can be attributed in part to excessive vascular remodeling resulting in elevated pulmonary vascular resistance (1, 5). The thickness and tissue mass of the pulmonary arterial walls are maintained by a balance between cell proliferation and apoptosis. A disruption of this balance in favor of proliferation can lead to thickening of the wall and to narrowing and eventually obliteration of the vessel lumen. Pulmonary vascular remodeling refers to the structural changes that lead to hypertrophy and/or luminal occlusion (6, 7). Evidence from multiple studies indicates that chronic hypoxia alters the ionic balance and Ca^{2+} homeostasis in pulmonary arterial smooth muscle cells (PASMCs), including causing membrane depolarization, elevating resting cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_{\text{cyt}}$), and changing the electrophysiological and Ca^{2+} responses of PASMCs to vasodilators and vasoconstrictors (8–11).

An increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in PASMCs is a major trigger for pulmonary vasoconstriction and is an important stimulus for cell proliferation and

migration, two major causes for pulmonary vascular remodeling. Intracellular Ca^{2+} is an important second messenger for cell proliferation (12, 13). When intracellular Ca^{2+} stores, such as the sarcoplasmic reticulum (SR), are depleted, store-operated Ca^{2+} channels (SOCs) mediate Ca^{2+} influx and increase $[\text{Ca}^{2+}]_{\text{cyt}}$ (14, 15). In HPH, increased expression of SOCs and enhanced store-operated Ca^{2+} entry (SOCE) contribute to increased $[\text{Ca}^{2+}]_{\text{cyt}}$ (16–18). SOCE is known to be important for cell proliferation and vascular remodeling in pulmonary hypertension, and studies from our lab have demonstrated increased expression of several proteins involved in SOCE, such as canonical transient receptor potential (TRPC) channels (TRPC3, TRPC6), stromal interaction molecule 2 (STIM2), and Orai2, in patients with pulmonary hypertension (16, 19–21). Recently, two studies have demonstrated Notch-dependent expression of TRPC6, raising the possibility that Notch signaling may transcriptionally regulate TRPC6 and may thereby play a critical role in HPH (22, 23).

Notch signaling is involved in vascular development, and Notch3 has recently been implicated in pulmonary hypertension (24, 25). Lung tissue from patients with pulmonary hypertension displays increased Notch3 and Notch3 intracellular domain expression when compared with normotensive patients (25). Additionally, Notch3 and Notch3 intracellular domain expression is increased in two animal models of pulmonary hypertension—HPH in mice and monocrotaline-induced pulmonary hypertension in rats—and knockout of Notch blocks the development of HPH in mice (25). We have recently shown that activation of Notch signaling via treatment with the Notch ligand Jagged-1 (Jag-1) enhances SOCE in PASMCs (26). In this study, we investigated the role of Notch signaling in the responses to acute and chronic hypoxia. Our results indicate that inhibition of Notch signaling attenuates acute HPV and chronic hypoxia-induced enhancement of SOCE in PASMCs. Additionally, we show that chronic hypoxia increases expression of SOCs and activates Notch signaling. Finally, we show that Notch intracellular domain (NICD) functionally interacts with TRPC6 and that blockade of TRPC6 inhibits acute HPV and the development of HPH in mice. These data are the first to

describe a dual role for Notch signaling of functional activation and up-regulation of Ca^{2+} channels in PASMCs in response to acute and chronic hypoxia.

Materials and Methods

Hypoxic Pulmonary Hypertension Mouse Model

All animal experiments were approved by the University of Illinois at Chicago Animal Care and Use Committee and were performed according to the guidelines of the University of Illinois at Chicago that comply with national and international regulations. Male C57BL/6N mice were exposed to hypoxia (10%) in a ventilated chamber for 4 weeks to induce pulmonary hypertension. After hypoxic exposure, right ventricular systolic pressure (RVSP) was measured by right ventricular catheterization with a pressure transducer catheter (SPF1030; Millar Instruments, Houston, TX) inserted through the right jugular vein. Detailed methods are available in the online supplement.

Assessment of Pulmonary Artery Thickness and Fulton Index

The left lung lobes were fixed in a 3% paraformaldehyde solution and paraffin embedded. Hematoxylin and eosin staining was performed on 3- μm sections according to common histopathological procedures.

Angiography

Detailed methods can be found in the online supplement.

Isolation of Mouse PASMCs

PASMCs were isolated from mouse lungs as described previously (27) using a modification of the method described by Marshall and colleagues (28).

Isolated Perfused/Ventilated Mouse Lung

The PAP of mice (three or four mice per group with repeated measurements) was measured using the isolated perfused/ventilated mouse lung system as previously described (29).

