ROLE OF CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR AND SPHINGOLIPIDS IN HYPOXIC PULMONARY VASOCONSTRICTION

by

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Hypoxic pulmonary vasoconstriction (HPV) optimizes ventilation-perfusion matching in the lung. As cystic fibrosis patients suffer from ventilation-perfusion mismatches, we probe for a potential role for cystic fibrosis transmembrane conductance regulator (CFTR) in HPV.

HPV in isolated mouse lungs is attenuated by CFTR inhibition or deficiency. In cultured human pulmonary artery smooth muscle cells (PASMC), CFTR inhibition blocks the hypoxia-induced $[Ca^{2+}]_i$ increase and the caveolar translocation of transient receptor potential canonical 6 (TRPC6) channels.

We also identify novel roles for sphingolipids in HPV. Ceramide production by neutral sphingomyelinase (nSMase) is required for TRPC6 translocation, and sphingosine-1-phosphste (S1P) signalling through S1P receptor 2 activates caveolae-associated TRPC6. Yet pulmonary vasoconstriction and TRPC6 translocation in response to exogenous nSMase are blocked by CFTR inhibition. CFTR function is likely required for TRPC6 trafficking or membrane stabilization, as CFTR associates with TRPC6 during hypoxia, which can be disrupted by CFTR inhibitor.

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List of Abbreviations

$[Ca2+]_i$ – intracellular Ca^{2+} concentration

AA – arachidonic acid

ABC transporter – ATP-binding cassette transporter

AMPK – adenosine monophosphate-activated protein kinase

Ang II – angiotensin II

ALI – acute lung injury

ARDS – acute respiratory distress syndrome

C1P – ceramide-1-phosphate

cAMP – cyclic adenosine monophosphate

Cav-1 – caveolin 1

CaM – calmodulin

CDase – ceramidase

CF – cystic fibrosis

CFTR – cystic fibrosis transmembrane conductance regulator

cGMP – cyclic guanosine monophosphate

COPD – chronic obstructive pulmonary disease

DAG – diacylglycerol

EET – epoxyeicosatrienoic acid

ER – endoplasmic reticulum

HPV – hypoxic pulmonary vasoconstriction

HAPH – high-altitude pulmonary hypertension

HAPE – high-altitude pulmonary edema

ILD – interstitial lung disease

IP_3 – inositol triphosphate

IPL – isolated and perfused lung

MLCK – myosin light chain kinase

MLCP – myosin light chain phosphatase

NADPH – nicotinamide adenine dinucleotide phosphate

NHERF – Na^+-H^+ exchanger regulatory factor

NSCC – non-selective cation channel

OLV – one-lung ventilation

P_aO_2 – arterial blood oxygen tension

P_{PA} – pulmonary arterial pressure

PA – pulmonary artery

PAEC – pulmonary artery (arterial) endothelial cell

PAF – platelet-activating factor

PASMC – pulmonary artery (arterial) smooth muscle cell

PH – pulmonary hypertension

PKC – protein kinase C

PLA_2 – phospholipase A2

PLC – phospholipase C

PTEN – phosphatase and tensin homolog

PVR – pulmonary vascular resistance

ROCC – receptor-operated Ca^{2+} channel

ROS – reactive oxygen species

S1P – sphingosine-1-phosphate

SCaMPER – sphingolipid calcium release-mediating protein of the ER

SK – sphingosine kinase

SMase – sphingomyelinase

SOCC – store-operated Ca^{2+} channel

SR – sarcoplasmic reticulum

TRPC6 – transient receptor potential canonical (classical) 6

V/Q – ventilation to perfusion ratio

VOCC – voltage-operated Ca^{2+} channel

1. Chapter 1 Introduction

1.1. Overview of hypoxic pulmonary vasoconstriction

Hypoxic pulmonary vasoconstriction (HPV) is a widely conserved, homeostatic and adaptive vasomotor response to low alveolar oxygen levels. It is a fundamental physiological adaptation that redirects blood flow from poorly ventilated areas to better-aerated regions of the lung. This redistribution of blood flow mediates ventilation-perfusion matching in order to optimize gas exchange and uphold systemic P_aO_2 levels. This phenomenon is intrinsic to the lung and juxtaposes the vasodilation in response to hypoxia observed in the systemic circulation[1-3]. Humans encounter hypoxia in a variety of disease and environmental conditions. HPV helps to optimize oxygenation in conditions such as regional airway obstruction and atelectasis, where HPV is localized to the affected regions of the lung. However, prolonged global hypoxia at high altitude or in hypoxic lung diseases like chronic obstructive pulmonary disease (COPD), HPV increases total pulmonary vascular resistance, and contributes to the development of pulmonary hypertension (PH) and *cor pulmonale*[4,5].

Observations resembling HPV were described well over a hundred years ago. Soon after the advent of techniques for measuring pulmonary arterial pressure (P_{PA}) in 1852, investigators noticed that interruption of ventilation in animals caused an increase in P_{PA}[6]. Although competing theories attempted to explain this phenomenon, the current understanding of HPV wasn't established until

1946, when von Euler and Liljestrand found that hypoxia without hypercapnia had a direct constrictive effect on pulmonary vessels[2,6]. Over the next half-century, pulmonary arterial smooth muscle cells (PASMC) of the pulmonary resistance arteries emerged as the effector cells in HPV[1,6]. Although PASMC are subject to modulation by other cell types such as endothelial cells, they contain the essential machinery responsible for oxygen sensing, downstream signal transduction, and elicitation of the vasomotor response. While individual pathways contributing to HPV have been identified, a unifying mechanistic concept has not yet emerged.

1.1.1. Physiological and pathophysiological significance of HPV

The concept of alveolar ventilation-perfusion relationship was alluded to as early as 1922 by the Scottish physiologist J. S. Haldane:

> *It is evident that in any particular air-sac system the mean composition of the contained air will depend on the ratio between the supply of fresh air and the flow of blood. If the supply of fresh air is unusually small in relation to the supply of venous blood, there will be a lower percentage of oxygen and higher percentage of carbon dioxide in the air of the air sac, and vice versa. It seems probable that by some means at present unknown to us a fair adjustment is maintained normally between air supply and blood supply. For instance, the muscular walls of bronchioles may be concerned in adjusting the air supply, or the arterioles or capillaries may contract or dilate so as to adjust the blood supply*[7].

The discovery of HPV by von Euler and Liljestrand thus addressed Haldane's question of how ventilation and perfusion are matched. Subsequent studies found regional hypoxic ventilation e.g. in one lobe or one lung, while maintaining normoxic ventilation to the remaining lung caused decreased perfusion to the hypoxic region[6,8]. The diversion of blood flow away from hypoxic regions represents a simultaneous increase of perfusion to better-ventilated areas of the lung, thus increasing the overall ventilation-perfusion ratio (V/Q).

The 2-compartment lung illustration by Sylvester *et al*. (Figure 1.1) concisely demonstrates the effect of HPV on ventilation-perfusion relationship and oxygen exchange. Under normoxic conditions (Figure 1.1 A), ventilation and perfusion are perfectly matched (V/Q = 1) in both compartments of the lung. If one compartment is completely obstructed without redistribution of perfusion by HPV (Figure 1.1 B), its V/Q will decrease to 0. While the other compartment is now receiving all of the ventilation without any change in perfusion, its V/Q will increase to 2. The blood flowing through the obstructed compartment will not be oxygenated, while the unchanged blood flow in the ventilated lung leads to no improvements in gas exchange. The result is an overall decrease in O_2 concentration of the systemic arterial blood. If HPV is allowed to reduce blood flow to the obstructed compartment by 60% (Figure 1.1 C), the blood flow to the obstructed and non-obstructed compartments are 20% and 80% of total perfusion, respectively, while the V/Q of the obstructed lung will remain 0, it will now be reduced from 2 to 1.25 in the non-obstructed compartment due to the increased perfusion diverted from the obstructed lung. This improvement in V/Q

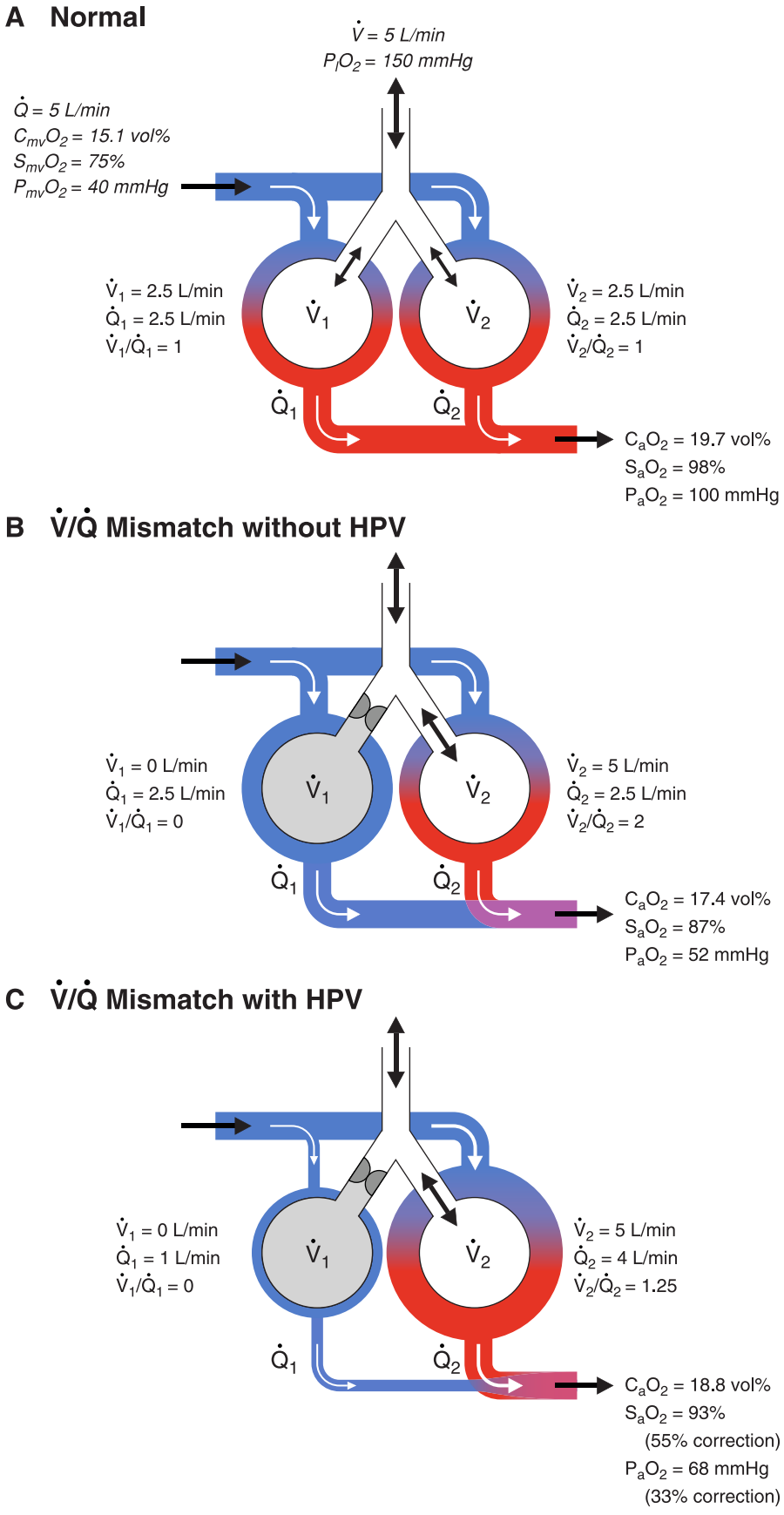


Figure 1.1 Effects HPV on ventilation-perfusion (V/Q) relationships and oxygen exchange in a 2-compartment lung[6]

matching leads to better oxygenation of the blood. Hence the HPV-induced blood flow redistribution reduced the severity of hypoxemia caused by the complete obstruction of one compartment. This 2-compartment lung model can be viewed as an analogy to selective atelectasis, or one-lung ventilation (OLV) that is frequently employed in thoracic surgery. During OLV, the collapsed lung is perfused without ventilation; therefore the shunt fraction might be expected to reach 40-50% of cardiac output without HPV. However, numerous clinical studies have shown that the actual shunt is only 20-25% with the help of HPV, which maintains the patient's P_aO_2 at safer levels[9].

In healthy individuals breathing at sea level during rest, HPV plays minimal role in V/Q matching[6,10]. However, HPV contributes significantly to ventilation-perfusion matching in conditions that involve regional hypoventilation or airway obstruction. In situations where HPV is disrupted and unable to contribute to V/Q matching, the resultant hypoxemia can be life threatening, especially in disease conditions. For example, HPV can be inhibited in acute lung injury (ALI)[8], sepsis[11] and pneumonia[12], and its disruption contributes to V/Q mismatches and resultant hypoxemia in CF patients[13,14].

The effectiveness of V/Q matching by HPV is maximal when hypoxic regions are relatively small, with sufficient normoxic areas for blood flow redistribution. The effect of HPV diminishes as hypoxic areas of lung increases[9]. Global hypoxia still induces an increase in P_{PA}; however, the resultant global lung vasoconstriction will not cause any redistribution of flow. In such conditions of global hypoxia,

sustained HPV contributes to the development of pulmonary hypertension. Regardless of the causal disease, the development of PH is associated with increased morbidity and mortality[6,15,16]. HPV causes increased vasomotor tone in chronic hypoxic diseases, most notably in COPD. It is estimated that 25-90% of COPD patients develop pulmonary hypertension[6,17,18]. PH is reported in around 20% of obstructive sleep apnea sufferers[4], and also prevalent in patients with interstitial lung disease (ILD)[4,6,19] and cystic fibrosis[20]. High-altitude pulmonary hypertension results from the increase in P_{PA} and PVR after only hours of exposure to high altitude[21,22] (higher than ~2000 meters[6]). After the persistence of HPV[23,24] due to prolonged exposure to hypoxia, pulmonary remodeling occurs and in some cases result in right heart failure or *cor pulmonale*[25,26]. In severe cases, robust and acute onset of HPV results in high-altitude pulmonary edema (HAPE)[4,6]. The prevalence of this potentially fatal condition is estimated to be as high as 16% at altitudes of 3,400 – 5,500 meters[6,19]. The HPV-induced rise in P_{PA} leads to increased capillary pressure and subsequent hydrostatic leak, resulting in edema and inflammatory responses[27,28].

Our understanding of the physiological and pathophysiological roles of HPV is still limited. Although the function of HPV is clear in ventilation-perfusion matching during regional lung hypoxia, the precise role of HPV is less understood in various disease conditions. For instance, in ALI and ARDS, there has been evidence both supporting[8] and opposing[29,30] impaired HPV. It is in fact unclear whether the changes observed in vasomotor tone in ARDS are caused entirely by HPV, as the disease may impart other vasoactive influences on the

pulmonary circulation[6], and may differ depending on injury conditions and ventilation[6]. The role of HPV in pulmonary hypertension is further complicated by the fact that the known mechanisms of HPV cannot fully account for the pathophysiology of PH in COPD and ILD. Moreover, in hypobaric hypoxic conditions, how HPV transitions from the acute phase developing minutes after ascent to altitude to a chronic phase contributing to the persistent increase of PVR and the development of PH is unknown. This lack of understanding of the precise role of HPV in these conditions is also reflected in the variability of treatment effectiveness that target HPV in these diseases[6]. In order to understand the pathophysiology of diseases involving HPV and to develop targeted and more effective treatment, we must first better comprehend underlying mechanisms of HPV.

1.1.2. Characteristics of hypoxic pulmonary vasoconstriction

HPV is a distinguishing characteristic of the pulmonary circulation, since the systemic arteries undergo vasodilation in response to hypoxia, in order to match perfusion supply to the metabolic needs of hypoxic tissues[31]. Furthermore, HPV is induced only by low airway and alveolar pO_2[32], and is unaffected by hypoxic perfusion[33]. Also in contrast to the systemic circulation, which can be subject to nervous input, HPV is initiated locally and independent of central and autonomic nervous control[34,35], evident by the persistence of HPV after lung denervation and transplantation in human[36], as well as in isolated lungs, vessels and cells.

However, autonomic and sensory nerves could participate in the modulation of HPV[6].

HPV is elicited by moderate hypoxia and has an occurrence threshold of around pO_2 80–90 mmHg, and reaches maximum at around pO_2 20–40 mmHg[6]. Depending on the severity of hypoxia, HPV can increase PVR by 50– 300% in humans[3]. HPV is initiated where gas exchange occurs, i.e. alveolar-capillary barrier and small pulmonary arteries. Vasopressor response is predominantly carried out by the smooth muscle cells of small resistance arteries of the precapillary[37], however, nonmuscular arterioles, capillaries and venules can also contribute[6]. Since some of these vessels contain no smooth muscles, it can be inferred that other cell types also participate in vessel constriction[6].

HPV observed in intact or isolated lungs amongst different species also have a consistent temporal profile. Moderate global hypoxia (pO_2 30–50 mmHg) in humans[38,39], dogs[40] and pigs[41] increased PVR to a maximum within 15 min, and maintained or reached a new plateau thereafter for up to 8 hours in humans[6]. In severe global hypoxia (pO_2 <30 mmHg) however, while maximum HPV is also achieved within 15 min, this is quickly followed by a 20–100% decrease at 15–50 min (phase 1 HPV) and then gradually increase again at 30–180 min (phase 2 HPV) up to near maximum levels of phase 1[6] (Figure 1.2). This biphasic response to severe hypoxia has been reported in whole lung and *ex vivo* isolated lungs from animals including dogs[40,42], pigs[43], rabbits[44,45], rats[46,47] and mice[48,49],

but is not necessarily observed consistently, due to differing experimental conditions.

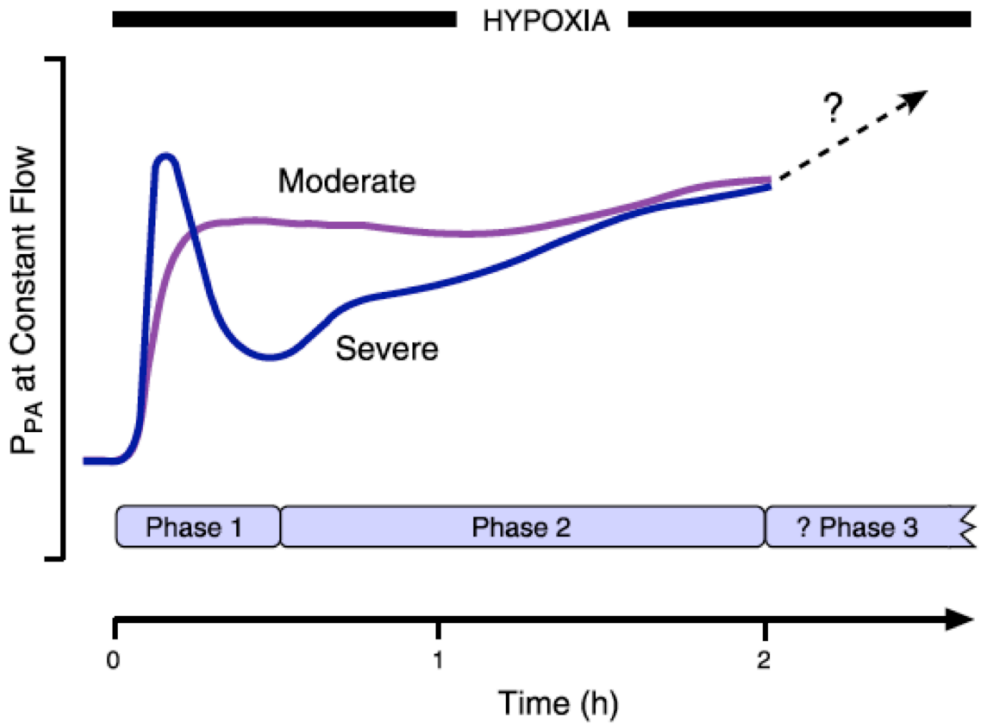


Figure 1.2 Representative time courses of lung pressor responses to moderate and severe hypoxia[6]

1.2. Mechanisms of hypoxic pulmonary vasoconstriction

Pulmonary artery smooth muscle cells (PASMC) are the main sensor and effector cells in HPV. The fact that hypoxia causes increase in intracellular Ca^{2+} and cell contraction in isolated PASMC confirm that these cells contain the necessary sensory, signal transduction and contractile machinery to elicit HPV[6,37]. A number of extrinsic modulators can facilitate or inhibit HPV[6].

