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CHOLESTEROL REGULATION OF PULMONARY ENDOTHELIAL CALCIUM ENTRY FOLLOWING CHRONIC HYPOXIA

Bojun Zhang
University of New Mexico

Bojun Zhang

Candidate

Cell Biology and Physiology

Department

This dissertation is approved, and it is acceptable in quality and form for publication:

Approved by the Dissertation Committee:

Thomas C. Resta Ph.D., Chairperson

Benjimen R. Walker Ph.D.

Nikki L. Jernigan Ph.D.

William S. Garver Ph.D.

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BY

Bojun Zhang

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DISSERTATION

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DEDICATION

Dedicated to my beloved mom who carried me all the way and gave me all her love.

I really miss you.

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B.S.

Ph.D.

ABSTRACT

Chronic hypoxia (CH)-induced pulmonary hypertension (PH) is associated with diminished ATP-induced endothelial Ca^{2+} entry as well as membrane cholesterol in pulmonary arteries. Store-operated Ca^{2+} entry (SOCE) and depolarization-induced Ca^{2+} entry are major components of the response to ATP and are similarly decreased after CH. Because endothelium-dependent vasodilation is closely associated with pulmonary endothelial $[Ca^{2+}]_i$, the blunted agonist-induced Ca^{2+} influx in pulmonary artery endothelial cells (PAEC) may contribute to the development of CH-induced PH. Interestingly, impaired agonist-induced Ca^{2+} influx in PAEC following CH can be restored by membrane cholesterol supplementation. In the current studies, we hypothesized that impaired Ca^{2+} entry in PAEC following CH is due to decreased membrane cholesterol.

We demonstrated that substitution of cholesterol with its functionally inactive epimer epicholesterol, greatly attenuated ATP-induced Ca^{2+} influx in PAEC from control rats. Whereas epicholesterol similarly blunted endothelial SOCE in PAEC from both groups, cholesterol supplementation restored diminished SOCE in PAEC from CH rats while having no effect in controls. Similar effects of cholesterol manipulation on T-type Ca^{2+}

channel-mediated Ca^{2+} influx were observed in PAEC. Additionally, the role of cholesterol in SOCE mediated by Orai1, a Ca^{2+} selective ion channel, was examined in PAEC. Whereas cholesterol restored endothelial SOCE in CH rats, both epicholesterol and the Orai1 inhibitor, AnCoA4, attenuated SOCE only in normoxic controls. The Orai1 inhibitor had no further effect in cells pretreated with epicholesterol. In cultured pulmonary endothelial cells, using pharmacological inhibition and siRNA knockdown of Orai1, we found that epicholesterol, AnCoA4 and Orai1 siRNA each inhibited SOCE compared to their respective controls. Epicholesterol had no additional effect following knockdown of Orai1. Finally, we found that endothelial stromal interaction molecule 1 (STIM1)-Orai1 interaction, which is essential for SOCE, requires membrane cholesterol. Our studies support a novel regulatory role for membrane cholesterol in agonist-induced Ca^{2+} entry and its components. Our observation also demonstrated that altered membrane cholesterol homeostasis may contribute to impaired endothelial Ca^{2+} influx pathways following CH.

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CHAPTER 1: Introduction

Mechanisms of chronic hypoxia-induced pulmonary hypertension

Pulmonary hypertension (PH) is the elevation of blood pressure in the pulmonary circulation and is a leading cause of morbidity and mortality in patients with a number of cardiovascular and respiratory diseases. Human PH is clinically defined as a mean arterial pressure ≥ 25 mmHg at rest (74). Multiple factors may lead to the development of PH, including genetic defects, disease, or environmental exposure. Interestingly, persistent or intermittent hypoxia is highly associated with many forms of PH, such as in chronic obstructive pulmonary disease, interstitial lung disease, and obstructive sleep apnea (65, 96, 138). The chronic hypoxia (CH)-induced PH animal model is one of the most commonly used to mimic human disease (165).

Under normal conditions, the pulmonary circulation is a low-resistance and low-pressure system. These characteristics allow blood to easily travel from the right ventricle through the lungs for gas exchange. Pulmonary vascular resistance (R) can be calculated as the ratio of the pressure gradient (ΔP) across all vessels to flow (Q):

$$R = \Delta P / Q$$

Thus, increased pulmonary arterial resistance would lead to elevated pulmonary arterial pressure that must be overcome by the right heart to pump blood through pulmonary circulation. There are various factors that can influence vascular resistance. Assuming a vascular system consists of straight, non-distensible cylindrical tubes that have laminar flow, then vascular resistance R of a single tube is equal to the product of the length of the tube (l) and viscosity (η) and a constant divided by the product of π and the fourth power of the internal radius:

$$R = (l * \eta * 8) / (\pi * r^4)$$

This is known as the Hagen-Poiseuille's law. Under pathological conditions such as prolonged exposure to hypoxia, however, there are functional and structural changes in the pulmonary circulation, including polycythemia, acute hypoxia-induced vasoconstriction of small pulmonary arteries, and pulmonary arterial remodeling with medial hypertrophy (2, 60). According to Hagen-Poiseuille's law, all of these changes lead to increase in pulmonary vascular resistance.

Polycythemia, an increase in the volume percentage of circulating red bloods cells in whole blood, results in increased in blood viscosity, thereby increasing vascular resistance. Chronic exposure to hypoxia may activate hypoxia inducible factor (HIF)-1α which induces erythropoiesis that leads to the polycythemic response (34). CH-induced active pulmonary vasoconstriction and arterial remodeling are the consequences of several complex physiological mechanisms. One major cause of these changes to the pulmonary vasculature is pulmonary arterial endothelial dysfunction, which is usually observed in the development of PH (10; 35). The dysfunction of endothelial cells may be triggered by multiple sources: shear stress, inflammation, hypoxia, and other unknown factors. This impaired endothelial function is generally believed to result in imbalanced production of various vasoactive factors (20, 49, 153). The increased production of vasoconstrictors with decreased production of vasodilators is common in most forms of PH. Although the pulmonary endothelium plays a major role in PH, the mechanisms by which impaired endothelial function leads to the development of PH are not well defined. Understanding the mechanisms of endothelial dysfunction leading to and contributing to PH is central to designing effective and specific treatment for these pathologies.

Endothelium in the pulmonary circulation

The vascular endothelium is the innermost layer of blood vessels and serves as a semi-selective barrier that allows exchange of fluid and macromolecules between the circulation and surrounding tissues. As a major part of the vascular system, the endothelium plays a crucial role in several physiological activities, such as maintaining tissue-fluid homeostasis, regulating angiogenesis and vascular tone, and preventing inflammation (54, 75, 107, 125, 139). In the pulmonary circulation, endothelial cells help maintain low resistance and prevent proliferation/migration of smooth muscle cells.

Pulmonary endothelial cells regulate vascular tone by the balanced production of various vasoactive compounds. Vascular tone reflects the result of smooth muscle contraction due to phosphorylation of myosin light chain (MLC). MLC phosphorylation is generally regulated by Ca^{2+}-dependent and -independent pathways: activation of myosin light chain kinase via Ca^{2+}/calmodulin or inhibition of myosin light chain phosphatase through activated small GTPase RhoA and Rho-associated protein kinase (15). Endothelium can produce vasoconstrictors like endothelin-1 and thromboxane that act as paracrine factors to stimulate G protein-coupled receptors and cause Ca^{2+} influx and subsequent contraction of smooth muscle cells (37, 131, 160). On the other hand, the pulmonary endothelium also releases vasodilators such as nitric oxide and prostacyclin in response to shear stress and agonist stimulation (52, 68). These vasodilators decrease both intracellular Ca^{2+} levels and Ca^{2+} sensitivity of smooth muscle cells, thereby causing vasorelaxation (86, 93).

NO is the most well studied vasodilator and plays an important role in maintaining low resistance in the pulmonary circulation (137). NO causes vasodilation primarily through activation of soluble guanylyl cyclase (cGC) in pulmonary artery smooth muscle cells (PASMC) (93), which leads to the production of cGMP from GTP. cGMP then activates protein kinase G (PKG). Intracellular calcium levels ($[Ca^{2+}]_i$) are crucial for myosin light chain (MLC) phosphorylation and contraction in PASMC. PKG activation causes smooth muscle relaxation primarily through lowering $[Ca^{2+}]_i$ via various mechanisms including inhibition of IP_3 receptors (129), activation of sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) (93, 123), or activation of plasma membrane Ca^{2+}-ATPase (113). Stimulated PKG may also lead to activation of Ca^{2+} -dependent K^+ channels (BK_{Ca}) and PASMC hyperpolarization (5, 40, 46, 167), which inhibits voltage-gated Ca^{2+} channel (VGCC) -mediated Ca^{2+} influx and promotes MLC dephosphorylation and subsequent vasorelaxation. cGMP may also inhibit calcium channels like TRPCs and VGCC thereby causing vasorelaxation. PKG activation also leads to vasorelaxation through Ca^{2+}-independent mechanisms. Studies suggest that PKG activates MLC phosphatase, which inhibits MLC phosphorylation and subsequent contraction (78, 97). PKG may also phosphorylate RhoA, thereby inhibiting RhoA-mediated Ca^{2+} sensitization (16, 17, 127). In addition, NO could directly activate BK_{Ca} and potentially cause smooth muscle relaxation independent of PKG (15). Besides its role in regulating vascular tone, endothelium-derived NO is also known to inhibit smooth muscle cell (SMC) proliferation and migration (62, 126), which is important in preventing vascular remodeling in responses to stimuli such as inflammation, oxidative stress and apoptosis.

Production of many endothelium-dependent vasoactive substances as well as regulation of membrane potential are largely a function of pulmonary endothelial $[Ca^{2+}]_i$ (1). The production of NO, for example, is catalyzed by endothelial nitric oxide synthase (eNOS) in the endothelium. Activation of eNOS requires increased endothelial $[Ca^{2+}]_i$ and formation of a Ca^{2+}/calmodulin complex (89). Along with other cofactors like NADPH and BH4, eNOS converts L-arginine into NO and L-citrulline (26, 89). Similarly, production of endothelium-derived prostacyclin requires intracellular Ca^{2+} and calmodulin (89, 130). The activation of cytosolic phospholipase A2, the key enzyme to release arachidonic acid from plasma membrane, is Ca^{2+}-dependent. Once arachidonic acid is liberated, it could be enzymatically converted by cyclooxygenase-1 to prostacyclin after multiple reactions. Arachidonic acid is also substrate for production of vasoconstrictor metabolites, such as thromboxanes, leukotrienes, and epoxyeicosatrienoic acids.

Regulation of endothelial Ca^{2+} levels

Endothelial Ca^{2+} is important second messenger in various signaling cascades and is carefully regulated by complex and dynamic pathways. Endothelial $[Ca^{2+}]_i$ reflects the sum of Ca^{2+} influx from extracellular space, Ca^{2+} release and sequestration by intracellular stores, and Ca^{2+} extrusion from the cell. Endothelial Ca^{2+} influx is mainly mediated by three mechanisms: 1) Ca^{2+} entry via receptor-mediated cation channels; 2) Ca^{2+} leak through cation channels down its electrochemical gradient; and 3) Ca^{2+} entry via stretch-activated cation channels. Steady-state $[Ca^{2+}]_i$ may be rapidly altered by stimuli such as shear stress or agonists, which activate membrane-bound receptors and cation channels and leads to Ca^{2+} entry (3, 161). This major Ca^{2+} influx signaling cascade also generates the

second messenger, IP_3, which can activate downstream ionotropic IP_3 receptors on the membrane of the ER, causing rapid and transient release of internal Ca^{2+} stores. Once ER Ca^{2+} stores are depleted, the ATPase pump refills the intracellular Ca^{2+} pool by sequestering Ca^{2+} from the cytosol, which may also be facilitated by a Ca^{2+} leak through cation channels on the plasma membrane (1). Meanwhile, the Na^{+}- Ca^{2+} exchanger on the plasma membrane helps remove excessive intracellular Ca^{2+} after agonist stimulation (23, 39, 122). Overall, these pathways work together to maintain $[Ca^{2+}]_i$ homeostasis in endothelial cells.

Agonist-induced Ca^{2+} entry

Receptor-mediated Ca^{2+} influx is one of the major pathways leading to increased $[Ca^{2+}]_i$. The binding of an agonist to its receptor mediates the activation of phospholipase C (PLC), which then leads to the production of diacylglycerol and IP_3 from hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (11). Cytosolic IP_3 then binds to IP_3 receptors on the membrane of the ER and causes Ca^{2+} efflux from the ER store. The depletion of ER stores triggers Ca^{2+} influx across the plasma membrane, which is known as store-operated Ca^{2+} entry (SOCE) (150). Agonist binding may also elicit Ca^{2+} entry through channels that are not activated by store emptying. This is known as receptor-operated calcium entry (ROCE) (120).

ATP, as one of the important agonists that meditates endothelial $[Ca^{2+}]_i$, serves as a regulator of vascular tone. Studies found that red blood cells (RBCs) and endothelial cells (ECs) can release ATP under certain conditions to maintain blood flow. In RBCs, when acutely exposed to low P_{O2}, low pH, or mechanical deformation, there is an increase of

ATP release (38, 92, 136). ATP then causes endothelium-dependent vasorelaxation in various species and tissues (25). It is likely that RBC- produced ATP binds to purinergic receptors on ECs and stimulates NO production (84). In endothelial cells, shear stress induces the production of ATP, which serves as an autocrine factor and stimulates endothelial NO production (4, 69). It is proposed that extracellular ATP binds to P_2Y purinergic receptors on plasma membrane of ECs, which leads to IP_3 production, internal Ca^{2+} store release, Ca^{2+} influx and subsequent increase in $[Ca^{2+}]_i$, leading to increased eNOS activation and NO production (50). Additionally, ATP may also enhance endothelial cell barrier function by Rac and cortactin-dependent activation of the cytoskeleton (59).

Store-operated Ca^{2+} entry

SOCE is a Ca^{2+} influx response when internal SR/ER Ca^{2+} stores are depleted. Under physiological conditions, agonists may bind to transmembrane receptors and activate PLC to produce IP_3 from hydrolysis of PIP_2. IP_3 then activates IP_3 receptors on membrane of SR/ER, leading to Ca^{2+} efflux, depletion of intracellular Ca^{2+} stores, and activation of cation channels on the plasma membrane (150). SOCE was first proposed in 1986 by Putney and colleagues and was later found in many cell types. In rat parotid acinar cells, the intracellular Ca^{2+} store depletion by muscarinic-cholinergic agonists causes Ca^{2+} influx (144). If the intracellular Ca^{2+} store is depleted independent of activation of PLC, Ca^{2+} influx is still observed (143). Later in mast cells, in response to IP_3 -induced Ca^{2+} store depletion, a highly Ca^{2+} selective inwardly rectifying current was found (57). This store-operated current (I_{soc}) with a high Ca^{2+} selectivity is known as calcium release -activated calcium (CRAC) current or I_{crac}.

SOCE has been demonstrated to be present in virtually all excitable and non-excitable cells and is involved in mediating many important physiological activities such as neurotransmitter secretion, skeletal muscle contraction, lymphocyte activation, and vascular tone (41, 79, 110, 146). Although the physiological importance of SOCE has been recognized for some time, the molecular mechanism of SOCE remained unclear until recent years. A key finding was the identification of stromal interacting molecule (STIM) proteins on the membrane of the ER. STIM1, as one isoform of STIM, was first found in a library screen which was developed to identify molecules in stromal cells that bind to pre-B lymphocytes (98). However, the role of STIM as the molecular component of SOCE was revealed more recently through RNA interference (RNAi) screens by two independent groups (83, 119). In *Drosophila* S2 cells, RNAi-mediated knockdown of STIM causes significantly reduction of Ca^{2+} entry induced by the SERCA inhibitor, thapsigargin (TG) (119). Similar observation that STIM1 and STIM2 are required for agonist- and TG-induced SOCE was found in human HeLa cell (83). Later, continued genome-wide RNAi screens in Drosophila S2 cells revealed Orai proteins as key components of the CRAC channel (111, 156, 164). The presence of STIM1 and Orai1 are sufficient to reconstitute functional SOCE in HEK293 cells (134).

The exploration for other potential components of SOCE also identified the involvement of non-selective Ca^{2+} -permeable mammalian transient receptor potential canocical (TRPC) channels (155). All TRPC channels are activated via the PLC signaling pathway (155). However, only TRPC1, TRPC3, and TRPC4 are activated mostly by store depletion, whereas the activation TPRC2, TRPC5, TRPC6, and TRPC7 is mainly through store-independent mechanisms (155). The finding that STIM1 also gates TPRC1 in SOCE

(58) generates a new question in regard to the role of TRPC1 and Orai1 in SOCE. Later studies then demonstrated that activation of TRPC1/STIM1-mediated SOCE requires functional Orai1 (67, 73). In PAEC, TRPC1 and TRPC4 also form heteromultimeric channels and interact with Orai1 via protein 4.1 in caveolin-rich membrane fractions (32). This TRPC1/4 complex and Orai1 interaction is important for channel activation and calcium selectivity (32). With the identification of these key components of SOCE, the molecular mechanism of SOCE is better understood (27). The EF-hand motif in the N terminus of STIM1 is localized in the ER lumen and senses ER Ca^{2+} stores. Upon the depletion of the ER Ca^{2+} store, the SAM domain near the EF-hand motif mediates STIM1 oligomerization and leads to translocation of STIM1 to the plasma membrane. This conformational change of STIM1 allows its interaction with Orai1 and forms ER-PM junctions, which are crucial in Ca^{2+} release-activated Ca^{2+} influx.

SOCE in both SMC and ECs is important in regulating vascular tone. In vascular SMC, Ca^{2+} influx induced by SR Ca^{2+} store depletion via SR Ca^{2+} -ATPase inhibition is associated with increased vascular tone in different blood vessels (79). Interestingly, SOCE-mediated vascular tone shows varying sensitivity to voltage-gated Ca^{2+} channel inhibitors between vascular beds. For example, most of the SOCE -induced contraction is nifedipine-sensitive in the rat aorta, which is not found in the rat pulmonary artery (79). These observations suggest that SOCE may modulate vascular tone in a direct or indirect manner. Many studies also indicate that increased $[Ca^{2+}]_i$ through SOCE contributes to smooth muscle proliferation and migration (13, 108, 141, 142). In cultured proliferative migratory rat aortic VSMCs, both SOCE and expression of STIM1 and Orai1 are elevated compared with non-proliferative controls (108). Knocking down STIM1 and Orai1 greatly

inhibits proliferation and migration of these active VSMCs (108). Besides its role in mediating vascular endothelial permeability (147), endothelial SOCE, as part of agonist-induced Ca^{2+} entry, significantly contributes to the increase in $[Ca^{2+}]_i$ and subsequent release of vasodilators like NO and prostacyclin (1, 70, 77, 82). In fact, pharmacological inhibition of SOC by SKF-96365 blunts the shear stress-induced NO response (4). Endothelial TRPC4 knockdown also impairs agonist-induced vasodilation by reducing SOCE (44).

Depolarization-induced Ca^{2+} entry

VGCCs are a group of Ca^{2+} permeable channels that are activated by depolarized membrane potential. Based on the requirement of a different level of depolarization for activation, these channels can be classified into two major groups, high voltage-activated (L-, P/Q-, N-, R- type) and low voltage-activated (T-type) calcium channels (150). These channels have been found in neurons, muscle, endocrine cells, and many other cell types (29, 145, 150, 162). They are involved in many different physiological activities including muscle contraction, excitation of neurons, upregulation of gene expression, release of hormones or neurotransmitters (28).