Cell Culture, Hypoxia, and Transfection

Human PASMCs were obtained from Lonza (Walkersville, MA) and were cultured in Medium 199 supplemented with 10% FBS

(Life Technologies, Grand Island, NY), 100 U/ml penicillin plus 100 μ g/ml streptomycin (Life Technologies), 50 μ g/ml D-valine (Sigma-Aldrich, St. Louis, MO), and 20 μ g/ml endothelial cell growth supplement (BD Biosciences, Franklin Lakes, NJ) at 37°C. For hypoxic experiments, PSMCs were cultured in an incubator with 3% O₂ for the indicated time. HEK-293 cells were transfected with pmaxGFP (Lonza) or Jag-1-HA pIRES (30) (Addgene plasmid 17336; a gift of Joan Conaway) using the Profection Mammalian Transfection System (Promega, Madison, WI).

[Ca²⁺]_{cyt} Measurement

The [Ca²⁺]_{cyt} measurement was performed as described previously (26).

Western Blot and Immunoprecipitation

Detailed methods can be found in the online supplement.

Antibodies

The following antibodies were used for Western blot and/or immunoprecipitation: NICD (07-1232; Millipore, Temecula, CA), Notch3 (sc-7424; Santa Cruz), β -actin (sc-81178; Santa Cruz), TRPC6 (PRS3897; Sigma-Aldrich), RBP-J κ (sc-28713; Santa Cruz), Orai1 (ACC-060; Alomone Labs, Jerusalem, Israel), Orai2 (ACC-061; Alomone Labs), STIM2 (S8572; Sigma-Aldrich), Jag-1 (2620; Cell Signaling Technologies, Beverly, MA), and β -tubulin (sc-9104; Santa Cruz).

Whole-Cell Patch Clamp

Briefly, Ca²⁺ currents were recorded with the whole-cell patch-clamp technique using an Axopatch-1D amplifier and a DigiData 1322 interface (Molecular Devices, Sunnyvale, CA). Detailed methods can be found in the online supplement.

Isometric Tension Measurements

The pulmonary arteries (PAs) of mice were isolated and cut into 2- to 3-mm-long rings. Isometric tension measurements were performed as previously described (27).

Statistical Analysis

Composite data are shown as the means $\pm$ SE. Statistical significance among groups was determined by one-way ANOVA followed by the Holm-Sidak method. Significant difference is expressed as **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

Results

Chronic Hypoxia Is Associated with Enhanced SOCE and the Development of Pulmonary Hypertension in Mice

To examine whether enhanced SOCE is involved in HPH *in vivo*, we measured and compared the amplitude of increases in [Ca²⁺]_{cyt} due to SOCE in PSMCs isolated from normoxic mice and from mice exposed to chronic hypoxia. In PSMCs isolated from normoxic mice, blockade of the SR Ca²⁺ ATPase pump with cyclopiazonic acid (CPA) (10 μ M) in the absence of extracellular Ca²⁺ (0 Ca) evoked a transient increase in [Ca²⁺]_{cyt} due apparently to Ca²⁺ mobilization or leakage from the SR. Restoration of extracellular Ca²⁺ in the continued presence of CPA that depletes Ca²⁺ from the SR evoked a second increase in [Ca²⁺]_{cyt}, which represents SOCE incurred as a result of store depletion (Figure 1A). Similar to results from previous studies in mice and rats (16–18), PSMCs isolated from mice exposed to chronic hypoxia (10% O₂, 4 wk) in a normobaric hypoxic chamber had significantly enhanced SOCE when extracellular Ca²⁺ was restored in PSMCs treated with CPA (Figure 1A). Mice exposed to chronic hypoxia developed significantly higher RVSP as compared with normoxic mice (Figure 1B). The increased RVSP in chronic hypoxic mice is associated with increased wall thickness of PAs with different outer diameters (Figure 1C, *upper* and *lower panels*). Using angiography, we demonstrated that chronic hypoxic mice had a decreased number of branches and junctions and a decreased total length of PAs compared with normoxic mice (Figure 1D, *upper* and *lower panels*). These data indicate that chronic hypoxia enhances SOCE in PSMCs and leads to the development of HPH in mice.

Exposure to Acute Alveolar Hypoxia Causes Acute HPV

To examine the effect of acute hypoxia on pulmonary vasoconstriction, we used the isolated perfused/ventilated mouse lung system. Acute alveolar hypoxia (3% O₂ for 5 min) significantly and repeatedly increased the pulmonary arterial pressure (PAP) in isolated perfused/ventilated lung, which returned to baseline under normoxic conditions; the acute hypoxia-induced increases in PAP were maintained at the

similar level after first hypoxic challenge (Figure 1E). These results demonstrate that acute alveolar hypoxia rapidly and reversibly causes pulmonary vasoconstriction.