Endothelial cells can play an important role in oxygen sensing at the alveolar-capillary barrier and signal transduction to upstream PASMC (own unpublished data); blood-borne factors and pulmonary nerves can also influence HPV[6]. Although it is clear that the core mechanisms reside within PASMC, the particular oxygen-sensor and its link to muscle contraction is still an area of contention and intense scientific research.

1.2.1 Current theories on O_2 sensing during HPV

Much of the current evidence points to the mitochondrion of PASMC as the primary O_2 sensor for HPV[6]. The impediment of oxidative phosphorylation by the lack of O_2 in the mitochondrial electron transport chain leads to an altered redox state. However it is debated whether reactive oxygen species (ROS) production is reduced or increased during hypoxia. Early evidence supported the decrease of ROS production by hypoxia lead to a more reduced PASMC[50] (Figure 1.3 A), causing the inhibition of voltage-gated K^+ (K_V) channels; subsequent membrane depolarization opens voltage-operated Ca^{2+} channels (VOCC) leads to muscle contraction[51,52]. Other proponents of the reduced redox state theory suggest that Ca^{2+} is released via ryanodine-sensitive channels from the sarcoplasmic reticulum, and a subsequent store-operated calcium entry[53-55].

While advocates of the reduced redox theory report decreased ROS production, other groups have repeatedly observed an increase in ROS during hypoxia[56-58]. Over the last decade, numerous studies utilizing ROS indicators, inhibitors of the

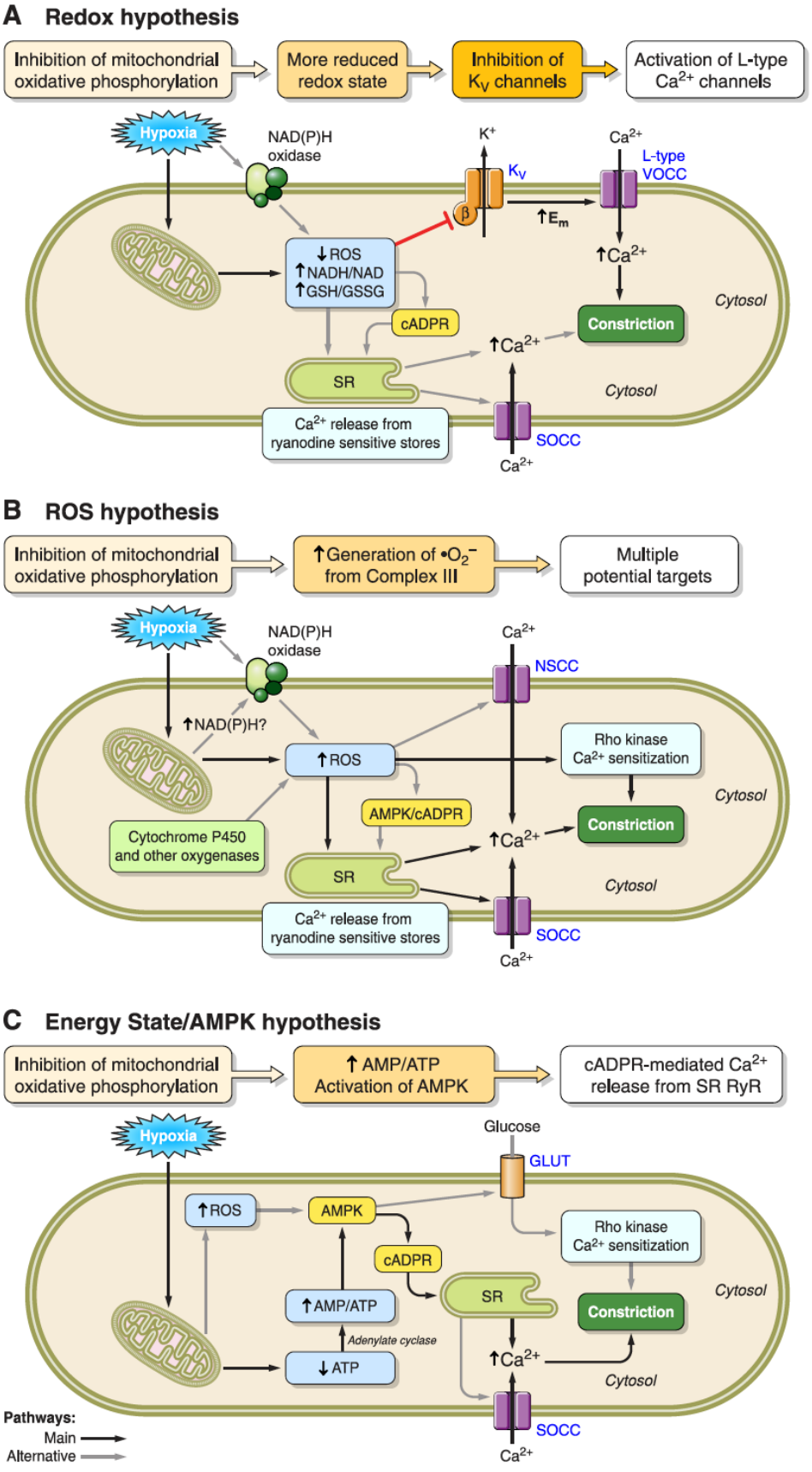


Figure 1.3 Current hypotheses of oxygen sensing in PASMC during hypoxic pulmonary vasoconstriction[6]

mitochondrial electron transport chain, and antioxidants have built up evidence in support of this latter view[6,37] (Figure 1.3 B). ROS can induce Ca2+ release from intracellular stores[59,60] and entry from nonselective cation channels (NSCC) including TRP channels[61,62]; activate Rho kinase-mediated Ca2+ sensitization[63], and a number of other pathways that may be involved in HPV. However, the precise targets and mechanisms of ROS signalling are poorly understood.

Furthermore, a third theory involving the activation of AMP-activated kinase (AMPK) during hypoxia has gained attention in recent years. AMPK has been shown to activate under severe hypoxia due to increased AMP/ATP ratio[64], and the activation of AMPK by inhibiting oxidative phosphorylation mimicked hypoxia[64]. The activation of AMPK is believed to cause a rise in the second messenger cyclic ADP-ribose (cADPR), which leads to a ryanodine-sensitive Ca2+ release from intracellular stores[6,37], and subsequent influx from store-operated Ca2+ channels (SOCC)[64] (Figure 1.3 C). The mechanism by which AMPK causes cADPR increase is not yet known, and whether ROS production activates AMPK is debated.

Other postulated O_2 sensors in HPV include membrane associated NADPH oxidase (NOX), which increases superoxide production in response to hypoxia. It seems the role of NOX in HPV is limited to phase 1, as pharmacological and genetic strategies of NOX inhibition did not alter phase 2 HPV[49]. Activity of members of the cytochrome P450 (CYP) superfamily can be altered by hypoxia[65-67]. Notably, CYP product 11, 12-EET plays a role in regulating TRPC6

channel translocation and Ca^{2+} entry, as well as the activation of Rho kinase. However, it is unclear whether CYP are directly involved in oxygen sensing, or is downstream of other sensors and ROS signaling[6].

Recent findings in our group suggest pulmonary arterial endothelial cells (PAEC) can also participate in oxygen sensing during HPV, as PASMC are absent from the site of gas exchange at the level of alveolar-capillary barrier. Wang *et al.* identified a critical role for pulmonary endothelial cells in retrogradely propagating depolarization signal through gap junctions from the alveolar barrier, to the upstream PASMC of muscularized arterioles. Numerous studies show that endothelial denudation decreases both phase 1 and phase 2 HPV[6], suggesting that the PAEC may contribute significantly in the mediation and modulation of HPV.

Current evidence provides the strongest support for the PASMC mitochondrion as primary O_2 sensors during HPV. ROS production occurs at the electron transport chain (complex III)[6,37], with possible synergistic contributions from NADPH oxidase. It is a topic of contention whether ROS actually increase or decrease during HPV, and the specific downstream targets of ROS and their mechanisms remain elusive.

1.2.2 Signal transduction and elicitation of contraction

Regardless of the debate revolving around the initial oxygen sensors, and despite the relative lack of knowledge of the precise mechanisms involved in downstream signalling pathways, it is clear that actin-myosin interactions in the PASMC are responsible for the vasopressor response, and the intracellular Ca^{2+} increase required for myosin light chain phosphorylation is well characterized[68]. Although the Ca^{2+} response is generally uncontended, many signalling pathways and ion channels have been implicated in HPV, and there is not yet an agreeable unifying concept of the complex interplay and sequence of events involved.

There are several sources of Ca^{2+} that contribute to the global cellular increase of $[Ca^{2+}]_i$. There is ample evidence suggesting Ca^{2+} release from sarcoplasmic reticulum (SR)[69,70], influx from voltage-dependent Ca^{2+} channels (VOCC)[71-73], store- and receptor-operated Ca^{2+} channels (SOCC and ROCC)[74-77] and non-selective cation channels (NSCC) all participate in Ca^{2+} signalling in HPV[6]. It has been proposed that changes in O_2 availability and thus alteration of redox state may directly or indirectly close K^+ channels leading to depolarization[52,78]. Concomitant Na^+ entry through activation of NSCC and perhaps Cl^- efflux[79,80] may compliment K^+ channel inhibition to trigger the activation of VOCC[6]. However, the mechanisms by which hypoxia leads to PASMC depolarization are still unclear, and how much membrane depolarization contributes to HPV response is uncertain[6].

$[Ca^{2+}]_i$ response in HPV has been extensively studied. Pharmacological inhibition[81,82] and facilitation[72,83] of VOCC indicate they are an important component, but not the only source of Ca^{2+} during HPV[70,74]. Ryanodine receptor- (RyR) and IP_3 receptor-dependent Ca^{2+} release from intracellular stores, the subsequent Ca^{2+} and Na^{2+} influx from SOCC and contribution to depolarization and activation of VOCC are also major constituents of the total $[Ca^{2+}]_i$ response[84-87]. Increasing evidence also point to a crucial role for ROCC in HPV[88-90], which upon agonist binding, depending on the protein, would either open its channel pore, or produce a second messenger response that may activate other ion channels, or induce SR/ER Ca^{2+} release[6].

It is thought that mainly transient receptor potential (TRP) channels constitute the NSCC (ROCC and SOCC) involved in HPV[6,75]. Particularly, TRPC1 and 6 are the most abundant TRPC channels found in distal pulmonary arteries of mice and rats[91,92], growing evidence suggest TRPC1 and TRPC6 make up most of the SOCC and ROCC found in PASMC, respectively[6,93,94]. TRPC6 seems to play an especially critical role in HPV since its deficiency and/or knockdown leads to the complete abolition of phase 1 HPV in isolated lungs, and complete or near complete inhibition of Ca^{2+} transient in PASMC during hypoxia[76,94] (see Section 1.3.3. for a more in-depth review of TRPC6 in HPV). Currently, it is frustratingly unclear the exact sequence of depolarization, VOCC and NSCC activation, store release, and the relative contribution of Ca^{2+} these different sources towards the total $[Ca^{2+}]_i$ response.

The next step in evoking PASMC contraction by hypoxia is the increased intracellular Ca^{2+} binding to calmodulin (CaM), which Ca^{2+}-CaM then binds to and activate myosin light-chain kinase (MLCK)[68]. Activated MLCK in turn phosphorylates myosin regulatory light chain (MLC_{20}), and *P*-MLC_{20} evokes actin-myosin interaction, and cell contraction[95]. On the other hand, myosin light-chain phosphatase (MLCP) is responsible for dephosphorylation of MLC_{20}, and the inhibition of MLCP during HPV is a major mechanism for calcium sensitization in PASMC[96,97]. MLCP is inhibited via phosphorylation by Rho kinase (RhoK) in PASMC during hypoxia[98,99]. Pharmacological inhibition of RhoK also attenuates HPV in isolated rat and mouse lungs[100-102]. Therefore, the complimentary $[Ca^{2+}]_i$ increase, Ca^{2+}-CaM-dependent actin-myosin interaction and Ca^{2+} sensitization by RhoA/RhoK are both required for cell contraction in PASMC during hypoxia. The exact mechanisms by which RhoK is activated during hypoxia are yet unclear.

As mentioned above, PASMC contain the necessary oxygen sensing and contractile machinery to carry out cell contraction in response to hypoxia. However a number of intrinsic and extrinsic factors and other cell types may contribute to the modulation of HPV. Particularly, the pulmonary arterial endothelial cells (PAEC) are especially important in HPV, as endothelium-denuded isolated pulmonary arteries have decreased vasoconstrictor response to hypoxia[103-106]. The inhibition or activation of PASMC contraction by endothelium-derived vasoactive factors such as nitric oxide (NO)[105,107] and endothelin-1 (ET-1)[108] may depress or facilitate the pressor response during

hypoxia[6]. HPV in pulmonary arteries requires intact endothelium, and ET-1 release by endothelial cells has been suggested to facilitate vasoconstriction by increasing Ca^{2+} sensitivity in PASMC[109,110]. As studies in our group suggest, PAEC may also have a critical involvement in the conduction of hypoxic signals from the site of hypoxia (alveolar-capillary barrier) to upstream PASMC via a retrograde propagation of depolarization and Ca^{2+} entry, and through the release of vasoactive factors such as arachidonic acids and EETs that stimulate the PASMC (unpublished data). In addition to the contribution by PAEC, other factors, such as various blood-borne agents[6], nerve input[111,112], blood and intracellular pH[113-115] have also been implicated in the modulation of HPV.

In summary, PASMC-dependent muscle contraction in HPV is ultimately elicited by actin-myosin interactions, whose activity is primarily regulated by intracellular Ca^{2+} levels, and Ca^{2+} sensitization by RhoK during hypoxia. The mechanism of oxygen sensing is an area of intensive research and controversy, and no single current hypothesis (redox state, ROS and AMPK) can fully account for the various and often conflicting observations. Downstream of O_2 sensing, numerous different ion channels, proteins, receptors and Ca^{2+} sources have been identified in the depolarization and Ca^{2+} response. While many appear to be crucial in HPV, such as L-type VOCC and TRPC6 channels, the mechanisms of their activation and the intricate interplay between all the players involved are poorly understood.

1.3. CFTR, sphingolipids and TRPC6 in HPV

Cystic fibrosis (CF) is caused by mutations in the *CFTR* gene. CF patients frequently suffer from hypoxemia[13], which has been attributed to severe ventilation perfusion (V/Q) mismatches[14] and intrapulmonary shunts[116], suggesting HPV in CF is impaired. Moreover, HPV can be compromised in sepsis and pneumonia caused by *Pseudomonas aeruginosa*[11,12], which can decreases CFTR abundance in the apical cell membrane by secreting CFTR inhibitory factor (Cif)[117,118]. CFTR is expressed in PASMC[119], and may thus play vasoregulatory roles in the lung. CFTR deficiency have been directly implicated in the homeostasis of sphingolipids ceramide[120] and S1P[121] that influence membrane properties and receptor signalling, which both relate to the regulation of TRPC6 channels. CFTR may also directly associate with TRPC6 in the plasma membrane, controlling its membrane abundance and conductivity[122].

1.3.1. Cystic fibrosis transmembrane conductance regulator

CFTR is a member of the ATP-binding cassette (ABC) membrane transporter superfamily. It forms a Cl^- channel essential for the Cl^- in the epithelial cells of a number of organs including the lung, kidney, intestines and pancreas[13,14,123,124]. Various forms of mutations in the *CFTR* gene cause the autosomal recessive disease cystic fibrosis. Absence or decrease in CFTR function in CF leads to abnormal transport of chloride and sodium across the epithelium[125,126] that afflict a number of organs with different symptoms. The most serious symptom and the hallmark of the disease is progressive dyspnea as a result of chronic lung

infection[123,127]. Ultimately, CF patients require lung transplantation as the disease advances.

As mentioned above, CF patients suffer from hypoxemia due to V/Q inequalities, as a result of HPV impairment. Disruption of membrane CFTR by virulent factor (Cif) is also associated with impaired HPV. However, no study has focused on the potential role of CFTR in HPV, therefore very little is known in this respect. Cl^- currents through the opening of Ca^{2+}-activated Cl^- channels have been implicated in the depolarization response to hypoxia in PASMC[6,79,80]. However, it is unlikely that CFTR contribute to HPV via its plasma membrane Cl^- channel activities. CFTR-mediated Cl^- current requires the phosphorylation of its regulatory R domain by protein kinase A (PKA)[128,129] or protein kinase II[130,131], which are dependent on cAMP and cGMP, respectively, and both of these cyclic nucleotides have been well-characterized in mediating vasorelaxation in the lung[6,132,133]. Rather, the role of CFTR as an ABC transporter channel may regulate the transport of other substrates and signaling molecules across the plasma membrane. Notably, CFTR is one of only two transporters known to facilitate the translocation of sphingosine-1-phosphate (S1P), and the only one of which that regulates S1P import into the cytosol in a cAMP-independent manner[121]. As this role of CFTR in S1P transport has been shown to regulate the vascular tone in the systemic circulation[134], there is a tantalizing possibility that it might contribute to HPV in a similar manner.

Other than transporting S1P, CFTR functions have been linked to the homeostasis of another sphingolipid, ceramide. CFTR deficiencies lead to abnormal levels of intracellular ceramide in the lung due to a vesicular pH imbalance as a result of CFTR dysfunction. Subsequent imbalance in the activities of pH sensitive sphingomyelinase and ceramidase causes a net accumulation of ceramide[135-138]. Membrane CFTR also regulates lipid raft ceramide levels[139,140]. As ceramide production has been directly implicated in HPV[127], the abnormal ceramide homeostasis in the absence of functional CFTR provides another possible reason for the requirement of CFTR in HPV.

In addition, S1P and ceramide can both potentially regulate the translocation and activation of the critical cation channel TRPC6 in HPV[141-143]. Most interestingly, CFTR was shown to physically interact with TRPC6 in the formation of a reciprocally coupled molecular complex in the plasma membrane[144]. CFTR was found to co-immunoprecipitate with TRPC6, and the knockdown of CFTR caused abnormal TRPC6-dependent Ca^{2+} response in epithelial cells[122].

