CFTR and sphingolipids mediate hypoxic pulmonary vasoonstriction

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Hypoxic pulmonary vasoonstriction (HPV) optimizes pulmonary ventilation-perfusion-matching in regional hypoxia, but promotes pulmonary hypertension in global hypoxia. Ventilation-perfusion mismatch is a major cause of hypoxia in cystic fibrosis. We hypothesized that cystic fibrosis transmembrane conductance regulator (CFTR) may be critical in HPV, potentially by modulating the response to sphingolipids as mediators of HPV. HPV and ventilation-perfusion mismatch were analyzed in isolated mouse lungs or in vivo. Ca\(^{2+}\) mobilization and transient receptor potential canonical 6 (TRPC6) translocation were studied in human pulmonary (PASMCs) or coronary (CASMCs) artery smooth muscle cells. CFTR inhibition or deficiency diminished HPV and aggravated ventilation-perfusion mismatch. In PASMCs, hypoxia caused CFTR to interact with TRPC6, whereas CFTR inhibition attenuated hypoxia-induced TRPC6 translocation to caveolae and Ca\(^{2+}\) mobilization. Ca\(^{2+}\) mobilization by sphingosine-1-phosphate (S1P) was also attenuated by CFTR inhibition in PASMCs, but amplified in CASMCs. Inhibition of neutral sphingomyelinase (nSMase) blocked HPV, whereas exogenous nSMase caused TRPC6 translocation and vasoonstriction that were blocked by CFTR inhibition. nSMase- and hypoxia-induced vasoonstriction, yet not TRPC6 translocation, were blocked by inhibition or deficiency of sphingosine kinase 1 (SphK1) or antagonism of S1P receptors 2 and 4 (S1P\(_{2/4}\)). S1P and nSMase had synergistic effects on pulmonary vasoonstriction that involved TRPC6, phospholipase C, and rho kinase. Our findings demonstrate a central role of TRPC6 and sphingolipids in HPV. Upon hypoxia, nSMase triggers TRPC6 translocation, which requires its interaction with CFTR. Concomitant SphK1-dependent formation of S1P and activation of S1P\(_{2/4}\) result in phospholipase C-mediated TRPC6 and rho kinase activation, which conjointly trigger vasoonstriction.

Significance

Hypoxic pulmonary vasoonstriction (HPV) is a physiological mechanism that protects against systemic hypoxia by redistributing blood flow from poorly to better ventilated areas of the lung, thereby minimizing ventilation-perfusion mismatch. However, in chronic hypoxia-associated lung disease, HPV contributes to pulmonary hypertension. In this study, we provide novel evidence for a dual role of sphingolipids as important signal mediators in HPV, which critically depends on the presence of functional cystic fibrosis (CF) transmembrane conductance regulator (CFTR). CFTR gene mutations cause CF, which is associated with profound pulmonary ventilation-perfusion mismatches. The present findings propel our current understanding of HPV, establish a previously undescribed mechanism for hypoxia in CF disease, and identify CFTR as a functional contributor to the pathologic changes in hypoxia-associated pulmonary hypertension.

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O2 diffusion (10). Intriguingly, HPV is also abrogated in pneumonia (11) or sepsis (12) caused by Pseudomonas aeruginosa, which phenocopy CF disease via secretion of the virulence factor CFTR inhibitory factor (Cif) (13). Robert et al. reported the expression of CFTR in PASMCs and demonstrated its involvement in the modulation of pulmonary arterial tone (14).

In the systemic circulation, CFTR-F508del, the most common mutation underlying human CF disease, has recently been associated with diminished Ca2+ release in vascular smooth muscle cells, decreased aortic tone, and responsiveness (15). Of specific relevance for HPV, CFTR was recently shown to directly interact with TRPC6, thus regulating TRPC6-dependent Ca2+ influx (16). Based on these considerations, we postulated that CFTR may play a crucial, yet so far unrecognized, role in hypoxia-induced Ca2+ mobilization underlying PASMC contraction and HPV.