Chronic Hypoxia-Induced Enhancement of SOCE and Acute HPV Are Dependent on Notch Signaling

We have previously reported that Notch3 is up-regulated in mice with HPH and that inhibition of Notch signaling with N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester (DAPT), a γ -secretase inhibitor, reverses the development of HPH in mice (25). Additionally, we have recently shown that activation of Notch signaling by treatment with Jag-1, a Notch ligand, enhances SOCE in human PSMCs (26). To investigate the involvement of Notch signaling in chronic hypoxia-enhanced SOCE, we treated human PSMCs with DAPT and examined hypoxia-induced SOCE. Human PSMCs were first exposed to normoxia (Nor) and hypoxia (Hyp, 3% O₂) for 48 hours, and then the increases of [Ca²⁺]_{cyt} induced by CPA-mediated passive depletion of intracellularly stored Ca²⁺ in the SR were measured in the absence or presence of DAPT. The chronic hypoxia-induced enhancement in SOCE is attenuated by inhibition of Notch signaling with DAPT (10 μ M) (Figures 2A and 2B), demonstrating that hypoxia-induced increase in SOCE in PSMCs is dependent on Notch signaling.

To determine whether Notch signaling is involved in acute HPV, we examined the response to acute hypoxia in isolated perfused/ventilated mouse lungs. Intrapulmonary arterial application of the vehicle (DMSO) had no effect on the acute HPV; however, intrapulmonary arterial application of DAPT significantly attenuated the amplitude of acute alveolar hypoxia-induced increase in PAP (Figures 2C and 2D, *left* and *right panels*). The increase phase of the curves of hypoxia-mediated increase in PAP was fitted by one exponential protocol, and the time constant for the activation phase of hypoxia-induced increase in PAP was significantly reduced by DAPT (Figure 2E). In the absence of hypoxia, DAPT alone caused a significant decrease in the baseline PAP, suggesting that a basal level of Notch signaling may be required to maintain pulmonary vascular tone (Figure 2F, *left* and *right panels*). These

Figure 1. Hypoxia causes pulmonary vasoconstriction, enhances store-operated Ca^{2+} entry (SOCE), and causes hypoxia-induced pulmonary hypertension (HPH) in mice. (A) Representative records (left panels) and summarized data (mean $\pm$ SE) (right panel) showing changes in $[Ca^{2+}]_{\text{cyt}}$ in response to cyclopiazonic acid (CPA) (10 μM)-induced store depletion in the absence of extracellular Ca^{2+} and subsequent SOCE upon replenishment of extracellular Ca^{2+} in pulmonary arterial smooth muscle cells isolated from normoxic (Nor) and hypoxic (Hyp) mice. $**P < 0.01$ versus Nor. (B) Representative record of right ventricular pressure (RVP) (left panels) and summarized data (means $\pm$ SE) (right panel) showing the peak value of right ventricular systolic pressure (RVSP) in Nor ($n = 6$) and Hyp ($n = 6$) mice. $*P < 0.05$ versus Nor. (C) Representative hematoxylin and eosin (H&E) images (upper panels) and summarized data (means $\pm$ SE) (lower panels) of small pulmonary arteries (PA) showing that the medial thickness is significantly increased in Hyp compared with Nor mice. $***P < 0.001$ versus Nor. (D) Representative image (upper panels) and summarized data (means $\pm$ SE) (lower panels) of whole-lung angiogram from Nor and Hyp mice. $**P < 0.01$ versus Nor. (E) Representative record (left panel) and summarized data (means $\pm$ SE) (right panel) of changes in pulmonary arterial pressure (PAP) in response to hypoxia (Hyp). $***P < 0.001$ versus Nor.

Figure 2. Inhibition of Notch signaling attenuates hypoxia-enhanced SOCE and hypoxic pulmonary vasoconstriction. (A) Representative records showing changes in $[Ca^{2+}]_{cyt}$ in response to CPA (10 μ M)-induced store depletion in the absence of extracellular Ca^{2+} and subsequent SOCE upon replenishment of extracellular Ca^{2+} in Nor and Hyp human pulmonary arterial smooth muscle cells with or without N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester (DAPT) (10 μ M). (B) Summarized data (mean $\pm$ SE) show the increases in $[Ca^{2+}]_{cyt}$ due to CPA-induced Ca^{2+} mobilization (Release) in the absence of extracellular Ca^{2+} (left panel) and to CPA-induced Ca^{2+} influx when extracellular Ca^{2+} was restored (SOCE-Peak) (right panel). ** $P < 0.01$ versus Nor and Hyp+DAPT. (C and D) Representative records (left panels) and summarized data (means $\pm$ SE) (right panels) of changes in PAP in isolated perfused/ventilated mouse lungs briefly ventilated with hypoxic gas (3% O_2 for 5 min) before and during intrapulmonary arterial application

results demonstrate that acute HPV is sensitive to inhibition of Notch signaling. Together, these data suggest that Notch signaling is involved in the responses of the pulmonary vasculature to chronic and acute hypoxia.