L-type VGCC were first described in neurons and cardiac cells and later found widely expressed in all excitable and some non-excitable cells (152). Ca^{2+} influx through L-type VGCC is the major source of Ca^{2+} entry in cardiac, skeletal, and smooth muscles. In cardiac muscle, activation of L-type VGCC is required to initiate Ca^{2+}-induced Ca^{2+} release and subsequent muscle contraction (14). In skeletal muscle, although L-type VGCC are not involved in the initiation of muscle contraction, they are important in mediating

Ca^{2+} release from intracellular stores, which then facilitates mobilization of the myofilaments and contraction (117). In vascular smooth muscle cells, Ca^{2+} influx through L-type VGCC also serves as one of the Ca^{2+} sources for Ca^{2+}/calmodulin-dependent phosphorylation of MLC, which leads to smooth muscle contraction. Indeed, L-type VGCC are important in mediating blood pressure and myogenic tone (35, 92).

Although L-type VGCC are considered the major VGCC that regulate the myogenic response, T-type VGCC in vascular smooth muscle cells are receiving more attention in recent years. In small resistance vessels, T-type VGCC are involved in regulating vascular tone (154). T-type VGCC are also involved in the development of CH-induced PH. Either chronic treatment of a T-type VGCC inhibitor or global deletion of the T-type VGCC gene ($Ca_v3.1$) protects mice from developing CH-induced PH (30).

T-type VGCC are normally found expressed in electrically excitable cells. However, their expression has also been described in pulmonary microvascular endothelial cells and PAEC from rats and mice (101, 157, 159, 166). In PMVECs, Wu et al (159) observed voltage-dependent currents with a more depolarized window current when compared to typical T-type channels. These currents were sensitive to pharmacological T-type channel blockers. Wang et al (157) found that acute hypoxia induced membrane depolarization and a subsequent increase of endothelial $[Ca^{2+}]_i$ in both alveolar capillaries and upstream arterioles. The endothelial $[Ca^{2+}]_i$ response to acute hypoxia was inhibited by the T-type channel blocker mibefradil in both pulmonary capillaries and arterioles. They proposed that membrane depolarization induced by acute hypoxia in lung capillaries is conducted upstream to arterioles via gap junctions and activates T-type channels. Increased endothelial $[Ca^{2+}]_i$ then leads to vasoconstriction by activation of Ca^{2+}-dependent

phospholipase A2-mediated vasoconstrictor production. Although T-type VGCC are not found in cultured rat PAEC (159), their expression and function have been described in freshly dispersed rat PACEs (101). Paffett et al. (101) have shown that T-type VGCCs contribute to depolarization-induced Ca^{2+} entry and ATP-induced Ca^{2+} entry. These findings suggest that T-type VGCCs are important mediators of depolarization-induced Ca^{2+} entry in the pulmonary endothelium.

Impaired pulmonary endothelial Ca^{2+} influx following CH

Impaired NO release is often observed in diseases such as chronic obstructive pulmonary disease and congestive heart failure and is a key feature of pulmonary hypertension (8, 90, 115, 140, 148). However, eNOS protein expression is increased in pulmonary hypertensive patients and CH-induced pulmonary hypertensive rats (48, 51, 116). The controversial observation that increased eNOS protein expression and impaired endothelium-derived vasorelaxation simultaneously occur in CH-induced PH rats could be the result of blunted eNOS activity. Murata et al confirmed this assumption by reporting that CH impairs posttranslational regulation of eNOS activity via blunting agonist-induced Ca^{2+} influx (95).

Previous work from our laboratory also shows that both basal $[Ca^{2+}]_i$ and agonist-induced Ca^{2+} influx are lower in PAEC from CH rats compared to those of control animals (99, 100). CH similarly inhibits endothelial SOCE and depolarization-induced Ca^{2+} influx through T-type VGCC, which are major components of agonist-induced Ca^{2+} entry in isolated PAEC (99, 101). These findings suggest that store-operated channels and T-type VGCC are important mediators of ATP-induced Ca^{2+} entry and that impaired Ca^{2+} entry and may contribute to reduced basal $[Ca^{2+}]_i$ in PAEC after CH, which may lead to

dysfunction of posttranslational eNOS regulation and subsequent impairment of endothelium-derived NO production in CH-induced PH.

Membrane cholesterol

Cholesterol is a major component of the plasma membrane. The importance of membrane cholesterol has been shown in regulating neurotransmission, cell signaling, and protein sorting (85, 128, 132). Additionally, many diseases such as type II diabetes (31), Alzheimer's disease (6), and cancer (91) are associated with abnormal cellular cholesterol levels. The homeostasis of cellular cholesterol levels is maintained mainly by four pathways: cellular cholesterol synthesis, cholesterol uptake from extracellular sources, cholesterol efflux, and esterification and storage in the lipid droplets (31). The ER is the major site of cholesterol *de novo* synthesis, which requires multiple enzymes and cofactors to covert acetyl-CoA to cholesterol in a complex series of reactions. Once synthesized, cholesterol is bound with caveolins and transported from the ER to plasma membrane. More than 90% of free cholesterol is located in the plasma membrane (66, 76) and is distributed to microdomains known as lipid rafts, which are rich in cholesterol, sphingolipids, and various proteins. Once in the plasma membrane, cholesterol maintains membrane integrity and regulates many signaling pathways.

Membrane cholesterol also serves as an important structural component of the plasma membrane by regulating membrane fluidity and permeability. Cholesterol may help maintains membrane fluidity by interfering with the interactions between phospholipid fatty acid chains. However, cholesterol may also increase membrane rigidity by reducing the flexibility of phospholipid fatty acid chains (55). This regulation of membrane fluidity

is crucial in controlling solute movement across the membrane when membrane composition or temperature are altered. In addition, positive electrostatic potential in the plasma membrane is increased when cholesterol content is augmented, which may change the membrane composition of charged components and alter the permeability of cations and anions (55). Membrane cholesterol is also required for the formation of specific lipid rafts known as caveolae. Caveolae are flask-shape invaginations of the plasma membrane that contain caveolin, cholesterol, sphingolipid, and various signaling molecules and receptors. They are involved in endocytosis, cholesterol transport, and signal transduction (42, 106). Besides caveolins, which serve as the structural component of caveolae (36, 112, 114), cholesterol is also essential in maintaining stable and functional caveolae. Both inhibition of cholesterol synthesis and acute membrane cholesterol depletion lead to caveolar disruption and inhibition of cell signaling (64, 94, 105, 124, 168).

Membrane cholesterol regulates ion channel function

Besides its structural role, membrane cholesterol is also important in regulating membrane-bound protein structure and function. Cholesterol is a polycyclic amphipathic molecule with a polar section consisting of a single β-hydroxyl group which can interact with membrane lipids or proteins through formation of hydrogen bonds (105). Various membrane-bound proteins have been shown to be regulated by membrane cholesterol, such as receptors, transporters, peptides, and ion channels (56, 80, 102, 151, 163).

The effect of membrane cholesterol on ion channel function may vary depending on the type of ion channel. Many studies demonstrate an inhibitory role of membrane cholesterol on ion channel activity via decreased open probability, unitary conductance,

and the number of active channels (80). For example, cholesterol decreases the open probability of many K^+ channels, as well as voltage-gated Na^+ and Ca^{2+} channels (33, 81, 149). In contrast, other ion channels such as nicotinic acetylcholine receptor, $GABA_A$ receptors, epithelial Na^+ channels and TRPC channels are inhibited by removal of membrane cholesterol (7, 9, 10, 72, 135), indicating that cholesterol may have an essential functional role in regulating these channels.

Cholesterol may additionally modulate the function of membrane proteins via either direct interaction or through altering the properties of lipid microdomains (80). Direct interaction between sterols and ion channels has been suggested by the sensitivity of inwardly rectifying potassium (K_{ir}) channels (118) and large conductance, Ca^{2+}-activated potassium (BK_{Ca}) channels (24) to various sterol analogues. In addition, cholesterol binding regions exist in both K_{ir} channels and BK_{Ca} channels (121, 133). Meanwhile, cholesterol may also indirectly regulate ion channels by modulating bilayer stiffness and hydrophobic interaction between the membrane proteins and the lipid bilayer (9). Thus, when determining the role of membrane cholesterol on channel function, extreme caution is needed to discriminate the different mechanisms by which cholesterol regulates certain types of ion channels. The enantiomer of cholesterol, epicholesterol, differs from cholesterol only in the stereochemistry of the hydroxyl group and has similar effects on membrane fluidity and lipid domain formation as those of cholesterol, but lacks the regulatory influences of cholesterol on membrane proteins (80). Thus epicholesterol has been used as a tool to distinguish the direct and indirect effect of cholesterol (80).

Membrane cholesterol regulates Ca^{2+} influx

The importance of membrane cholesterol in regulating Ca^{2+} entry has been shown in many different types of cells. In VSMCs, membrane cholesterol depletion by methyl-β-cyclodextrin reduces TRPC1-mediated Ca^{2+} entry in response to endothelin-1 (9). Similarly, cholesterol depletion decreases STIM1 clustering, prevents activation of TRPC1, and blunts SOCE in HSG and HEK293 cells (9). In addition, depletion of membrane cholesterol also disrupts the association of Orai1 with TRPC1 and STIM1, and attenuates SOCE in human platelets (61). This observation is also confirmed in lymphocytes, in which a reduction of membrane cholesterol decreases SOCE. Cholesterol enrichment, on the other hand, increases agonist-induced Ca^{2+} influx in cultured VSMCs (12).

Under pathological conditions, the importance of membrane cholesterol in regulating endothelial Ca^{2+} influx has also been revealed. Our laboratory's previous studies show that both membrane cholesterol levels and ATP- induced Ca^{2+} entry are reduced in PAEC following CH (100). Interestingly, cholesterol repletion restores CH-impaired endothelial ATP- induced Ca^{2+} influx (100), suggesting that membrane cholesterol may facilitate endothelial agonist-induced Ca^{2+} entry. In PAEC, ATP induces both SOCE and T-type VGCC blocker-sensitive Ca^{2+} influx (101). Although the role of membrane cholesterol in regulating SOCE has been implicated in many studies (10, 47), the effect of membrane cholesterol in regulation of endothelial depolarization-induced Ca^{2+} entry is unknown. Considering that SOCE and depolarization-induced Ca^{2+} entry are major components of ATP-induced Ca^{2+} entry, and that both Ca^{2+} influx pathways are blunted

following CH (99, 101), it is possible that membrane cholesterol also regulates these two components of the ATP-induced Ca^{2+} response.

Rationale and specific aims

As summarized above, endothelial dysfunction is closely associated with many forms of PH. In CH-induced PH, for example, increased pulmonary vascular resistance and myogenic tone are observed with decreased production of endothelial vasodilatory factors (21, 63, 95, 158). $[Ca^{2+}]_i$ is a key regulator of the synthesis of many endothelial vasoactive factors. CH decreases $[Ca^{2+}]_i$ by impairing Ca^{2+} influx in pulmonary artery endothelium (99–101). Diminished endothelial Ca^{2+} entry may contribute to elevated vascular tone by limiting the activity of eNOS and other Ca^{2+} -sensitive vasodilatory pathways (26, 43, 53, 87, 88, 95). Many Ca^{2+} channels are localized in cholesterol-enriched lipid rafts (19, 45, 94, 104). Cellular cholesterol homeostasis affects membrane lipid content and thus the function of Ca^{2+} channels and subsequent signaling pathways (10, 18, 22, 103, 109) by mechanisms that are not fully understood.

There are multiple Ca^{2+} entry pathways that could directly affect intracellular Ca^{2+} levels in the endothelium. Agonist-induced calcium entry, as one of the major Ca^{2+} entry pathways, has several components including SOCE and receptor-operated Ca^{2+} entry (ROCE). Studies from our laboratory have shown that both endothelial SOCE and ROCE are reduced in CH-induced PH (99, 101). Our previous work also identified a membrane potential-sensitive Ca^{2+} entry pathway, which contributes to ROCE under control conditions, but is lacking in CH-induced PH (101). This depolarization-induced Ca^{2+} entry appears to involve T-type VGCCs which are expressed mesenteric and cerebral

endothelium but not reported in endothelium from other systemic vascular beds (71). Additionally, CH leads to diminished membrane cholesterol that can be restored by cholesterol supplementation (100). Interestingly, impaired agonist-induced Ca^{2+} entry is also rescued by cholesterol restoration (100). However, it is not clear if membrane cholesterol affects different Ca^{2+} entry pathways similarly. Furthermore, it is unknown whether the effect of membrane cholesterol on Ca^{2+} entry is due to altered interaction of channels with cholesterol directly or with other factors. Understanding the effect of CH on cholesterol homeostasis and its impact on endothelial function may lead to new treatment strategies for PH. Therefore, the proposed studies will test the *central hypothesis* (Figure 1) that **impaired Ca^{2+} entry in pulmonary artery endothelial cells following chronic hypoxia is due to decreased membrane cholesterol**.

To test this hypothesis, we addressed the following Specific Aims:

Specific Aim 1: Determine the role of membrane cholesterol homeostasis in impaired pulmonary endothelial Ca^{2+} entry following CH (Figure 2).

We hypothesized that 1) *membrane cholesterol facilitates SOCE and depolarization-induced Ca^{2+} entry in PAEC*; and 2) *reduced endothelial Ca^{2+} influx following CH is due to loss of membrane cholesterol*. To test these hypotheses, we administered cholesterol or epicholesterol to acutely isolated PAEC from control and CH (4 wk, 380 Torr) rats to either supplement or replace native cholesterol, respectively. The efficacy of membrane cholesterol manipulation was confirmed by filipin staining. Ca^{2+} influx in PAEC was measured in response to ATP, store-depletion, or a depolarizing stimulus. Additional experiments examined the effect of cholesterol manipulation on endothelial caveolar number in cultured pulmonary endothelial cells.

Specific Aim 2: Identify the contribution of membrane cholesterol to regulation of Orai1-mediated SOCE in pulmonary endothelial cells (Figure 3).

We hypothesized that *cholesterol facilitates the interaction of Orai1 with STIM1 to mediate SOCE in PAEC*. Experiments in this aim assessed the role of cholesterol in Orai1-mediated SOCE using CH exposure in rats as a physiological stimulus to decrease PAEC cholesterol. Effects of Orai1 inhibition with AnCoA4 on SOCE were examined in isolated PAEC sheets from control and CH rats following cholesterol supplementation, epicholesterol substitution, or vehicle treatment. We further determined the role of cholesterol in Orai1-mediated SOCE by measuring SOCE in cholesterol-manipulated cultured pulmonary endothelial cells following either pharmacological inhibition of Orai1 or Orai1 siRNA knockdown. In addition, the effect of cholesterol manipulation on STIM1-Orai1 interaction was assessed by proximity ligation assay.

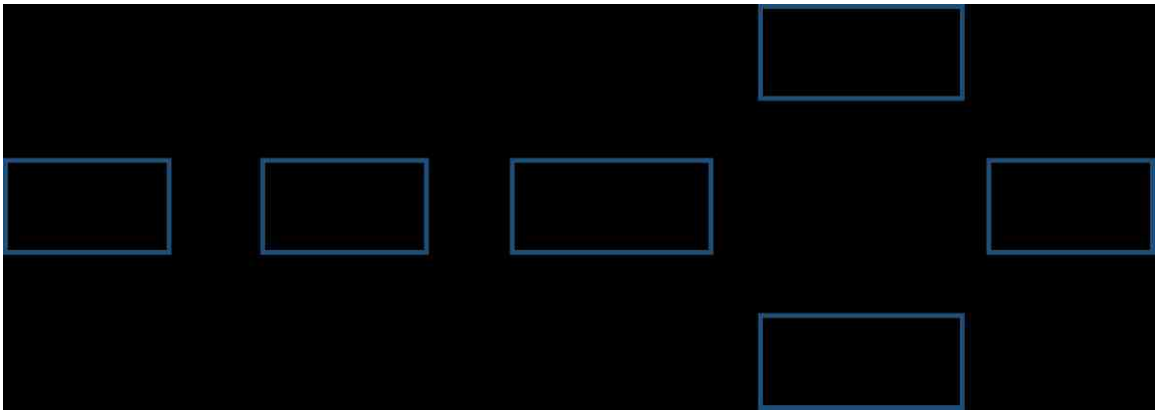

Figure 1: Schematic representation of the central hypothesis. We hypothesized that chronic hypoxia (CH) reduces membrane cholesterol levels in pulmonary artery endothelial cells and thus impairs agonist-induced Ca^{2+} entry and its components, store-operated Ca^{2+} entry and depolarization-induced Ca^{2+} entry. Changes of these Ca^{2+} entry pathways lead to reduced $[Ca^{2+}]_i$ in pulmonary artery endothelial cells following CH.

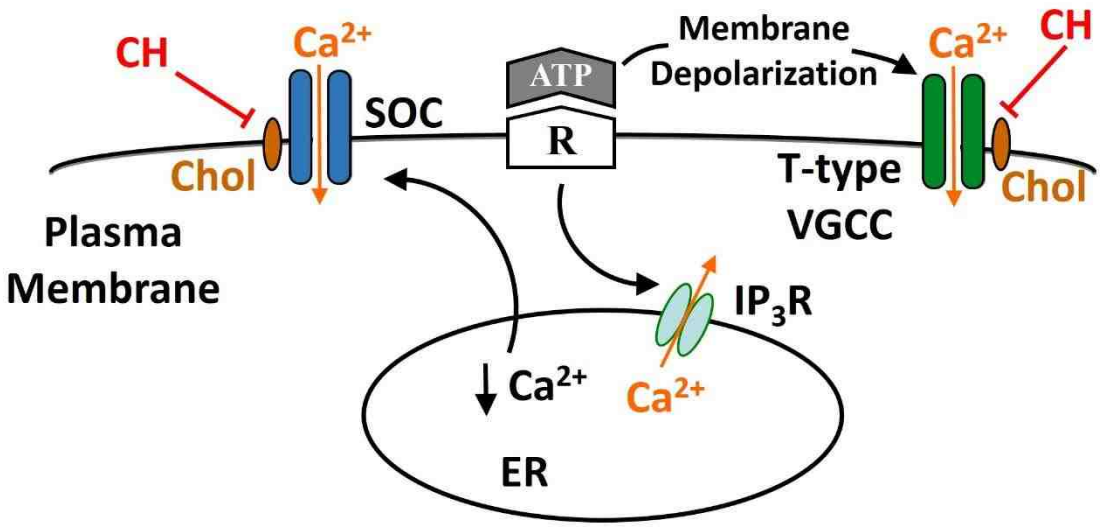

Figure 2: Schematic representation of Aim 1. Cholesterol (Chol), endoplasmic reticulum (ER), inositol trisphosphate receptor (IP_3R), store-operated Ca^{2+} channel (SOC), T-type voltage gated calcium channel (T-type VGCC), receptor (R).

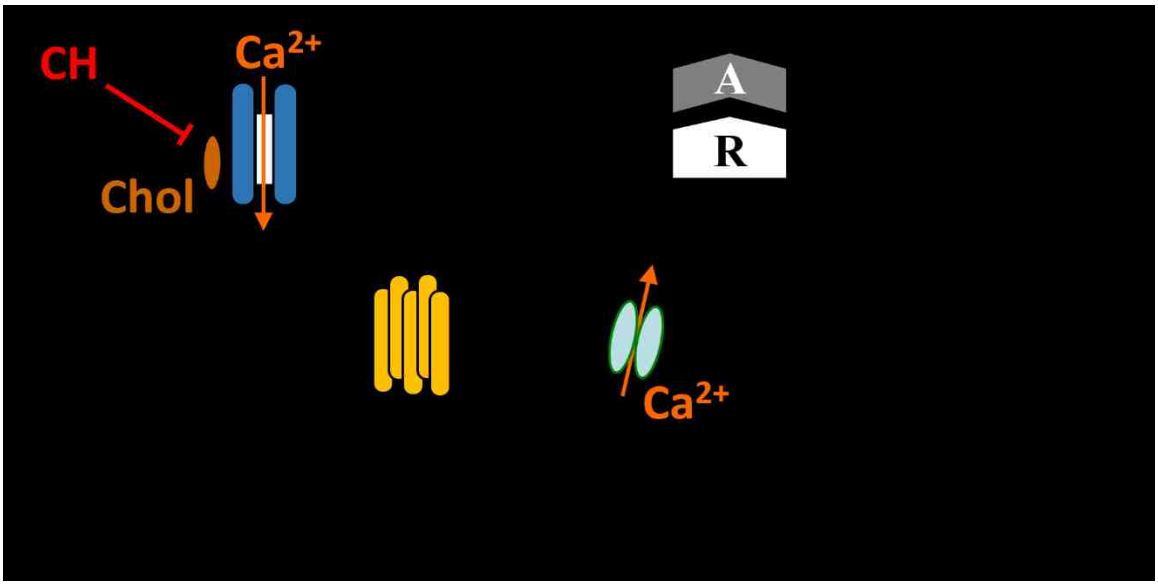

Figure 3: Schematic representation of Aim 2. Cholesterol (Chol), endoplasmic reticulum (ER), inositol trisphosphate receptor (IP_3R), agonist (A), receptor (R).