Interestingly, CFTR is considered to regulate homeostasis and lipid raft concentrations of sphingolipids (17, 18), which have recently become implicated in HPV in that HPV is blocked by inhibition of neutral sphingomyelinase (nSmase), which releases ceramide from sphingomyelin (19). nSmase is activated upon oxidative stress (20), possibly via arachidonic acid liberation by phospholipase A2 (21), which all have been linked to HPV (1). In addition, ceramide accumulates in PASMCs upon hypoxia (22), mediates caveolar TRPC6 translocation in lung endothelial cells (23), and contributes to constriction of pulmonary artery rings (19, 24). Although ceramide may thus potentially act as a direct mediator of HPV, it may also serve as substrate for the formation of other, bioactive sphingolipids, most notably sphingosine-1-phosphate (SIP). SIP is generated by conversion of ceramide to sphingosine and its subsequent phosphorylation by sphingosine kinase (SphK) (25), which is known to be up-regulated upon hypoxia (26). In the lung, SphK1 is the predominant SphK isoform (27) and has been shown to modulate pulmonary vascular responsiveness and remodeling (28). SIP acts intracellularly as a second messenger or extracellularly via activation of five G protein-coupled receptors (GPCRs) termed S1P1-5 (29), of which SIP2 (29) and SIP4 (30) mediate pulmonary vasoconstriction. Because SIP2 (29, 31) and SIP4 (32) receptor engagement activates PLC and RhoK, and S1P is a known activator of TRPCS (33), we hypothesized that SIP may trigger both central pathways of HPV: DAG formation and consecutive TRPC6-induced Ca2+ mobilization, as well as RhoK-mediated Ca2+ sensitization (1). A potential role of SIP in HPV is particularly intriguing in consideration of its potential tie to CFTR, in that CFTR is one of only two transporters shown to translocate SIP across biological membranes (17). In the present study, we hence probed for the functional role of CFTR and its mechanistic link to sphingolipid signaling in HPV.

Results

HPV Requires CFTR. First, we probed for the functional relevance of CFTR for an intact HPV response. In isolated perfused mouse lungs, acute hypoxia (1% O2) triggered the characteristic increase in pulmonary arterial pressure (Ppa), which was attenuated by ~50% by either pharmacological inhibition or genetic deficiency of CFTR, respectively (Fig. 1 A, Fig. 1 A, both of which had no effect on basal Ppa. Similarly, in an in vivo model of regional hypoventilation following partial airway occlusion with saline, CFTR-deficient (CFTR−/−) mice had more severe hypoxemia compared with wild-type (WT) mice (Fig. 1B), further consolidating a critical role for CFTR in HPV. Conversely, angiotensin II-induced increases in Ppa were little affected by CFTR inhibition and unaltered in CFTR−/− mice, indicating that the functional role of CFTR in pulmonary vasoconstriction is rather specific for the response to hypoxia (Fig. 1 C). Cl− channels have been implicated in the PASMC response to hypoxia (34); however, HPV responses in lungs perfused with Cl− free perfusion did not differ from control lungs, suggesting that the functional relevance of CFTR was not attributable to its function as a Cl− channel (Fig. 1D). Western blot analyses showed unabated CFTR expression in cultured PASMCs exposed to 2-24 h of hypoxia (1% O2; Fig. 1E), or in pulmonary arteries of WT mice exposed to 5 wk of normoxia or hypoxia [10% (vol/vol) O2] (F). Values are given as mean and SEM; n = 6, 5, 5, 4-5, 8, and 4 per group for A–F, respectively. **P ≤ 0.05, ***P ≤ 0.1 vs. control group.

CFTR Deficiency Partially Protects from Hypoxia-Induced PH and Pulmonary Arterial Remodeling. To evaluate whether CFTR is also crucial for long-term vascular adaptation to chronic hypoxia, Cftrtm1Unc Tg(FABPCFTR)1Jaw/J mice (which have normal longevity due to intestinal CFTR expression but lack CFTR in the lungs) and corresponding WT mice were exposed to 5 wk of chronic hypoxia (10% O2). Compared with normoxic mice, WT hypoxic mice showed elevated right ventricular systolic pressures (RVSP; Fig. 2A); increased pulmonary arterial medial wall
thickness of small (20–50 μm), medium-sized (50–100 μm), and large pulmonary arteries (100–150 μm) (Fig. 2 B and C); and a higher degree of muscularization in small pulmonary arteries (Fig. 2D). In mice deficient in pulmonary CFTR, the hypoxia-induced increases in RVSP, medial wall thickness, and small vessel muscularization were markedly attenuated compared with hypoxia-treated WT mice (Fig. 2), whereas right ventricular hypertrophy assessed by Fulton index did not differ between CFTR-deficient (0.36 ± 0.02, mean ± SEM) and WT mice (0.35 ± 0.02).