Chronic Hypoxia Activates Notch Signaling, which Leads to Interaction with and Up-Regulation of SOCs in PSMCs

TRPC6 has been shown to be up-regulated in PSMCs isolated from mice and rats exposed to chronic hypoxia (16, 17). We therefore examined the protein expression level of SOCs (e.g., TRPC6, Orai, and STIM) and NICD after exposure to chronic hypoxia. In human PSMCs, chronic hypoxia up-regulated protein expression of TRPC6, Orai1, Orai2, and STIM2 (Figures 3A and 3B). Additionally, chronic hypoxia increased the NICD protein level, indicating that Notch signaling is activated by hypoxia (Figure 3A). These data show that exposure to chronic hypoxia increases expression of proteins involved in SOCE in association with Notch signaling. To investigate the molecular mechanism behind the enhancement of SOCE by chronic hypoxia, we conducted immunoprecipitation studies in human PSMCs. Our data show that chronic hypoxia causes an increase in the amount of RBP-J κ bound to NICD (Figure 3C). RBP-J κ is a nuclear transcription factor known to interact with NICD, and increased binding of RBP-J κ with NICD suggests elevated activation of Notch signaling. After exposure to chronic hypoxia, NICD also binds TRPC6, suggesting that Notch signaling functionally interacts with TRPC6 (Figure 3C). We next examined the possibility that Notch signaling regulates expression of TRPC6 by overexpression of Jag-1, a Notch ligand. Overexpression of Jag-1 resulted in increased protein expression of TRPC6 as compared with control cells transfected with green fluorescent protein (GFP) (Figure 3D). Together, these results indicate that Notch signaling mediates chronic hypoxia-induced enhancement of SOCE

through direct functional interaction and up-regulation of TRPC6.

Activation of Notch Signaling by Jag-1 Can Mimic the Effects of Chronic and Acute Hypoxia on $[Ca^{2+}]_{\text{cyt}}$ in PSMCs and PAP in Isolated Perfused Lung

To determine if activation of Notch signaling was sufficient to mimic the effects of hypoxia, we treated human PSMCs with Jag-1 to activate Notch signaling (by up-regulating SOCs). Long-term treatment (48 h) of human PSMCs with Jag-1 resulted in activation of Notch signaling, as indicated by increased protein levels of NICD (Figure 3E), and significantly enhanced the peak and plateau phases of SOCE (Figure 3F) (26). In human PSMCs treated with Jag-1, extracellular application of 2-aminoethoxydiphenyl borate (2-APB) (40 μM), a blocker of TRPC6 channels (see below), abolished the Jag-1-mediated increase in SOCE (Figure 3G, *left* and *right panels*). These data indicate that chronic treatment of PSMCs with Jag-1 up-regulates TRPC6, Orai1/2, and STIM2 and enhances SOCE; the Jag-1-mediated augmentation of SOCE is mainly due to Ca^{2+} influx through 2-APB-sensitive cation channels (e.g., TRPC6).

To determine if Notch signaling was involved in acute HPV, we investigated the effect of Jag-1 on PAP in isolated perfused/ventilated mouse lung. Treatment with the control peptide (vehicle) had little effect, whereas intrapulmonary arterial application of Jag-1 resulted in a significant increase in PAP, demonstrating that Jag-1 can mimic acute hypoxia to cause pulmonary vasoconstriction (Figure 3H). Together, these data demonstrate that activation of Notch signaling by Jag-1 can mimic chronic and acute hypoxia to enhance SOCE and cause pulmonary vasoconstriction.

Blockade of TRPC6 Inhibits the Development of HPH in Mice

To determine if Notch signaling-mediated up-regulation of TRPC6 and enhancement of SOCE contribute to the development of HPH in mice, we treated normoxic and chronically hypoxic mice with 2-APB,

a TRPC channel antagonist. We first determined the effect of 2-APB on whole-cell cation currents in HEK-293 cells transfected with the human TRPC6 gene. Transient transfection of the TRPC6 gene into HEK-293 cells significantly increased the protein level of TRPC6 (Figure 4A) in comparison to cells transfected with GFP (Mock) and resulted in a significant increase in cation currents, which are mainly due to cation influx through TRPC6 (Figure 4B). Extracellular application of 2-APB markedly decreased the whole-cell TRPC6 currents at -90 or $+90$ mV (Figure 4C). In these experiments, the whole-cell cation currents, elicited by test potentials ranging from -100 to $+100$ mV (with a holding potential of 0 mV to inactivate voltage-gated cation and anion channels), were significantly increased in TRPC6-transfected cells compared with cells transfected with a GFP vector (Mock) (Figure 4B). In TRPC6-transfected cells, external application of 2-APB (100 μM) significantly decreased the currents (Figure 4C). These data indicate that 2-APB is a potent blocker of TRPC6 channels.