Much past research in CFTR has focused on its role in the pathogenesis of CF, and the potential functions for CFTR in the mediation of HPV may have been overlooked. This work will investigate whether CFTR is required for HPV, and the underlying mechanisms of mediation.

1.3.2. Sphingolipids

Sphingolipids are a class of lipids that derive their name from the Greek word "sphinx" due to their enigmatic nature. It is a fitting name because many of their biological functions are still unclear. There are hundreds of known species of sphingolipids[145,146], and some of the best studied include sphingomyelin, ceramide and sphingosine-1-phosphate (S1P). These lipids are fundamental constituents of the plasma membrane. They regulate a variety of cellular and physiological responses including apoptosis and cell survival[147], immune responses[148,149], vascular tone[150] and permeability[151], and HPV[127,152].

Sphingolipids are structurally important and modulate the physical properties of lipid bilayers and the activity of membrane proteins[151,153] . Sphingolipids are enriched in lipid rafts and caveolae – separate microdomains of the plasma membrane that function as platforms for receptors and proteins[146]. Sphingomyelinase (SMase) releases ceramide from membrane sphingomyelin, and ceramide release through SMase activity promotes lipid raft formation[146] and controls recruitment of receptors to rafts[143,154]. There are several different classes of SMase that generate ceramide (aSMase, nSMase1, 2 and 3)[154], and they have distinct cellular localizations, compartmentally controlling the balance of sphingolipids. Interestingly, nSMase2 is a major isozyme localized at the plasma membrane[149], and can be activated by H_2O_2[155,156].

nSMase have been implicated in HPV by regulating Kv channels, PKCζ and ROS production[127,152,157]. Recent finding in pulmonary arterial endothelial cells

showed that upon PAF stimulation, acid sphingomyelinase activation and ceramide production induced rapid TRPC6 translocation to the caveolae[143]. As TRPC6 associate with caveolin 1[158] and translocate to the caveolae under hypoxia[159], a similar ceramide-mediated mechanism may regulate the membrane trafficking of TRPC6 in PASMC during HPV.

Other than their structural importance in the membrane and raft formation, sphingolipids can be potent bioactive molecules through both intra- and extracellular signaling. The metabolite of ceramide, sphingosine-1-phosphate (S1P) is only produced via release from ceramide by ceramidase[160], and increases upon activation of SMase[161,162]. It modulates vascular tone in both systemic and pulmonary circulation[134,163]. S1P has 5 cell surface G protein-coupled receptors ($S1P_{1-5}$)[164], of which $S1P_2$ and $S1P_4$ have been implicated in vasoregulatory roles in the pulmonary circulation[163,165]. S1P receptors can couple with PLC and Rho[142], both of which are involved in the mediation of HPV[90,98], and exogenous S1P induces pulmonary vasoconstriction in a $S1P_2$/Rho-dependent manner[163]. In addition to receptor signaling, S1P also has a number of intracellular targets[160]. Interestingly, S1P activates TRPC3 via receptor signaling[166], and TRPC5 through both receptor and intracellular signaling, and TRPC5 was proposed as an ionotropic receptor for intracellular S1P[167]. Given that PLC activation via G protein-coupled receptor signaling and subsequent DAG generation is required in TRPC6 activation during HPV[90], S1P is an attractive candidate in TRPC6 signaling in PASMC.

It has been described that CFTR mutations cause imbalance of intracellular ceramide homeostasis[120,127], and the membrane localization of CFTR regulates lipid raft formation and ceramide levels[139,140,168], which may be required for the membrane localization of TRPC6. CFTR is also involved in the transport of S1P across the plasma membrane, and regulates its degradation[121,134]. Accordingly, sphingolipid-mediated TRPC6 trafficking and/or activation may constitute the missing link between CFTR and HPV.

1.3.3. Transient receptor potential canonical 6

Transient receptor potential or TRP channels were first discovered in *Drosophila melanogaster*[169,170]. Mammalian homologues of TRP channels form a superfamily of cation channels including TRPC, TRPV, TRPA, TRPM, TRPP, and TRPML. In general, TRP proteins consist of 6 transmembrane domains; the loop between S5 and S6 is believed to form the channel pore[93,171]. TRP channels form homo and heterotetramers that are permeable to Na^+, K^+, Cs^+, Li^+, Ca^{2+}, Mn^{2+}, and Mg^{2+} ions[172,173].

TRPC channels are named classical or canonical TRP channels since they were the first mammalian channels identified that are closely related to fruit fly TRP channels[93,169]. TRPC can be further divided into three subfamilies according to amino acid homology: TRPC1, TRPC4/5, and TRPC3/6/7[93,172]. TRPC3, 6 and 7 share 70-80% sequence homology, and form functional heteromultimers[172]. In

addition to having a caveolin 1-binding site, TRPC6 protein also has two glycosylation sites, which affect basal channel activity[93,171,172].

Nonselective cation channels (NSCC), which include both SOCC and ROCC, play a major role in total Ca^{2+} influx in HPV[6]. NSCCs in HPV are believed to be multimers of TRP channels[174,175]. TRPC6 is widely expressed in all smooth muscle cells, but is most richly expressed in the lung, and particularly highly expressed in distal pulmonary arterial smooth muscle cells[170]. Studies using TRPC-deficient mice showed a complete absence of phase 1 HPV, while vasoconstriction responses to thromboxane mimetic was unchanged[76], suggesting TRPC6 is specifically required for acute HPV. The abolition of phase 1 HPV was accompanied by a lack of $[Ca^{2+}]_i$ increase in TRPC6$^{-/-}$ PASMC in response to hypoxia[76]. Therefore, TRPC6 channels appear to be indispensible for the Ca^{2+} response in PASMC and subsequent vasoconstriction in HPV. Given the predominant role of VOCC in Ca^{2+} influx during HPV, and that TRP6 has a relatively low permeability to Ca^{2+} in comparison to Na^{+}[176], it is likely that the cation influx through TRPC6 drives membrane depolarization, inhibits K_v channels, and lead to a VOCC-driven Ca^{2+} influx[170,177]. Other than a role in acute HPV, TRPC6 channels have been suggested to contribute to the development of chronic hypoxic pulmonary hypertension, as TRP6 expression was around 3-fold higher in chronic hypoxic rat PASMC[178].

During hypoxia, intracellular TRPC6 translocate to caveolin 1 rich lipid rafts[159]. A concomitant increase in the formation of TRPC6 activator diacylglycerol (DAG)

by phospholipase C (PLC) triggers cation influx through TRPC6[90]. Further, H_2O_2 was found to induce TRPC6 trafficking to the membrane and sensitize it to DAG via an unknown intermediate protein[179]. Intriguingly, as mentioned above, nSMase2 is activated by H_2O_2[155,156]. Further, S1P is an excellent candidate for TRPC6 activation, as there is currently no known G protein-coupled receptor agonist implicated in the activation of PLC during hypoxia, a critical step in activating TRPC6 and eliciting HPV[90].

CFTR may influence TRPC6 signaling during HPV in two ways. First, the regulation of intracellular levels of sphingolipids may influence lipid raft formation and therefore cause impediment to TRPC6 localization in the caveolae. Second, CFTR physically associates with TRPC6 in the formation of a reciprocally coupled molecular complex in the plasma membrane[144], and therefore potentially influence its membrane abundance or channel activity. In this study, we explore these possibilities.

2. Chapter 2 Rationale and objectives

2.1. Rationale

Hypoxic pulmonary vasoconstriction optimizes ventilation-perfusion (V/Q) matching in the lung. HPV is fundamental to the physiological and pathophysiological processes involved a number of disease and clinical conditions. Although our understanding of HPV has advanced greatly since it was first described, and a number of individual pathways have been identified, we still lack a unifying mechanistic concept.

HPV is central to the regulation of pulmonary gas exchange, the pathogenesis of high-altitude pulmonary edema, pulmonary hypertension, and heart failure. V/Q mismatches resulting from the dysregulation of HPV can be a significant cause of hypoxemia in cystic fibrosis patients[13,14]. Moreover, end stage CF patients frequently develop pulmonary hypertension and *cor pulmonale* as a result of chronic hypoxia induced pulmonary hypertension[125]. However, there is no current literature addressing dysregulation of HPV in CF patients, nor the potential role of cystic fibrosis transmembrane conductance regulator (CFTR) in HPV.

CFTR plays an important role in sphingolipid homeostasis by regulating intracellular levels of both ceramide and S1P[120,121]. Therefore, CFTR may contribute to HPV by facilitating sphingolipid signalling. Sphingomyelinase

(SMase) activity and its product ceramide have been implicated in HPV[127,152] and the recruitment of TRPC6 to caveolae[143]. The metabolite of ceramide, sphingosine-1-phosphate (S1P) regulates vascular tone[134] and induces pulmonary vasoconstriction[163]. Moreover, S1P receptors can couple with PLC and Rho[141,142,164] to potentially induce DAG-dependent TRPC6 activation, as well as to mediate RhoK-dependent Ca^{2+} sensitization during HPV.

Intracellular Ca^{2+} increase in PASMC is a crucial stage in HPV, and it is critically dependent on transient receptor potential canonical 6 (TRPC6)[76]. TRPC6 channels may also be regulated by CFTR and sphingolipids, as CFTR associates with TRPC6 in the plasma membrane[122] and as SMase regulates the recruitment of TRPC6 to caveolae[143]. The regulation of sphingolipids ceramide and S1P by CFTR, and its membrane association with TRPC6 by may thus form the missing link between CFTR, TRPC6 and HPV, and may represent a significant signaling pathway, filling a knowledge gap of the fragmented understanding of HPV. In this study, we investigate the role of CFTR and sphingolipids in TRPC6 translocation and activation in HPV.

2.2. Hypothesis

CFTR is required for sphingolipid-mediated TRPC6 translocation and activation in pulmonary arterial smooth muscle cells (PASMC) during HPV.

2.3. Objectives

2.3.1. Objective #1: Investigate whether functional CFTR is required for an intact HPV response through TRPC6 signaling

Specific aim #1: Examine the effect of CFTR inhibition or deficiency on HPV

Specific aim #2: Examine the role of CFTR in nonhypoxia-induced pulmonary vasoconstriction

Specific aim #3: Examine the role of CFTR as a Cl^- channel in HPV

Specific aim #4: Examine the role of CFTR in Ca^{2+} signaling in PASMC during hypoxia

Specific aim #5: Examine the role of CFTR in TRPC6 trafficking in PASMC

2.3.2. Objective #2: Investigate mechanisms of sphingolipid regulation of TRPC6 activation in HPV, and the role of CFTR in this regulation

Specific aim #1: Examine the effect of SMase inhibition on HPV

Specific aim #2: Examine the role of SMase in TRPC6 trafficking in PASMC

Specific aim #3: Examine the effect of CFTR inhibition on SMase-induced pulmonary vasoconstriction and TRPC6 translocation in PASMC

Specific aim #4: Examine the role of ceramide vs. S1P in TRPC6 activation and HPV

Specific aim #5: Examine the role of S1P receptor signaling in TRPC6 activation and HPV

Specific aim #6: Examine the synergistic effects of ceramide and S1P

2.3.3. Objective #3: Investigate mechanisms of CFTR regulation of the sphingolipid-mediated TRPC6 activation in hypoxia

Specific aim #1: Investigate the mechanism of CFTR regulation of hypoxia-induced TRPC6 trafficking

3. Chapter 3 Results

3.1. Objective #1: CFTR in HPV and TRPC6 signaling

The first step in our study was to establish a role for CFTR in the regulation of hypoxic pulmonary vasoconstriction. Although studies have suggested CF patients suffer from hypoxemia as a result of V/Q mismatches[13,14], there are no studies in existing literature addressing the potential role of CFTR in HPV. Here, we hypothesized that CFTR is critical in HPV, and disruptions in CFTR functions has a direct impact on the vasomotor response during hypoxia.

3.1.1. CFTR is required for intact HPV (Specific aim #1)

To determine whether CFTR participates in HPV, we used an *ex vivo* approach to quantify HPV in the intact lung, as well as an *in vivo* experiment to demonstrate the physiologically relevant role of CFTR in HPV and V/Q matching. First, using the isolated perfused and ventilated mouse lung model (IPL, see section 5.2.), we assessed HPV by measuring the increase in pulmonary arterial perfusion pressure when switching ventilation from 21% O_2 to 1% O_2. In both the specific[180] CFTR channel inhibitor CFTRinh-172 (10 μM, Sigma-Aldrich, St. Louis, MO) pretreated WT lungs and in CFTR-deficient lungs, HPV responses were attenuated by 47.5% and 69.2%, respectively compared to the vehicle-treated WT control (Figure 3.1). The reduction of HPV in $CFTR^{-/-}$ lungs was highly

statistically significant (P=0.0022). The baseline PAP of CFTR inhibited and deficient lungs did not differ from control.

In an *in vivo* experiment of regional hypoventilation, mice were anesthetized and ventilated with room air and challenged with 25 µL of saline by tracheal administration. While at baseline the P_aO_2 did not differ between $CFTR^{-/-}$ and WT mice, partial airway occlusion with saline instillation resulted in severe hypoxemia in $CFTR^{-/-}$ but not in WT mice (Figure 3.2). These results establish a highly novel role for CFTR in HPV and demonstrate its importance in ventilation-perfusion matching.

3.1.2. CFTR is specific for HPV (Specific aim #2)

In order to rule out the possibility that CFTR inhibition or deficiency has a general vasodilatory effect in the lung that is not specific for HPV, we used a bolus 1 µg angiotensin II (Ang II, Sigma-Aldrich, St. Louis, MO) stimulation to elicit nonhypoxia-induced vasoconstriction in isolated mouse lungs. 10 µM CFTRinh-172 pretreatment and CFTR deficiency did not have any inhibitory effect (P>0.05) on Ang II-induced vasoconstriction in isolated mouse lungs (Figure 3.3), suggesting CFTR is specifically required for HPV.

3.1.3. Role of CFTR as a Cl^- channel in HPV (Specific aim #3)

CFTR is a Cl^- channel that transports Cl^- ions across the plasma membrane down their electrochemical gradient[181]. Absence of functional CFTR will therefore

impact the anion flow and possibly alter membrane potential and therefore affect membrane depolarization during HPV. To examine whether alterations in the Cl^- gradient would affect HPV, we substituted Cl^- with NO_3^- in lung perfusate in our IPL experiments. We found that removing Cl^- from perfusate had no impact on the magnitude of HPV (Figure 3.4), suggesting CFTR most likely contributes to HPV independent of its Cl^- transporting functions.

3.1.4. CFTR is required in Ca^{2+} signaling in PASMC during hypoxia (Specific aim #4)

Increase in intracellular Ca^{2+} is prerequisite for contraction in PASMC during hypoxia; it is a crucial step for the activation of contractile machinery. We used ratiometric imaging of fura-2 to assess $[Ca^{2+}]_i$. Fura-2 (Life Technologies, Carlsbad, CA)-loaded human PASMC were cultured on 22 mm glass coverslips and loaded in a heated imaging chamber (Warner Instruments, Hamden, CT) and perfused with normoxic and subsequently hypoxic Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO) (see section 5.9.). We found that 10 μM CFTRinh-172 blocked the $[Ca^{2+}]_i$ increase in response to hypoxia (Figure 3.5), suggesting CFTR functions are crucial for the Ca^{2+} signaling in HPV.

3.1.5. CFTR is required for TRPC6 trafficking in PASMC during hypoxia (Specific aim #5)

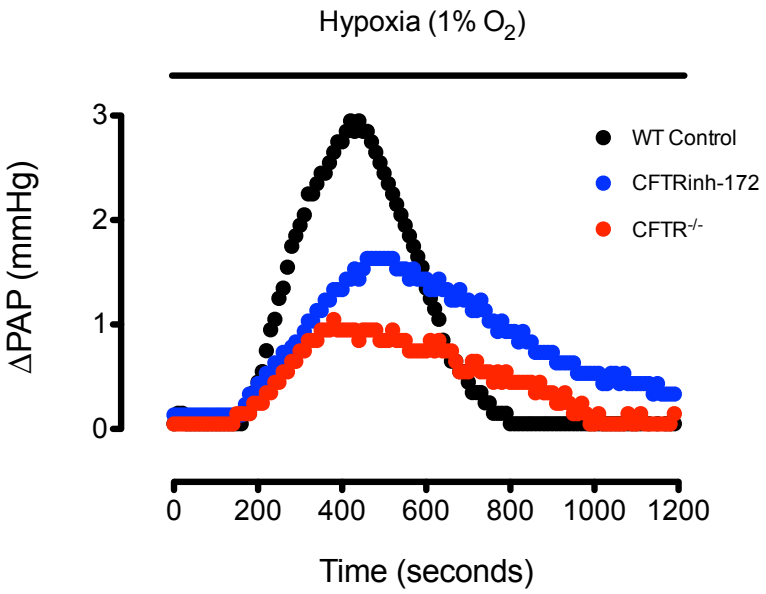
In addition to voltage-operated Ca^{2+} channels and intracellular Ca^{2+} stores, TRPC6 channels have been shown to be critical for intracellular Ca^{2+} increase

during HPV, as TRPC6 deficiency leads to the complete abolition of $[Ca^{2+}]_i$ increase in PASMC during hypoxia as well as phase 1 HPV in isolated lungs[76]. In agreement with previous findings, we found an attenuation of HPV in isolated mouse lungs treated with two specific TRPC6 inhibitors 2910-0498[88] (25 μM) and larixol acetate (5 μM) (Figure 3.6). TRPC6 channels translocate to caveolae during hypoxia[159], thus the regulation of its trafficking directly influences its membrane abundance and consequent cation flux. Using sucrose density gradient ultracentrifugation, we isolated caveolae fractions from PAMSC whole cell lysate. Caveolae fractions were identified by western blot analysis using anti-caveolin 1 (cav-1) and anti-flotillin 1 (flot-1) antibodies[182,183]. Using the protocol described in section 5.6, fractions 4-6 from the 10-fraction sucrose density gradient were the only fractions that contained both markers (Figure 3.7), and were subsequently probed for proteins of interest using western blot.

PASMC treated with 1% O_2 for 15 minutes showed a significant increase in TRPC6 channel abundance in their caveolar fractions. This upregulation of caveolar TRPC6 during hypoxia was attenuated by 10 μM CFTRinh-172 pretreatment (Figure 3.8). This finding confirms that TRPC6 channels are indeed regulated by translocation during hypoxia, and also suggests that CFTR functions are required for the trafficking of TRPC6.

Taken together, these results indicate CFTR functions are critically important for the Ca^{2+} signaling in PASMC and HPV by regulating TRPC6 trafficking in PASMC during hypoxia.