**CFTR Is Required for Hypoxia-Induced Ca\(^{2+}\) Signaling and TRPC6 Translocation.** To dissect the mechanistic role of CFTR in HPV, we analyzed the effect of CFTR inhibition on hypoxia-induced Ca\(^{2+}\) mobilization in PASMCs. Hypoxia (1% O\(_2\)) caused a rapid increase in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) in human PASMCs, which was completely attenuated by CFTR inhibition (Fig. 3 A and B). Compared with human PASMCs, Ca\(^{2+}\) mobilization in response to hypoxia was markedly lower in isolated murine PASMCs, but was again significantly reduced in CFTR\(^{-/-}\) compared with corresponding WT mice (Fig. 3C). Although the [Ca\(^{2+}\)]\(_i\) increase in PASMCs exceeded the response to hypoxia in coronary artery smooth muscle cells (CASMCs) by almost one magnitude, a finding that is in line with the selectivity of hypoxic vasoconstriction to the pulmonary circulation. CFTR inhibition attenuated the response in smooth muscle cells (SMCs) of both vascular beds (Fig. 3D). Based on the emerging recognition of sphingolipids as mediators of HPV, we also tested for the role of CFTR in the SMC response to SIP. Intriguingly, CFTR inhibition attenuated the SIP-induced [Ca\(^{2+}\)] increase in PASMCs, yet amplified the respective response in CASMCs (Fig. 3E), suggesting an important site-specific role for CFTR in the regulation of sphingolipid-mediated vasoactive responses.

The effects of pharmacological or genetic CFTR deficiency are strongly reminiscent of the complete inhibition of the hypoxic [Ca\(^{2+}\)]\(_i\) response in PASMCs of TRPC6-deficient mice (3), which prompted us to probe for a potential functional connection between CFTR and TRPC6. To this end, we first confirmed the functional role of TRPC6 in HPV by use of the TRPC6 inhibitor larixol acetate (35), which blocked the pulmonary pressure response to hypoxia almost completely (Fig. 4A). Next, we tested whether TRPC6 translocation to caveolae, which is an essential prerequisite for its functional role in the PASMC [Ca\(^{2+}\)]\(_i\) response to hypoxia, may depend on CFTR. Analysis of caveolin-1-rich fractions showed a distinct translocation of TRPC6 to caveolae in response to hypoxia, which, was, however, blocked by CFTR inhibition (Fig. 4B). Because TRPC6 is activated by DAG (7), we also tested whether CFTR may modulate this pathway. However, pulmonary vasoconstriction induced by a DAG analog was not affected by CFTR inhibition (Fig. S1), suggesting that the requirement of the PASMC [Ca\(^{2+}\)]\(_i\) response to hypoxia for CFTR relates to CFTR’s role in the translocation rather than in the activation of TRPC6.

**CFTR Interacts with TRPC6 in a Hypoxia-Dependent Manner.** To better comprehend the functional link between CFTR and the caveolar translocation of TRPC6, we considered the possibility that both proteins may interact directly as described in epithelial cells (16). In coimmunoprecipitation experiments, we identified an interaction between both proteins that was specific for hypoxic PASMCs, but absent in normoxic PASMCs and blocked by
CFTR inhibition (Fig. 4C), suggesting that CFTR may facilitate TRPC6 translocation by direct protein–protein interaction.

nSMase-Dependent Pulmonary Vasoconstriction and TRPC6 Recruitment to Caveolae Require CFTR. nSMase has been identified as an important contributor to HPV in rats (19), a notion that could be confirmed in the present study for the isolated mouse lung in that inhibition of nSMase, yet not acid sphingomyelinase (aSMase), blocked HPV (Fig. 5A). Likewise, nSMase inhibition decreased the hypoxia-induced accumulation of TRPC6 in PASMC caveolae (Fig. 5B), indicating that nSMase acts upstream of TRPC6 and, potentially, also CFTR in HPV. The latter view was confirmed in isolated lungs in that the vasoconstrictor response to hypoxia could be mimicked by exogenous addition of bacterial nSMase and that nSMase-induced vasoconstriction was again largely attenuated by inhibition of CFTR or TRPC6, respectively (Fig. 5C). Analogously, nSMase induced TRPC6 translocation to caveolae, which was again decreased by CFTR inhibition (Fig. 5D).