We next treated normoxic and chronic hypoxic mice with 2-APB and examined and compared the RVSP (Figure 4D); the Fulton Index, calculated as the ratio of the weight of right ventricle to the weight of left ventricular and septum $[RV/(LV+S)]$ (Figure 4E); and the medial thickening of PAs. Chronic hypoxia significantly increased RVSP and caused right ventricular (RV) hypertrophy (Figure 4F). The hypoxia-induced increases in RVSP and RV hypertrophy were associated with significant pulmonary vascular remodeling; the vascular medial wall thickness of small PAs (outer diameter < 100 μm) in chronic hypoxic mice was significantly greater than in normoxic mice (Figures 4G and 4H). Intraperitoneal injection of 2-APB (1 mg/kg/d) had little effect on RVSP and $RV/(LV+S)$ in normoxic mice but significantly attenuated the increase in RVSP (Figures 4D and 4E) and $RV/(LV+S)$ (Figure 4F) in chronic hypoxic mice. Consistent with the effects on RVSP, 2-APB significantly inhibited the medial thickening of small PA in chronic hypoxic mice (Figures 4G and 4H).

Figure 2. (Continued). of DAPT (10 μM) (C) or vehicle (DMSO) (D). (E) Averaged data (means $\pm$ SE) demonstrating the activation phase of the hypoxia-induced increase in PAP in isolated perfused/ventilated lungs before (Cont) and during (DAPT) acute treatment with DAPT. (F) Representative record (*left panel*) and summarized data (means $\pm$ SE) (*right panel*) demonstrating the levels of PAP before (Cont), during (DAPT), and after (Wash) application of DAPT alone. $**P < 0.01$ versus Cont and Wash.

Figure 3. Hypoxia-induced activation of Notch signaling leads to up-regulation of SOC and enhancement of SOCE, and Jagged (Jag)-1-mediated activation of Notch signaling mimics the hypoxic effect in human pulmonary arterial smooth muscle cells (PASMCs). (A) Representative Western blot analysis of canonical transient receptor potential (TRPC) 6 and Notch intracellular domain (NICD) in PASMCs exposed to normoxia (for 48 h) and to hypoxia for the indicated time (24–72 h). (B) Representative Western blot analyses of Orai1, Orai2, and STIM2 in Nor and Hyp (for 48 h) human PASMCs. (C) Representative Western blot analysis of TRPC6 and RBP-Jk (as control) in Nor and Hyp PASMC lysates immunoprecipitated with NICD. (D) Representative Western blot analysis of Jag-1 and TRPC6 in HEK293 cells overexpressing green fluorescent protein (GFP) or Jag-1 (72 h). (E) Representative Western blot analysis of Notch1 intracellular domain (NICD) in PASMCs treated with control peptide (Cont) or Jag-1 (50 μ M, 48 h). (F) Representative record (left panels) and summarized data (means $\pm$ SE) (right panel) demonstrating increases in $[Ca^{2+}]_{cyt}$ due to CPA-mediated SOCE in PASMCs treated with vehicle or Jag-1 (48 h). $^{**}P < 0.01$ versus vehicle bars. (G) Representative records (left panels) and summarized data (means $\pm$ SE) (right panel) showing increases in $[Ca^{2+}]_{cyt}$ due to CPA-mediated SOCE in PASMCs treated with vehicle (Veh) or Jag-1 (for 48 h) with (+2-aminooethoxydiphenyl borate [+2-APB]) or without (Cont) extracellular application of 40 μ M 2-APB. $^{\#}P < 0.05$ versus Veh; $^{*}P < 0.05$ versus Cont–Jag-1. (H) Representative records (left panels) and summarized data (means $\pm$ SE) (right panel) showing changes in PAP in wild-type (WT) mice before and during intrapulmonary arterial superfusion of a control peptide (Vehicle) and Jag-1 (10 μ M). Alveolar hypoxia (Hyp, 3% O_2) was applied to the isolated perfused/ventilated mouse lung via a mini ventilator. $^{**}P < 0.01$ versus Hyp and Jag-1.