A



B

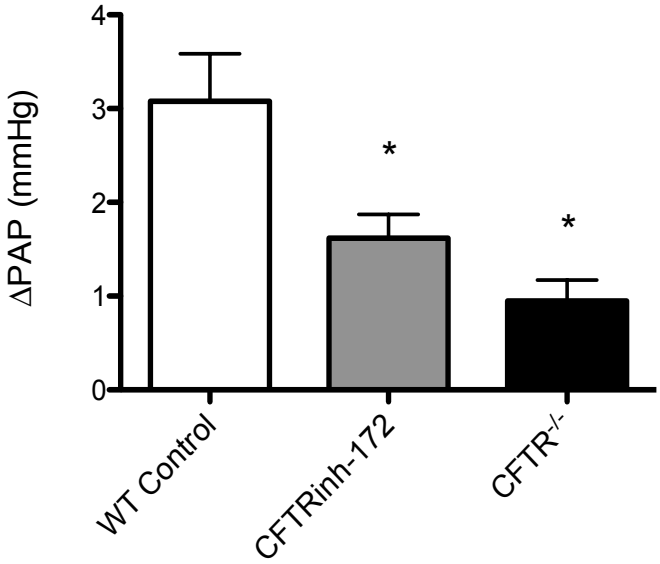


Figure 3.1 HPV requires CFTR: (A) representative HPV response in untreated isolated WT lungs (black), CFTRinh-172 treated WT lungs (blue) and CFTR$^{-/-}$ lungs (red). (B) Hypoxia-induced increase in PAP in isolated mouse lungs was attenuated by CFTR inhibition (CFTRinh-172,) and in lungs of CFTR-deficient mice. n=6 each

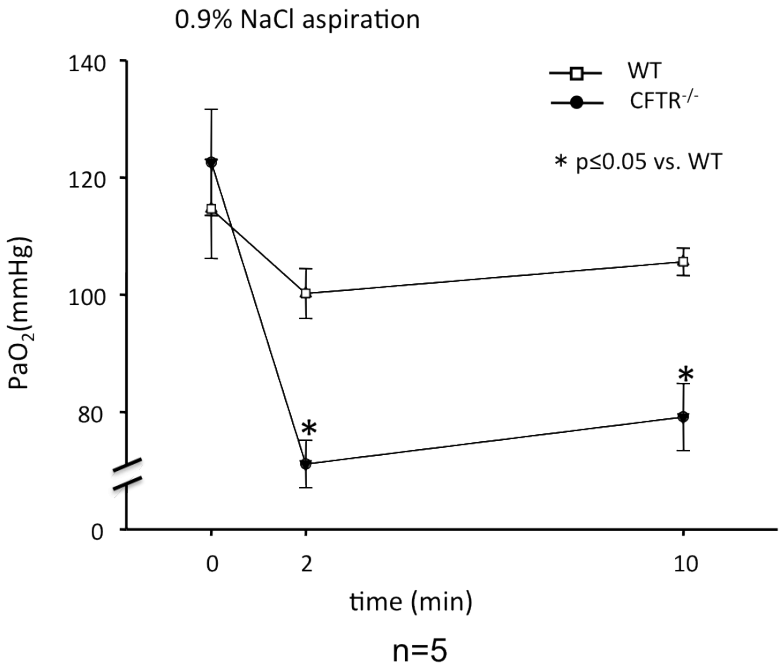


Figure 3.2 CFTR-deficient mice suffer from hypoxemia during regional hypoventilation: partial airway occlusion with 25 µL of saline by tracheal administration led to a more severe hypoxemia in $CFTR^{-/-}$ mice compared to WT control. n=5 each

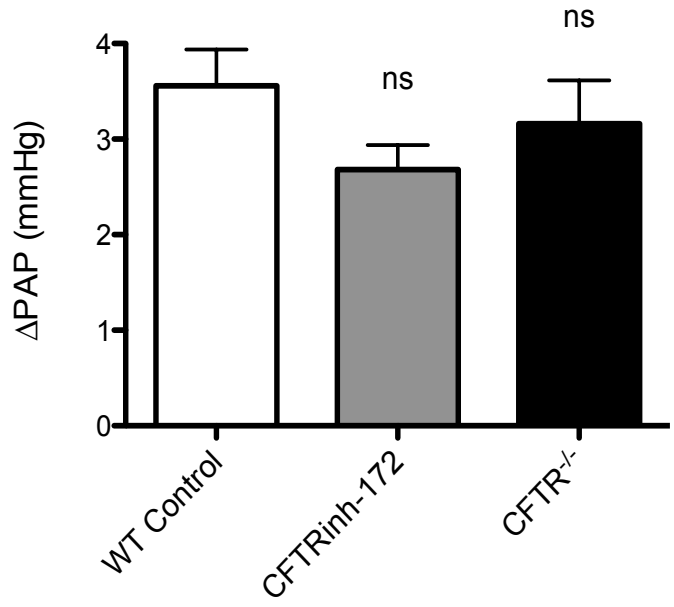


Figure 3.3 CFTR is specifically required for HPV: CFTR inhibition or deficiency did not affect the increase in PAP in response to angiotensin II. n=5 each

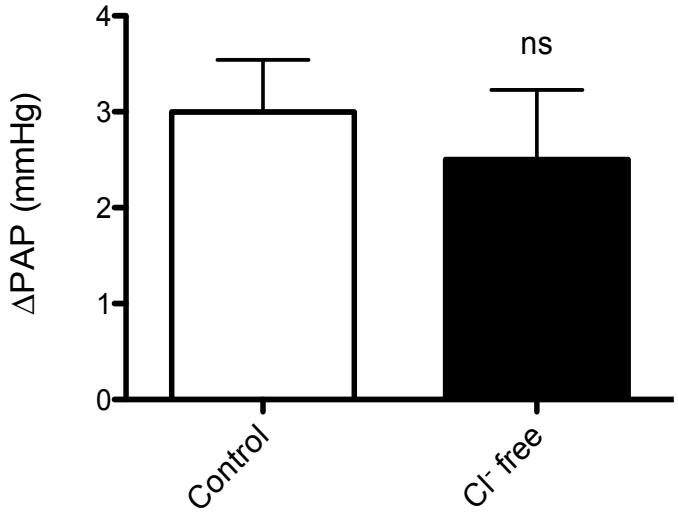
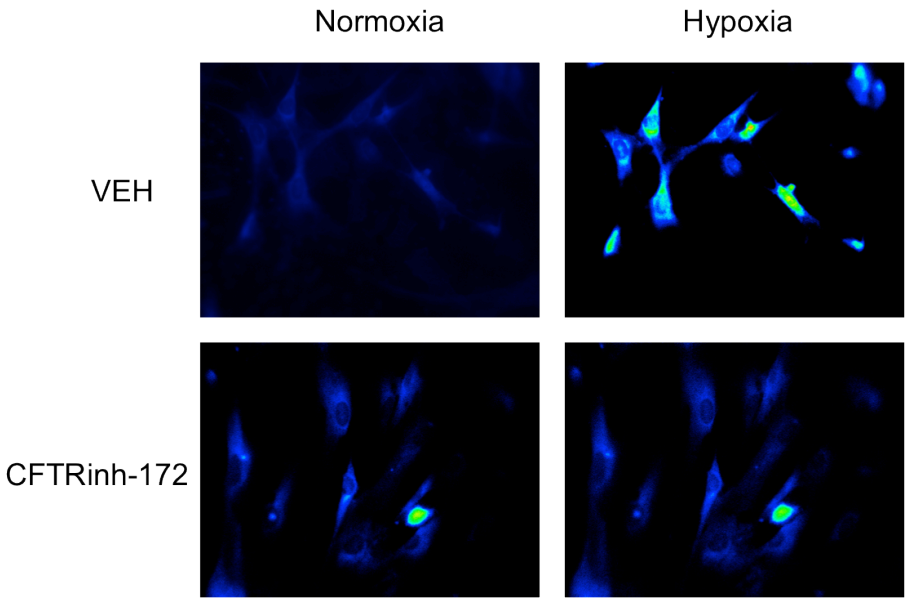


Figure 3.4 The role of CFTR in HPV does not relate to its Cl^- channel functions: Cl^- free perfusate does not affect the hypoxia-induced increase in PAP. n=5 each

A



B

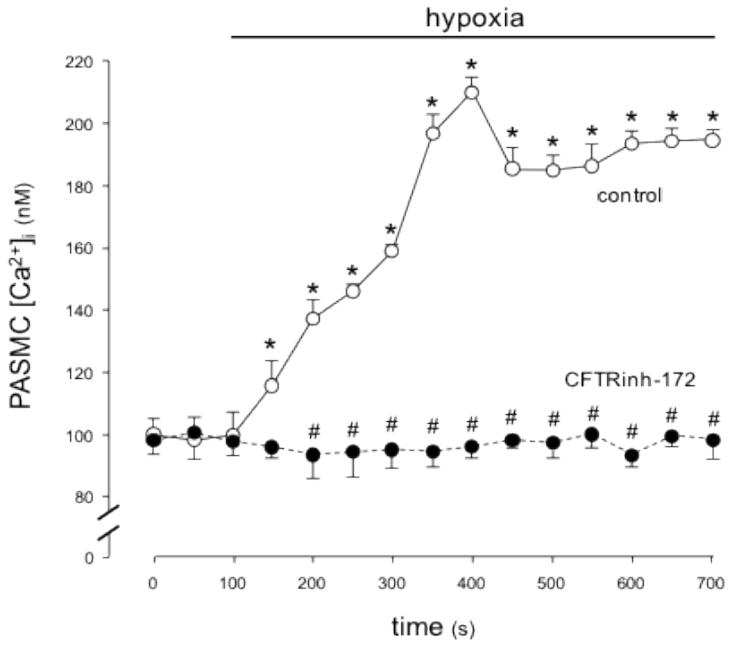
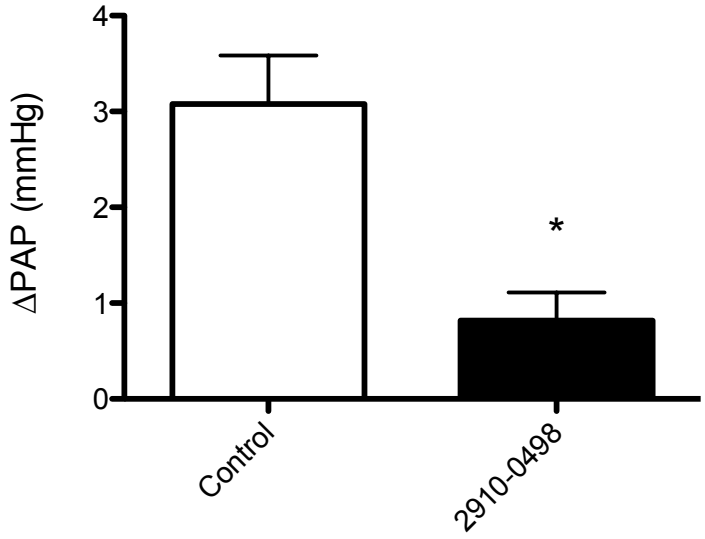


Figure 3.5 Ca^{2+} mobilization in PASMC during hypoxia requires CFTR: (A) Representative images of $[Ca^{2+}]_i$ response to hypoxia in PASMC (B) Increase in $[Ca^{2+}]_i$ in fura-2 loaded PASMC in response to hypoxia was attenuated by CFTR inhibition. n=5 each

A



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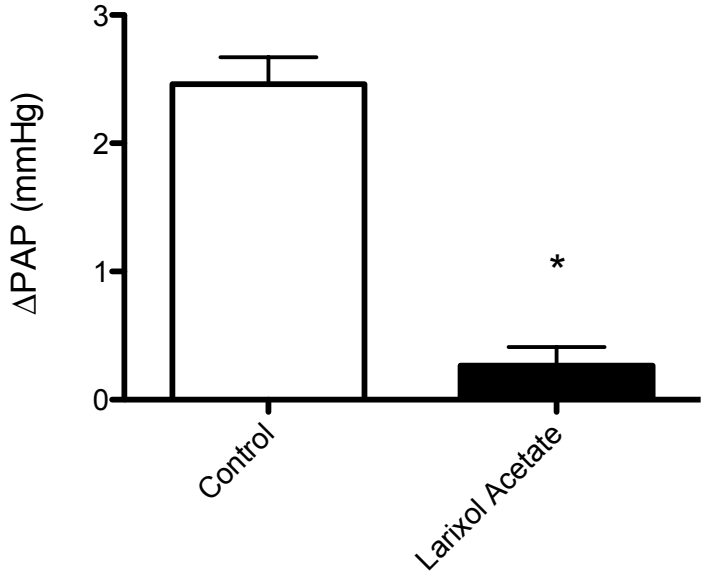


Figure 3.6 HPV requires TRPC6: TRPC6 specific inhibitors (A) 2910-0498 (25 μM) and (B) larixol acetate (5 μM) attenuated hypoxia-induced PAP increase. n=5 each

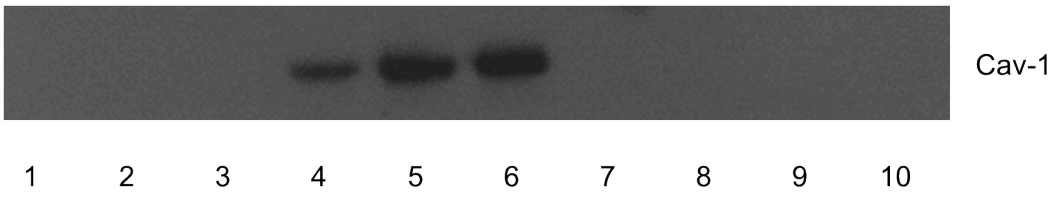


Figure 3.7 Fractions 4 – 6 contain caveolae: western blot analysis of sucrose density gradient fractions confirmed fractions 4, 5, and 6 contain caveolae marker cav-1.

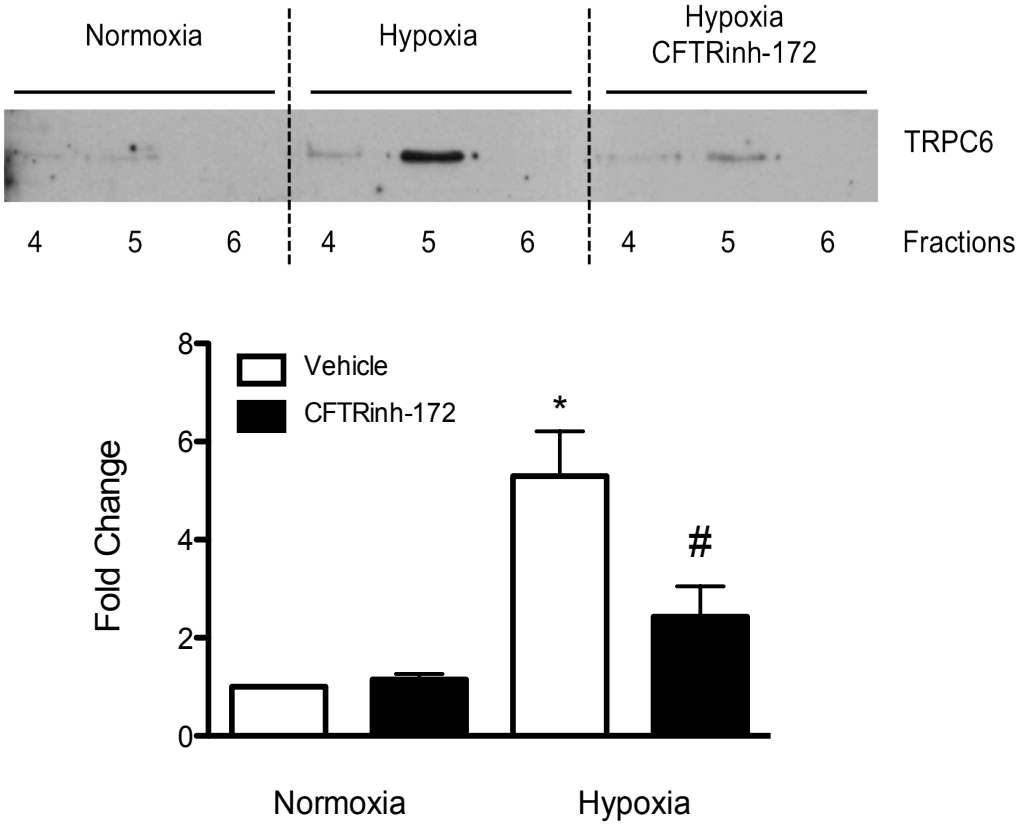


Figure 3.8 TRPC6 translocation during hypoxia requires CFTR: CFTRinh-172 treatment significantly decreased TRPC6 presence in caveolae during hypoxia. n=3 each *p<0.05 vs. vehicle normoxia #p<0.05 vs. vehicle hypoxia

3.2. Objective #2: Sphingolipids in HPV and TRPC6 signaling

Here, we used various pharmacological inhibitors as well as exogenous sphingolipids to identify the role of sphingolipids in HPV in isolated lungs and TRPC6 trafficking in PASMC. Due to the highly hydrophobic nature and thus insolubility of naturally occurring long-chain ceramides, we used a bacterial homologue of nSMase, to mimic the effects endogenous ceramide release from sphingomyelin.

3.2.1. Role of SMase in HPV (Specific aims #1 & #3)

Recent finding by our group shows that in endothelial cells, acid sphingomyelinase (aSMase) is required for TRPC6 translocation[143], in discord with Cogolludo's findings that neutral sphingomyelinase (nSMase), but not aSMase is required for HPV[143]. Since sphingolipids may be subject to distinctive spatial and temporal control in response to different stimuli and in different cell types, we first sought to elucidate the type of SMase required in our experimental model of HPV.

Shown in Figure 3.9, pretreatment with nSMase inhibitor GW4869[168] (10 μM, Sigma-Aldrich, St. Louis, MO) but not the aSMase inhibitor ARC39[184] (10 μM Dr. Christoph Arenz, Institute for Chemistry, Humboldt Universität Berlin) blocked the PAP increase in isolated mouse lungs in response to hypoxic ventilation. This finding is consistent with prior studies that nSMase but not aSMase is necessary

for HPV. Treatment with exogenous SMase (100 U/L, Sigma-Aldrich, St. Louis, MO) from *Bacillus cereus*, a homologue of mammalian nSMase, also predictably induced pulmonary vasoconstriction in isolated mouse lungs. This SMase (and presumably through ceramide production)-induced pulmonary vasoconstriction was blocked by 10 μM CFTRinh-172 (Figure 3.10) pretreatment in isolated mouse lungs, suggesting CFTR functions are required for the SMase-mediated pulmonary vasoconstriction, as a potential mechanism by which it regulates HPV.

3.2.2. Role of SMase in TRPC6 trafficking in PASMC (Specifics aim #2 & 3)

Prior studies showed that ceramide production by activated nSMase regulate HPV by modulating Kv channels, PKCζ and ROS production[127,152,157]. However, this does not exclude the possibility that ceramide production under hypoxia also alters the properties of caveolae, and influence the recruitment and clustering of membrane receptors, protein channels, and the formation of molecular complexes. We hypothesized that nSMase activation and ceramide production during hypoxia has a direct impact on TRPC6 translocation to the caveolae, similar to our prior findings in lung endothelial cells.

First, we established that SMase-induced pulmonary vasoconstriction is indeed TRPC6-dependent. Figure 3.11 shows that 5 μM larixol acetate pretreatment significantly attenuated the PAP increase in response to 100 u/L exogenous SMase stimulation in isolated lungs. Next, we found nSMase inhibitor GW4869 (10 μM) decreased TRPC6 abundance in caveolae during hypoxia (Figure 3.12)

in PASMC, suggesting that nSMase activation is required for TRPC6 translocation during hypoxia. Exogenous SMase alone (100 U/L) was also sufficient to trigger TRPC6 translocation caveolae of PASMC; importantly, this SMase-induced TRPC6 translocation was blocked by CFTR inhibition (Figure 3.13).