SIP Signaling Is Required for Hypoxia- and nSMase-Induced Pulmonary Vasoconstriction. To further identify the signaling pathways that link hypoxia and nSMase to CFTR and TRPC6, we focused on the role of SIP as a bioactive downstream product of nSMase-derived ceramide. Inhibition of the SIP synthesizing enzyme SphK blocked HPV by >50%, suggesting a critical involvement...
of S1P in the hypoxia response (Fig. 6A). This notion was confirmed by experiments demonstrating that JTE-013, which blocks S1P receptors 2 and 4, both of which have been shown to promote pulmonary vasoconstriction (29, 30), similarly inhibited HPV (Fig. 6A). In parallel, inhibition of SphK or S1P2/4 also reduced nSMase-induced pulmonary vasoconstriction (Fig. 6B). HPV was also attenuated by genetic deficiency of SphK1, the main extranuclear isoform of SphK (Fig. 6C). Sphk1−/− mice also showed a moderately decreased basal Ppa (11.5 ± 0.2 cmH2O) compared with WT mice (13.1 ± 0.2 cmH2O); however, as shown (28), this difference in basal Ppa does not mitigate their general vascular responsiveness to vasoconstrictive stimuli other than hypoxia. Unexpectedly, however, HPV was neither reduced by deletion of S1P2 nor of S1P1 alone (Fig. 6C), while basal Ppa mean were unaltered in S1P2−/− or S1P1−/− mice. More surprisingly, S1P signaling was not required for TRPC6 translocation, because S1P1 alone did not increase the caveolar abundance of TRPC6 channels (Fig. 6D), and SphK inhibition did not alter nSMase-induced TRPC6 translocation (Fig. 6E).

**Following nSMase-Mediated TRPC6 Translocation, S1P Induces Vasoconstriction Through TRPC6, PLC, and RhoK Activation.** Because S1P does not exert its effects on HPV via TRPC6 translocation, we further analyzed nSMase- and S1P-induced downstream signaling events. Although nSMase-evoked pulmonary vasoconstriction requires PLC (Fig. 7A) and TRPC6 (as shown in Fig. 5C), S1P alone did not elicit pulmonary vasoconstriction through activation of TRPC6 or PLC, but rather in a RhoK-dependent manner (Fig. 7B). We considered that this preponderance of RhoK-dependent vasoconstriction may relate to the lack of TRPC6 translocation following S1P stimulation and, therefore, devised an approach in which we would stimulate TRPC6 translocation by nSMase while blocking the conversion of nSMase-derived ceramide to S1P, and then reassess the mechanisms by which S1P elicits pulmonary vasoconstriction. As shown before, nSMase induced a vasoconstrictor response that was largely blocked by SphK inhibition. However, when S1P was added to lungs treated with both nSMase and SphK inhibitor, the vasoconstrictive response to S1P was amplified by approximately threefold, indicating a synergistic action of nSMase-derived ceramide and exogenous S1P (Fig. 7C). Furthermore, in the presence of nSMase and SphK inhibition, the S1P-induced vasoconstrictive response became sensitive to inhibition of TRPC6, PLC, or RhoK with an additive effect of the inhibitor combination, thus mimicking the characteristic pharmacological profile of the intact HPV response (Fig. 7D).