Figure 4. Blockade of TRPC6 with 2-APB inhibits the development of HPH in mice. (A) Representative Western blot of TRPC6 in PSMCs transfected with GFP and *TRPC6* gene. (B) Representative whole-cell currents (left panels) and the current–voltage (*I*–*V*) relationship curves (right panel) in mock-transfected (transfected with GFP) or *TRPC6*-transfected cells. (C) Representative whole-cell currents (left panels) and amplitudes of currents at -90 and $+90$ mV in *TRPC6*-transfected cells before (Control) and during (2-APB) application of $100\ \mu\text{M}$ 2-APB. $**P < 0.01$ and $***P < 0.001$ versus control. (D and E). Representative record of RVP (D) and summarized data (mean $\pm$ SE) (E) showing the peak value of RVSP (E) in Nor ($n = 6$) and Hyp ($n = 6$) mice that were treated with vehicle or 2-APB (+2-APB; $1\ \text{mg/kg/d}$). (F) Averaged Fulton index, calculated as the ratio of the weight of right ventricle to the weight of left ventricular and septum [RV/(LV+S), mean $\pm$ SE] in Nor and Hyp mice treated with or without 2-APB. $**P < 0.01$ versus Hyp alone (red bars). (G and H) Representative H&E images (G) of small pulmonary arteries and summarized data (means $\pm$ SE) (H) of the medial thickness of pulmonary arteries with a diameter (ϕ) less than $50\ \mu\text{m}$ (left panel) and between 51 and $100\ \mu\text{m}$ (right panel) in Nor ($n = 6$) and Hyp mice ($n = 6$) that are treated with vehicle or 2-APB. $**P < 0.01$ versus Hyp alone (red bars).

These data imply that the chronic hypoxia-induced increase in expression of TRPC6 and enhancement of SOCE in PSMCs mediated by Notch signaling contributes to the development of HPH in mice and that intraperitoneal injection of the TRPC6 antagonist is an efficient therapeutic approach for HPH.

Inhibition of TRPC6 Attenuates Acute HPV and PA Contraction

To determine if TRPC6 channels are involved in acute HPV, we examined the effect of blockade of TRPC6 on acute HPV. Intrapulmonary arterial application of 2-APB ($40\ \mu\text{M}$) markedly attenuated the

acute alveolar hypoxia-induced increase in PAP, whereas application of the vehicle had no effect on the hypoxia-induced increase in PAP (Figures 5A and 5B, left and right panels). The activation kinetics of the hypoxia-induced increase in PAP show that inhibition of TRPC6 with 2-APB significantly attenuated the activation rate of HPV (Figure 5C). Additionally, in the absence of hypoxia, 2-APB alone caused a significant decrease in the baseline PAP (Figure 5D, left and right panels), similar to treatment with DAPT (Figure 2F, left and right panels). These data demonstrate that activation of TRPC6 contributes to acute alveolar hypoxia-induced pulmonary vasoconstriction.

To further understand the role of TRPC6 in pulmonary vasoconstriction, we examined the effect of TRPC6 blockade in isolated mouse PA. The active tension was measured by an isometric tension transducer in isolated mouse PA rings precontracted with phenylephrine (PE). Acute application of 2-APB in the presence of PE produced a significant decrease in PE-induced active tension at low ($100\ \mu\text{M}$) and high ($300\ \mu\text{M}$) doses (Figures 5E and 5F). Inhibition of TRPC6 with 100 and $300\ \mu\text{M}$ 2-APB resulted in decreases in active tension of 30 and 95% , respectively (Figure 5F). These results indicate that TRPC6 (or other cation channels sensitive to 2-APB) plays an important role in

Figure 5. Inhibition of TRPC6 with 2-APB attenuates acute hypoxia-induced pulmonary vasoconstriction. (A and B) Representative records (left panels) and summarized data (means $\pm$ SE) (right panels) of changes in PAP in isolated perfused/ventilated mouse lungs briefly ventilated with hypoxic gas (3% O_2 for 5 min) before (Cont), during (2-APB), and after (Wash) intrapulmonary arterial application of 2-APB (40 μ M) (A) or vehicle (DMSO) (B). (C) Averaged data demonstrating the activation phase of the hypoxia-induced increase in PAP in isolated perfused/ventilated lungs before (Cont) and during (2-APB) intrapulmonary arterial application of 2-APB. (D) Representative record (left panel) and summarized data (means $\pm$ SE) (right panel) demonstrating decreased basal PAP after 2-APB treatment. * $P < 0.05$ versus Cont and Wash. (E) Representative tracing showing changes in isometric tension before, during, and after application of 100 μ M (left panel) or 300 μ M (right panel) 2-APB in isolated pulmonary artery rings precontracted with phenylephrine (PE) (100 nM). (F) Summarized data (means $\pm$ SE) showing change in PE-induced active tension in isolated pulmonary artery rings before (Cont), during (2-APB), and after (Wash) application of 100 μ M (left panel) or 300 μ M (right panel) 2-APB. * $P < 0.05$ and ** $P < 0.01$ versus Cont and Wash.

agonist-mediated pulmonary vasoconstriction. Together, these data demonstrate that TRPC6 channels are involved in acute HPV.