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CHAPTER 2:

Reduced Membrane Cholesterol Limits Pulmonary Endothelial Ca^{2+} Entry Following Chronic Hypoxia[1]

Bojun Zhang, Jay S. Naik, Nikki L. Jernigan, Benjimen R. Walker and Thomas C. Resta

Vascular Physiology Group

Department of Cell Biology and Physiology

University of New Mexico Health Sciences Center

Albuquerque, NM

Running Head: Pulmonary Endothelial Ca^{2+} Entry Following Chronic Hypoxia

Corresponding Author: Thomas C. Resta, Ph.D.

Department of Cell Biology and Physiology

University of New Mexico Health Sciences Center

MSC 08-4750, 1 University of New Mexico

Albuquerque, NM 87131-0001

Phone: 505-272-8822

Fax: 505-272-6649

e-mail: TResta@salud.unm.edu

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Abstract

Chronic hypoxia (CH)-induced pulmonary hypertension is associated with diminished production of endothelium-derived Ca^{2+}-dependent vasodilators such as nitric oxide. Interestingly, ATP-induced endothelial Ca^{2+} entry as well as membrane cholesterol (Chol) are decreased in pulmonary arteries from CH rats (4 wk, P_B = 380 Torr) compared to normoxic controls. Store-operated Ca^{2+} entry (SOCE) and depolarization-induced Ca^{2+} entry are major components of the response to ATP and are similarly decreased after CH. We hypothesized that membrane Chol facilitates both SOCE and depolarization-induced pulmonary endothelial Ca^{2+} entry, and that CH attenuates these responses by decreasing membrane Chol. To test these hypotheses, we administered Chol or epicholesterol (Epichol) to acutely isolated pulmonary arterial endothelial cells (PAEC) from control and CH rats to either supplement or replace native Chol, respectively. The efficacy of membrane Chol manipulation was confirmed by filipin staining. Epichol greatly reduced ATP-induced Ca^{2+} influx in PAEC from control rats. Whereas Epichol similarly blunted endothelial SOCE in PAEC from both groups, Chol supplementation restored diminished SOCE in PAEC from CH rats while having no effect in controls. Similar effects of Chol manipulation on PAEC Ca^{2+} influx were observed in response to a depolarizing stimulus of KCl. Furthermore, KCl-induced Ca^{2+} entry was inhibited by the T-type Ca^{2+} channel antagonist, mibefradil, but not the L-channel inhibitor, diltiazem. We conclude that PAEC membrane Chol is required for ATP -induced Ca^{2+} entry and its two components, SOCE and depolarization-induced Ca^{2+} entry, and that reduced Ca^{2+} entry after CH may be due to loss of this key regulator.

Key Words: pulmonary hypertension, T-type calcium channels, ATP, store-operated $Ca2^{+}$ entry, depolarization-induced Ca^{2+} entry.

New & Noteworthy: This research is the first to examine the direct role of membrane cholesterol in regulating pulmonary endothelial agonist -induced Ca^{2+} entry and its components. The results provide a potential mechanism by which chronic hypoxia impairs pulmonary endothelial Ca^{2+} influx which may contribute to pulmonary hypertension.

Introduction

Pulmonary vascular dysfunction resulting from chronic hypoxia (CH) leads to increased vascular resistance and pulmonary hypertension in patients with chronic lower respiratory diseases, sleep apnea, and in residents at high altitude. Vasoconstriction and vascular remodeling associated with dysregulation of endothelium-derived mediators are the major components of elevated vascular resistance in CH-induced pulmonary hypertension. Production of many endothelium-dependent vasoactive substances as well as regulation of membrane potential are largely a function of pulmonary endothelial intracellular calcium levels ($[Ca^{2+}]_i$). For example, $[Ca^{2+}]_i$ is a key regulatory factor in the activity of endothelial nitric oxide synthase (9, 17, 35, 42) phospholipase 2 (PLA_2) (52, 53), and the small and intermediate conductance Ca^{2+}-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively) that are responsible for endothelial cell hyperpolarization upon activation by agonists (22, 30). Diminished pulmonary endothelial $[Ca^{2+}]_i$ may limit production of endothelium-derived vasodilators and anti-mitogenic substances including nitric oxide, prostacyclin, and endothelium-derived hyperpolarizing factors. Although endothelial dysfunction and an associated reduction in pulmonary artery endothelial $[Ca^{2+}]_i$ and endothelium-derived nitric oxide (33, 38–40) may be contributing factors to the development of CH-induced pulmonary hypertension, the mechanisms by which endothelial $[Ca^{2+}]_i$ is reduced following CH are incompletely understood.

Our previous studies demonstrate that both basal $[Ca^{2+}]_i$ and agonist-induced Ca^{2+} influx are lower in pulmonary artery endothelial cells (PAEC) from CH rats compared to those of control animals (38, 39). CH similarly inhibits store-operated calcium entry (SOCE) and depolarization-induced Ca^{2+} influx through T-type voltage-gated Ca^{2+}

channels (VGCC), which are major components of agonist-induced Ca^{2+} entry in isolated PAEC (38, 40). Furthermore, agonist-induced Ca^{2+} influx along with membrane cholesterol levels are reduced in PAEC from rats exposed to CH. This impaired agonist-induced Ca^{2+} influx in PAEC can be restored by both membrane cholesterol supplementation and by administration of a caveolin-1 (Cav-1) scaffolding domain peptide (39). The number and structure of caveolae, however, are not altered in PAECs from CH rats compared to controls (39). These data suggest that cholesterol *per se* may affect Ca^{2+} entry in these cells. However, questions remain as to whether cholesterol directly modulates endothelial agonist-induced Ca^{2+} entry and whether its major components, SOCE and depolarization-induced Ca^{2+} entry, are differentially affected.

The membrane cholesterol depleting agent, methyl-β-cyclodextrin (MβCD), has been used to investigate the importance of membrane cholesterol in cellular signaling pathways (58). However, by removing membrane cholesterol, MβCD can also disrupt caveolar structure (41). Consequently, this approach often raises the question of whether membrane cholesterol regulates signaling pathways by direct interaction with membrane proteins or rather by altering the properties of lipid microdomains. To address this problem, the enantiomer of cholesterol (epicholesterol), which has similar effects on membrane fluidity and lipid domain formation as those of cholesterol but lacks the regulatory influences of cholesterol on ion channel function, has been used as a tool to study cholesterol-ion channel interaction (1, 20, 29, 56). In the present study, we hypothesized that 1) membrane cholesterol facilitates SOCE and depolarization-induced Ca^{2+} entry in PAEC; and 2) reduced endothelial Ca^{2+} influx following CH is due to loss of membrane cholesterol. We tested this hypothesis by examining the effect of either membrane

cholesterol supplementation or cholesterol substitution with epicholesterol on endothelial Ca^{2+} entry in freshly isolated PAEC cells from normoxic and CH rats.

Methods

Animals and Chronic Hypoxic Exposure Protocol

Male Sprague-Dawley rats (200-250 g) were used for all studies. Rats exposed to CH were placed in a hypobaric chamber with barometric pressure maintained at ≈380 Torr for 4 wk. Age-matched control rats were housed in similar cages under ambient barometric pressure (≈630 Torr). The hypobaric chamber was opened three times per week to provide fresh rat chow, water, and clean bedding. All animals were maintained on a 12:12-h light-dark cycle. All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center.

Preparation of cholesterol and epicholesterol solutions

MβCD is a cyclic oligomer of glucose that when saturated with either cholesterol or epicholesterol, can effectively deliver these sterols to the plasma membrane (20). This approach has been used to enrich or substitute endogenous membrane cholesterol in neurons (50) and aortic endothelial cells (45). MβCD -cholesterol or -epicholesterol complexes were generated as described earlier (10). Briefly, the cyclodextrin-sterol solutions were prepared by the addition of sterols to MβCD (10 mM) in the molar ratio of 1:5 and dissolution in HEPES buffer containing the following (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4). Each solution was vortexed and sonicated using a bath sonicator for 10-15 min. The saturated cyclodextrin-sterol solution

was then placed in a rotating incubator at 37 °C overnight. This stock solution was filtered through a 0.22 μm syringe filter, aliquoted, and stored at -80 °C.

Isolation and preparation of pulmonary artery endothelial cells

Following CH or normoxic exposure, rats were euthanized with pentobarbital sodium (200 mg/kg ip) and the heart and lungs were exposed by midline thoracotomy. The left lung was rapidly excised and placed in ice-cold HEPES buffer solution. Intrapulmonary arteries (3rd and 4th order, 200-400 μm inner diameter) were dissected from the superior region of the left lung, and the parenchymal lung tissue was carefully removed. Arteries were then cut longitudinally and treated with 0.2 mg/ml dithiothreitol and 2 U/ml papain in HEPES buffer for 45 min at 37 °C. Vessels were carefully removed from the digestion solution and placed in 1 ml of HEPES buffer containing 2 mg/ml bovine serum albumin. Pulmonary artery endothelial cell sheets were then released by gentle trituration with a small-bore fire-polished Pasteur pipette and stored at 4 °C. One to two drops of the solution containing freshly isolated rat PAEC were placed on a poly-L-lysine-coated glass cover slip and incubated at 37 °C in the presence vehicle, cholesterol, or epicholesterol. Cholesterol supplementation was performed in previously untreated endothelial sheets isolated from rats by incubation with cholesterol:MβCD (Chol:MβCD) solution for 30 min at 37 °C. Epicholesterol substitution was similarly achieved by incubating isolated PAEC with epicholesterol:MβCD (EpiChol:MβCD) solution for 30 min at 37 °C.

Membrane cholesterol content

Rat PAEC were freshly isolated and prepared on poly-L-lysine-coated glass cover slips before treatment with vehicle, cholesterol, or epicholesterol. Briefly, cholesterol supplementation and epicholesterol substitution were performed by incubating PAEC with Chol:MβCD or EpiChol:MβCD solutions, respectively, for 30 min at 37 °C. Treated PAEC were then washed with PBS and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. Endothelial cell membrane cholesterol was detected by incubating cells with the fluorescent cholesterol marker filipin III (Sigma, 20 μg/ml) for 15 min at room temperature under light-protected conditions, and coverslips were mounted on the slides using mounting media (39). Slides were air-dried at 4 °C and stored at -20 °C until analysis. The samples were imaged by fluorescence confocal microscopy (Zeiss LSM 510 AxioObserver; Göttingen, Germany) using 405-nm laser (excitation), a 420-nm long pass filter (emission), and a Plan-Neofluor X40/1.3 oil objective. Filipin staining was quantified using NIH Image J. A total of 25-100 cells per rat were analyzed. Fluorescence intensity was quantified by setting a threshold using blank control (filipin-untreated group). Fluorescence of each PAEC sheet was calculated and averaged to determine mean fluorescence for each animal.

Endothelial caveolar number

Cultured rat pulmonary microvascular endothelial cells (PMVEC, passage 6, cultured in MCDB-131 complete media, VEC Technologies) were treated with vehicle, MβCD, Chol:MβCD, or EpiChol:MβCD at 37 °C for 30 min. Cells were washed in HEPES buffer for 10 min and prepared for transmission electron microscopy (TEM) by fixation with 3% formaldehyde, 2% glutaraldehyde, and 1.5 mM $CaCl_2$ in 0.1 M sodium cacodylate

buffer. Cells were post-fixed in reduced osmium tetroxide (1% OsO_4 and 0.5% potassium ferrocyanide), dehydrated, embedded in epoxy resin, sectioned, and stained with uranyl acetate (saturated, aqueous).

Caveolae between 60 and 100 nm in diameter were counted at the membrane of endothelial cells and divided by the length of cell membrane in μm using NIH Image J software. A total of 100 images encompassing 38 cells and 438 μm of membrane were analyzed by a person who was blinded to treatment.

Endothelial fura-2 loading

Following vehicle, cholesterol, or epicholesterol treatment, freshly isolated PAEC sheets were plated and loaded with fura-2 AM (3 μM and 0.05% pluronic acid) in HEPES buffer for 7 min at room temperature (≈23 °C) and washed for 15 min at 37 °C. Ratiometric changes in endothelial cell $[Ca^{2+}]_i$ were determined by alternating a xenon arc lamp light source between 340- and 380- nm bandpass filters at 1 Hz (IonOptix Hyperswitch), and the interleaved fura-2 fluorescence emissions at 510 nm were detected with a photomultiplier tube.

Agonist-induced Ca^{2+} influx and SOCE in freshly isolated endothelial cells

Agonist-induced Ca^{2+} influx and SOCE were measured in PAEC sheets as described previously (39). After fura-2 loading and washing, fura-2-loaded endothelial sheets were superfused with Ca^{2+}-free HEPES buffer for 5 min, then stimulated with ATP (20 μM) or cyclopiazonic acid (CPA, sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, 10 μM) to deplete intracellular Ca^{2+} stores. Calcium entry was then induced by repletion

of extracellular Ca^{2+} (1.8 mM) in the continued presence of ATP or CPA. Ca^{2+} influx was quantified as the area under curve (AUC) for the 5 minutes following reintroduction of extracellular Ca^{2+}.

Measurement of PAEC Membrane Potential (E_m)

Endothelial E_m was measured in *en face* small pulmonary arteries superfused with physiological salt solution (37 °C, equilibrated with 10% O_2, 6% CO_2) using glass microelectrodes (tip resistance 40–80 MΩ) filled with 3 M KCl. A Neuroprobe amplifier (A-M Systems) was used for recording E_m. Analog output from the amplifier was low pass filtered at 1 kHz and recorded and analyzed using Axoscope software (Axon Instruments). E_m was measured under baseline conditions and in response to 10 μM ATP. Criteria for acceptance of E_m recordings included: 1) an abrupt negative deflection of potential as the microelectrode was advanced into a cell; 2) a stable membrane potential for at least 1 min; and 3) an abrupt change in potential to 0 mV after the electrode was retracted from the cell.

Depolarization-induced Ca^{2+} influx in freshly isolated endothelial cells

The ratiometric changes in fura-2 fluorescence represent the sum of changes in intracellular $[Ca^{2+}]_i$, which includes Ca^{2+} influx, Ca^{2+} uptake by organelles, and Ca^{2+} extrusion from the cell. To specifically assess Ca^{2+} entry, Mn^{2+} is used as a Ca^{2+} surrogate due to its unique property to traverse most Ca^{2+} -permeable channels (2), irreversibly bind to fura-2, and quench fura-2 fluorescence (wavelength at ~360 nm). Endothelial cell depolarization-induced Ca^{2+} influx was determined by Mn^{2+} quenching of fura-2 fluorescence in freshly isolated PAEC sheets as describe previously (40). This preparation

was excited at the isosbestic wavelength (360 nm), and emission recorded at 510 nm. Similar to the previous protocol, fura-2-loaded endothelial sheets were superfused with Ca^{2+}-free HEPES buffer for 5 min and administered KCL (60 mM) to elicit membrane depolarization. Calcium entry represented by the influx of the Ca^{2+} surrogate Mn^{2+} was then determined upon addition of extracellular Mn^{2+} (500 μM) in the continued presence of KCl. Depolarization-induced Ca^{2+} entry was quantified by the percentage of the Mn^{2+} quenched fluorescence at 120s after administration of Mn^{2+}.

Calculations and statistics

All data are expressed as means ± SE. Values of n refer to the number of cells for experiment examining caveolar number or to the number of animals for other experiments. Percentage data were converted to normal distributions by arcsine transformation before parametric analysis. An unpaired t-test, one-way ANOVA, two-way ANOVA or Kruskal-Wallis H test were used where appropriate for statistical comparisons. If differences were detected by ANOVA or the Kruskal-Wallis H test, individual groups were compared with the Student-Newman-Keuls or Dunn's multiple comparison tests, respectively. A probability of < 0.05 was accepted as statistically significant for all comparisons.

Results

Effect of cholesterol manipulation on endothelial membrane cholesterol content

Filipin is a polyene antibiotic that binds to membrane cholesterol via hydrophobic interaction (37), but cannot bind to epicholesterol (14). Consistent with our previously published data (39), filipin fluorescence was less in cells from CH rats compared with

normoxic rats (Fig. 1). Cholesterol supplementation restored diminished membrane cholesterol in cells from CH animals while having no effect in normoxic controls. Furthermore, epicholesterol treatment significantly reduced filipin fluorescence in freshly isolated PAEC from both control and CH rats. These data suggest that epicholesterol treatment is an effective approach to substitute endogenous membrane cholesterol for epicholesterol in this preparation.

Effect of cholesterol manipulation on endothelial caveolar number

Effects of cholesterol depletion, supplementation or substitution treatments on caveolar number and structure were examined in cultured rat PMVEC using transmission electron microscopy. MβCD, as a cholesterol carrier, can sequester membrane cholesterol and disrupt caveolar structure when used alone (21). Consistent with previous findings, MβCD decreased the incidence of caveolae (Fig. 2). In contrast, neither cholesterol supplementation nor epicholesterol substitution altered caveolar number compared to vehicle control. These results support an effect of epicholesterol treatment to substitute rather than deplete endogenous membrane cholesterol.

Epicholesterol substitution attenuates endothelial ATP-induced Ca^{2+} entry

ATP-induced Ca^{2+} entry and SOCE were assessed using ratiometric fura-2 measurement as described previously (38, 39). Ca^{2+} entry was quantified as the AUC for the 5 minutes following reintroduction of extracellular Ca^{2+}, in the continued presence of ATP or CPA (Fig. 3). ATP-induced Ca^{2+} entry was diminished in isolated PAEC from normoxic rats after EpiChol:MβCD treatment compared with vehicle controls, but was not

significantly altered by cholesterol supplementation (Chol:MβCD) (Fig.4). These data suggest that endothelial membrane cholesterol is necessary for agonist-induced Ca^{2+} entry.

Cholesterol supplementation restores endothelial SOCE following CH

To assess the importance of membrane cholesterol in SOCE, we examined effects of cholesterol supplementation and epicholesterol substitution on CPA-induced Ca^{2+} influx in isolated PAEC. SOCE was diminished in cells from CH rats compared with controls and was rescued by cholesterol repletion (Fig.5). Furthermore, epicholesterol substitution greatly inhibited CPA-induced Ca^{2+} entry in PAEC from both normoxic and CH rats when compared with their respective vehicle controls.

ATP causes endothelial membrane depolarization

We have previously demonstrated that CH inhibits depolarization-induced Ca^{2+} entry in PAEC (40). However, it is unclear if ATP elicits depolarization in these cells that could evoke this pathway. Consistent with this possibility, we found that ATP caused membrane depolarization in endothelium of freshly isolated pulmonary arteries from control rats (Fig. 6).

Decreased membrane cholesterol leads to reduced endothelial depolarization-induced Ca^{2+} entry following CH

Endothelial cell depolarization-induced Ca^{2+} influx was determined by Mn^{2+} quenching of fura-2 fluorescence in freshly isolated PAEC. Introduction of Mn^{2+} caused a steady decline in fura-2 fluorescence in the absence of KCl in PAEC from both control and

CH rats, indicative of basal Ca^{2+} entry (Fig. 7). A depolarizing stimulus of KCl (60 mM) increased entry of the Ca^{2+} surrogate in isolated PAECs from control rats. However, this response to KCl was absent in in PAECs from CH rats.