**Discussion**

In this study, we identified a critical role for CFTR in HPV in that CFTR promotes nSMase-mediated TRPC6 abundance in caveolin-rich lipid rafts, possibly via CFTR/TRPC6 protein complex formation. Additionally, and in parallel to nSMase-mediated TRPC6 translocation, sphingolipid signaling contributes to HPV via formation of S1P and subsequent activation of TRPC6 via PLC and of RhoK, presumably by acting through S1P2/4 (Fig. 8). Sphingolipids thus exert a dual action on TRPC6 in HPV, in that they both translocate (nSMase-derived ceramide) and activate (S1P) TRPC6 channels.
CFTR inhibition or deficiency had no effect on basal perfusion pressures in the isolated lung, a finding that is in line with the virtual lack of basal vascular tone in this preparation (36). However, both interventions markedly reduced the HPV response in isolated mouse lungs. A functional role of CFTR in ventilation-perfusion matching was further substantiated by the fact that CFTR deficiency abrogated systemic hypoxia upon regional hypoventilation, in line with regional \( V_A/Q \) inequalities as a result of impaired HPV. CFTR inhibition blocked caveolar TRPC6 translocation and \( Ca^{++} \) mobilization in response to hypoxia in PASMCs, demonstrating that CFTR is essential for the role of TRPC6, a central signal transducer in HPV (3, 7). In line with this view, CFTR was shown to associate with TRPC6 under hypoxia, whereas CFTR inhibition led to disruption of this protein complex, indicating that protein–protein interaction with CFTR is required for TRPC6 translocation in response to hypoxia. Both the amino- and carboxyl-terminal tails of CFTR can interact with a multitude of binding partners, which can regulate protein expression and function by Western blot analysis and as documented by RT-PCR and immunocytochemistry (14), and mortality and fibrotic lung remodeling (41). CFTR expression has been shown to develop less lung fibrosis, and treatment with the known detrimental effects of CF disease (44). CFTR inhibition led to disruption of this protein complex, indicating that protein–protein interaction with CFTR is required for TRPC6 translocation in response to hypoxia. Both the amino- and carboxyl-terminal tails of CFTR can interact with a multitude of binding partners, which can regulate protein expression and function by Western blot analysis and as documented by RT-PCR and immunocytochemistry (14), and mortality and fibrotic lung remodeling (41). CFTR expression has been shown to develop less lung fibrosis, and treatment with the known detrimental effects of CF disease (44).

Importantly, CFTR deficiency not only attenuated the acute pulmonary vascular response to hypoxia, but also mitigated the hypoxia-induced opening of \( Cl^- \) channels in PASMCs has been proposed to contribute to right ventricular hypertrophy in this model is independent of CFTR and not exclusively the result of increased afterload. This latter interpretation is in line with a recent study demonstrating distinct differences in right ventricular pathology and function between rat models of either simply mechanical pressure overload or angioproliferative PH (39). Notably, the reported role of CFTR in PH and the proposed link between CFTR and S1P signaling are in line with recent data demonstrating a critical role of the SphK1–S1P–SIP axis in pulmonary arterial hypertension (40). Although not directly tested in the present study, the identified role of CFTR and sphingolipids may equally pertain to other hypoxia-related lung diseases commonly associated with PH such as e.g., chronic obstructive pulmonary disease (COPD) or lung fibrosis. In line with this notion, \( SphK1^-/- \) mice have been shown to develop less lung fibrosis, and treatment with the SphK inhibitor SKI II 7 d after bleomycin challenge reduces mortality and fibrotic lung remodeling (41). CFTR expression in cultured SMCs from rat intrapulmonary arteries had been documented by RT-PCR and immunocytochemistry (14), and was confirmed in the present study for human PASMCs as protein expression and function by Western blot analysis and as CFTR-dependent \([Ca^{++}]_i\) signaling, respectively. CFTR expression levels were not decreased by hypoxia, indicating that the functional roles of CFTR in the pulmonary vascular response to hypoxia are not abated by an adaptive down-regulation of the channel. It is noteworthy, however, that the proposed mechanistic role of CFTR for PH is not unequivocally evident in CF patients, which have a similar prevalence of mild or moderate PH as patients with COPD and a comparable reduction in forced expiratory volume (FEV\(_1\)) (42, 43). Consequently, the relevance of pharmacological CFTR inhibition as a therapeutic strategy for PH patients remains doubtful and is likely outweighed by the known detrimental effects of CF disease (44).

CFTR is a well-characterized channel for monovalent anions including \( Cl^- \) and \( HCO_3^- \) (45), and hypoxia-induced opening of \( Cl^- \) channels in PASMCs has been proposed to contribute...
to HPV by causing membrane depolarization and activation of voltage-gated Ca\textsuperscript{2+} channels (34). However, CFTR-related effects on the pulmonary vasopressor response are presumably independent of its Cl\textsuperscript{−} channel activity, because HPV was independent of the transmembrane Cl\textsuperscript{−} gradient in the isolated perfused lung. Furthermore, activity of CFTR as a Cl\textsuperscript{−} channel requires phosphorylation of its regulatory domain via cAMP or cGMP (46, 47), which, however, trigger PASMC relaxation rather than constriction (48). Along these lines, CFTR-mediated Cl\textsuperscript{−} flux has been proposed to mediate pulmonary artery vasodilatation, rather than constriction (14). However, in the present study, CFTR inhibition or deficiency had little or no effect on pulmonary vasoconstriction induced by angiotensin II, suggesting a particular role for CFTR in hypoxic pulmonary vasoconstriction.