These results indicate that CFTR regulates SMase-induced TRPC6 translocation to caveolae in PASMC during hypoxia, and strongly suggest this to be the mechanistic link between CFTR and HPV.

3.2.3. Role of ceramide vs. S1P in HPV and TRPC6 activation (Specific aim #4)

Sphingosine-1-phosphate (S1P), the phosphorylated form of sphingosine, is a derivative of ceramide, and is only produced from the degradation of ceramide[148,153]. Given that its levels rise rapidly upon nSMase activation[161], and being a highly potent signaling molecule involved in vascular regulation[134,163,167,185] and transported via CFTR[121], we could not ignore their potential signaling role in HPV.

To determine whether S1P is required for HPV, we inhibited S1P production by blocking sphingosine phosphorylation in isolated mouse lungs using a sphingosine kinase inhibitor[162] (SKI II, 5 μM, Sigma-Aldrich, St. Louis, MO). Pretreatment with SKI II significantly attenuated hypoxia-induced PAP increase

in isolated lungs (Figure 3.14), suggesting S1P production is required for HPV. Similarly, SKI II blunted exogenous nSMase-induced pulmonary vasoconstriction (Figure 3.15); indicating the phosphorylation of sphingosine downstream of ceramide production is a critical step in eliciting vasoconstriction.

S1P has been implicated in the activation of other TRPC channels such as TRPC5 by both receptor and direct-binding activation[167], and by modulating stress fibre formation and increasing TRPC1 association with lipid rafts[186]. Since TRPC6 translocation to caveolae appears to be a principal mechanism of activation during hypoxia, we tested whether S1P is required for TRPC6 translocation to the caveolae. Treatment with S1P alone (10 μM, Cayman Chemical, Ann Arbor, MI) did not result in any increase in caveolar abundance of TRPC6 (Figure 3.16 A), and sphingosine kinase inhibition by 5 μM SKI II did not block the exogenous SMase-induced TRPC6 translocation (Figure 3.16 B).

Given that S1P does not induce TRPC6 translocation, and exogenous SMase could still trigger TRPC6 translocation without the production of S1P, it is likely that ceramide alone (or in combination with sphingosine) is sufficient to promote TRPC6 recruitment. Next, we investigated alternative pathways of S1P signaling that may be crucial in HPV.

3.2.4. Role of S1P receptor signaling in HPV (Specific aim #5)

S1P has five extracellular G-protein coupled receptors, $S1P_{1-5}$[141,164]. Notably, $S1P_2$ has been implicated in S1P-induced pulmonary vasoconstriction[163]. In addition to coupling with Rho via $G_{12/13}$[141,142,164], which may lead to Ca^{2+} sensitization crucial for HPV, $S1P_2$ couples to phospholipase C (PLC) via $G_{q/11}$[141,142,164]. PLC activation generates diacylglycerol (DAG), which is the direct activator of TRPC6[187]. The activation of PLC has been shown to be critical for HPV via activation of TRPC6 channels[90], yet no receptor or agonist have yet been identified. We hypothesized S1P generated downstream of nSMase activation contribute to HPV through $S1P_2$ receptor signalling via Rho and/or PLC activation.

Pretreatment with the $S1P_2$ receptor antagonist JTE-013[188] (10 μM, Sigma-Aldrich, St. Louis, MO) had a strong inhibitory effect on both hypoxia and exogenous SMase-induced pulmonary vasoconstriction in isolated mouse lungs (Figures 3.14 & 3.15), suggesting S1P signalling through $S1P_2$ is a key step in HPV. SMase-induced pulmonary vasoconstriction was also ablated by pretreatment with10 μM PLC inhibitor U73122[189] but not by its inactive analogue, U73343 (both Sigma-Aldrich, St. Louis, MO) (Figure 3.17), suggesting PLC activation is important downstream of nSMase activation.

Treatment with 10 μM S1P alone in isolated mouse lungs induced robust pulmonary vasoconstriction. However, this vasoconstriction was not mediated through TRPC6 or PLC activation, as inhibition with 5 μM larixol acetate or 10

μM U73122 did not block this vasoconstriction (Figure 3.18 & 3.19). Rather, this vasoconstriction was blocked by 10 μM Y27632[190] (Sigma-Aldrich, St. Louis, MO), suggesting S1P signals largely through Rho (Figure 3.20), which is in accordance with previous findings[163]. Nevertheless, as S1P alone does not induce TRPC6 translocation, the seemingly predominant Rho-mediated vasoconstriction may not be the only signaling pathway during hypoxic conditions, where TRPC6 channels are inserted into the caveolae downstream of nSMase activation, then possibly activated through $S1P_2$-PLA-DAG signaling. This notion is further examined in the next section.

3.2.5. Synergistic effects of ceramide and S1P (Specific aim #6)

The balance between ceramide and S1P is tightly regulated. Relative levels of ceramide and S1P have often been described as a "rheostat"[191,192], the balance of which can regulate a number of cellular processes such as cell survival, motility and inflammation[193]. In addition to their antagonizing effects, ceramide (and ceramide-1-phosphate) and S1P have also been shown to act synergistically in embryonic stem cell differentiation[194] and prostaglandin E_2 production[191].

As my findings in previous sections suggest, ceramide production by nSMase activation during hypoxia is both necessary and sufficient for TRPC6 translocation to caveolae. However, S1P signaling through $S1P_2$ is further required to elicit vasoconstriction. Therefore, we hypothesized that, upon hypoxia

stimulation, TRPC6 channels are recruited to caveolae by ceramide production, and further stimulation by DAG generated by PLC coupled to $S1P_2$ is required for channel activation. In addition, the Rho pathway downstream of $S1P_2$ may also represent a significant component for eliciting the full HPV response.

Since we have shown that S1P production is vital for the SMase-induced pulmonary vasoconstriction (Figure 3.15), the application of exogenous S1P should rescue this attenuation. Although sphingosine kinase inhibition abolishes nSMase-induced vasoconstriction, TRPC6 channels should be inserted into the caveolae as a result of ceramide production (Figure 3.16 B). If S1P rescues this blunted SMase-induced vasoconstriction; the corresponding response should reflect a direct effect of S1P signaling. This provides a model to study and identify downstream signaling pathways of S1P relating to our hypothesis of bipolar activation (translocation of intracellular TRPC6 followed by extracellular receptor activation) of TRPC6 by ceramide and S1P.

The following findings are summarized in Figure 3.21. Treatment with exogenous nSMase and S1P while blocking endogenous S1P production resulted in strong vasoconstriction, demonstrating exogenous S1P can indeed rescue the effect of endogenous S1P production blockade during nSMase treatment. Next, we showed that this rescue is related to the activation of TRPC6 channels, as TRPC6 inhibitor larixol acetate decreased the vasoconstrictive response significantly. Moreover, inhibition of PLC by U73122 also decreased the rescued vasoconstriction, suggesting PLC activation is downstream of S1P receptor

activation. Expectedly, Rho is also involved downstream of S1P, since S1P alone without nSMase also acted via Rho.

Here, we demonstrated ceramide and S1P act together for TRPC6 activation. S1P-induced vasoconstriction in conjunction with nSMase is dependent on TRPC6 and PLC signaling, where as S1P alone without ceramide signals through neither TRPC6 nor PLC. Whether Rho signaling downstream of S1P is related to TRPC6 activation is unclear, but RhoK is known to play a major role in HPV as it inhibits myosin light chain phosphatase in compliment to Ca^{2+} influx that leads myosin light chain kinase activation. Together, sphingolipid signaling through TRPC6 and Rho promote myosin light chain phosphorylation and vasoconstriction.

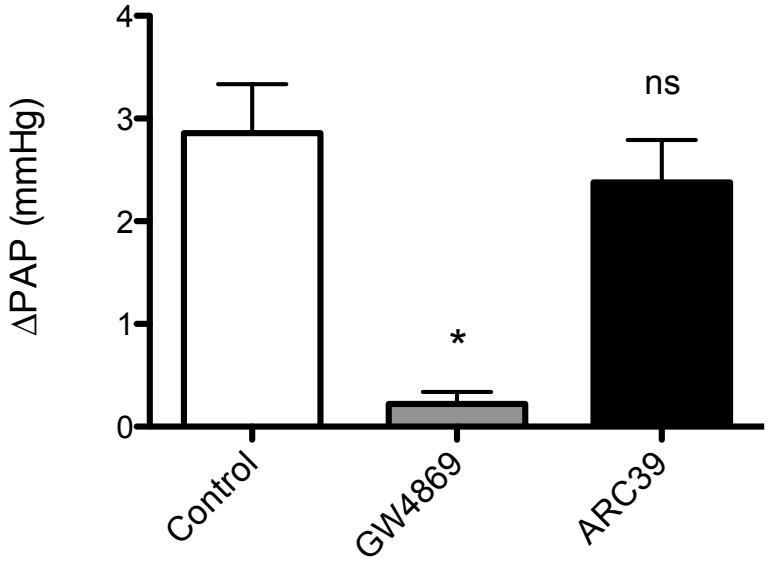


Figure 3.9 nSMase is required for HPV: nSMase inhibitor (GW4869) but not aSMase inhibitor (ARC39) blocked hypoxia-induced PAP increase. n=5 each

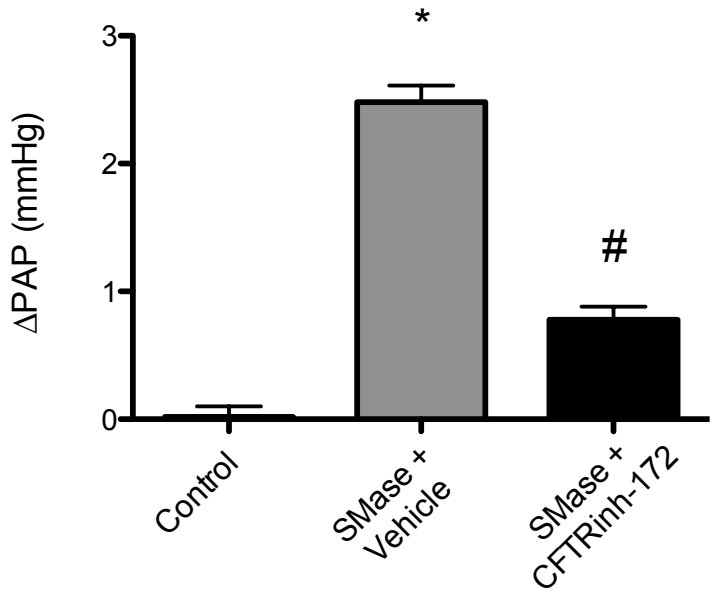


Figure 3.10 CFTR is required for nSMase-induced pulmonary vasoconstriction: exogenous nSMase increased PAP and was blocked by CFTRinh-172. n=5 each *p<0.05 vs. control #p<0.05 vs. SMase + vehicle

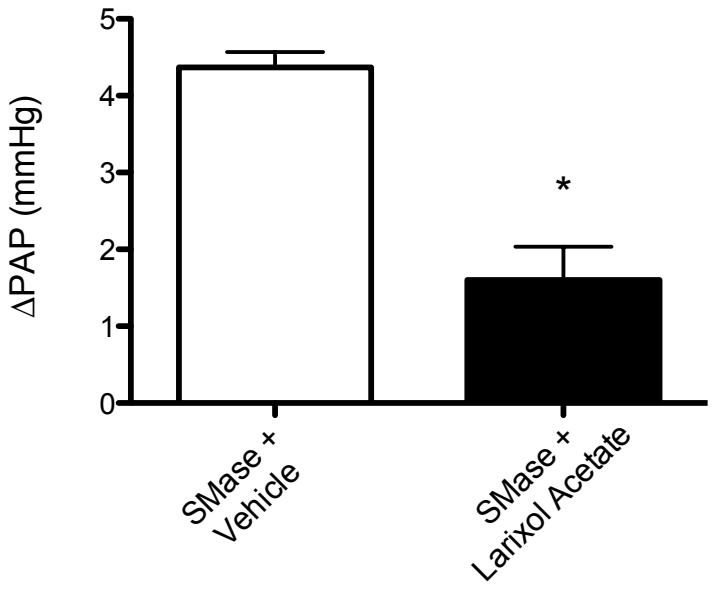


Figure 3.11 nSMase-induced pulmonary vasoconstriction is TRPC6-dependent: TRPC6 inhibitor larixol acetate (5μM) attenuated nSMase-induced PAP increase. n=3 each

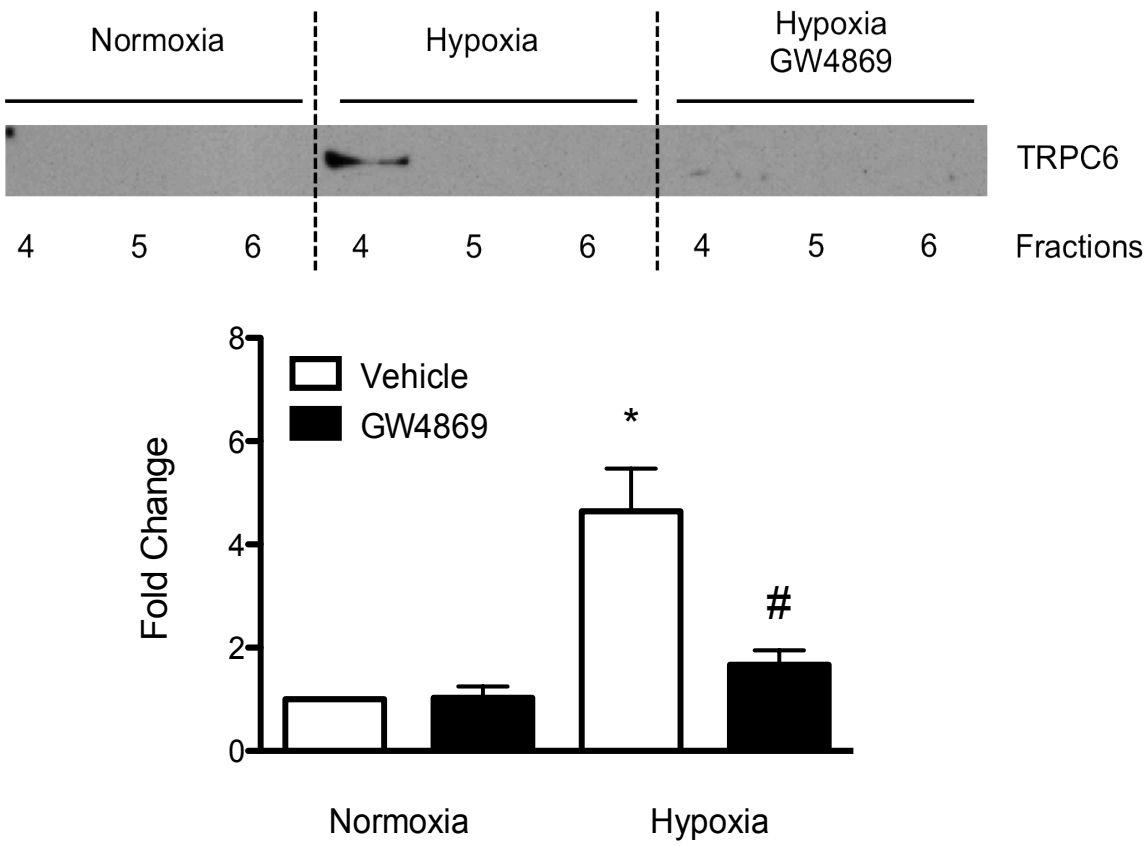


Figure 3.12 Hypoxia-induced TRPC6 translocation requires nSMase: GW4869 significantly decreased TRPC6 presence in caveolae during hypoxia. n=4 each *p<0.05 vs. vehicle normoxia #p<0.05 vs. vehicle hypoxia

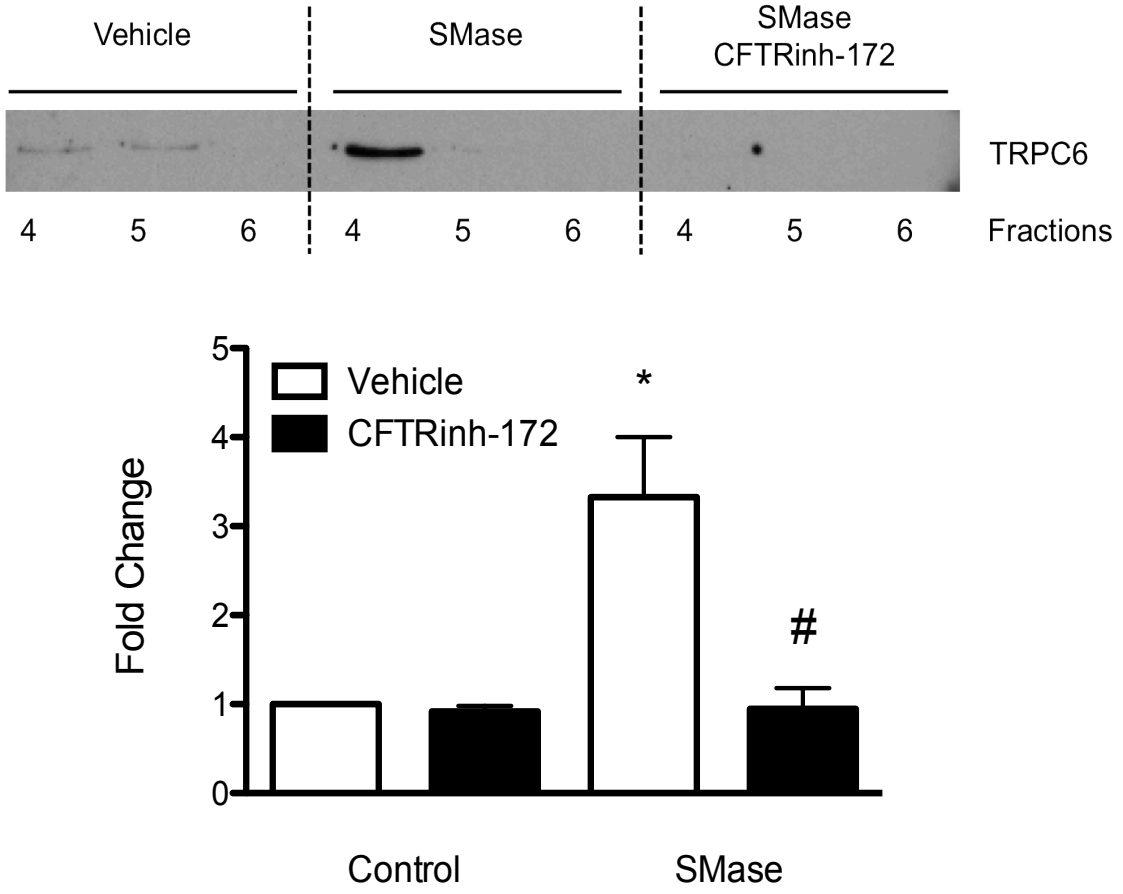


Figure 3.13 nSMase-induced TRPC6 translocation requires CFTR: CFTRinh-172 significantly decreased TRPC6 presence in caveolae during nSMase stimulation n=3 each *p<0.05 vs. vehicle control #p<0.05 vs. vehicle SMase