To confirm the involvement of T-type VGCCs in depolarization-induced Ca^{2+} influx (40), we repeated these experiments in the presence of the L-type Ca^{2+} channel inhibitor, diltiazem, or the T-type inhibitor, mibefradil. Whereas diltiazem did not affect responses to KCl in cells from either normoxic or CH rats, mibefradil abolished KCl-induced Mn^{2+} quenching of fura-2 fluorescence in PAEC from normoxic controls, while having no effect in cells from CH rats (Fig. 8).

Consistent with a requirement for membrane cholesterol in depolarization-induced Ca^{2+} entry, epicholesterol substitution prevented depolarization-induced Mn^{2+} entry in PAEC from normoxic rats, whereas cholesterol supplementation was without effect in these cells (Fig. 9). In addition, cholesterol repletion partially restored the KCl-induced Mn^{2+} influx in PAEC from CH rats, suggesting that reduced membrane cholesterol contributes to impaired depolarization-induced Ca^{2+} entry following CH.

Discussion

Our laboratory has previously shown that reduced membrane cholesterol following CH is associated with decreased ATP-induced Ca^{2+} entry in intrapulmonary artery endothelial cells. However, it is unclear if membrane cholesterol regulates endothelial Ca^{2+} entry through direct interaction with signaling molecules or through changes in physical properties of the plasma membrane. The goal of the present study was to determine the contribution of membrane cholesterol to agonist-induced Ca^{2+} entry and its major components, store-operated Ca^{2+} entry and Ca^{2+} entry through voltage-gated Ca^{2+} channels.

The major findings from this study are that substitution of endogenous membrane cholesterol with its epimer, epicholesterol, attenuates ATP-induced Ca^{2+} entry, SOCE and depolarization-induced Ca^{2+} entry in PAEC. In addition, decreased endothelial SOCE and depolarization-induced Ca^{2+} entry following CH are largely restored by cholesterol supplementation. However, neither cholesterol supplementation nor epicholesterol substitution alters endothelial caveolar number and structure. The results from this study suggest that membrane cholesterol directly regulates agonist-induced Ca^{2+} entry and its components and further demonstrate that impaired endothelial Ca^{2+} entry following CH is due to altered membrane cholesterol homeostasis. These findings provide an improved mechanistic understanding of factors that contribute to endothelial dysfunction and associated PH resulting from long-term hypoxic exposure.

Many cells release ATP in response to mechanical signals including shear stress, extracellular fluid movement, and changes of cell volume (16, 51, 57). The activation of G protein-coupled purinergic receptors by released ATP may regulate myogenic tone and vascular remodeling (15, 25). In endothelial cells, shear stress-induced production of ATP serves as an autocrine factor and stimulates endothelial NO production (3). ATP as an agonist achieves its various roles through controlling intracellular Ca^{2+}. Intracellular Ca^{2+} is a crucial second messenger affecting various cellular processes and is regulated by different pathways (6). Receptor-mediated Ca^{2+} influx is one of the major pathways to increase $[Ca^{2+}]_i$. The binding of an agonist to its receptor mediates activation of phospholipase C and production of inositol 1,4,5 trisphosphate (IP_3). Cytosolic IP_3 then binds to IP_3 receptors on endoplasmic reticulum (ER), leading to depletion of the ER Ca^{2+} store and subsequent SOCE (44). Agonist binding may also elicit Ca^{2+} entry through

receptor-operated cation channels that are not activated by store emptying (47). Interestingly, agonists like ATP may further mediate depolarization-induced Ca^{2+} entry through VGCC secondary to activation of various nonselective cation channels, including transient receptor potential canonical (TRPC) channels and the ionotropic P2X receptor (11).

Cholestrol is one of the key components of the plasma membrane and may affect cellular signaling. Direct interaction between sterols and ion channels has been suggested by the sensitivity of inwardly rectifying potassium (K_{ir}) channels (45) and large conductance, Ca^{2+}-activated potassium (BK_{Ca}) channels (8) to various sterol analogues. In addition, cholesterol binding regions exist in both K_{ir} channels and BK_{Ca} channels (48, 49). However, the effect of membrane cholesterol on ion channel function may vary depending on the type of ion channel. For example, cholesterol decreases the open probability of many K^+ channels, as well as voltage-gated Na^+ and Ca^{2+} channels (13, 27, 52). In contrast, other ion channels such as epithelial Na^+ channels and TRPC channels are inhibited by removal of membrane cholesterol (4, 5, 26). Regulation of SOCE by membrane cholesterol has been implicated in different cell types in previous studies (5, 19), in which membrane cholesterol depletion by MβCD impaired SOCE. However, the effect of membrane cholesterol in regulation of endothelial depolarization-induced Ca^{2+} entry is unknown.

The importance of membrane cholesterol in agonist-induced Ca^{2+} entry has been suggested in cultured vascular smooth muscle cells, in which cholesterol enrichment augments agonist-induced Ca^{2+} influx (7). Our previous findings that cholesterol repletion enhances impaired ATP- induced Ca^{2+} entry in PAECs following CH (39) also suggests that membrane cholesterol may facilitate endothelial agonist-induced Ca^{2+} entry. ATP

induces both SOCE and T-type VGCC blocker -sensitive Ca^{2+} influx in PAECs (40). In this study, we confirmed that ATP causes membrane depolarization in pulmonary artery endothelium, which may lead to T-type channel activation and Ca^{2+} influx. Considering that SOCE and depolarization-induced Ca^{2+} entry are major components of ATP- induced Ca^{2+} entry, and that both Ca^{2+} influx pathways are blunted following CH (38, 40), it is possible that membrane cholesterol also regulates these two components of the ATP-induced Ca^{2+} response.

Considering the wide range of effects of MβCD such as altering caveolar structure and disrupting other lipid microdomains (21), the direct contribution of membrane cholesterol to regulation of ion channels remains unclear. To investigate the direct role of membrane cholesterol in mediating endothelial Ca^{2+} influx, we used epicholesterol, the enantiomer of cholesterol, to substitute membrane cholesterol. Although epicholesterol has effects similar to cholesterol on physical properties of the cell membrane (56), few studies have examined the effect of EpiChol:MβCD on caveolae. Here we provide evidence that MβCD alone nearly abolished the incidence of caveolae, whereas neither Chol:MβCD nor EpiChol:MβCD affected caveolar number in cultured PMVEC. It has previously been shown that administration of an EpiChol:MβCD solution to bovine aortic endothelial cells effectively substitutes epicholesterol for endogenous membrane cholesterol (46). To verify this observation in our preparation, we assessed effects of epicholesterol substitution on membrane cholesterol content in freshly isolated PAEC using the fluorescent cholesterol marker, filipin. Epicholesterol does not interact with filipin due to a different orientation of the 3-hydroxyl group compared to cholesterol (14, 37). As expected, we found that epicholesterol treatment greatly decreased filipin fluorescence intensity in isolated PAEC

from both normoxic and CH rats, consistent with epicholesterol substitution of endogenous membrane cholesterol. In addition, cholesterol treatment restored membrane cholesterol content in PAEC from CH rats to the level of normoxic controls, consistent with our previous findings (39). These findings suggest that EpiChol:MβCD treatment substitutes endogenous membrane cholesterol for epicholesterol without disrupting caveolae. Consequently, the observed effects of epicholesterol treatment on Ca^{2+} influx are likely due to loss of ion channel regulation by cholesterol, rather than to changes in caveolar density.

Using epicholesterol to substitute endogenous cholesterol, we demonstrated a direct role of membrane cholesterol to facilitate ATP-induced Ca^{2+} entry in PAEC. Our data are consistent with findings that depletion of membrane cholesterol impairs this Ca^{2+} response in PAEC (39). We also provide evidence that SOCE, as the major component of the ATP-induced Ca^{2+} response, is similarly regulated by membrane cholesterol. This observation implies that membrane cholesterol may either directly affect store-operated cation channels (SOCs) or interact with a signaling pathway that activates SOCs. The finding that reduced endothelial SOCE in pulmonary arteries following CH was acutely restored by membrane cholesterol supplementation suggests that CH limits SOCE by reducing membrane cholesterol rather than by decreasing ion channel expression. Although this study has not identified the specific cation channel(s) involved in pulmonary endothelial SOCE, candidates ion channels include TRPC1, TRPC4 and Orai1, each of which have been implicated in endothelial SOCE and demonstrate cholesterol-sensitivity (5, 12, 23, 32).

Wu et al (54) first demonstrated that cultured rat PMVECs express mRNA of $Ca_V3.1$ and possess voltage-dependent current that are sensitive to T-type channel blockers. They also reported that T-type VGCCs contribute to both agonist-induced Ca^{2+} entry and

SOCE in PMVECs. Although T-type VGCCs are not found in cultured rat PAECs (54), their expression and function are described in freshly dispersed rat PAECs (40). Paffett et al showed that T-type VGCCs contribute to depolarization-induced Ca^{2+} entry and receptor-operated Ca^{2+} entry in response to ATP. Following exposure to CH, the mibefradil sensitive component of ATP-induced Ca^{2+} entry is greatly reduced. The effect of CH on this Ca^{2+} entry is not due to altered membrane K^+ permeability, since the impaired endothelial depolarization-induced Ca^{2+} entry following CH persists even when membrane K^+ permeability is equivalently clamped by K^+ ionophore, valinomycin. These findings suggest that T-type VGCCs are important mediators of depolarization-induced Ca^{2+} entry and that impaired Ca^{2+} entry via these channels may contribute to reduced basal $[Ca^{2+}]_i$ in PAECs after CH (40). Using the Mn^{2+} quenching technique to selectively assess Ca^{2+} entry, we confirmed that depolarization-induced Ca^{2+} entry was sensitive to T-type VGCC inhibition and Ca^{2+} entry was eliminated following CH. Additionally, depolarization-induced Ca^{2+} entry following CH was partially restored by cholesterol supplementation. A similar effect of cholesterol on VGCCs has been reported in other cell types (55). Although studies on cholesterol regulation of VGCC are limited, our finding that epicholesterol substitution abolished depolarization-induced Ca^{2+} entry in control cells suggests that there may be a direct interaction between membrane cholesterol and T-type VGCCs that controls channel activity.

Two potential mechanisms of decreased membrane cholesterol following CH include: 1) CH inhibits *de novo* cholesterol biosynthesis; and 2) CH induces membrane cholesterol oxidation. Mukodani *et al*. first reported that hypoxia induces lipid accumulation and impairs cholesterol synthesis in cultured rabbit skin fibroblasts (31). The

mechanism by which hypoxia affects cholesterol synthesis was later explored by Nguyen et al (36). They reported that hypoxia induces accumulation of cholesterol biosynthetic intermediates and activates HIF1-α-mediated induction of ER membrane proteins called insulin-induced gene. These two signaling pathways lead to rapid degradation of HMG-CoA reductase and subsequently limit synthesis of cholesterol (36). Chronic hypoxia can also increase the production of reactive oxygen species (ROS) (18, 28), which may facilitate membrane cholesterol oxidation. Because filipin cannot be used to label oxidized cholesterol (43), it is possible that reduced filipin staining in PAECs of CH group is the result of membrane cholesterol oxidation by hypoxia-induced ROS. Cholesterol oxidation not only has potential to disrupt the interaction between cholesterol and ion channels and many regulatory proteins, but may also to inhibit *de novo* cholesterol synthesis (34). Future studies are required to evaluate the potential contributions of these mechanisms to reduced PAEC membrane cholesterol following CH.

Regulation of intracellular Ca^{2+} levels is a complex process. It is interesting that CH impairs agonist-induced Ca^{2+} entry in PAEC via altered cholesterol regulation of potential ion channels. This decreased endothelial ATP-induced Ca^{2+} influx following CH is not associated with changes in ATP-induced Ca^{2+} mobilization (39), suggesting that attenuated Ca^{2+} entry is not due to altered Ca^{2+} release of Ca^{2+} loading of the ER after CH exposure. However, whether CH similarly affect other intracellular Ca^{2+} handling pathways like Ca^{2+} sequestration and efflux remains to be determined. It should also be noted that the impaired Ca^{2+} entry following CH is limited to endothelial cells of large pulmonary arteries and may not reflect those of smaller arteries and arterioles that likely contribute more to regulating vascular resistance in hypertensive pulmonary circulation.

Besides, both SOCE and receptor-operated Ca^{2+} entry in response to P_2Y receptor agonist UTP are also reduced in pulmonary artery smooth muscle cells (PASMC) following CH (24). It rises another interesting question that whether CH impairs agonist-induced Ca^{2+} influx in PASMC through similarly mechanism involving membrane cholesterol observed in PACE. Future studies are needed to evaluate whether these effects of CH to alter calcium influx pathways are conserved across the pulmonary circulation and the impact of these responses to the development of CH-induced PH.

In conclusion, the current study provides evidence that membrane cholesterol facilitates pulmonary endothelial Ca^{2+} entry likely through interaction with membrane ion channels. Our findings also demonstrate that impaired SOCE and depolarization-induced Ca^{2+} entry following CH are associated with reduced membrane cholesterol levels and are restored by cholesterol supplementation. This membrane cholesterol associated decrease of endothelial Ca^{2+} entry following CH may not only affect regulation of vascular tone, but also contribute to physiological changes, such as endothelial migration/proliferation and apoptosis. Our studies contribute to the understanding of the effect of CH on membrane cholesterol homeostasis and subsequent impact on endothelial $[Ca^{2+}]_i$ in pulmonary arteries, which could shed light on developing potential therapeutic treatments for PH that target membrane cholesterol. Future studies will focus on identifying specific ion channels that interact with membrane cholesterol in pulmonary endothelial cells.

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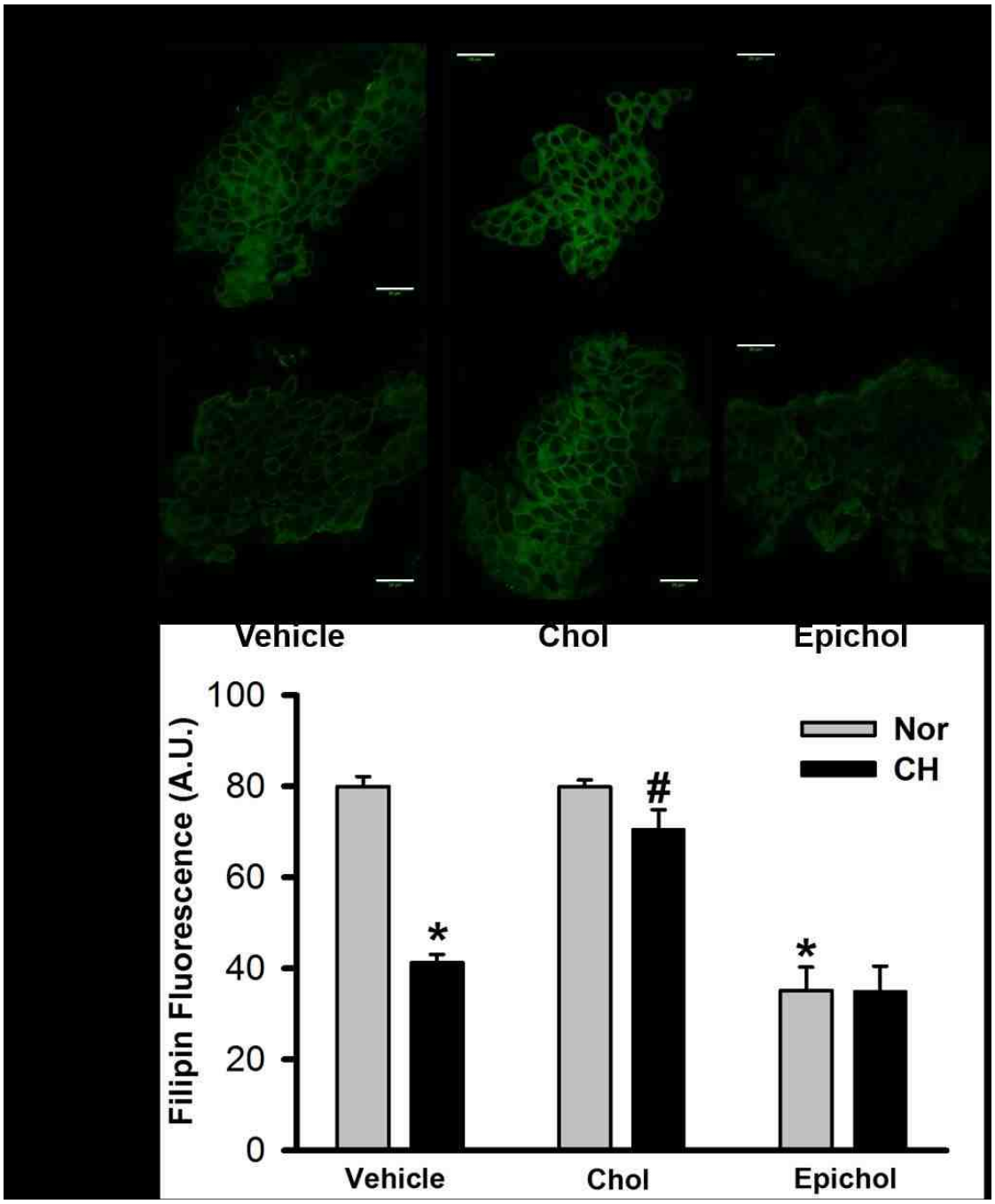

Figure 1. Epicholesterol reduces endogenous membrane cholesterol of freshly isolated PAEC sheets from both normoxic (Nor) and CH rats. A) Representative images of membrane cholesterol indicated by filipin fluorescence in PAEC isolated from each group. Cells were pretreated with vehicle, cholesterol (Chol), or epicholesterol (Epichol). Scale bars = 20 μm. B) Mean filipin fluorescence (arbitrary units; A.U.) in PAEC sheets from each group. Two-way ANOVA followed by the Student-Newman-Keuls post-hoc test was used to compare between groups. Values are means ± SE; n = 3 animals/group. *$P < 0.05$ vs. Nor vehicle; $^{\#}P < 0.05$ vs. CH vehicle.