CFTR has been implicated in the homeostasis of sphingolipids (18, 49), which have been shown to trigger caveolar TRPC6 translocation in lung endothelial cells (23) and hypoxia-induced vasoconstriction in isolated rat pulmonary arteries (19, 22). Consistent with the latter reports (19), we found HPV to require nSMase but not aSMase. The functional role of nSMase in HPV relates on the one hand to the CFTR-dependent recruitment of TRPC6 to caveolae in response to hypoxia, because hypoxia-induced TRPC6 translocation was prevented by nSMase inhibition and, conversely, mimicked by exogenous nSMase. These effects were independent of SphK and, hence, SIP, because SphK inhibition did not prevent TRPC6 translocation in response to exogenous nSMase, and SIP alone did not induce TRPC6 translocation. These data are essentially in agreement with our recent finding that SMase-derived ceramide triggers caveolar recruitment of TRPC6 in lung microvascular endothelial cells in response to platelet-activating factor (23). However, nSMase-derived sphingolipid signaling in HPV also involves a critical role for SIP. Albeit SIP was not required for TRPC6 recruitment to caveolae, SphK inhibition or SphK1 deficiency markedly inhibited HPV, demonstrating that TRPC6 translocation alone is not sufficient to elicit a full HPV response, which instead requires additional SIP signaling. In line with previous data demonstrating that SIP can trigger pulmonary vasoconstriction by acting through either SIP\textsubscript{1} (29) or SIP\textsubscript{3} (30), we found HPV similarly inhibited by the dual SIP\textsubscript{1,3} inhibitor JTE-013. Intriguingly, while SIP increased [Ca\textsuperscript{2+}]\textsubscript{i} in smooth muscle cells of both the pulmonary and the systemic circulation, CFTR inhibition attenuated SIP-induced Ca\textsuperscript{2+} mobilization in PASMCs, but amplified the response in CASMCs. The latter response is in line with recent data demonstrating that CFTR—by acting as an uptake channel for SIP that is essential for its subsequent intracellular degradation by SIP-phosphohydrolase 1—counterregulates SIP-mediated increases in systemic vascular tone (50). Conversely, our present data identify CFTR as an amplifier of SIP-induced Ca\textsuperscript{2+} signaling in PASMCs, suggesting an important role of CFTR as a site-specific regulator of vasomotor responses that conceptually aligns with its novel implication in HPV. Unexpectedly, however, HPV was unmitigated in mice with single knockouts of either SIP\textsubscript{1} or SIP\textsubscript{3}, indicating functional redundancy between both receptors. Both GPCRs, SIP\textsubscript{2} (via G\textsubscript{q11}) (51) and SIP\textsubscript{4} (via G\textsubscript{q13}) (52), activate PLC, which catalyzes the conversion of phosphatidylinositol 4,5-bisphosphate (PIP\textsubscript{2}) into inositol 1,4,5-trisphosphate (IP\textsubscript{3}) and diacylglycerol (DAG), the classical activator of TRPC6 (4), and signal in parallel via G\textsubscript{q11} to activate RhoA (51, 52). Consistent

![Diagram A](https://via.placeholder.com/150)