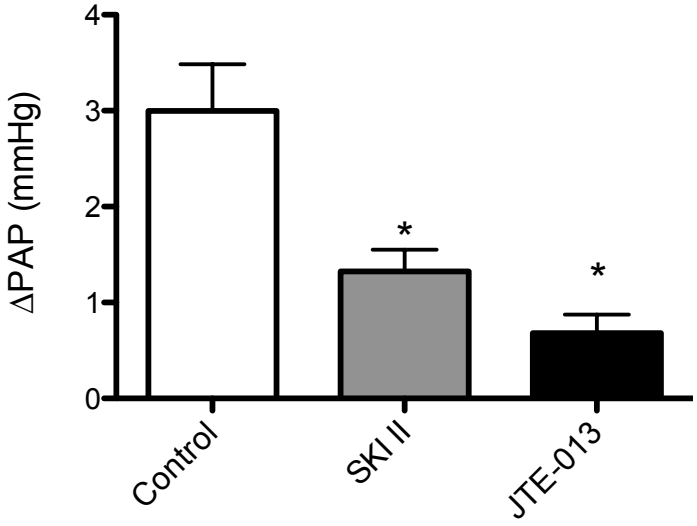


Figure 3.14 HPV requires S1P production and signaling through its receptors: hypoxia-induced PAP increase was attenuated by sphingosine kinase inhibitor (SKI II), and S1P receptor 2 antagonist (JTE-013). n=5 each

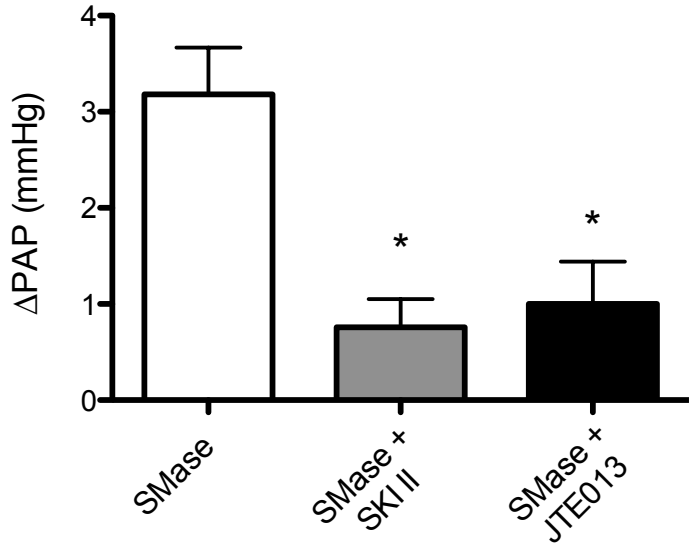
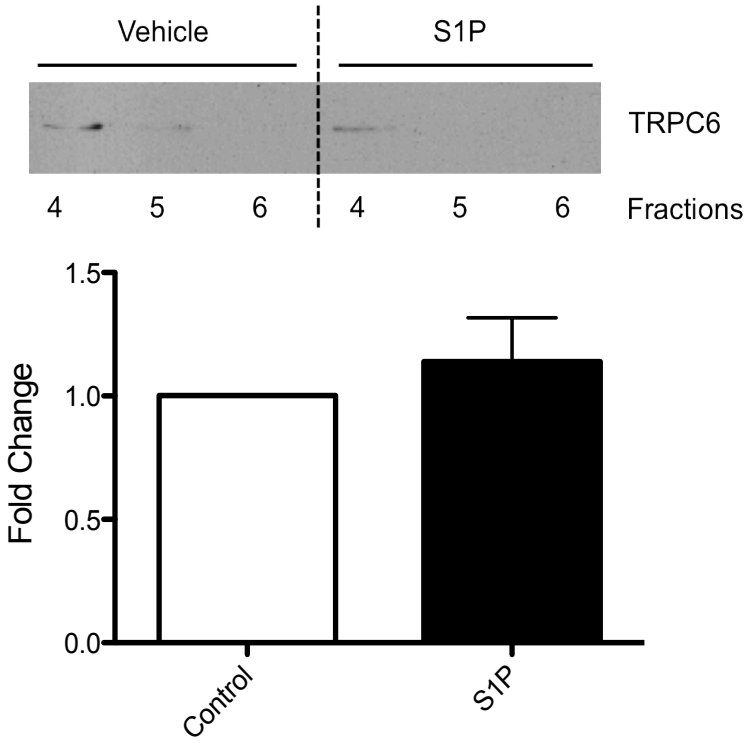


Figure 3.15 nSMase-induced pulmonary vasoconstriction requires S1P: nSMase-induced PAP increase was attenuated by sphingosine kinase inhibitor and S1P receptor 2 antagonist. n=5 each

A


Vehicle
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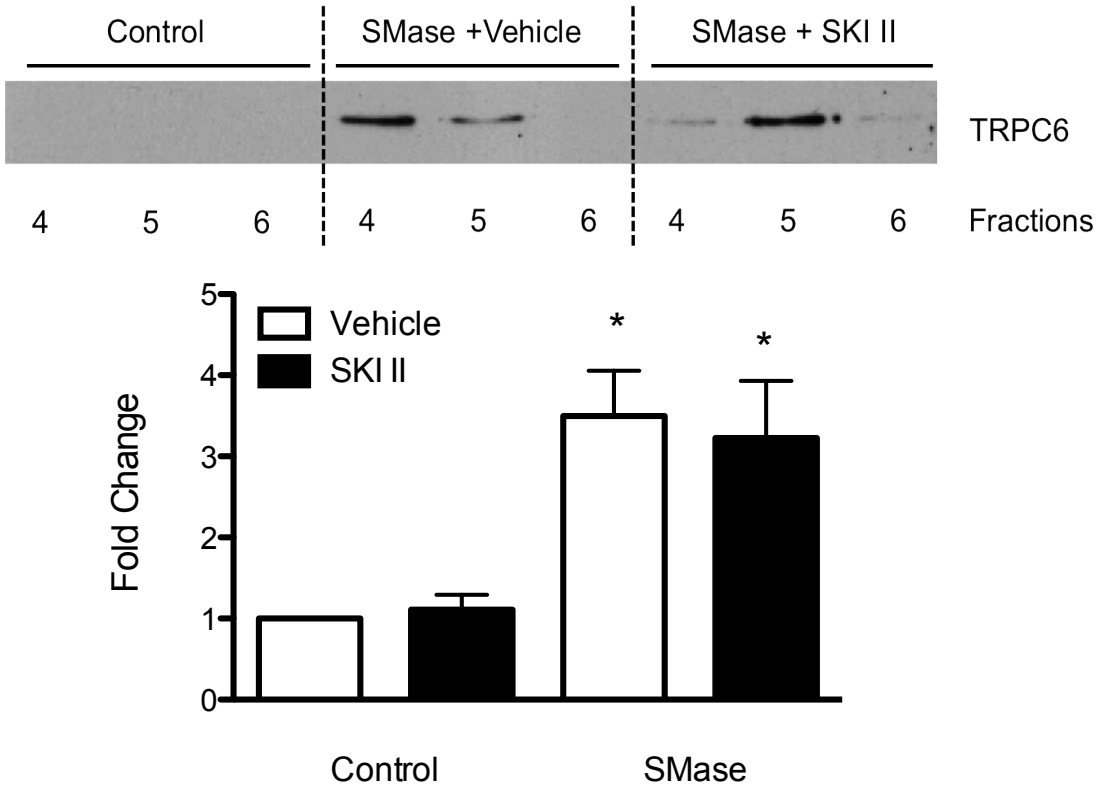
Control
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4
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Fold Change
Vehicle
SKI II
*
*
Control
SMase

Figure 3.16 S1P is not required for TRPC6 translocation: (A) S1P alone did not induce TRPC6 translocation to the caveolae. n=3 each (B) nSMase-induced TRPC6 translocation to the caveolae was not affected by sphingosine kinase inhibition. n=3 each *p<0.05 vs. vehicle control

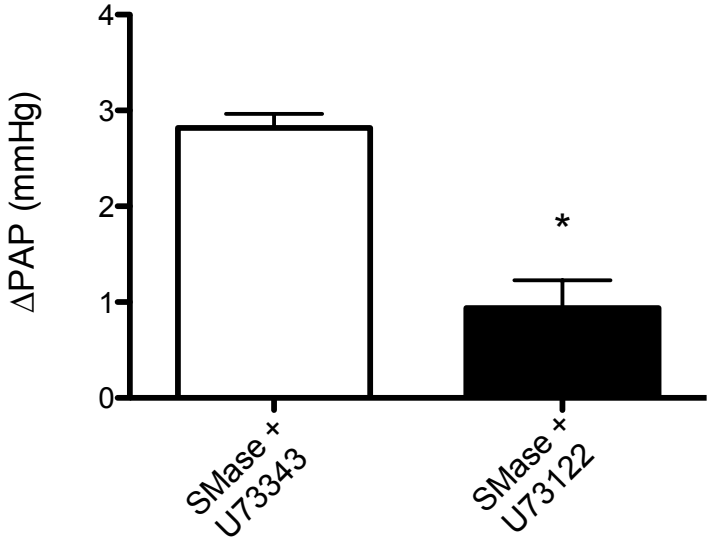

Figure 3.17 nSMase-induced pulmonary vasoconstriction requires PLC: phospholipase C inhibitor U73122 but not its inactive analogue U73343 attenuated the nSMase-induced PAP increase. n=5 each *p<0.05 vs. SMase+U73343

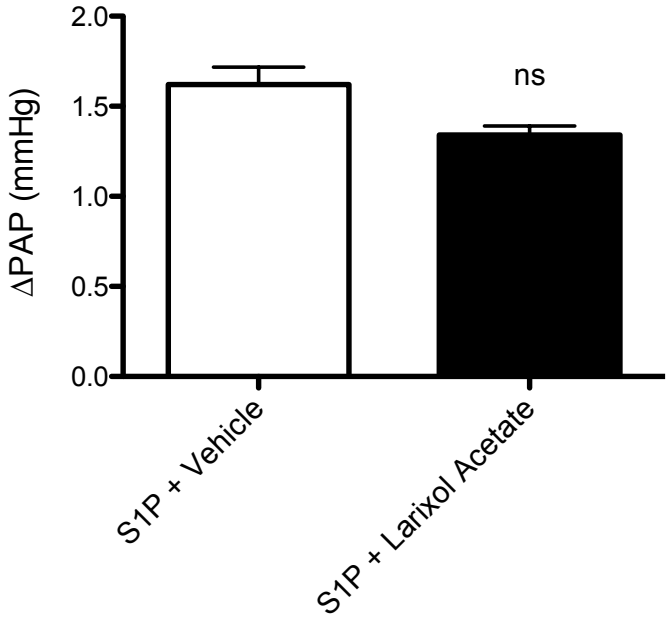

Figure 3.18 TRPC6 is not required for exogenous S1P-induced pulmonary vasoconstriction: S1P-induced pulmonary vasoconstriction was not blocked byTRPC6 inhibitor larixol acetate. n=5 each

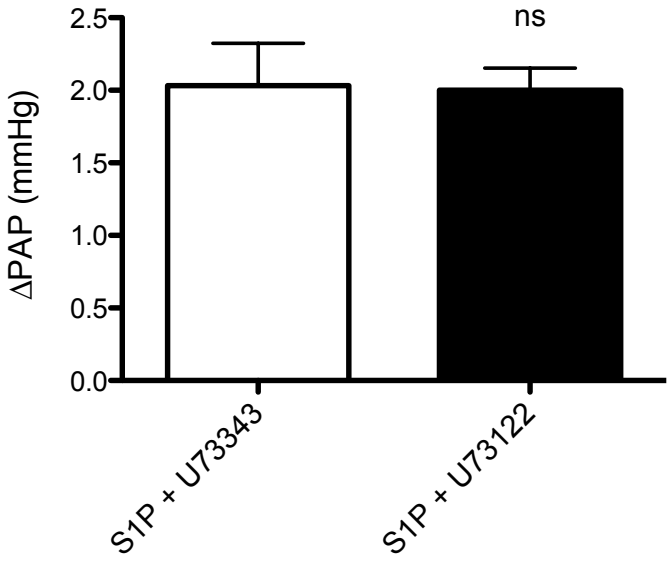

Figure 3.19 PLC is not required for exogenous S1P-induced pulmonary vasoconstriction: S1P-induced pulmonary vasoconstriction was not blocked by PLC inhibitor U73122. n=5 each

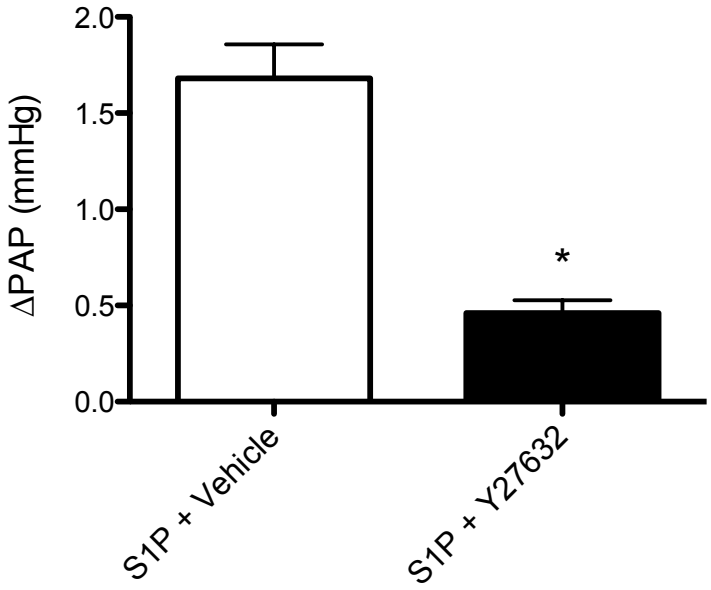

Figure 3.20 S1P-induced pulmonary vasoconstriction requires Rho-kinase signaling: Rho-kinase inhibitor Y27632 (10 μM) attenuated pulmonary vasoconstriction in response to exogenous S1P (10 μM). n=5 each *p<0.05 vs. S1P+Y27632

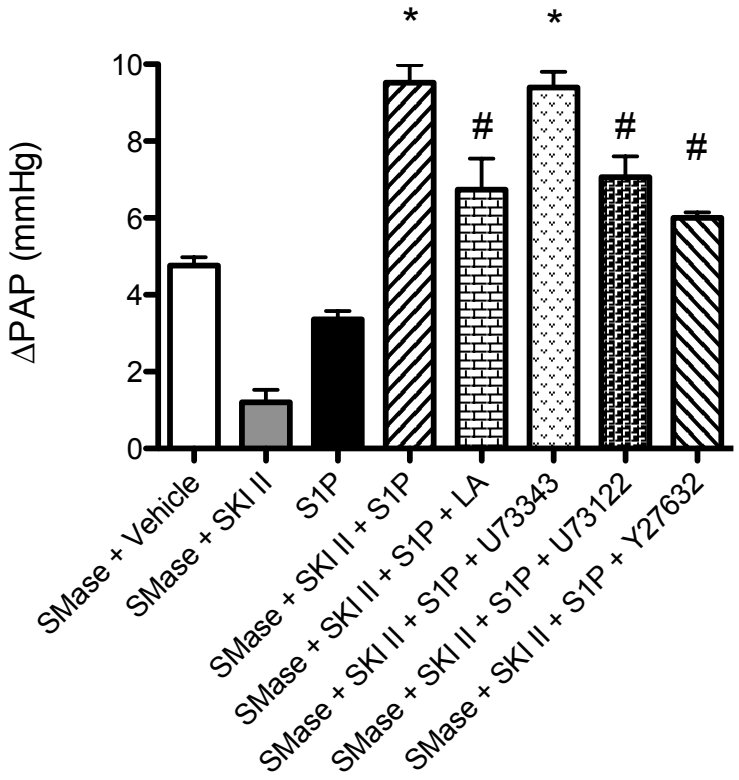

Figure 3.21 Synergistic effects of ceramide and S1P through TRPC6 and Rho-kinase activation: Exogenous S1P rescued the blunted SMase-induced pulmonary vasoconstriction caused by sphingosine kinase inhibition. Endogenous ceramide production coupled with exogenous S1P treatment revealed that TRPC6, PLC and Rho-kinase activities contribute to the synergistic ceramide and S1P signaling.

Inhibitors larixol acetate (LA), U73122 and Y27632 significantly decreased the ceramide- and S1P-induced pulmonary vasoconstriction.

n=5 each *$p<0.05$ vs. SMase + SKI II #$p<0.05$ vs. SMase + SKI II + S1P

3.3. Objective #3: Mechanism of CFTR regulation of TRPC6 activation in HPV

3.3.1. Investigate the mechanism of CFTR regulation of hypoxia and SMase-induced TRPC6 trafficking (Specific aim #1)

Recent study reported that CFTR and TRPC6 formed molecular complexes in the plasma membrane of epithelial cells, reciprocally regulating each other's channel activities[122,144]. CFTR may thus form similar complexes with TRPC6 in PASMC. Hypoxia may trigger the formation of CFTR-TRPC6 complexes in the caveolae that are functionally important for cation influx or channel stabilization. CFTR may either function as a molecular chaperone for TRPC6 translocation, or is required for membrane stabilization of TRPC6. We hypothesized that hypoxia induces the formation of CFTR-TRPC6 complexes in PASMC.

Co-immunoprecipitation experiments revealed under normoxic conditions, there is no CFTR-TRPC protein interaction in PASMC. After 15 min hypoxia (1% O_2) treatment, there is a clear and robust CFTR-TRPC6 interaction. This protein complex formation is prevented by pretreatment with CFTRinh-172. Considered together with previous findings that CFTR inhibition abolished TRPC6 translocation, these results suggest that CFTR-TRPC6 association may be critically important for TRPC6 localization in the caveolae during HPV, and CFTRinh-172 disrupts this association.