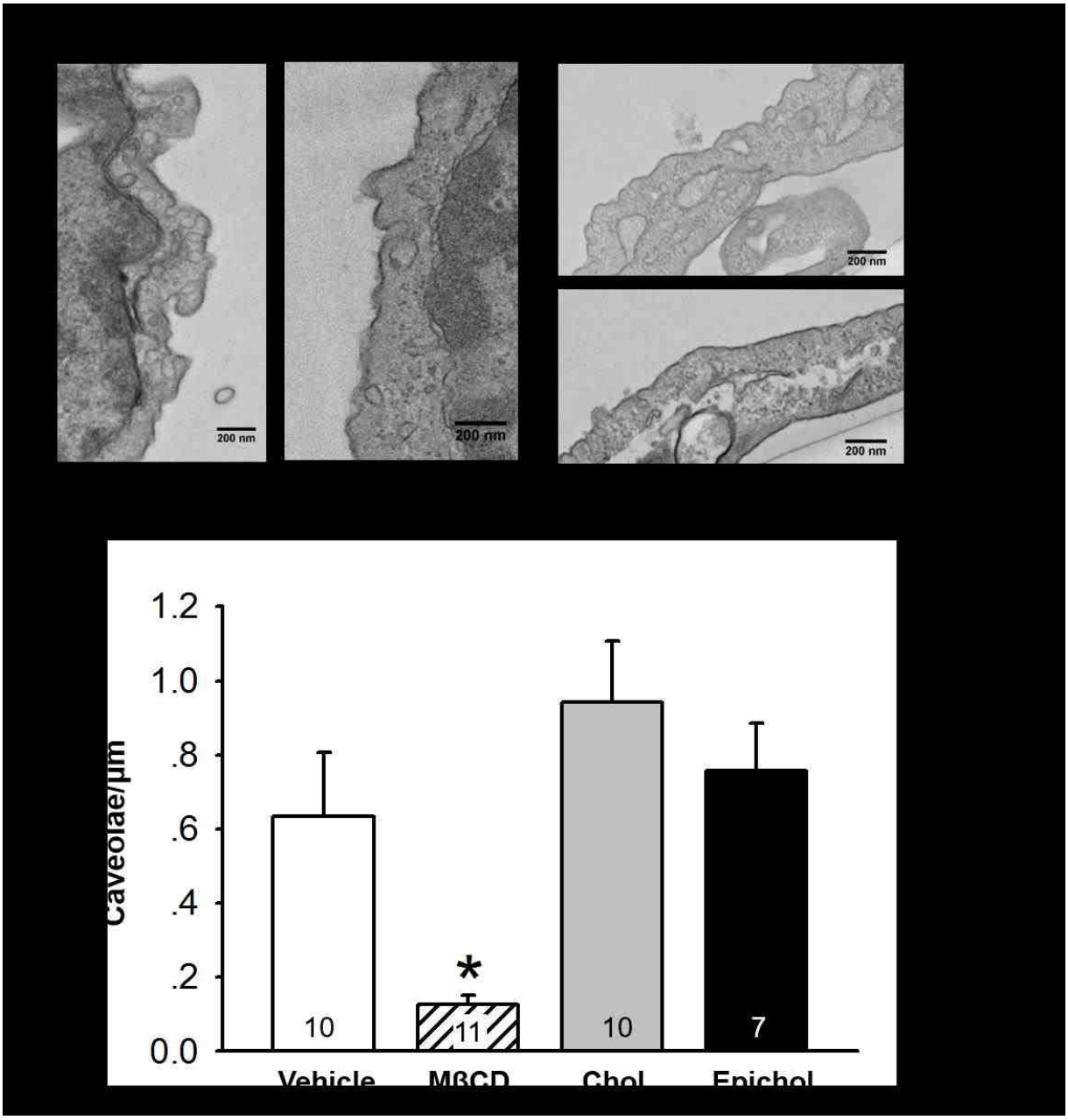

Figure 2. Caveolae number is reduced by cholesterol depletion with MBCD, but not by cholesterol or epicholesterol treatment in cultured PMVEC. A) Representative images of caveolae in cultured PMVEC from each treatment group. B) Mean number of caveolae per length of cell membrane (caveolae/µm). Data were compared by the Kruskal-Wallis H test and Dunn's multiple comparison test. Values are means ± SE; n=7-11 cells (indicated in bars); *$P < 0.05$ versus all other treatments. A total of 100 images encompassing 38 cells and 438 µm of membrane were analyzed.

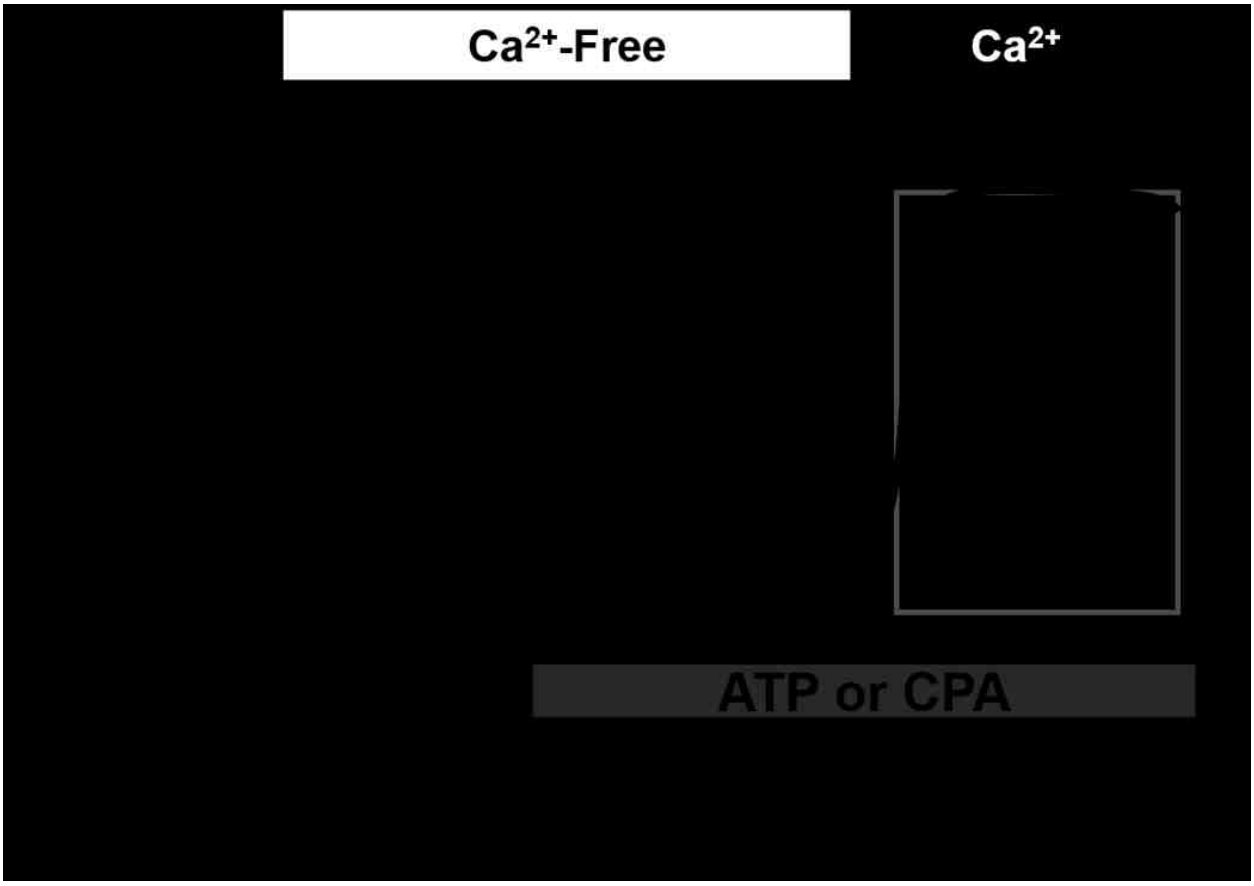

Figure 3. Experimental protocol for measuring ATP- and CPA- induced Ca^{2+} entry in isolated PAEC sheets. Depletion of intracellular Ca^{2+} store was induced by 20 μM ATP or 10 μM CPA in a Ca^{2+}-free HEPES buffer. Intracellular Ca^{2+} is expressed as the fura-2 340/380 nm emission ratio. Ca^{2+} entry was assessed by calculating area under the curve (AUC) for the 5 min following reintroduction of extracellular Ca^{2+} (indicated by rectangle).

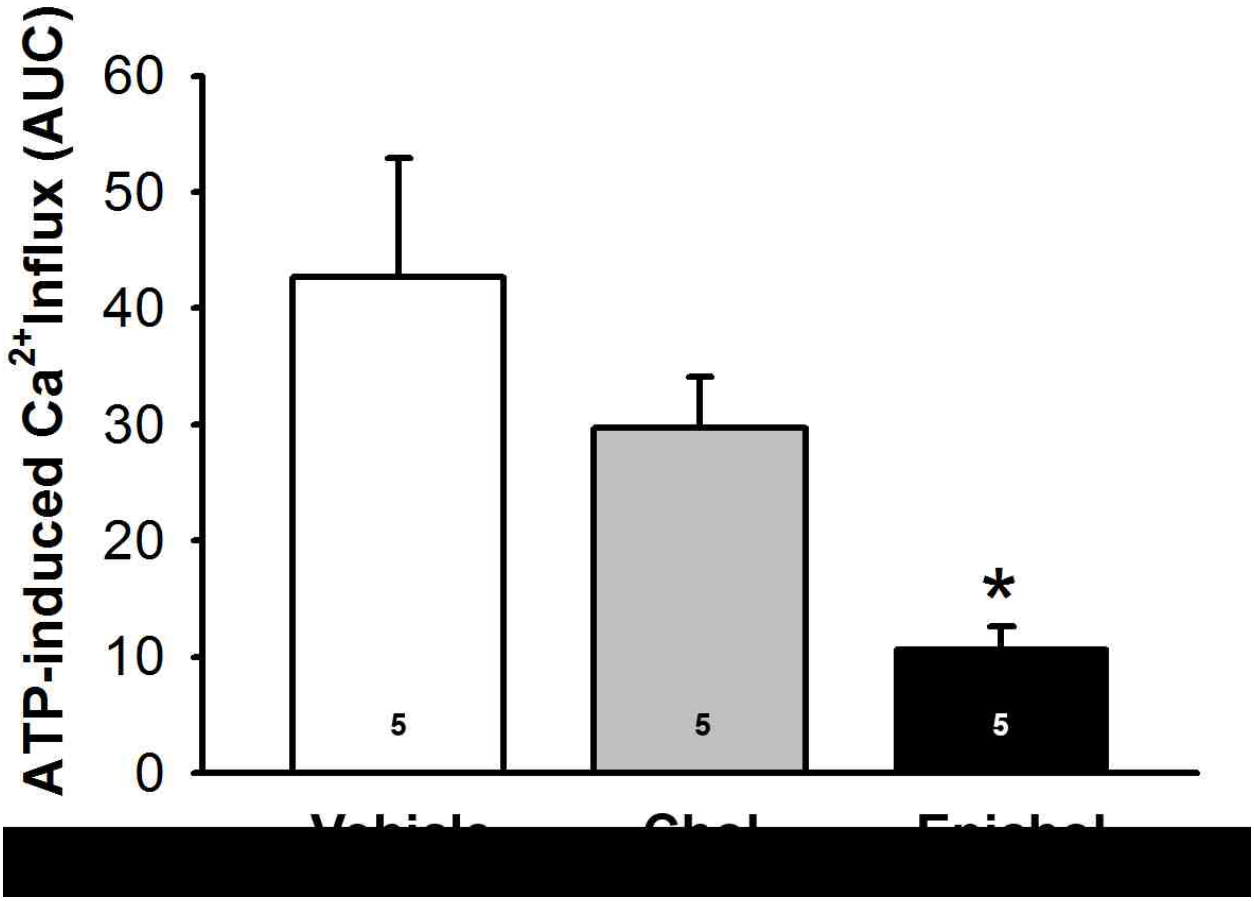

Figure 4. Epicholesterol substitution reduces ATP-induced Ca^{2+} entry in PAEC sheets from normoxic rats. Ca^{2+} influx was assessed by ratiometric analysis of fura-2 fluorescence. One-way ANOVA followed by the Student-Newman-Keuls test was used to compare between groups. Values are means ± SE; n = 5 animals/group. *P < 0.05 vs. vehicle.

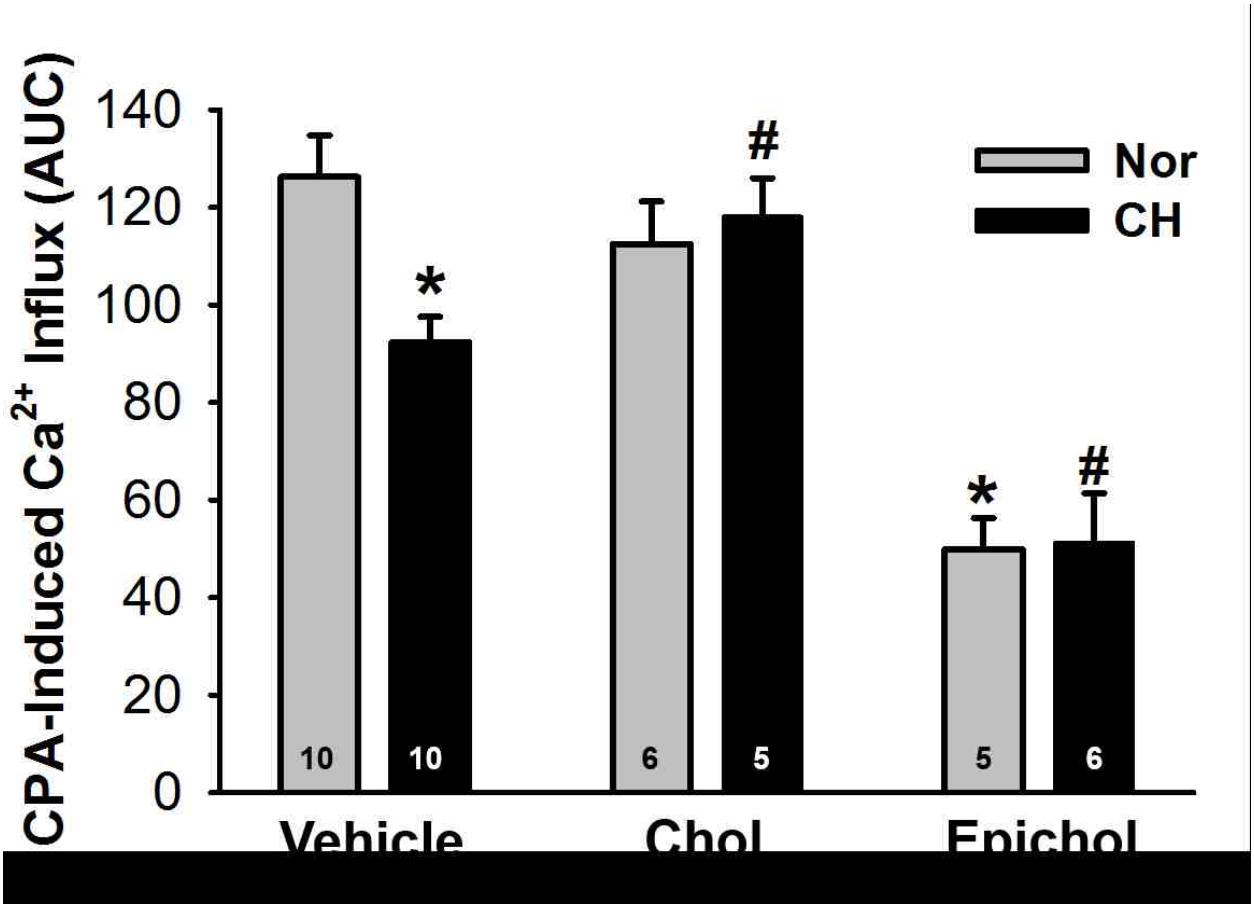

Figure 5. Decreased membrane cholesterol leads to reduced endothelial SOCE following CH. Ca^{2+} influx was assessed by ratiometric analysis of fura-2 fluorescence in PAEC sheets. Cholesterol supplementation restored CPA-induced Ca^{2+} entry following CH, whereas epicholesterol substitution significantly inhibited CPA-induced Ca^{2+} entry in PAEC sheets from both normoxic and CH rats. Groups were compared by two-way ANOVA followed by multiple comparisons testing using the Student-Newman-Keuls test. Values are means ± SE; n = 5-10 animals/group (indicated in bars). $^{*}P$ <0.05 vs. Nor vehicle; $^{\#}P$ <0.05 vs. CH Vehicle.

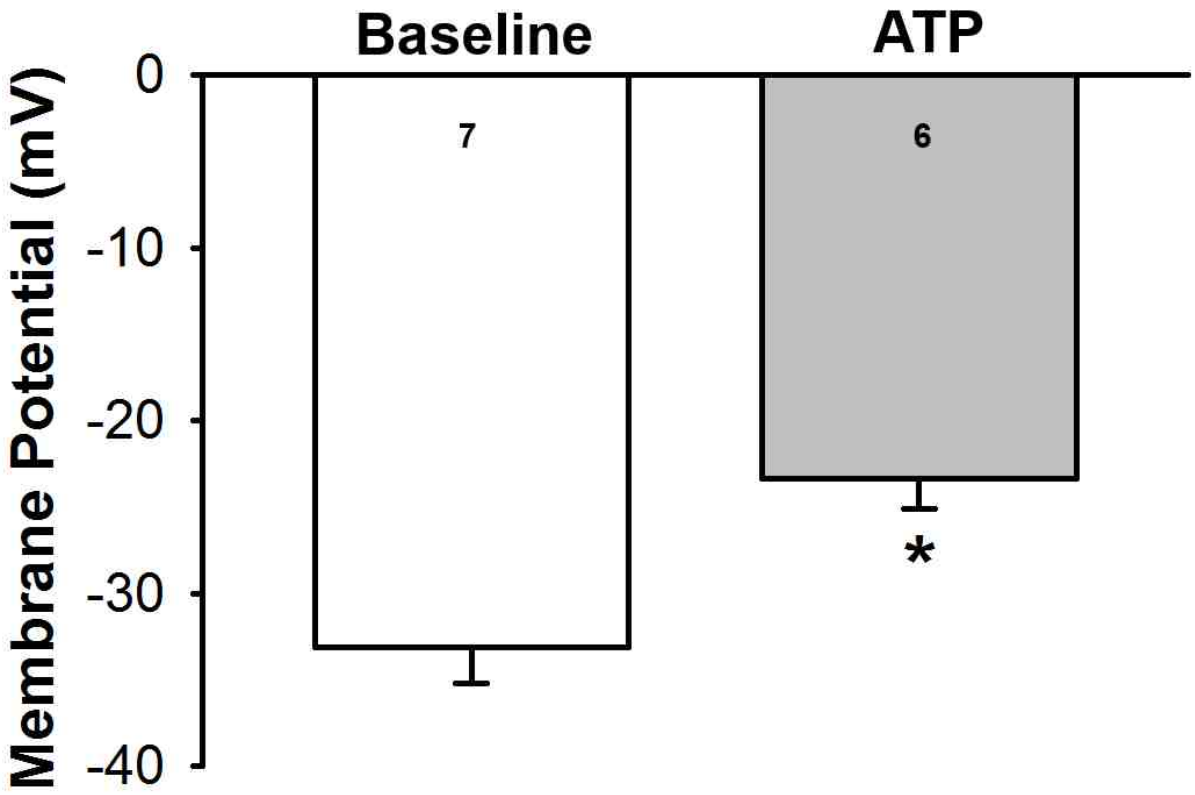

Figure 6. ATP induces membrane depolarization in pulmonary artery endothelium. Endothelial membrane potential was measured under baseline conditions and in response to 10 μM ATP in *en face* small pulmonary arteries from control rats. An unpaired t-test was used to compare between two groups. Values are means ± SE; n = 7, Baseline; n=6, ATP (3-6 E_m recordings were conducted per artery and averaged for an n=1). *P <0.05 vs. Baseline.