Knockout of TRPC6 Attenuates Acute HPV, SOCE, and the Development of HPH

To examine whether TRPC6 channels are required for acute HPV and chronic HPH, we compared acute HPV in isolated perfused/ventilated lung from wild-type (WT) and *Trpc6*^{-/-} mice. Acute alveolar hypoxia reversibly increased PAP in WT mice, and the acute hypoxia-mediated increase in PAP was significantly inhibited in *Trpc6*^{-/-} mice (Figure 6A, left and right panels). Intrapulmonary arterial application of DAPT markedly reduced the amplitude of acute alveolar hypoxia-induced increase in PAP in WT mice but not in *Trpc6*^{-/-} mice (Figure 6B, left and right panels). These data demonstrate that TRPC6 is required for acute HPV in mice.

It has been well reported that Orai1/2 and STIM1/2 contribute to SOCE in many cell types (14, 15). To test if TRPC6 also contributes to SOCE, we measured the increase in $[Ca^{2+}]_{cyt}$ due to SOCE in PSMCs isolated from WT and *Trpc6*^{-/-} mice. Deletion of TRPC6 significantly decreased the resting $[Ca^{2+}]_{cyt}$ and inhibited SOCE in PSMCs (Figures 6C and 6D); these data indicate that TRPC6 is necessary for, or at least involved in, SOCE in PSMCs.

To determine if TRPC6 channels are required for the development of HPH, we exposed WT and *Trpc6*^{-/-} mice to chronic hypoxia. Exposure of WT mice to chronic hypoxia increased RVSP compared with normoxic WT mice ($P < 0.001$) (Figures 6E–6H). In *Trpc6*^{-/-} mice, however, the RVSP during chronic hypoxia was significantly lower than the RVSP in WT mice ($P < 0.001$; $n = 5$) (Figures 6E–6G). Global knock-out of TRPC6 alone had little effect on baseline RVSP (Figures 6E and 6G) or on the Fulton Index (Figure 6H), but deletion of TRPC6 significantly attenuated the hypoxia-mediated increase in RVSP and RV hypertrophy measured by the RV/(LV+S) ratio (Figure 6H). These data indicate that up-regulation of TRPC6 and enhancement of SOCE in PSMCs play an important role in the development of HPH.

Figure 6. Acute hypoxia-induced pulmonary vasoconstriction and chronic hypoxia-induced pulmonary hypertension are attenuated in *Trpc6*^{-/-} mice. (A and B) Representative records (left panels) and summarized data (means ± SE) (right panels) of changes in PAP in isolated perfused/ventilated lungs from WT and *Trpc6*^{-/-} mice briefly ventilated with hypoxic gas (3% O₂ for 5 min) alone (A) and during intrapulmonary arterial application of DAPT (40 μM) (B). ** $P < 0.01$ versus WT, Control. (C and D) Representative records (C) and summarized data (means ± SE) (D) showing the

Discussion

Acute hypoxia causes pulmonary vasoconstriction, and persistent hypoxia causes sustained pulmonary vasoconstriction and vascular remodeling, leading to pulmonary hypertension. An increase in $[Ca^{2+}]_{cyt}$ in PSMCs due to Ca^{2+} influx through SOCs, receptor-operated Ca^{2+} channels, and voltage-dependent Ca^{2+} channels is a major trigger for pulmonary vasoconstriction and an important stimulus for pulmonary vascular medial thickening. In this study, we found that (1) chronic hypoxia enhances SOCE by Notch signaling-mediated up-regulation of TRPC6, (2) acute HPV is mediated by Notch signaling-mediated functional activation of TRPC6, (3) hypoxia increases expression of TRPC6 and *Orai1* and activates Notch signaling, (4) Notch interacts with and transcriptionally up-regulates TRPC6, (5) activation of Notch signaling can mimic acute and chronic hypoxia to cause pulmonary vasoconstriction and vascular remodeling, and (6) pharmacological blockade of TRPC6 channels and deletion of the TRPC6 gene significantly inhibit acute HPV and markedly attenuate the development of HPH in mice. Collectively, these data imply that hypoxia-induced activation of Notch signaling enhances SOCE by direct functional interaction with and transcriptional up-regulation of TRPC6, leading to HPV and the development of HPH. This is the first study to report that hypoxia-mediated Notch signaling has dual effects of functional activation and transcriptional up-regulation of Ca^{2+} channels, which are associated with the development of pulmonary vascular remodeling in experimental models of pulmonary hypertension.

The canonical Notch signaling pathway is an evolutionarily conserved pathway that dictates cell fate and influences cell proliferation, cell differentiation, and apoptosis (31, 32). Canonical Notch signaling is important for the regulation of growth, apoptosis, migration, and differentiation of vascular smooth muscle

cells (VSMCs) and is a key mediator of vascular morphogenesis (33–35). Notch is required for arterial–venous differentiation during embryonic development and regulates arterial specification of VSMCs (36, 37). Notch3 is only expressed in VSMCs of arteries; it is not expressed in veins (24). We have previously demonstrated that Notch3 is up-regulated in patients with pulmonary hypertension and in animal models of experimental pulmonary hypertension (25).