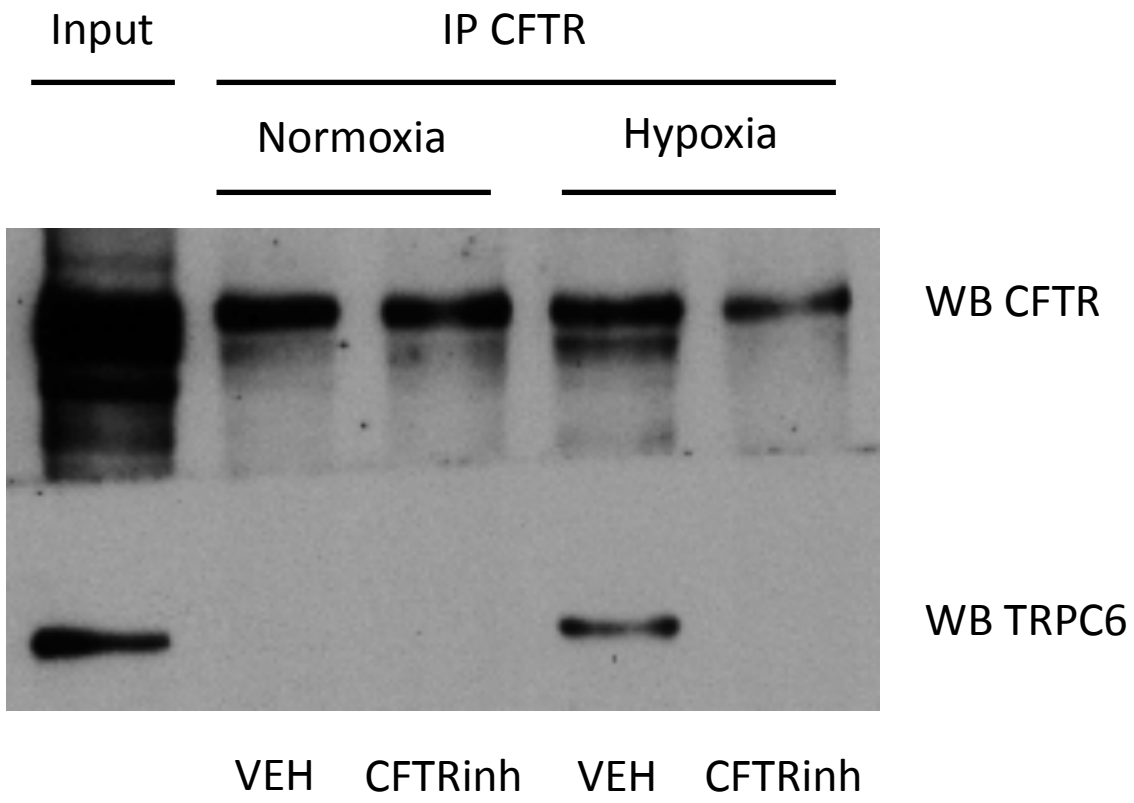

Figure 3.22 CFTR and TRPC6 interact under hypoxia: Immunoprecipitation for CFTR and subsequent western blotting for TRPC6 revealed the two proteins form complex upon hypoxia treatment (1% O_2). This protein interaction is abolished by CFTRinh-172 (10μM) pretreatment.

4. Chapter 4 Discussion

4.1. CFTR is required in HPV by mediating TRPC6 translocation

CF patients suffer from hypoxemia as a result of V/Q mismatches[13,14], therefore CFTR dysfunction may be linked to impaired HPV. In this study, we confirmed that CFTR is crucial for full HPV response in mice. Both CFTR inhibition and deficiency resulted in severely blunted HPV in isolated lungs. $CFTR^{-/-}$ mice suffered hypoxemia much more severe compared to WT mice when challenged with airway saline droplets. Moreover, CFTR inhibition or deficiency did not affect Ang II-induced pulmonary vasoconstriction. Clearly, CFTR functions have a direct influence on the regulation of pulmonary vascular pressor responses specifically to hypoxia. The role of CFTR in HPV in the intact lung is ultimately linked to the requirement for CFTR functions for Ca^{2+} response in PASMC during hypoxia, as CFTRinh-172 completely abolished the $[Ca^{2+}]_i$ increase in PASMC.

CFTR has been previously implicated in mediating endothelium-independent vasorelaxation in PASMC in response to cAMP[119], thereby regulating vascular tone in the lung. The Cl^- channel activity of CFTR is activated by cAMP/cGMP-dependent protein kinase phosphrylation[128,129]. Since cAMP and cGMP have been well characterized in mediating pulmonary vasorelaxation[119,132,133], it is unlikely that Cl^- conductance by CFTR contributes to membrane depolarization and vasoconstriction. This notion is further strengthened by the fact that removal of Cl^- from lung perfusate did not impact HPV in isolated lungs. Therefore CFTR is required in HPV independent of its Cl^- channel functions.

Increasing evidence show TRPC6 is critically important for Ca^{2+} response in acute HPV. Mechanisms of activation appear to be both increasing abundance of proteins by insertion into the caveolae[159], and a receptor-mediated DAG formation[90]. This study confirmed the translocation of TRPC6 to caveolae in PASMC is a principal mechanism of activation, and this translocation requires CFTR. For the first time, we have established a role for CFTR, as well as the mechanistic link between CFTR functions and HPV.

4.2. TRPC6 translocation during hypoxia requires nSMase

In agreement with previous findings, nSMase inhibition, but not aSMase inhibition attenuated HPV. We found that TRPC6 translocation to the caveolae also requires nSMase, and exogenous SMase was sufficient to induce pulmonary vasoconstriction (in a TRPC6-dependent manner), as well as TRPC6 translocation, suggesting ceramide production by nSMase recruits TRPC6 to caveolae. Yet this nSMase-induced translocation also requires CFTR functions, as inhibition of CFTR attenuates TRPC6 trafficking. We also demonstrated that although S1P is required for HPV, it is not need for TRPC6 recruitment.

Ceramide production at the plasma membrane is thought to promote the formation of large platforms by reorganizing small lipid rafts[195]. The formation of a larger raft “traps” and clusters surface proteins[196,197], which is believed to be important in initiating or amplifying a specific signal[195]. Although the exact

mechanisms are unknown, intact lipid raft domains are required for Ca^{2+} entry through TRPC6[198]. Lipid rafts are also required for the clustering of Ca^{2+} sensor stromal interaction molecule (STIM1) and TRPC channels, which regulate store-operated Ca^{2+} entry[199], as well as the heteromultimeric formation of TRPC channels[200].

TRPC6 has a caveolin 1 binding site[93,170], and is shown to co-localize with caveolin 1 in co-immunoprecipitation experiments[158], which is probably required for its insertion and stabilization in the caveolae. It is likely that SMase activation downstream of hypoxia releases ceramide, which promotes the formation of TRPC6-cav-1 complexes in the modified lipid rafts.

nSMase1 is found in the ER and golgi, while nSMase2 is primarily localized at the plasma membrane[201,202], therefore most likely to contribute to ceramide production at the cell surface. nSMase2 can be activated by oxidative stress[156,203-205], which is a probable mechanism during HPV. It can also be regulated by phospholipase A2 (PLA_2) activities and the formation of arachidonic acid (AA)[206,207]. PLA_2, arachidonic acids and its metabolites are implicated in HPV[208-210], AA metabolite 11,12-EET was shown to promote translocation of TRPC6 to caveolae by an unknown mechanism[159]. nSMase activation and resultant TRPC6 translocation may be implicated in these observations. Furthermore, the release of AAs and EETs by endothelial cells may constitute a mechanism by which the PAEC mediate PASMC contraction through nSMase activation, relating to the recent findings by our group (Wang *et al*.).

Regardless of the signal for activation of nSMase during hypoxia, we have clearly demonstrated a critical role for nSMase in HPV that is related to the recruitment of TRPC6 to caveolae, as well as downstream production of S1P, and signaling through S1P receptor.

4.3. S1P signaling in HPV

This study clearly identified a critical role for S1P signaling during HPV, downstream of nSMase activation. Inhibition of sphingosine phosphorylation by sphingosine kinase inhibition in hypoxia or SMase treatment severely blunted vasoconstriction, although S1P is not required for TRPC6 translocation to caveolae. These findings suggest that TRPC6 translocation alone is not sufficient to elicit vasoconstriction, and further signaling by S1P is required.

S1P has diverse biological effects due to the differential expression patterns and distinct signal transduction of its five receptors[142,164]. Enzymes that dictate the balance of sphingolipids also tightly regulate its synthesis, degradation, and localization. S1P levels increase upon nSMase activation[161], although we have not examined this pathway in PASMC. There is also evidence indicating sphingosine kinase may be activated by hypoxia[211] in vascular smooth muscle cells. S1P receptors $S1P_{1\text{-}4}$ are expressed in PASMC[165,212,213], $S1P_2$ and $S1P_4$ have reported vasoregulatory functions in the pulmonary vasculature[163,165]. Both $S1P_2$ and $S1P_4$ can activate PLC and Rho via $G_{q/11}$, G_i, and $G_{12/13}$ by $S1P_2$[141,142],

and G_i, and $G_{12/13}$ by $S1P_4$[214]. $S1P_2$ was reported to mediate Rho kinase-mediated vasoconstriction[163], and $S1P_4$ was reported to cause pulmonary vasoconstriction via PLC-dependent Ca^{2+} release and store-operated entry, and Rho kinase-mediated Ca^{2+} sensitization[165].

We hypothesized that $S1P_2$ activation of PLC is a major component in HPV by DAG synthesis and TRPC6 activation. In agreement with previous findings, using $S1P_2$ antagonist JTE-013, we identified a role for $S1P_2$ signaling through Rho kinase in eliciting pulmonary vasoconstriction. S1P alone did not induce TRPC6- or PLC-dependent vasoconstriction, and is probably explained by the fact that basal levels of caveolae-associated TRPC6 are very low. Accordingly, when TRPC6 channels are translocated by nSMase activation, the following S1P stimulation also activated these translocated TRPC6 channels via PLC activation and DAG synthesis.

There are probably additional effects of S1P receptor signaling - IP_3 accumulation downstream of PLC activation, which then activates IP_3 receptors and cause intracellular store release, and followed by store-operated Ca^{2+} entry. PKC activation by DAG accumulation and Ca^{2+} release may in turn lead to Ca^{2+} sensitization during HPV[85,215].

S1P can act in either autocrine or paracrine manner[216]. Isolated PASMC directly exposed to hypoxia respond by activating nSMase, resulting in ceramide production, and its subsequent conversion into S1P by ceramidase and

sphingosine kinase. S1P is released through ABCC1[217] to signal surface receptor. SK1 is the cytosolic isozyme, which upon activation translocate to membrane lipid rafts[218]. It is likely this lipid raft-associated form is responsible for the phosphorylation of sphingosine released from ceramide produced by membrane nSMase.

This study does not focus on the role of S1P released from endothelial cells, but endothelial expression of sphingosine kinase 1 (SK1) in lung endothelial cells far exceed SK1 levels in the PASMC[219]. We can speculate that S1P released by endothelial cells may constitute a substantial portion of total S1P produced during HPV. As endothelium-derived mediators are highly important in the mediation and maintenance of HPV, particularly an unknown mediator in phase 2 HPV is thought to induce Rho kinase-dependent Ca^{2+} sensitization[220]. S1P may fulfill the criterion for this pulmonary specific vasoconstrictor.

4.4. Mechanisms of TRPC6 regulation by CFTR

In this study, we first identified a role for CFTR in the nSMase-mediated TRPC6 translocation to caveolae, therefore establishing a critical link between CFTR functions and the mediation of HPV. CFTR inhibition attenuated the SMase-induced TRPC6 translocation, indicating that CFTR functions are either required for SMase activities, upstream of SMase activation, or that CFTR is somehow required for a critical step downstream of SMase activation.

Co-immunoprecipitation experiments indicate that CFTR increases physical interactions with TRPC6 during hypoxia, suggesting the formation of CFTR-TRPC6 complexes may be an important regulatory mechanism of TRPC6 channel localization and activities. Further, CFTRinh-172 is able to block hypoxia and SMase-induced vasoconstriction and TRPC6 translocation, presumably by inhibiting CFTR-TRPC6 interactions. It is yet unclear how CFTRinh-172 dissociates CFTR and TRPC6 during hypoxia. The exact mechanism of inhibition of CFTR by CFTRinh-172 is not known, although it is believed that CFTRinh-172 allosterically regulate CFTR channel activities by imparting conformational changes that cause a decrease in channel open probability[180,221]. The extent of this allosteric change is unclear, but it is possible that the inhibitor may change the conformation or cause an obstruction at the TRPC6 (or adaptor protein) binding site.

Antigny *et al.* showed in airway epithelial cells CFTR and TRPC6 regulate each other's ion transport within the same membrane complex[122]. Although the mechanism of this reciprocal coupling is not clear, perhaps the channel activity (or the conformation of pore/gate) of CFTR is required for its coupling with TRPC6. Regardless of the mechanism of CFTRinh-172 on the inhibition of CFTR-TRPC6 interaction, it is clear that it is capable of disassociating the two proteins and down-regulating TRPC6 trafficking to caveolae, and attenuating HPV.

The importance of the formation of CFTR-TRPC6 complex during hypoxia is also unclear. Since CFTR inhibition attenuates TRPC6 translocation, it is possible that CFTR may act as a chaperone protein, assisting the caveolar insertion of TRPC6. Further, the surface abundance of TRPC6 is not only regulated by channel insertion, but also by endocytic recycling through endosomal pathways, which are important for the intracellular trafficking of TRPC6[222]. Therefore, CFTR-TRPC6 interactions may thus increase TRPC6 insertion, or decrease TRPC6 endocytosis and degradation. Alternatively, the interaction may be required for the stabilization of TRPC6 in the plasma membrane, making CFTR an adaptor or anchoring protein for TRPC6. There may be some evidence supporting this notion.

PTEN-TRPC6 interactions are important for the stabilization of TRPC6 to the plasma membrane in endothelial cells[223]. It was proposed that PTEN serves as a membrane anchor to stabilize TRPC6, whose cell surface expression is required for channel activity[224]. The PDZ binding domain is crucial for the stabilization of PTEN at the plasma membrane, interestingly, PTEN lacking the PDZ binding domain also failed to interact with TRPC6. Since TRPC6 does not contain PDZ domain, this suggests there maybe an intermediate protein in the same complex containing the PDZ domain, facilitating PTEN-TRPC6 interactions[223].

Interestingly, CFTR protein also contains a PDZ binding domain, which is required for the functional expression of CFTR at the apical membrane[225,226], and also the stabilization of CFTR in the lipid raft[139]. Both PTEN and CFTR are

known to interact with the membrane anchor protein Na^+-H^+ exchanger regulatory factor 1 (NHERF1) via PDZ interaction[225,227,228].
Intriguingly, TRPC proteins contain a NHERF1-binding domain, which is important in the membrane localization of TRPC4 and TRPC5[229,230].

We speculate that CFTR may directly or indirectly via adapter proteins such as NHERF1, associate with TRPC6, thereby stabilizing the complex within the lipid raft domains of the plasma membrane. In essence, this would make CFTR an adaptor protein for TRPC6 stabilization in the plasma membrane during hypoxia.

4.5. Limitations and future directions

4.5.1. CFTR as a Cl^- channel

As discussed above, CFTR requires activation by cyclic nucleotides cAMP/cGMP, which are implicated in mediating pulmonary vasodilation[132,133], and therefore theoretically making the Cl^- transporting functions of CFTR unlikely in HPV. To further examine the role of Cl^- flux during HPV, we removed Cl^- ions from lung perfusate and found that it did not affect the magnitude of the vasopressor response to hypoxia. However, the removal of extracellular Cl^- would in fact facilitate its efflux from cells, and thus contribute to membrane depolarization. This experiment therefore did not conclusively exclude the role of CFTR as a Cl^- channel, but since the removal of Cl^- neither increased nor decreased HPV response, it shows that in our model of HPV, extracellular Cl^- have negligible contributions to vasoconstriction. Taken together with our

findings that CFTR regulates TRPC6 trafficking, it is clear that CFTR contributes to HPV in a manner not exclusively as the Cl^- transporter it is classically perceived.

4.5.2. Caveolae isolation

To study proteins associated with caveolae lipid rafts, we isolated lipid rafts using sucrose density gradient ultracentrifugation. Lipid raft membranes are typically defined as insoluble in cold, non-ionic detergents such as Brij 56, which due to their buoyancy, migrate to lighter density sucrose after centrifugation[231,232]. The detergent isolation method is sometimes thought to be inconsistent. Detergents are believed to solubilize some proteins weakly associated with rafts, as well as disrupting cytoskeletal-membrane associations, therefore may cause artefacts in results[233-235]. However, TRPC6 has a caveolin 1 biding site[93], and co-immunoprecipitates with cav-1[143]. Therefore we believe that TRPC6 association with caveolae is strong, and unlikely to be disrupted by detergent.

Furthermore, due to slight inconsistencies with each gradient preparation, there is variability in the raft protein and proteins associated with raft (i.e. TRPC6) expression in fractions 4-6 between experiments. However, in all experiments, only fractions 4, 5 and 6 were positive for caveolae marker cav-1, and it is usually most highly expressed in fraction 4. However, due to the above-mentioned minor variability, the expression of proteins in these three fractions may differ between experiments. However, TRPC6 protein presence always

corresponds with cav-1 expression in these fractions, and is never found in fractions where cav-1 is not expressed.

4.5.3. Mechanisms of CFTR-TRPC6 interactions

In this work, we identified a potential role for CFTR in regulating the membrane localization of TRPC6 in PASMC during HPV. The mechanisms of interaction are unclear, and whether CFTR-TRPC6 interaction actually promotes the recruitment or prevents the recycling of TRPC6 in caveolae is not known. There is also the possibility that other proteins are present in this complex, which are critical for the localization or function of these proteins. To this end, we will probe for CFTR-TRPC6 co-localizations in caveolae by immunofluorescence microscopy. Alternatively, we can perform FRET analysis of CFY- and YFP-tagged proteins, and identify relevant binding sites by targeted mutation analysis.

The mechanism by which CFTRinh-172 disrupts CFTR-TRPC6 signaling is unknown, and further studies are needed to clarify this mechanism. However, we here show that the inhibitor is sufficient to disrupt protein interaction, and can therefore inhibit TRPC6 translocation by this mechanism.

4.5.4. Role of CFTR in regulating sphingolipid levels in HPV

We showed CFTR bind to TRPC6 during hypoxia as a potential mechanism of regulating Ca^{2+} response. However, there is still a potential role for CFTR in

regulating ceramide and S1P levels in PASMC. Numerous studies showed CFTR dysfunction leads to an imbalance in sphingolipid homeostasis[120,135], and its localization in the lipid raft influences membrane ceramide composition[139,236]. To elucidate the role of CFTR in the hypoxia-induced sphingolipid formation, we can quantify levels of ceramide, sphingosine and S1P in lungs and PASMC by lipid chromatography-mass spectrometry[135], and probe for the role of CFTR by pharmacological inhibition and knockdown strategies.

Further, we hope to differentiate the role of endothelial cells and smooth muscle cells in sphingolipid generation during HPV. In the event that S1P is predominantly derived from the endothelium, the role of CFTR in S1P uptake into PASMC will be tested, and the intracellular role of S1P[216] in HPV could be another potentially crucial signaling pathway, as S1P can induce intracellular Ca^{2+} release by activating a calcium channel termed sphingolipid calcium release-mediating protein of the ER (SCaMPER)[237].

4.5.5. Role of S1P receptors in HPV

We have demonstrated a critical role for $S1P_2$ in mediating PLC-dependent TRPC6 activation, and Rho kinase-dependent Ca^{2+}-sensitization in PASMC during HPV. However, our data solely relies on the $S1P_2$ receptor antagonist JTE-013, whose specificity for $S1P_2$ has recently been called into question. JTE-013 was shown to block S1P signaling through $S1P_4$[238,239], a receptor that has been recently implicated in mediating S1P-induced pulmonary

vasoconstriction[165]. To address this issue, we will test HPV responses using $S1P_4$ specific antagonist[240], as well as in $S1P_2$-defiencent mice.