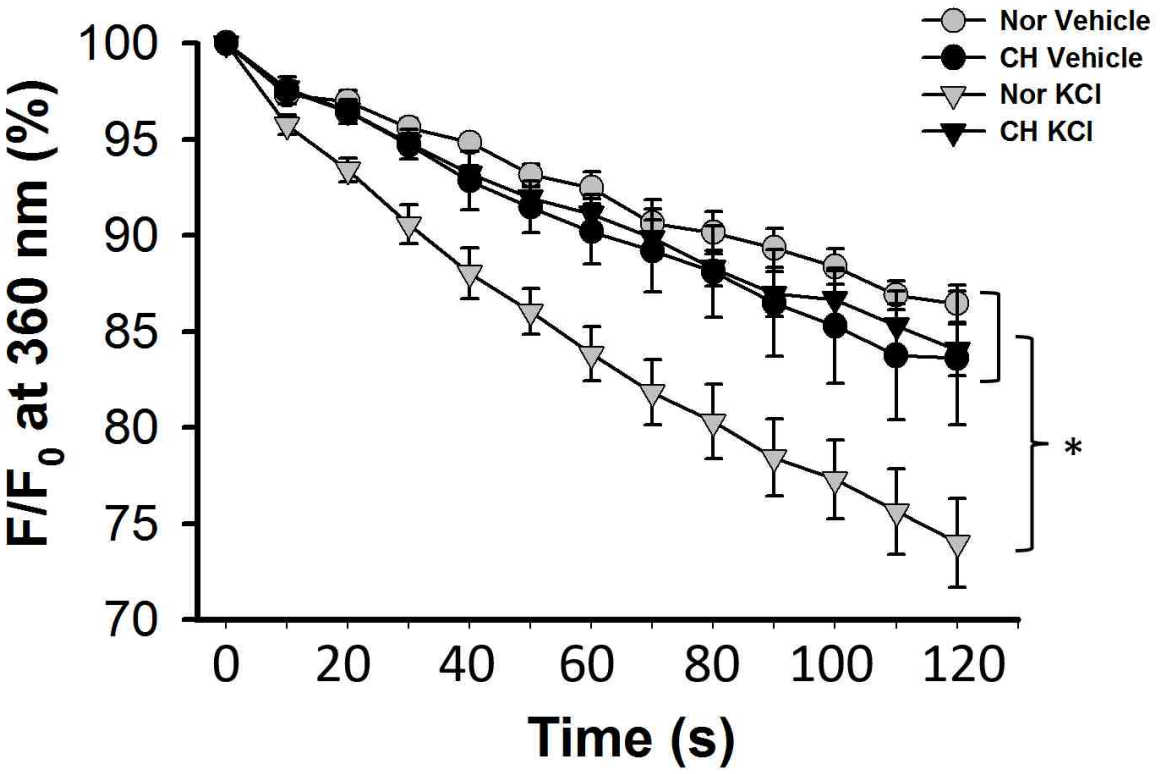

Figure 7. Exposure to CH abolishes endothelial depolarization-induced Ca^{2+} entry. A measure of endothelial Ca^{2+} entry was assessed by Mn^{2+} quenching of fura-2 fluorescence in PAEC sheets from control and CH rats treated with either vehicle (time control) or 60 mM KCl (depolarizing stimulus). F, fluorescence intensity at 360 nm excitation; F_0, fluorescence intensity at time zero. Two-way ANOVA and the Student-Newman-Keuls test were used to compare between groups at each time point. Values are means ± SE; n = 5, Nor Vehicle; n = 5, CH Vehicle; n = 7, Nor KCl; n = 6, CH KCl. *P < 0.05 vs. Nor KCl over the range of 20 – 120 s.

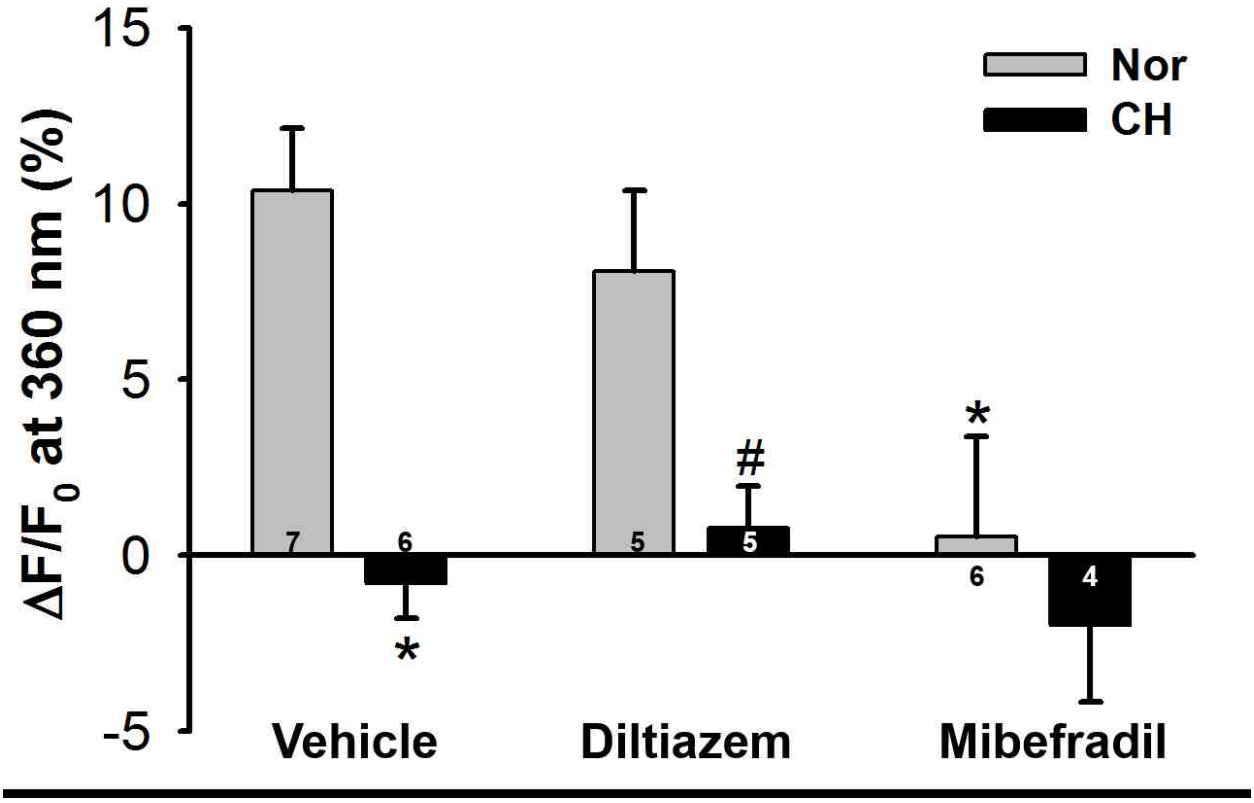

Figure 8. CH attenuates depolarization-induced Ca^{2+} entry through T-type Ca^{2+} channels in PAEC. Data represent Mn^{2+}-quenched fura-2 fluorescence (at 90 s time point) in response to KCl (60 mM) in PAEC sheets from control and CH rats. Diltiazem (50 μM) and mibefradil (10 μM) were used to selectively inhibit L-type and T-type Ca^{2+} channels, respectively. Data are expressed as $\Delta F/F_0$ (%) from time control. Two-way ANOVA and the Student-Newman-Keuls test were used to compare between groups. Values are means ± SE; n = 4-7 animals/group (indicated in bars). *$P < 0.05$ vs. Nor vehicle; $^{\#}P < 0.05$ vs. Nor diltiazem.

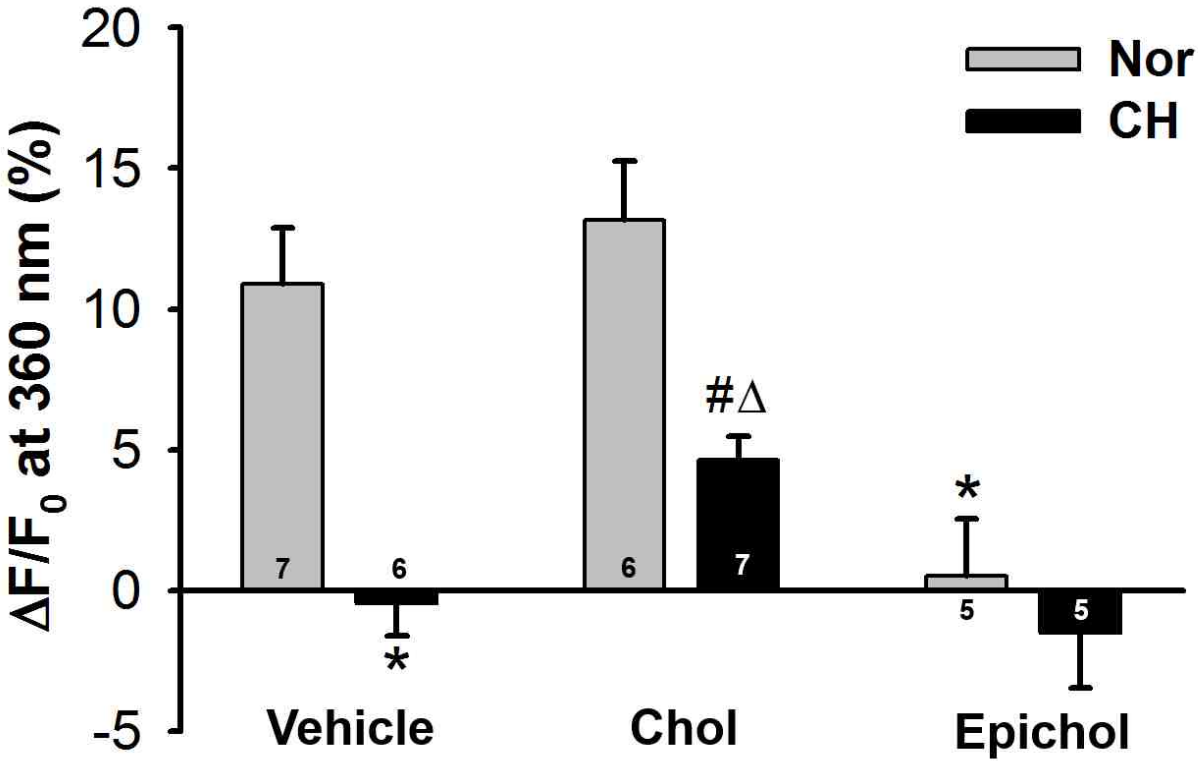

Figure 9. Decreased membrane cholesterol leads to reduced endothelial depolarization-induced Ca^{2+} entry following CH. Data represent Mn^{2+}-quenched fura-2 fluorescence (at 90 s time point) in response to KCl (60 mM) in PAEC sheets from control and CH rats. Cholesterol repletion partially restored depolarization-induced Ca^{2+} entry following CH. Epicholesterol greatly inhibited KCl-induced Ca^{2+} entry in PAEC from both normoxic and CH rats. Data are expressed as $\Delta F/F_0$ (%) from time control. Statistical comparisons were made using two-way ANOVA and the Student-Newman-Keuls post-hoc test. Values are means ± SE; n = 5-7 animals/group (indicated in bars). $^{*}P < 0.05$ vs. Nor vehicle; $^{\#}P < 0.05$ vs. Nor chol; $^{\Delta}P < 0.05$ vs. CH vehicle.

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CHAPTER 3:

Reduced Membrane Cholesterol Following Chronic Hypoxia Limits Orai1-Mediated Pulmonary Endothelial Ca^{2+} Entry[2]

Bojun Zhang, Jay S. Naik, Nikki L. Jernigan, Benjimen R. Walker and Thomas C. Resta

Vascular Physiology Group

Department of Cell Biology and Physiology

University of New Mexico Health Sciences Center

Albuquerque, NM

Running Head: Pulmonary Endothelial Ca^{2+} Entry Following Chronic Hypoxia

Corresponding Author: Thomas C. Resta, Ph.D.

Department of Cell Biology and Physiology

University of New Mexico Health Sciences Center

MSC 08-4750

1 University of New Mexico

Albuquerque, NM 87131-0001

Phone: 505-272-8822

Fax: 505-272-6649

e-mail: TResta@salud.unm.edu

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Abstract

Endothelial dysfunction in chronic hypoxia (CH)-induced pulmonary hypertension is characterized by reduced store-operated Ca^{2+} entry (SOCE) and diminished Ca^{2+}-dependent production of endothelium-derived vasodilators. We recently reported that SOCE in pulmonary arterial endothelial cells (PAEC) is tightly regulated by membrane cholesterol, and that decreased membrane cholesterol is responsible for impaired SOCE following CH. However, the ion channels involved in cholesterol-sensitive SOCE are unknown. We hypothesized that cholesterol facilitates SOCE in PAEC through the interaction of Orai1 and stromal interaction molecule 1 (STIM1). The role of cholesterol in Orai1-mediated SOCE was initially assessed using CH exposure in rats (4 wk, 380 mmHg) as a physiological stimulus to decrease PAEC cholesterol. Effects of Orai1 inhibition with AnCoA4 on SOCE were examined in isolated PAEC sheets from control and CH rats following cholesterol supplementation, substitution of endogenous cholesterol with epicholesterol (Epichol), or vehicle treatment. Whereas cholesterol restored endothelial SOCE in CH rats, both Epichol and AnCoA4 attenuated SOCE only in normoxic controls. The Orai1 inhibitor had no further effect in cells pretreated with Epichol. Using cultured pulmonary endothelial cells to allow better mechanistic analysis of the molecular components of cholesterol-regulated SOCE, we found that Epichol, AnCoA4 and Orai1 siRNA each inhibited SOCE compared to their respective controls. Epichol had no additional effect following knockdown of Orai1. Furthermore, Epichol substitution significantly reduced STIM1-Orai1 interaction assessed by proximity ligation assay. We conclude that membrane cholesterol is required for the STIM1-Orai1 interaction

necessary to elicit endothelial SOCE. Furthermore, reduced PAEC membrane cholesterol following CH limits Orai1-mediated SOCE.

Key Words: pulmonary hypertension, STIM1, store-operated Ca^{2+} entry.

New & Noteworthy: This research demonstrates a novel contribution of cholesterol to regulate the interaction of Orai1 and STIM1 required for pulmonary endothelial agonist-induced Ca^{2+} entry. The results provide a mechanistic basis for impaired pulmonary endothelial Ca^{2+} influx following CH that may contribute to pulmonary hypertension.

Introduction

Endothelial dysfunction in pulmonary hypertension is characterized by imbalanced production of endothelium-derived vasocontrictors and vasodilators leading to vasoconstriction, vascular remodeling, and subsequent elevation of pulmonary vascular resistance (8, 21, 75). The production of many endothelium-derived vasodilators and anti-mitogenic compounds, including nitric oxide (NO), prostacyclin, and endothelium-derived hyperpolarizing factors is closely regulated by endothelial intracellular calcium levels ($[Ca^{2+}]_i$) (9, 17, 25, 45, 47). Diminished pulmonary endothelial $[Ca^{2+}]_i$ and limited production of endothelium-derived NO are associated with the development of chronic hypoxia (CH)-induced pulmonary hypertension (51, 56). However, the mechanisms of reduced pulmonary endothelial $[Ca^{2+}]_i$ following CH exposure are not well-investigated.

Endothelial store-operated Ca^{2+} entry (SOCE) is required for endothelium-dependent vasorelaxation in systemic arteries (28), in part through production of NO (3). SOCE is initiated when a stimulated G protein-coupled receptor activates phospholipase C (PLC), which produces inositol 1,4,5 trisphosphate (IP_3) by hydrolyzing phosphatidylinositol bisphosphate (PIP_2). IP_3, as a second messenger, activates IP_3 receptors on the endoplasmic reticulum (ER) membrane, leading to rapid ER Ca^{2+} release. ER Ca^{2+} store depletion then activates store-operated cation channels (SOC), which leads to sustained Ca^{2+} influx (64). Stromal interaction molecule 1 (STIM1) has been identified as an ER Ca^{2+} sensor and was suggested as the essential link between Ca^{2+} store depletion and SOC activation (72). As ER Ca^{2+} levels fall, STIM1 molecules oligomerize on the ER membrane and interact with SOC on the cell membrane, which include Orai channels (15). Orai1, one of three isoforms of Orai found in mammalian cells, mediates SOCE when

activated by STIM1 (86). The STIM1-Orai1 interaction occurs in cholesterol-rich caveolae on the cell membrane. Interestingly, intact caveolae are required for activation of Orai1-mediated SOCE in platelets (14). Depletion of membrane cholesterol not only inhibits SOCE, but also interupts STIM1 clustering near the cell membrane (59). However, whether membrane cholesterol directly faciliates STIM1-Orai1 interaction and SOCE activation or indirectly modulates this Ca^{2+} entry pathway by contributing to lipid raft integrity is not clear.

We have previously reported that decreased endothelial SOCE following CH is associated with diminished membrane cholesterol and this reduced SOCE is restored by cholesterol supplementation (56, 57, 84). Furthermore, to study the functional role of membrane cholesterol in regulating SOCE, epicholesterol (Epichol) was used to replace native cholesterol. As the enantiomer of cholesterol, Epichol has similar effects on membrane fluidity and lipid domain formation as those of cholesterol but lacks the regulatory influences of cholesterol on ion channel function (22, 65, 83). Substitution of endogenous cholesterol with Epichol mimics the effect of CH in reducing endothelial SOCE while having no effect on caveolar number (84). However, the mechanisms by which reduced endothelial membrane cholesterol limits SOCE following CH are not clear. In the present study, we hypothesized that cholesterol facilitates the interaction of Orai1 with STIM1 to mediate SOCE in PAEC. We tested this hypothesis by examining the effects of either cholesterol or Epichol treatment combined with Orai1 inhibition on SOCE in both freshly isolated pulmonary arterial endothelial cells (PAEC) from normoxic and CH rats and in cultured pulmonary microvascular endothelial cells (PMVEC).

Methods

Animals and Chronic Hypoxic Exposure Protocol

Male Sprague-Dawley rats (200-250 g) were used for *in vivo* and *ex vivo* studies. Rats exposed to CH were placed in a hypobaric chamber with barometric pressure maintained at ≈ 380 mmHg (inspired PO_2 ≈ 70 mmHg) for 4 wk (84). Age-matched control rats were housed in similar cages under ambient barometric pressure (≈ 630 mmHg in Albuquerque, NM). The hypobaric chamber was opened three times per week to provide fresh rat chow, water, and clean bedding. All animals were maintained on a 12:12-h light-dark cycle. All protocols used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center.

Isolation and preparation of pulmonary artery endothelial cells

Following CH or normoxic exposure, rats were euthanized with pentobarbital sodium (200 mg/kg ip) and the heart and lungs were exposed by midline thoracotomy. The left lung was rapidly excised and placed in ice-cold HEPES buffer solution. Intrapulmonary arteries (3rd and 4th order, 200-400 μm inner diameter) were dissected from the superior region of the left lung, and the parenchymal lung tissue was carefully removed. Arteries were then cut longitudinally and treated with 0.2 mg/ml dithiothreitol and 2 U/ml papain in HEPES buffer for 45 min at 37 °C. Vessels were carefully removed from the digestion solution and placed in 1 ml of HEPES buffer containing 2 mg/ml bovine serum albumin. PAEC sheets were then released by gentle trituration with a small-bore fire-polished Pasteur pipette and stored at 4 °C. One to two drops of the solution containing freshly isolated rat PAEC were placed on a poly-L-lysine-coated glass cover slip and incubated at

37 °C for 30 min prior to experimentation (84). PAEC sheets collected using this approach are identified by their distinct morphology (57, 84) and uptake of Dil-Ac-LDL (56).

Cell culture

To complement experiments using freshly isolated PAEC sheets, cultured pulmonary microvascular endothelial cells (PMVEC; VEC Technologies) were also used in this study. Cultured cells permit the use of genetic manipulation with siRNA and determination of protein-protein interactions (Orai1-STIM1) using proximity ligation assay for better mechanistic analysis of the molecular components of cholesterol-regulated SOCE. PMVEC were maintained in a humidified incubator at 37 °C with 5% CO_2 in MCDB-131 complete media (VEC Technologies). Cultures were split every 4-6 days and cells studied between passages 5 and 10. Depending on the experimental protocol, cells were seeded onto round poly-L-lysine-coated glass coverslips in 6-well plates, 6-well plates coated with attachment factors, or 18-well slides coated with attachment factors.

Preparation of cholesterol and epicholesterol solutions

Solutions were prepared by saturating methyl-β-cyclodextrin (MβCD) with either cholesterol or Epichol, as decribed previously (11). Briefly, the cyclodextrin-sterol solutions were prepared by the addition of sterols to MβCD (10 mM) in a molar ratio of 1:5 and dissolution in HEPES buffer containing the following (in mM): 150 NaCl, 6 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, and 10 glucose (pH 7.4). Each solution was vortexed and sonicated using a bath sonicator for 10-15 min. The saturated cyclodextrin-sterol solution was then placed in a rotating incubator at 37 °C overnight. This stock solution was filtered through a 0.22 μm syringe filter, aliquoted, and stored at -80 °C.