In this study, we show that Notch signaling is activated by hypoxia in PSMCs. Exposure to hypoxia results in stabilization of hypoxia-inducible factor (HIF)-1 α , and several studies have described cross-talk between HIF-1 α and Notch. It has been reported that Notch can potentiate HIF-1 α -mediated transactivation of hypoxia-inducible genes in tumorigenesis and cellular differentiation (38, 39). Additionally, NICD has been shown to interact with factor inhibiting HIF-1 and to act as a molecular sink, allowing for enhanced HIF-1 α function (39). Conversely, HIF-1 α has been shown to enhance Notch signaling by several mechanisms. HIF-1 α can stabilize NICD under normoxic and hypoxic conditions, resulting in transcriptional up-regulation of Notch target genes (40). Several studies have demonstrated that reactive oxygen species can activate Notch signaling (41–43). Given that hypoxia increases reactive oxygen species, it is possible that Notch signaling is activated in response by reactive oxygen species in hypoxic conditions (44). It has previously been shown that hypoxia requires Notch signaling to keep cells in an undifferentiated state (45). Hypoxia induces proliferation of PSMCs, and these proliferative PSMCs exhibit an undifferentiated phenotype (46, 47). PSMCs are not terminally differentiated, and Notch signaling controls PSMC differentiation and modulates expression of contractile genes (48–51). Vascular remodeling caused in part by increased proliferation of PSMCs contributes to increased pulmonary vascular resistance in HPH. Together these findings support our

hypothesis that Notch mediates the pulmonary vascular remodeling seen in HPH.

Here, we provide evidence for a novel dual role of Notch signaling. NICD directly interacts with and activates TRPC6, leading to enhanced SOCE and to an increase in $[Ca^{2+}]_{cyt}$ in PSMCs. In our previous study, we demonstrated that short-term (5–10 min) treatment with Jag-1 activates Notch signaling and enhances SOCE. These data, together with our current study showing the effects of Notch signaling on acute HPV, demonstrate a rapid response to activation of Notch signaling in PSMCs. This suggests that the interaction of NICD and TRPC6 is independent of any transcriptional functions of Notch signaling, which would require hours instead of minutes. Therefore, the functional interaction of Notch and TRPC6 is likely a noncanonical form of Notch signaling. The earliest evidence of noncanonical Notch signaling came from *in vitro* studies in myoblast cells, which demonstrated that increased Notch1 levels inhibited differentiation into muscle cells (52). Noncanonical Notch signaling has been shown to regulate β -catenin signaling in stem and progenitor cells in which membrane-bound Notch physically associates with β -catenin and negatively regulates accumulation of active β -catenin (53). Additionally, noncanonical Notch signaling has been shown to inhibit apoptosis by activating Akt signaling. NICD binds to mTOR and Rictor, leading to the activation of Akt and inhibition of apoptosis (54).

In addition to functional interaction with TRPC6 by noncanonical Notch signaling, activation of canonical Notch signaling regulates expression of TRPC6. Previous studies in neuronal cells have demonstrated NICD-dependent transcription of TRPC6 (23) and that TRPC6 is a key mediator of Notch-driven growth and invasiveness of glioblastoma cells (22). We show in this study that blockade of the hypoxia-induced increase in TRPC6 expression and function inhibits acute HPV and the development of HPH in mice. These findings support our novel

Figure 6. (Continued). resting or basal $[Ca^{2+}]_{cyt}$ and changes in $[Ca^{2+}]_{cyt}$ due to SOCE induced by CPA (10 μ M) in PSMCs isolated from WT and *Trpc6*^{-/-} mice. ****P* < 0.001 versus WT. (E–G) Representative records of RVP (E and F) and summarized data (means $\pm$ SE) (G) showing RVSP in WT and *Trpc6*^{-/-} mice exposed to normoxia and hypoxia (10% O₂ for 4 wk). (H) RV/(LV+S) in WT and *Trpc6*^{-/-} mice exposed to normoxia and hypoxia. **P* < 0.05 and ****P* < 0.001 versus WT-hypoxia.

hypothesis that hypoxia activates canonical and noncanonical Notch signaling to enhance SOCE through direct functional interaction and regulation of expression of Ca^{2+} channels, leading to HPV and

contributing to the development of HPH. This study not only demonstrates the involvement of Notch signaling in the regulation of $[\text{Ca}^{2+}]_{\text{cyt}}$ but will also provide a framework for the design of new

combination therapies for the treatment of HPH. ■

Author disclosures are available with the text of this article at www.atsjournals.org.

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