**Fig. 7.** Downstream signaling pathways of nSMase and SIP in isolated mouse lungs. (A) nSMase-induced pulmonary vasoconstriction requires PLC: The PLC inhibitor U73122, but not its inactive analog U73343 (each 10 μmol/L), attenuated the nSMase (100 μL)-induced increase in ΔPpa. (B) SIP (10 μmol/L)-induced pulmonary vasoconstriction was not blocked by the TRPC6 inhibitor LA (5 μmol/L) or the PLC inhibitor U73122 (10 μmol/L), but inhibited by the RhoK inhibitor Y27632 (10 μmol/L). (C and D) Following nSMase-induced TRPC6 translocation, SIP induced an amplified vasoconstrictive response through activation of PLC, TRPC6, and RhoK: In the presence of the SphK inhibitor SKI II (5 μmol/L), the lung vasomotor response to exogenous nSMase (100 μL) was largely blunted, yet the vasoconstrictive effect of SIP (10 μmol/L) was amplified in the presence of nSMase and SKI II (C). The resulting synergistic response was sensitive to inhibition of TRPC6 by LA (5 μmol/L), PLC by U73122 yet not U73343 (both 10 μmol/L), and RhoK by Y27632 (10 μmol/L) (D), with combined inhibition (combi) of all three pathways showing an additive effect. Values are given as mean and SEM; n = 5 per group. *P ≤ 0.05, **P ≤ 0.01 vs. indicated group.
Pulmonary Vascular Responsiveness in Isolated Perfused and Ventilated Mouse Lungs. Murine lungs were prepared as described (57). Briefly, lungs were perfused with 37 °C sterile Krebs–Henseleit hydroxyethyl aminoelystein buffer (1 mL min⁻¹; Serag-Wiesen) in a nonrecirculating fashion, and left atrial pressure was adjusted to +2.2 cmH₂O. After isolation, lungs were ventilated with negative pressure in a closed chamber, volume-controlled with a tidal volume of ~9 mL/kg, an end-expiratory pressure of ~2 cmH₂O, and a respiratory rate of 90 breaths per min. Hyperinflation (~24 cmH₂O) was performed at 4-min intervals. Mean pulmonary arterial pressure (Ppa mean) was continuously monitored, and vasoconstrictive responses were assessed as the maximal difference in Ppa mean (ΔPpa mean). Indomethacin (30 μmol/L) and 1-Nitro-

sphingolipid signaling in HPV, in that nSMase-derived ceramide mediates TRPC6 translocation in a CFTR-dependent manner, whereas subsequent SphK1-dependent conversion to S1P and signaling via S1P₂ receptors, thereby triggering Ca²⁺ influx via PLC and subsequent Ca²⁺ sensitization via RhoK, ultimately leading to PASMC contraction.

These findings identify a dual role for sphingolipid signaling in HPV, in that nSMase-derived ceramide mediates TRPC6 translocation in a CFTR-dependent manner, whereas subsequent SphK1-dependent conversion to S1P and signaling via S1P₂ receptors triggers vasoconstriction by concomitant activation of TRPC6, likely via PLC-dependent DAG formation, and consecutive Ca²⁺ influx, and simultaneous Ca²⁺ sensitization via RhoA/RhoK.

Materials and Methods
Reagents. All reagents were purchased from Sigma-Aldrich unless stated otherwise.

Mice. C57BL/6J WT mice were purchased from The Jackson Laboratory. For analysis of HPV and oxygenation during regional hypoxia, CFTR-deficient mice (CFTR⁻/⁻) generated by insertion mutagenesis targeted to exon 10 (CFTRtm1HGU) on an MF1/129 genetic background (53), and their corresponding WT littermates were provided by H. Schulz (Heilmhzent Centre, Munich). For analysis of chronic hypoxia-induced PH, the more robust CFTR⁻/⁻ mice that reexpress CFTR in the gastrointestinal tract under control of the rat fatty acid binding protein 2, intestinal (Fabp2) promoter (CFTRtm1Jaw/J, N12) (54) but lack CFTR in lungs, and their corresponding C57BL/6J controls (CFTR⁺/⁺) were purchased from The Jackson Laboratory. Mice homozygote deficient in SphK1 (SphK1⁻/⁻) (55), S1P₆ (S1P₆⁻/⁻) (56), and S1P₆ (S1P₆⁻/⁻) (52) were provided by M. Kress (Department of Physiology and Medical Physics, Medical University of Innsbruck, Innsbruck, Austria), R. L. Proia (Genetics of Development and Disease Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda), and M. Li (Department of Immunogenetics and Immunogenetics, Max-Delbrück-Center for Molecular Medicine, Berlin), respectively. Generation and genotyping of these mice have been described (32, 54–56).