Endothelial cell membrane cholesterol manipulation

Cholesterol supplementation was performed in untreated PAEC or PMVEC by incubation with the cholesterol/MβCD solution for 30 min at 37 °C (84). Epichol substitution was similarly achieved by incubating isolated PAEC or cultured PMVEC with the Epichol/MβCD solution under the same conditions. Preliminary studies revealed that, in contrast to effects of membrane cholesterol manipulation in freshly dispersed PAEC from normoxic rats (84), cholesterol supplementation increased SOCE in cultured PMVEC and conferred sensitivity to Epichol substitution (data not shown), suggesting that membrane cholesterol content is reduced under cell culture conditions compared to the native state. Therefore, cultured PMVEC were pretreated with cholesterol/MβCD solution prior to experimentation to mimic responses of native PAEC cells.

Ca^{2+} influx in freshly isolated and cultured pulmonary endothelial cells

Following vehicle, cholesterol, or Epichol treatment, freshly isolated PAEC sheets or cultured PMVEC (passages 5-10) were loaded with fura-2 AM (3 μM and 0.05% pluronic acid) in HEPES buffer for 7 min at room temperature (≈23 °C) and washed for 15 min at 37 °C. SOCE, depolarization-induced Ca^{2+} influx, and receptor-operated Ca^{2+} influx were measured by Mn^{2+}-quenching of fura-2 fluorescence in PAEC sheets or PMVEC as previously described (84). Mn^{2+} enters the cell as a Ca^{2+} surrogate and reduces fura-2 fluorescence upon binding to the fluorophore. The preparation was excited at the isosbestic wavelength (360 nm) at 1 Hz (IonOptix Hyperswitch), and emission recorded at 510 nm with a photomultiplier tube. At this excitation wavelength, fura-2 fluorescence intensity is not influenced by changes in $[Ca^{2+}]_i$, thus providing a measure of Ca^{2+} influx as reflected by Mn^{2+} uptake independent of ER Ca^{2+} release, sequestration or efflux across the cell

membrane (31). Fura-2-loaded endothelial cells were superfused with Ca^{2+}-free HEPES buffer in the presence of vehicle or AnCoA4 (20 μM, Orai1 inhibitor (67)) for 5 min and administered the sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor, cyclopiazonic acid (CPA, 10 μM) to deplete intracellular Ca^{2+} stores and activate store-operated channels. To confirm the specificity of AnCoA4 as an inhibitor of SOCE, KCl (60 mM) or 1-oleoyl-2-acetyl-sn-glycerol (OAG, diacylglycerol analog, 50 μM) were applied in separate studies to examine the effect of AnCoA4 on depolarization-induced and receptor-operated Ca^{2+} influx (58), respectively. Ca^{2+} entry represented by influx of the Ca^{2+} surrogate Mn^{2+} was then determined upon addition of extracellular Mn^{2+} (500 μM) in the continued presence of AnCoA4 or vehicle. SOCE was quantified by the percentage of the Mn^{2+} quenched fluorescence at 120 s after administration of Mn^{2+}.

Membrane cholesterol content

We have previously used the fluorescent cholesterol marker, filipin, to determine the efficacy of cholesterol manipulation protocols in freshly dispersed PAEC (84). To confirm similar effectiveness of cholesterol manipulation in PMVEC, cultures (passage 6) were treated with vehicle, MβCD, cholesterol:MβCD, or Epichol:MβCD at 37 °C for 30 min, then washed with PBS and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. Endothelial cell membrane cholesterol was detected by incubating cells with filipin III (Sigma, 20 μg/ml) for 15 min at room temperature under light-protected conditions, and coverslips were mounted on the slides using mounting media (57). Slides were air-dried at 4 °C and stored at -20 °C until analysis. The samples were imaged by fluorescence confocal microscopy (Zeiss LSM 510 AxioObserver; Göttingen, Germany) using 405-nm laser (excitation), a 420-nm long pass filter (emission), and a Plan-Neofluor

X40/1.3 oil objective. Filipin fluorescence intensity was quantified using NIH Image J, and calculated as that above threshold assessed using a blank control (filipin-untreated group). Fluorescence of each PMVEC was calculated and averaged to determine mean fluorescence for each treatment group. Cellular cholesterol content was also examined using an Amplex Red cholesterol assay kit (Molecular Probes) following manufacturer instructions.

Orai1 siRNA knockdown

A cocktail of three different Orai1 siRNAs was used (Thermofisher, RSS357633-357635). Transfection in PMVEC (passages 6-10) was achieved using lipofectamine (Invitrogen) according to the manufacturer's instructions. A scrambled sequence (Dharmacon) was used as a non-targeting (NT) control. Cells seeded onto either round glass coverslips (for Ca^{2+} imaging) or 6-well plates (for Western blotting) were transfected with 9 μg of siRNA in each well and were assayed 72 hr after transfection. There were no apparent effects of either siRNA or NT treatments on cell morphology.

Western blotting for Orai1

PMVEC were homogenized in a buffered solution (255 mM sucrose, 10 mM Tris HCl, 2 mM EDTA, 12 μM leupeptin, 1 μM pepstatin A, and 0.3 μM aprotinin) and centrifuged at 500 x g for 5 min at 4 °C. Cell lysate protein content was quantified using a NanoDrop (NanoDrop 2000, Thermofisher) and 50 g of protein were separated by SDS-PAGE (12% Tris/glycine) and transferred onto polyvinylidene fluoride membranes. After blocking with 5% nonfat milk dissolved in Tri-buffered saline with 0.1% Tween 20 (TBS-T) for 1 hr at room temperature, the membrane was probed with primary antibody (1:400, rabbit anti-Orai1, ACC-062, Alomone Labs) in TBS-T containing 5% non-fat milk

overnight at 4 °C. After washing, the membrane was incubated with secondary antibody (IgG-horseradish peroxidase-conjugated goat anti-rabbit, 1:3000, Bio-Rad) in TBS-T containing 0.5% non-fat milk for 1 h at room temperature. Anti-β-actin (1:5000) was used for loading control experiments in which the same membrane probing for Orai1 above was washed and reprobed for β-actin. Detection was performed with the enhanced chemiluminescence reagent (ECL Western blotting detection reagents, Pierce) and chemiluminescence-sensitive film (GeneMate). All bands of targeted size were quantified by densitometry using the ImageJ software.

Duolink proximity ligation assay

STIM1-Orai1 interaction is required for the activation of Orai1 channels and SOCE. To determine whether cell membrane cholesterol regulates this pivotal step in SOCE, the interaction of STIM1 and Orai1 was assessed in PMVEC using the Duolink *in situ* proximity ligation assay (PLA) according to manufacturer's instructions (Sigma-Aldrich). Briefly, PMVEC were plated on 18-well slides (Ibidi) and grown to 80-90% confluency. PMVEC pretreated with cholesterol or Epichol were then treated with either vehicle or CPA (10 μM; 5 min) before fixing with 2% paraformaldehyde. PMVEC were incubated with Duolink blocking buffer for 30 min at 37°C then incubated overnight with rabbit anti-STIM1 (1:250; ab106531, Abcam) and goat anti-Orai1 (1:100; sc-74778, Santa Cruz Biotechnology). Cells were then incubated with anti-rabbit PLUS and anti-goat MINUS PLA probes (1:5) for 1 hr at 37°C. Negative controls were completed by *1*) omission of primary antibody, and *2*) incubation with each primary antibody individually. Samples were amplified with Duolink *In Situ* Detection Reagent Orange (excitation/emission: 554/579 nm; Sigma-Aldrich) for 100 min at 37°C. SYTOX Green

(1:5,000; Invitrogen) was used as a nuclear stain. Samples were mounted with Duolink mounting media and Z-stack images of the PLA interaction were acquired using a confocal microscope (TCS SP5; Leica). The number of puncta per cell were determined using Image J (NIH).

Calculations and statistics

All data are expressed as means ± SE. Values of n refer to the number of animals for experiments using freshly isolated PAEC or to the number of groups as indicated in figure legends for other experiments. Percentage data were converted to normal distributions by arcsine transformation before parametric analysis. An unpaired t-test, one-way ANOVA, two-way ANOVA or Kruskal-Wallis H test were used where appropriate for statistical comparisons. If differences were detected by ANOVA or the Kruskal-Wallis H test, individual groups were compared with the Student-Newman-Keuls or Dunn's multiple comparison tests, respectively. A probability of < 0.05 was accepted as statistically significant for all comparisons.

Results

Impaired pulmonary endothelial SOCE following CH is restored by cholesterol supplementation

The importance of membrane cholesterol in diminished SOCE following CH was confirmed by examining effects of cholesterol supplementation and Epichol substitution on CPA-induced Ca^{2+} influx in freshly isolated PAEC from control and CH rats using the Mn^{2+} quenching technique. Although cholesterol treatment did not affect endothelial SOCE in normoxic rats, Epichol substitution greatly inhibited this Ca^{2+} entry pathway (Fig.

1A). In contrast, cholesterol repletion increased SOCE in PAEC of CH rats (Fig. 1B). Treatment with Epichol did not further attenuate SOCE in PAEC from CH rats compared to vehicle treated cells (Fig. 1B). These data are summarized in Figure 1C. Exposure to CH significantly blunted SOCE compared to normoxic controls, which was restored by cholesterol supplementation (Fig. 1C). In cells from normoxic animals, treatment with Epichol reduced SOCE, mimicking the effects of CH exposure.

Orai1 mediates pulmonary endothelial SOCE

We initially assessed the contribution of Orai1 to pulmonary endothelial SOCE using the Orai1 inhibitor, AnCoA4 (REF). AnCoA4 was originally identified as an inhibitor of Orai1 using minimal functional domains of Orai1 and STIM1 to screen small-molecule microarrays. This compound was found to directly bind to the C-terminus of Orai1 and interfere not only with channel gating but also with the interaction of Stim1 and Orai1. The specificity of AnCoA4 to inhibit SOCE was confirmed by comparing its effectiveness to attenuate SOCE vs. other forms of Ca^{2+} entry not linked to Orai1, including depolarization-induced Ca^{2+} entry mediated by T-type voltage-gated Ca^{2+} channels (REF) and receptor-operated Ca^{2+} entry in response to the diacylglycerol analog, OAG (REF). In PAEC from normoxic rats. AnCoA4 significantly attenuated CPA-induced SOCE without affecting Ca^{2+} entry to a depolarizing stimulus of KCl (Fig. 2A). AnCoA4 similarly reduced SOCE without affecting receptor-operated Ca^{2+} influx elicited by OAG (Fig. 2B) in cultured PMVEC where this pathway of influx is more demonstrable. Together, these data establish the specificity of AnCoA4 as a SOCE-specific inhibitor.

CH impairs Orai1-mediated pulmonary endothelial SOCE: role of membrane cholesterol

The effect of CH on Orai1-mediated endothelial SOCE was examined using AnCoA4 in freshly isolated PAEC from normoxic and CH rats. Orai1 inhibition significantly reduced endothelial SOCE in PAEC from normoxic rats (Fig. 3A, B), while having no effect in cells from CH animals (Fig. 3A, C). However, AnCoA4-sensitive SOCE was restored by cholesterol supplementation in PAEC from CH rats (Fig. 3C), mirroring the efficacy of Orai1 inhibition in cholesterol-treated cells from normoxic rats (Fig. 3B). In contrast, Orai1 inhibition was without effect on Ca^{2+} influx following Epichol substitution in PAEC from either normoxic (Fig. 3B) or CH rats (Fig. 3C).

Effect of cholesterol manipulation on membrane cholesterol content in cultured PMVEC.

To further explore the mechanism by which membrane cholesterol regulates Orai1-mediated endothelial SOCE, we focused on cultured PMVEC where genetic approaches and assessment of protein-protein association are technically more feasible. We first examined the effect of cholesterol manipulation on endothelial cell membrane cholesterol levels. Consistent with previous observations in freshly isolated PAEC (84), both MβCD and Epichol treatment significantly reduced filipin fluorescence (Fig.4A, B). Interestingly, cholesterol supplementation increased filipin fluorescence in cultured PMVEC (Fig.4A, B), which is different from our previous observation in freshly isolated PAEC where cholesterol treatment did not further augment filipin fluorescence (57). We also examined endothelial cholesterol content using an Amplex Red cholesterol assay. Consistently, cholesterol treatment augmented endothelial cholesterol content while MβCD and Epichol treatment reduced native cholesterol content (Fig.4C). Since these results suggest that cultured cells are relatively cholesterol deplete, PMVEC used in the following studies were

first supplemented with cholesterol prior to subsequent manipulation to better mimic native cells.

Epicholesterol substitution reduces SOCE in PMVEC

SOCE was assessed in cultured PMVEC after cholesterol manipulation. Similar to that observed in native cells, Epichol substitution significantly reduced endothelial SOCE compared to the cholesterol-treated group (Fig. 5).

Orai1 siRNA knockdown inhibits membrane cholesterol-mediated SOCE in PMVEC

Orai1 siRNA was employed as a genetic approach to complement pharmacological inhibition studies using AnCoA4 and further control for potential off-target effects of the inhibitor. The efficacy of Orai1 siRNA knockdown was confirmed by western blotting. Orai1 siRNA had a moderate but significant effect to reduce Orai1 protein expression compared with the NT control siRNA (Fig. 6A, B). Consistent with pharmacologic Orai1 inhibition in both native and cultured cells (Fig. 2A, B), Orai1 siRNA knockdown attenuated SOCE in PMVEC (Fig. 6C). Furthermore, Epichol substitution greatly reduced endothelial SOCE of the NT group without affecting that of Orai1 siRNA-treated cells (Fig. 6C). Together these findings confirm the involvement of Orai1 in cholesterol-sensitive SOCE.

Epicholesterol substitution reduces STIM1-Orai1 interaction in PMVEC

The role of membrane cholesterol in regulating STIM1-Orai1 interaction was examined in PMVEC. CPA-induced ER store depletion increased STIM1-Orai1 co-localization (i.e., increased the number of red puncta) (Fig. 7A, B). Epichol treatment, however, nearly abolished STIM1-Orai1 co-localization when compared to the cholesterol-

treated group (Fig.7A, B), supporting a role for membrane cholesterol to facilitate the interaction of STIM1 and Orai1 in PMVEC.

Discussion

Our laboratory has previously shown that reduced membrane cholesterol following CH is associated with impaired SOCE in intrapulmonary artery endothelial cells, and that endothelial membrane cholesterol facilitates SOCE through direct interaction with signaling molecules. However, the mechanism by which membrane cholesterol-regulated endothelial Ca^{2+} entry via SOC has not previously been addressed. The goal of the present study was to determine the contribution of membrane cholesterol to Orai1-mediated SOCE in pulmonary endothelial cells. The major findings from this study are that 1) restoration of SOCE by cholesterol supplementation in PAEC from CH rats is sensitive to Orai1 inhibition; 2) both substitution of endogenous membrane cholesterol with its epimer, Epichol, and inhibition of Orai1 by AnCoA4 attenuate SOCE without additive effects in isolated PAEC and cultured PMVEC; 3) Epichol treatment does not further reduce SOCE in PMVEC following Orai1 siRNA knockdown; and 4) membrane cholesterol is required for the interaction of STIM1 and Orai1 in response to ER Ca^{2+} store depletion in PMVEC. The results from this study suggest that membrane cholesterol directly regulates Orai1-mediated endothelial SOCE by facilitating the interaction of STIM1 and Orai1, and further demonstrate that impaired pulmonary endothelial Ca^{2+} entry following CH is due to altered membrane cholesterol homeostasis that limits Orai1 activity.

One of the hallmarks of CH-induced pulmonary hypertension is pulmonary arterial endothelial dysfunction. Pulmonary arterial smooth muscle cell contraction and

proliferation is regulated by vasoactive factors secreted from the endothelium. The increased production of vasoconstrictors/proliferative factors and decreased synthesis of vasodilatory/anti-mitogenic factors contribute to enhanced vascular tone and remodeling in CH-induced pulmonary hypertension (71). Production of many endothelium-dependent vasodilators as well as regulation of membrane potential are largely a function of pulmonary endothelial intracellular calcium levels ($[Ca^{2+}]_i$). The activity of endothelial nitric oxide synthase (9, 17, 53, 62), phospholipase 2 (PLA_2) (43, 69), and the small and intermediate conductance Ca^{2+}-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively) that are responsible for endothelial cell hyperpolarization upon activation by agonists (25, 45) are regulated by $[Ca^{2+}]_i$. Thus, diminished pulmonary endothelial $[Ca^{2+}]_i$ may limit production of these endothelium-derived vasoactive factors. In CH-induced pulmonary hypertension, for example, posttranslational regulation of eNOS activity is impaired due to reduced agonist-induced Ca^{2+} influx (51). Our previous work also shows that both basal $[Ca^{2+}]_i$ and agonist-induced Ca^{2+} influx are lower in PAEC from CH rats compared to those of control animals (56, 57). CH similarly inhibits endothelial SOCE, receptor-operated Ca^{2+} entry, and T-type VGCC-mediated depolarization-induced Ca^{2+} influx, which are major components of agonist-induced Ca^{2+} entry in isolated PAEC (56, 58). These findings suggest that store-operated channels and T-type VGCCs are important in determining endothelial Ca^{2+} influx, and impaired Ca^{2+} entry through these channels may contribute to reduced basal $[Ca^{2+}]_i$ in PAEC after CH. Previous studies from our laboratory suggest that CH inhibits endothelial Ca^{2+} influx through alterations in membrane lipid domains, which represent key regulatory sites of ion channel function in PAEC (57, 84).

Cholesterol is a polycyclic amphipathic molecule with a polar section consisting of a single β-hydroxyl group that can interact with membrane lipids or proteins through formation of hydrogen bonds (61). Cholesterol-enriched caveolar microdomains are signal transduction platforms where many ion channels and their regulatory factors reside (71). Membrane cholesterol can inhibit some ion channels by decreasing open probability, unitary conductance, and the number of active channels (38). In contrast, ion channels such as the nicotinic acetylcholine receptor, GABA receptors, epithelial Na^+ channels and TRPC channels are inhibited by removal of membrane cholesterol (4–6, 32, 73), indicating an important role for cholesterol in normal channel function. Consistent with this regulatory function, our previous studies show that membrane cholesterol facilitates major Ca^{2+} entry pathways in PAEC, including agonist-induced Ca^{2+} entry, SOCE, and depolarization-induced Ca^{2+} entry, and that CH impairs endothelial Ca^{2+} influx by reducing membrane cholesterol levels (84). However, the mechanisms by which cholesterol regulates endothelial Ca^{2+} influx are not well-investigated.

The current study focused on exploring the ion channel involved in membrane cholesterol-dependent endothelial SOCE, a major component of agonist-induced Ca^{2+} entry, in the pulmonary circulation. Orai1 and transient receptor potential canonical channels (TPRC) have been reported as putative SOC (18, 40, 60, 82, 87). Depletion of ER Ca^{2+} stores causes STIM1 oligomerization (15, 39), which recruits Orai1 into microdomains and ensures the physical interaction between STIM1 and Orai1, leading to Orai1 activation and Ca^{2+} influx (86). TRPC1 and TRPC4 channels also localize to caveolar microdomains and regulate endothelial Ca^{2+} entry in murine PMVEC (50). However, TRPC1-mediated SOCE is less Ca^{2+} selective compared to Orai1-mediated

SOCE (35) and requires functional Orai1 (34). Orai1 also interacts with TPRC4 and regulates TRPC1/4 heterotetramer channel activation and Ca^{2+} selectivity in lung endothelial cells (12). The current study investigated Orai1 as a candidate ion channel in mediating cholesterol-dependent endothelial SOCE. We found that either pharmacological inhibition or gene silencing of Orai1 significantly decreased endothelial SOCE, demonstrating that Orai1 contributes to SOCE in both PAEC and PMVEC. Furthermore, AnCoA4-sensitive SOCE was abolished in PAEC from CH rats, suggesting that the reduction in SOCE following CH results from impaired Orai1 activity. Interestingly, our finding that cholesterol replenishment rescues Ca^{2+} entry in PAEC from CH rats suggests that altered expression of Orai1 or STIM1 does not explain this deficit, but rather regulation of Orai1 activity by the molecular composition of the membrane. Such influences of cholesterol may occur through direct interaction with Orai1 and STIM1, indirectly through regulation of caveolin-1 or other lipid raft components, or by altering Orai1/STIM1 trafficking and membrane localization.