There may also be a heirarchial sequence of activation of TRPC6 and Rho kinase downstreamd of S1P receptor activation. Recent studies indicated TRPC6 activation by DAG, combined with the subsequent Ca^{2+} influx, jointly activate PKC, which inturn activates RhoA[241,242].

Thus, further studies should be conducted to investigate the underlying mechanisms of CFTR and sphingolipids in regulating TRPC6 and HPV.

4.6. Summary

This work established a critical role for CFTR and sphingolipids in HPV. nSMase is activated in PASMC in response to hypoxia. Ceramide is released from sphingomyelin, which alters membrane composition, and promotes the recruitment of TRPC6 to caveolin 1-rich lipid raft domains. The stabilization of TRPC6 in the caveolae is likely dependent on its interactions with CFTR, as they form complexes under hypoxia, and CFTR inhibition or deficiency disables the protein complex formation, abolishes TRPC6 translocation to caveolae and HPV. Ceramide released from sphingomyelin is further broken down into sphingosine by ceramidase, and sphingosine is phosphorylated by sphingosine kinase to form S1P. S1P then acts on its extracellular receptor $S1P_2$ in an autocrine or paracrine manner, which activates PLC to release DAG, triggering cation influx

through TRPC6. Concomitant Rho kinase activation by $S1P_2$ inhibits myosin light chain phosphatase (MLCP). Therefore, Ca^{2+} and Rho signaling synergistically promote actin-myosin interaction, eliciting smooth muscle contraction.

This work will hopefully advance the understanding of one of many pathways contributing to HPV, and establish a mechanistic link for hypoxemia in CF patients. Additional studies are warranted to provide translational benefits for treating HPV impairments associated with CFTR dysfunction, as well as chronic hypoxia-induced pulmonary hypertension.

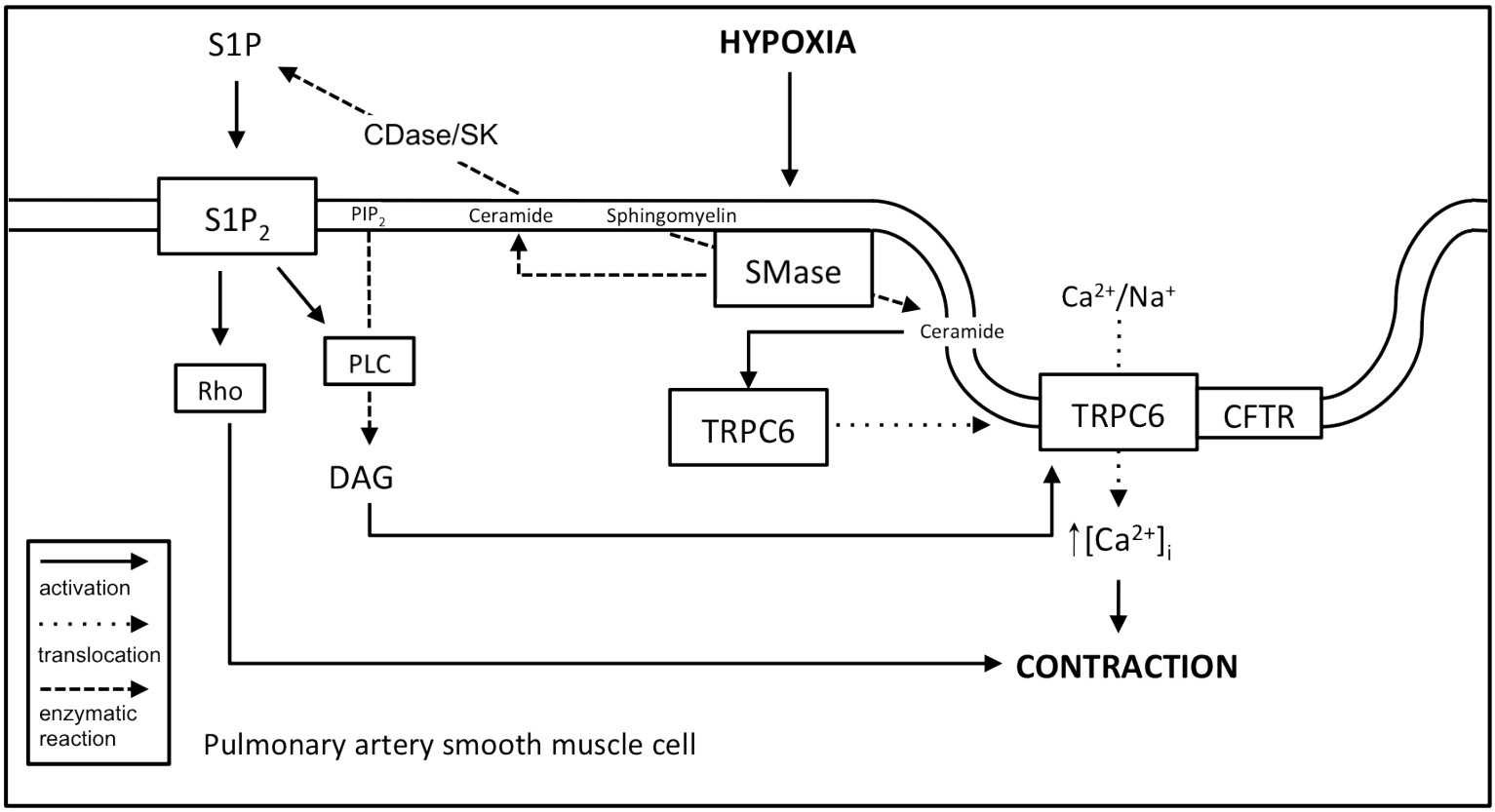

Figure 4.1 – Schematic overview of CFTR and sphingolipids in TRPC6 signaling and HPV

5. Chapter 5 Materials and Methods

5.1. Animals

Male C57BL/6 (25-30g) (The Jackson Laboratory, Bar Harbor, ME) were used for all isolated and perfused lung experiments unless otherwise specified. CFTR-KO mice (*Cftr*^{tm1Unc} Tg(FABPCFTR)1Jaw/J) weighing between 25-30g were purchased from The Jackson Laboratory. All animals were housed at the St. Michael's Hospital Li Ka Shing Knowledge Institute Animal Vivarium. All animal experiments were approved by the hospital Animal Care and Use Committee.

5.2. Isolated, perfused and ventilated mouse lungs

Male mice (body weight 20–35 g) were anaesthetized by intraperitoneal injection of ketamine/xylazine (200/10 mg/kg body weight) and placed in a water-jacketed chamber heated to 37°C (Isolated perfused lung size I; Hugo Sachs Elektronik, March-Hugstetten, Germany). After tracheostomy and intubation, the mice were ventilated using positive pressure volume-controlled ventilation (MiniVent 845; Hugo- Sachs Elektronik, March-Hugstetten, Germany) with a tidal volume of 10 ml/kg body weight, a positive end-expiratory pressure of 2 cm H_2O, and a respiratory rate of 90 breaths/min. The normoxic gas mixture consisted of 21% O_2, 5% CO_2, and 74% N_2 (Praxair, Hamilton, ON). Following a laparotomy, the diaphragm was removed and the lungs were exposed by a right parasternal thoracotomy. The animals were heparinized and subsequently exsanguinated by

a cut through the inferior vena cava. After placement of a ligature around the ascending aorta and the main pulmonary artery, a stainless steel cannula (1 mm inner diameter) was inserted into the pulmonary artery via the right ventricle, and fixed by suture ligation. Another stainless steel cannula (1 mm inner diameter) was inserted into the left atrium *via* the apex of the left ventricle across the mitral valve to drain pulmonary venous efflux. Lungs were perfused at a constant flow rate of 50 ml/kg/min using a peristaltic pump (Ismatec Laboratoriumstechnik GmbH, Wertheim-Monfeld, Germany), the left atrial pressure was held constant at 2 mmHg throughout the experiment.

Pulmonary artery pressure (PAP) and left atrial pressure (LAP) were measured using saline-filled membrane pressure transducers (Hugo Sachs Elektronik, March-Hugstetten, Germany) connected to a side port of the inflow and outflow cannula, respectively. Data were recorded using PULMODYN® Pulmonary Mechanics Data Acquisition Software (Hugo Sachs Elektronik, March-Hugstetten, Germany), the system was calibrated before each experiment.

The perfusate consisted of Hank's Balanced Salt Solution (Sigma-Aldrich, St. Louis, MO) with 5% bovine serum albumin (Calbiochem, Merck KGaA, Darmstadt, Germany) and 5% dextran (Sigma-Aldrich, St. Louis MO) to prevent pulmonary edema[243]. 30 µM Indomethacin (Sigma-Aldrich, St. Louis MO) and 1 mM *N_ω-Nitro-L-arginine methyl ester* (Sigma-Aldrich, St. Louis MO) were added to inhibit endogenous prostaglandin and nitric oxide synthesis, respectively[243].

The oxygen partial pressure of the perfusate was measured before and immediately after each experiment during normoxic ventilation and was found to range between 120 and 155 mm Hg. Previous study showed that the oxygen partial pressure within this range does not affect hypoxic pulmonary vasoconstriction[244]. Lungs were only included in this study if perfusion pressure was stable and below 10 mm Hg during the initial 10-min baseline perfusion period and if lungs had no signs of hemostasis, atelectasis, or edema.

5.3. *In vivo* regional hypoventilation

Mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg bw) and xylazine (10mg/kg bw), tracheotomized, intubated and ventilated with room air (tidal volume of 10 mL/kg bw, 90 breaths/min). A catheter was inserted into the left carotid artery. Ventilation-perfusion mismatch was induced by intratracheal instillation of 25 µl of saline (Baxter, Deerfield, IL) causing partial occlusion of the larger airways. Arterial blood samples were obtained at baseline as well as 2 and 10 min after tracheal saline instillation, and blood gas analyses were performed (RapidLab 348; Chiron Diagnostics GmbH, Fernwald, Germany).

5.4. Cell culture

Primary human pulmonary artery smooth muscle cells (PASMC) were purchased from Lonza (Clonetics™ PASMC, Lonza, Basel, Switzerland) and cultured according to instructions. Culture media kits (SmGM™-2 BulletKit™, CC-3182) and subculture supplies including HEPES Buffered Saline Solution (CC-5024),

Trypsin/EDTA Solution (CC-5012) and Trypsin Neutralizing Solution (CC-5002) were all purchased from Lonza. All cells were used within the first ten passages of growth.

5.5. Hypoxia treatment of cells in culture

Hypoxia treatment of PASMC was performed in a temperature controlled hypoxia chamber (37°C, 1% O_2). Pre-equilibration of hypoxic culture media (pO_2: ~10 mmHg) was achieved in a hypoxic cell incubator (37°C, 1% O_2, 5% CO_2) and was applied to the cells inside the hypoxia chamber for 15 minutes and rinsed twice with ice-cold hypoxic PBS (pO_2: ~10 mmHg). Cell lysis procedures were all carried out inside the hypoxia chamber according to experimental protocol. Hypoxia treatment for sucrose density gradient ultracentrifugation and co-immunoprecipitation experiments were all carried out as described.

5.6. Caveolae isolation by sucrose density gradient ultracentrifugation

Sucrose solutions were made by dissolving sucrose (Sigma-Aldrich, St. Louis, MO) in TKM buffer (50 mM Tris, 20 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA. pH 7.4), to give final concentrations of 5%, 30% and 80% sucrose solutions (w/v). The sucrose solutions were prepared at room temperature on the same day then chilled to ice-cold prior to use. PASMC were grown on 100-mm culture dishes; four confluent plates were combined and used for each raft preparation. Cells

were washed twice with ice cold PBS and 1 mL of ice-cold 0.5% (w/v) Brij 56 (Sigma-Aldrich, St. Louis, MO) lysis buffer containing protease inhibitors sodium vanadate; PMSF and Complete Mini inhibitor cocktail (Roche Applied Science, Penzberg, Germany) were added to the cells. After 1 minute in lysis buffer on ice, the cells were scraped and lysis buffer was transferred sequentially from one plate to the next for all for plates in order to concentrate lysate and limit the total volume to 1mL. The cell lysate was next transferred to an ice-cold loose-fitting Dounce homogenizer and subject to 10 strokes with the homogenizer, and mixed on a rotating platform at 4°C for 20 min.

After lysis, 800 µL of the cell homogenate was transferred to the bottom of a 5 mL ultracentrifugation tube (Beckman Instruments, Brea, CA). An equal volume of 80% sucrose was added and mixed with the homogenate to yield a 40% sucrose solution. 1.60 mL of 30% sucrose was then slowly layered on top of the bottom 40% sucrose and lysate to avoid any mixing. Finally, 1.60 mL of 5% sucrose was slowly layered on top of the 30% sucrose solution. There should be a total of 4.80 mL of solution in a 5 mL centrifuge tube, and make sure that it is almost full to the top the tube, as too much free space may cause the tube to collapse when rotating at high speeds. The tube was then placed in a pre-chilled swing-out rotor (Model SW55Ti, Beckman Instruments, Brea, CA) and centrifuged at 4°C at 180,000 G for 18 hours in an ultracentrifuge (Model L8M, Beckman Instruments, Brea, CA)

After centrifugation, 10 equal-volume fractions were collected from the tube and probed for caveolae markers cav-1 and flotillin-1 by western blot. Fractions containing caveolae were identified to be fractions 4, 5, and 6. These fractions were further probed for proteins of interest. Total protein concentrations from each fraction were quantified using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) and 5 µg of protein from each fraction were loaded onto 10% SDS polyacrylamide gels and analyzed by western blot.

5.7. Immunoprecipitation

PASMC grown on 100 mm dish were lysed in 1 mL of RIPA buffer containing protease inhibitors and homogenized by passing through a 30-gauge needle several times. Cell lysates were rotated for 10 min in 4°C to ensure protein solubilization. Supernatant was supplemented with 3 mL NET buffer (50 mM Tris- HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.05% Nonidet P-40), and pre-cleared for 1 h at 4°C. After pre-clearance, 2 mg of CFTR antibody (monoclonal anti- human CFTR antibody, IgG_{2A} clone # 24–1, R&D Systems, Minneapolis, MN) was added to supernatant and incubated over night at 4°C. 3 mg of protein A/G-sepharose beads (Thermo Fisher Scientific, Waltham, MA) was added to samples and incubated on a rotating platform for 1 h at 4°C to precipitate immune complexes. Bead-bound complexes was then washed 3 times with ice-cold NET buffer, and denatured by boiling 5 min in Laemmli buffer. Samples were separated on SDS-PAGE (8% polyacrylamide gel) and analyzed by western blot.

5.8. Western blot

Protein concentration for each sample was determined by the bicinchoninic acid (BCA) method using the Pierce® BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA). Samples were loaded on a polyacrylamide gel and separated by electrophoresis. Nitrocellulose membranes were blocked for 1 h at room temperature in tris-buffered saline (TBS) and 0.1% (v/v) Tween-20 containing 5% (w/v) skim milk. Membranes were subsequently incubated overnight at 4°C in blocking solution containing primary antibodies (see list of antibodies below). Membranes are washed 3 times with TBS containing 0.1% Tween-20 and incubated with HRP-linked secondary antibody for 2 h at room temperature. After washing 3 times, bands were detected using a Pierce ECL chemiluminescence kit (Thermo Fisher Scientific, Waltham, MA). The density of the band of interest was measured and normalized to a control protein.

5.9. Ca^{2+} imaging in PASMC

50 µg Fura-2 AM (Life Technologies, Carlsbad, CA) dissolved in 30 µL Pluronic F-127 (20%) solution in DMSO (Life Technologies, Carlsbad, CA). 5 µM Fura-2 AM was applied to PASMC grown on coverslips (22 mm x 22 mm) in a six-well cell culture plate containing growth medium. After 45 minutes incubation at 37°C, the coverslip was loaded onto a recording chamber (Warner Instruments, Hamden, CT) heated to 37°C with temperature controller (Warner Instruments, Hamden, CT). The cells were subsequently washed with Hank's Balanced Salt

Solution (Sigma-Aldrich, St. Louis, MO) for 15 minutes at a rate of 0.5 mL/min. A baseline reading was obtained under normoxic HBSS (pO_2: ~150 mmHg) perfusion for 10 min. The cells subsequently underwent hypoxia treatment by perfusion with hypoxic HBSS (pO_2: ~10 mmHg) for 20 min. Fluorescence emission was collected in 10 s intervals through an upright intravital microscope (Axiotech^{Vario} 100 HD; Zeiss, Jena, Germany) equipped with an apochromat objective (UAPO 40x W2/340; Olympus, Hamburg, Germany) and appropriate dichroic and emission filters (Zeiss, Jena, Germany) by a CCD camera (Sensicam; PCO, Kelheim, Germany) and subjected to digital image analysis (TILLvisION 4.01; T.I.L.L. Photonics). Fluorescence images were recorded at excitation wavelengths of λ=340, 360 and 380 nm, and emission of λ=510.

Normoxic and hypoxic HBSS solutions were prepared by bubbling in parafilm-sealed bottles with gas mixtures of 21% O_2, 5% CO_2, balanced N_2 and 1% O_2, 5% CO_2, balanced N_2 respectively, and heated in a 37°C water bath. The heated and gas-equilibrated HBSS was driven by a peristaltic pump (Ismatec Laboratoriumstechnik GmbH, Wertheim-Monfeld, Germany) through the imaging chamber at a rate of 0.5 mL/min.

5.10. hTRPC6-YFP transient transfection

Human TRPC6-YFP cDNA constructs were kindly provided by Dr. Alexander Dietrich, Univ. of Munich.

PASMC were cultured on 22mm x 22mm coverslips in six-well cell culture plates and grown to 30 – 40% confluency prior to transfection. For transfection of each well, 1 µg cDNA was added to 2.5 µL of FuGENE HD (Roche Applied Science, Penzberg, Germany) in 200 µL Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA) at room temperature and incubated for 15 minutes at room temperature. The cDNA-FuGENE-DMEM mixture was added drop wise to cells. Experiments were carried out 48 hours post transfection. Transfection efficiency was approximately 30%.

5.11. List of antibodies

Primary antibodies

Caveolin 1 (BD Transduction Laboratories, Franklin Lakes, NJ)

Source: mouse monoclonal

Concentration: 1: 500

CFTR (R&D Systems, Minneapolis, MN)

Source: mouse monoclonal

Concentration: 1: 200

Flotillin-1 (Cell Signaling Technology, Beverly, MA)

Source: rabbit polyclonal

Concentration: 1: 1000

TRPC6 (Alomone Labs, Jerusalem, Israel)

Source: rabbit polyclonal

Concentration: 1: 200

Secondary antibodies

Goat anti-mouse, HRP-conjugated (Bio-Rad, Hercules, CA)

Concentration: 1: 5000

Donkey anti-rabbit, HRP-conjugated (GE Healthcare, Little Chalfont, UK)

Concentration: 1: 5000

5.12. Data and statistical analysis

All data are expressed as mean±SEM, where n is the number of independent experiments. Nonparametric Mann-Whitney U-test was used for the comparison of two groups. For multiple group comparisons, a nonparametric 1-way ANOVA (Kruskal-Wallis) test was performed. If there was significant difference between test groups, Mann-Whitney U-test was utilized to test for differences between individual groups. Differences were considered significant if $P<0.05$. Statistical analysis was performed using Prism 5 (Graphpad).

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