Sphk1⁻/⁻ mice were backcrossed >12 generations on a C57BL/6J background. S1P₆⁻/⁻ and S1P₆⁻/⁻ mice were backcrossed 2 and 10 generations, respectively, on a BALB/c background. As corresponding controls for SphK1⁻/⁻ and S1P₆⁻/⁻, C57BL/6J and BALB/c WT mice, respectively, were purchased from Charles River Laboratories. S1P₆⁻/⁻ littermates served as controls for S1P₆⁻/⁻. All mice were housed under specific pathogen-free conditions. All procedures of this study were conducted after approval by the Institutional Animal Care and Use Committee of St. Michael’s Hospital or by the local State Office of Health and Social Affairs (LAGeSo, Berlin).

Chronic Hypoxia Experiments. CFTR⁺/⁺ and CFTRtm1WgAfAPC(CFTR)Jaw/w, N12 mice lacking pulmonary CFTR expression were housed at either normoxia (21% O₂) or hypoxia (10% O₂) as described (2). After 5 wk, RVSP was determined by a 1.4 F microtip Millar catheter. Hearts were excised and weighed for assessment of Fulton index (right ventricular weight/ left ventricular + septum weight). Pulmonary arterial medial wall thickness and muscularization of small (20–50 μm) pulmonary arteries was assessed from 5-μm-thick H&E-stained lung sections as described (63).
Hypoxia or nSMase Treatment of PASMC and Caveolar Isolation. PASMCs were cultured as described above and grown on 100-mm culture dishes. For hypoxia treatment, PASMCs were stored in a hypoxia chamber (37 °C, 1% O2) in which hypoxic culture medium (pO2 ~ 10 mmHg) was applied to the cells for 15 min. For nSMase or S1P treatment, homolog nSMase (100 U/mL) or S1P (10 μM) was administered to the culture medium 10 min before treatment. PASMCs were rinsed twice with ice-cold PBS and lysed for 1 min with 0.5% Brij 56 lysing buffer containing sodium vanadate (1 mM), phenylmethylsulfonylfluoride (1 mM), and Complete Mini (Roche Applied Science). The cell lysate was homogenized (Dounce homogenizer) and mixed (4 °C, 20 min). Next, 800 μL of the homogenate were mixed with 800 μL of 80% sucrose Tris-potassium-magnesium (TKM) buffer solution in an ultracentrifugation tube. Sixteen hundred microliters of 30% sucrose TKM buffer solution were then layered on top, followed by 1600 μL of 5% sucrose TKM buffer solution. After ultracentrifugation (4 °C, 180,000 g, 18 h; L8M, Beckman Coulter), 10 equal-volume fractions were collected from the tube and probed for the caveolar marker caveolin-1 by Western blot. Fractions containing caveolae were identified to be fractions 4, 5, and 6 (Fig. 52) and were further analyzed for TRPC6 abundance by Western blot.

Western Blot. For analyses of caveolar fractions, 5 μg of protein from each fraction were loaded on SDS polyacrylamide gels and separated by electrophoresis. Nitrocellulose membranes were blocked for 1 h at room temperature in Tris-buffered saline (TBS) and 0.1% Tween 20 and incubated with primary antibodies against caveolin-1 (1:500; monoclonal; Transduction Laboratories) or TRPC6 (1:200; rabbit polyclonal; Lot ACC017AN1425; Alomone Labs). Membranes were washed three times with TBS containing 0.1% Tween 20 and incubated with HRP-conjugated secondary goat anti-mouse (1:5,000; Bio-Rad) or donkey anti-rabbit (1:10,000; Jackson ImmunoResearch) primary antibodies, followed by incubation overnight (rotated at 4 °C) with 2 mg of anti-human CFTR antibody (mouse monoclonal, IgG2a clone 24–21; R&D Systems). After incubation (1 h, 4 °C) with 3 μg of protein A/G Sepharose beads (Thermo Fisher Scientific), bead-bound complexes were washed with NET buffer, and proteins were separated by electrophoresis and analyzed by Western blot for CFTR (1:200; mouse monoclonal; R&D Systems) and TRPC6 (1:200; rabbit polyclonal; Lot ACC017AN1425; Alomone Labs).

Statistical Analysis. Mann–Whitney U test for nonparametrical data, two-way ANOVA for comparison of continuously measured data between groups, and one-way ANOVA followed by Bonferroni’s post hoc test for multiple comparisons were performed by using GraphPad Prism 4.0 (GraphPad Software). P values presented as <0.05, **<0.01, and ***<0.001 were considered statistically significant.

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