Although AnCoA4 is characterized as a selective inhibitor of Orai1 (ref), whether AnCoA4 similarly inhibits Orai2 or Orai3 is unknown. These related Ca^{2+} channel isoforms have been implicated as store-operated channels in a variety of cell types (16, 33, 68, 77), and mediate SOCE when over-expressed with STIM1 in heterologous expression systems (48). However, in contrast to the established contribution of Orai1 to SOCE in pulmonary endothelial cells, store-depletion induced Ca^{2+} entry is independent of Orai2 and Orai3 in endothelial cells from several vascular beds (30, 74, 85). Furthermore, whether these channels are expressed and play a functional role in PAECs remains to be established. Therefore, while we cannot exclude a possible inhibitory effect of AnCoA4 on

Orai2 or Orai3 in the present study, the similar effects of AnCoA4 and Orai1 siRNA to inhibit SOCE are supportive of a contribution of Orai1 to this response.

Cholesterol may modulate the function of membrane proteins *via* either direct interaction or through altering the properties of lipid microdomains (38). Several groups have reported that membrane cholesterol depletion by MβCD attenuates SOCE in a variety of cell types (14, 20, 24, 29, 59). Interestingly, however, recent reports by Derler et al (13) and Pacheco et al (55) reported cholesterol-binding sites on Orai1 and STIM1, respectively, and demonstrated an inhibitory effect of membrane cholesterol on SOCE in cultured human embryonic kidney 293 cells and rat basophilic leukemia 2H3 cells. Both studies further showed that the cholesterol-Orai1 and cholesterol-STIM1 interaction was attenuated by mutating the cholesterol-binding site of the target protein. Although many studies have employed the cholesterol-depleting agents MβCD or filipin to evaluate functional roles for membrane cholesterol, an important limitation of these agents is that they may exert off-target effects by disrupting caveolar stucture, thereby altering biophysical properties of the plasma membrane (2, 61, 84). Thus, these approaches may interrupt SOCE in a non-specific manner unrelated to direct interaction of membrane cholesterol with ion channels, which may limit the interpretation of how cholesterol regulates SOC. In our current studies, we substituted endogenous cholesterol with Epichol, an approach that alters membrane cholesterol content without disrupting caveoli (84), and found that cholesterol is required for Orai1-mediated SOCE in both isolated PAEC and cultured PMVEC. We also showed that Epichol substitution significantly reduced the STIM1-Orai1 interaction, suggesting that membrane cholesterol regulates endothelial SOCE by facilitating this interaction that is required for Orai1 activation. The reason for

the conflicting observations of how membrane cholesterol regulates SOCE in our current studies and those of other groups (13, 55) is not clear, but may be due to heterogeneity between either different cell types or between transfected/cultured cells and native endothelial cells. Additionally, since SOCE is a complex muti-step signaling pathway, it is also possible that membrane cholesterol affects components of SOCE differently. Galan et al and Pani et al (20, 59) suggested that membrane cholesterol is required for STIM1 oligmerization and subsequent interaction with and activation of Orai1. After the STIM1-Orai1 complex is formed, however, cholesterol may inhibit SOCE through direct interaction with Orai1 and STIM1 (13, 20, 55). Additional experiments are required to investigate if membrane cholesterol directly regulates endothelial STIM1 clustering, STIM1-Orai1 interaction, and Orai1 activation in PAECs.

Although our studies indicate that membrane cholesterol plays a functional role in Orai1-mediated SOCE in pulmonary endothelial cells, these findings do not preclude a potential role for cholesterol to regulate TRPC channels independent of Orai1 and STIM1. Despite a lack of evidence for direct regulatory cholesterol binding sites on TRPC channel sequences, the membrane cholesterol sensitivity of TRPC channels has been reported in several cell types (1, 6, 37, 50, 81). For example, the extraction of membrane cholesterol by cyclodextrins impairs TRPC1 signaling processes (6, 42). Although cholesterol may play a pivotal regulatory role in TRPC1-mediated SOCE by regulating membrane structure, direct effects of cholesterol on TRPC functions are possible.

Our studies also suggest that loss of membrane cholesterol contributes to impaired endothelial SOCE in CH-induced pulmonary hypertension (84). Due to the limitation of the filipin staining approach to detect oxidized cholesterol (63), reduced membrane

cholesterol following CH is possibly the result of impaired cholesterol *de novo* synthesis (54), oxidative modification of cholesterol (52) or decreased membrane cholesterol trafficking. Nguyen et al (54) have explored the mechanism by which hypoxia affects *de novo* cholesterol biosynthesis. They reported that hypoxia induces accumulation of cholesterol biosynthetic intermediates and rapid degradation of HMG-CoA reductase, which contribute to a reduction of cholesterol production. Given the importance of reactive oxygen species to the development of CH-induced pulmonary hypertension (19, 26, 27, 41), endothelial dysfunction in this setting may alternatively result from oxidative modifications to cholesterol that alter membrane lipid domains and interfere with ion channel function.

Based on our findings that CH attenuates pulmonary endothelial Ca^{2+} entry by depleting membrane cholesterol, it may be predicted that targeted depletion of PAEC cholesterol would minimally or adversely affect the development of PH in this setting. In apparent contrast to this prediction, HMG-CoA reductase inhibitors (statins) have demonstrated beneficial effects in attenuating the development of CH-induced pulmonary hypertension in animal models (23, 36, 49). However, the mechanisms by which statins mediate this protective influence are thought to be independent of their cholesterol lowering properties (10, 46, 70, 78, 80). These include inhibition of RhoA that can not only decrease vascular smooth muscle contractility (76), proliferation and migration (79), but also increase eNOS expression and activity (70). Additional evidence suggests that statins increase NO production by inhibiting expression and activity of NADPH oxidase subunits, and thus production of endothelial reactive oxygen species (44, 78, 80), rather than through effects on endothelial cholesterol content. Such non-specific effects of statins likely result

from decreased production of intermediates of the mevalonate pathway that are necessary for posttranslational modification of many signaling proteins (7). In contrast to these animal studies, however, clinical trials of statin therapy in patients with pulmonary arterial hypertension have been largely inconclusive (66).

In conclusion, the current study demonstrates a novel effect of membrane cholesterol to regulate pulmonary endothelial SOCE by facilitating the interaction of STIM1 and Orai1 (Figure 8). These observations advance our basic understanding of how membrane cholesterol regulates endothelial $[Ca^{2+}]_i$ homeostasis, and have potentially broader implications for cholesterol-dependent regulation of a wide range of vasoactive and mitogenic pathways. These findings may additionally provide a mechanistic basis to explain the CH-induced diminution of endothelial Ca^{2+} influx and associated endothelial dysfunction that is central to the pathogenesis of pulmonary hypertension. Challenges of future studies are to identify the potential contribution of reactive oxygen species to decreased endothelial cholesterol following CH, either through direct cholesterol oxidation or impaired sterol trafficking, and the contribution of these responses to increased vasoconstrictor reactivity, arterial smooth muscle mitogenesis, and the development of pulmonary hypertension.

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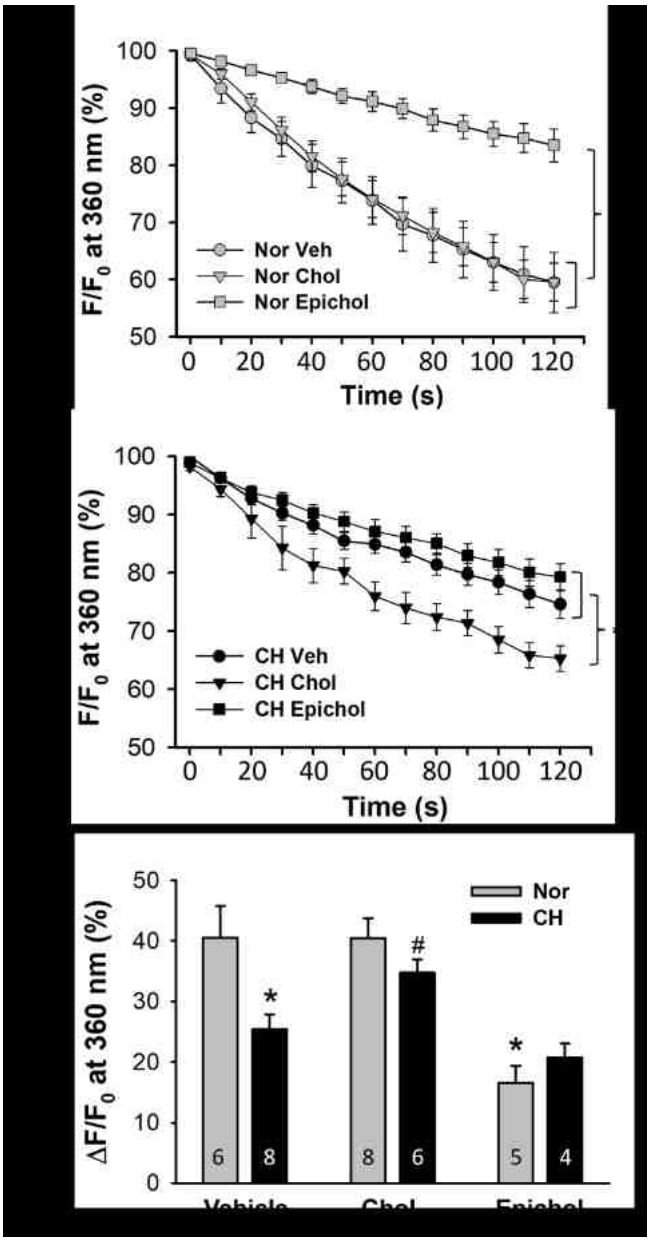

Figure 1. Impaired pulmonary endothelial store-operated Ca^{2+} entry (SOCE) following chronic hypoxia (CH) is restored by cholesterol supplementation. Cyclopiazonic acid (CPA)-induced Ca^{2+} entry was assessed by Mn^{2+} quenching technique in freshly isolated pulmonary artery endothelial cell (PAEC) sheets from control and CH rats. Cells were pre-treated with vehicle, cholesterol (Chol), or epicholesterol (Epichol). F, fluorescence intensity at 360 nm excitation; F_0, fluorescence intensity at time zero. **A)** SOCE in PAEC from normoxic rats.; n = 5-8 animals/group. *P <0.05 vs. Veh and Chol over the range of 30 - 120 s. **B)** SOCE in PAEC from CH rats. n = 4-8 animals/group. *P <0.05 vs. Veh and Epichol over the range of 60 - 120 s. **C)** SOCE in PAEC from each group 120 s after onset of quenching. *P <0.05 vs. Nor Vehicle; #P <0.05 vs. CH Vehicle. Values are means ± SE. A One-way ANOVA (A,B) or two-way ANOVA (C) followed by the Student-Newman-Keuls test were used to compare between groups.

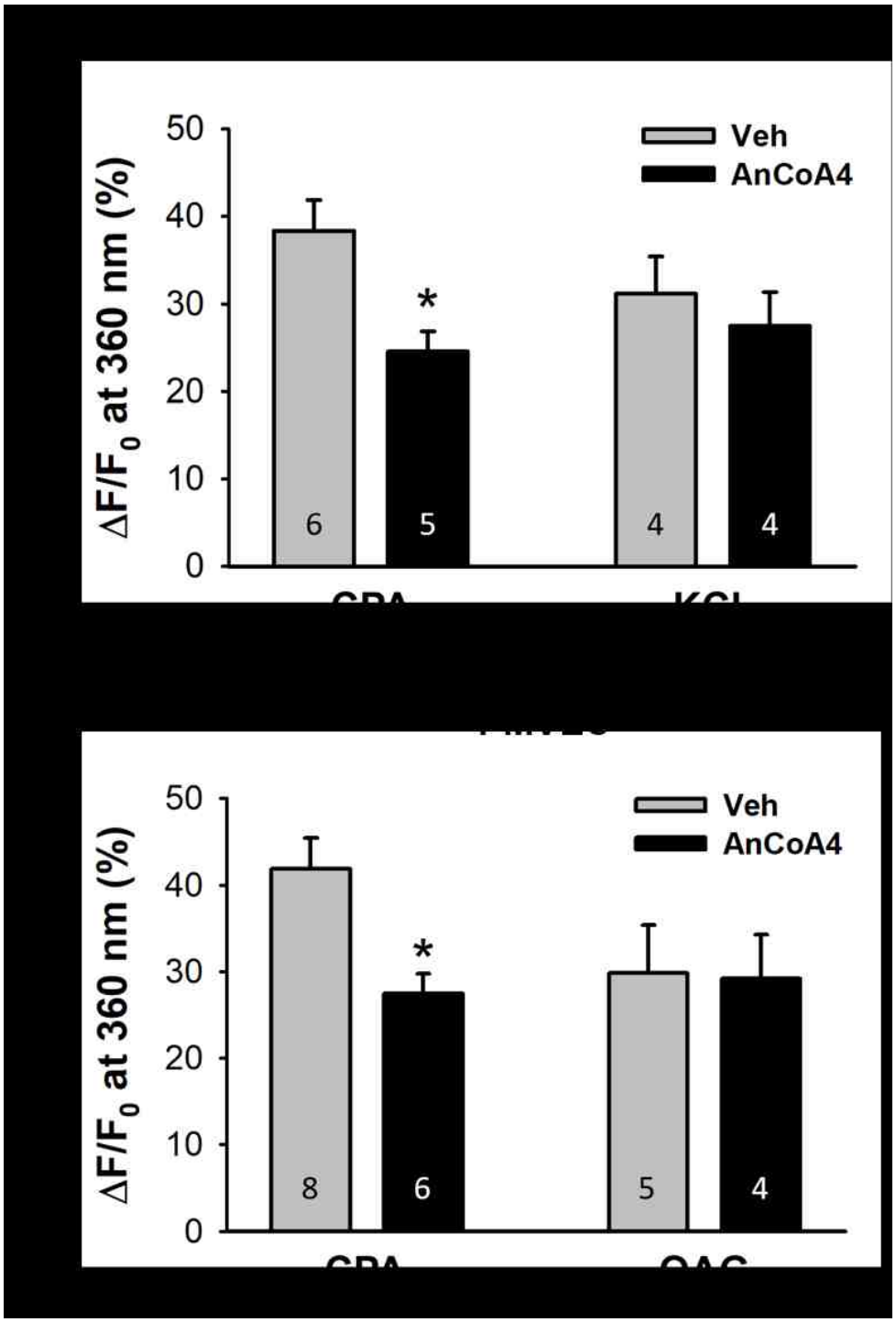

Figure 2. Orai1 mediates pulmonary endothelial SOCE. **A)** AnCoA4 inhibits SOCE but not depolarization-induced Ca^{2+} entry in freshly dispersed PAEC from normoxic rats. **B**) AnCoA4 attenuates SOCE but not OAG (diacylglycerol analog)-induced Ca^{2+} entry in cultured pulmonary microvascular endothelial cells (PMVEC). An unpaired t-test was used to compare between groups at 120 s after onset of quenching. *$P < 0.05$ vs. Veh CPA.

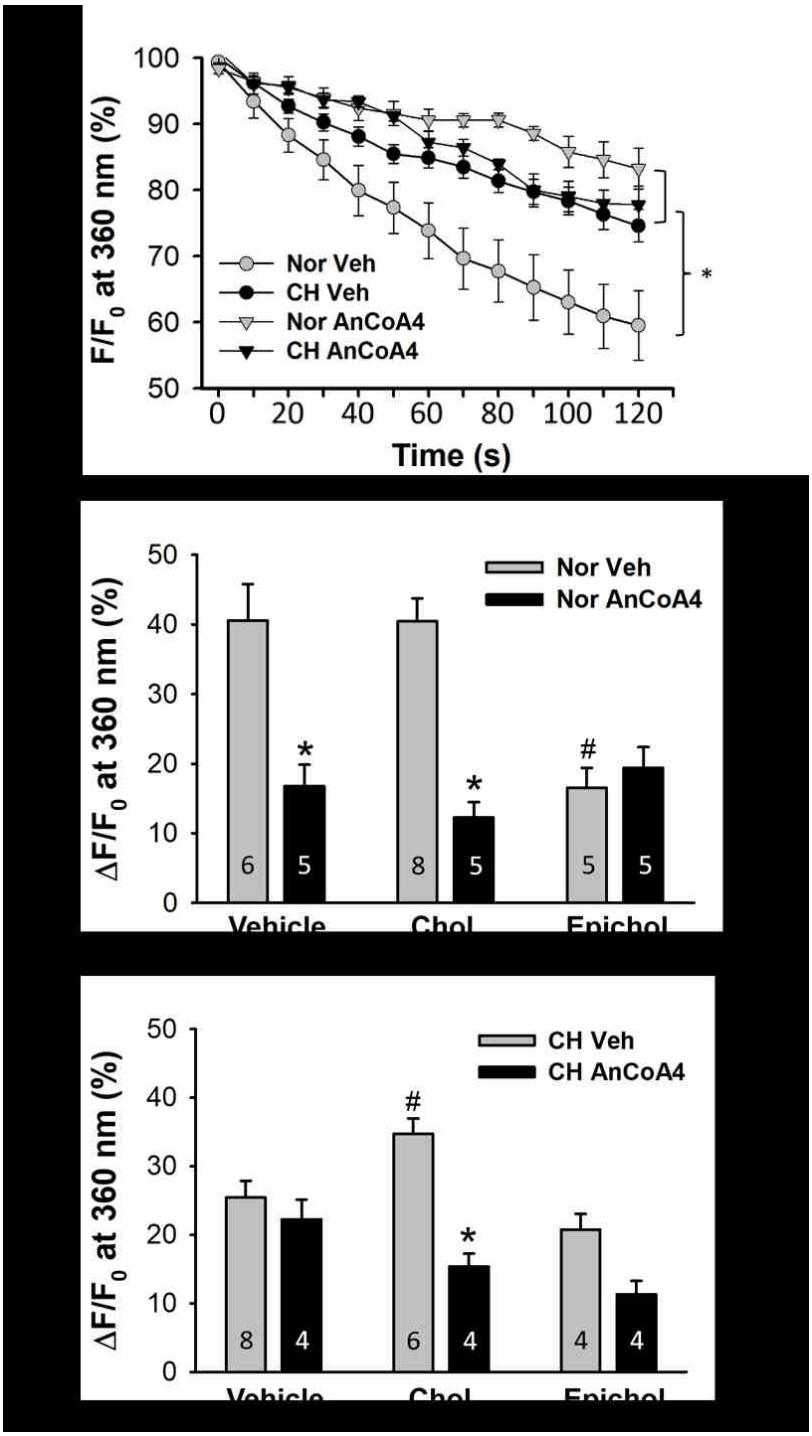

Figure 3. Membrane cholesterol-sensitive Orai1 contributes to impaired SOCE in PAEC following CH. **A)** SOCE was assessed in PAEC from normoxic and CH rats pretreated with either the Orai1 inhibitor, AnCoA4, or vehicle. n = 6, Nor Veh; n = 8, CH Veh; n = 5, Nor AnCoA4; n = 4, CH AnCoA4. *P < 0.05 vs. Nor-Veh over the range of 40 - 120 s. Effects of AnCoA4 on SOCE (120s after onset of quenching) in freshly dispersed PAEC from normoxic **(B)** and CH **(C)** rats following membrane cholesterol manipulation. *P <0.05 vs. Veh within groups; $^{\#}P$ <0.05 vs. Veh-Vehicle.

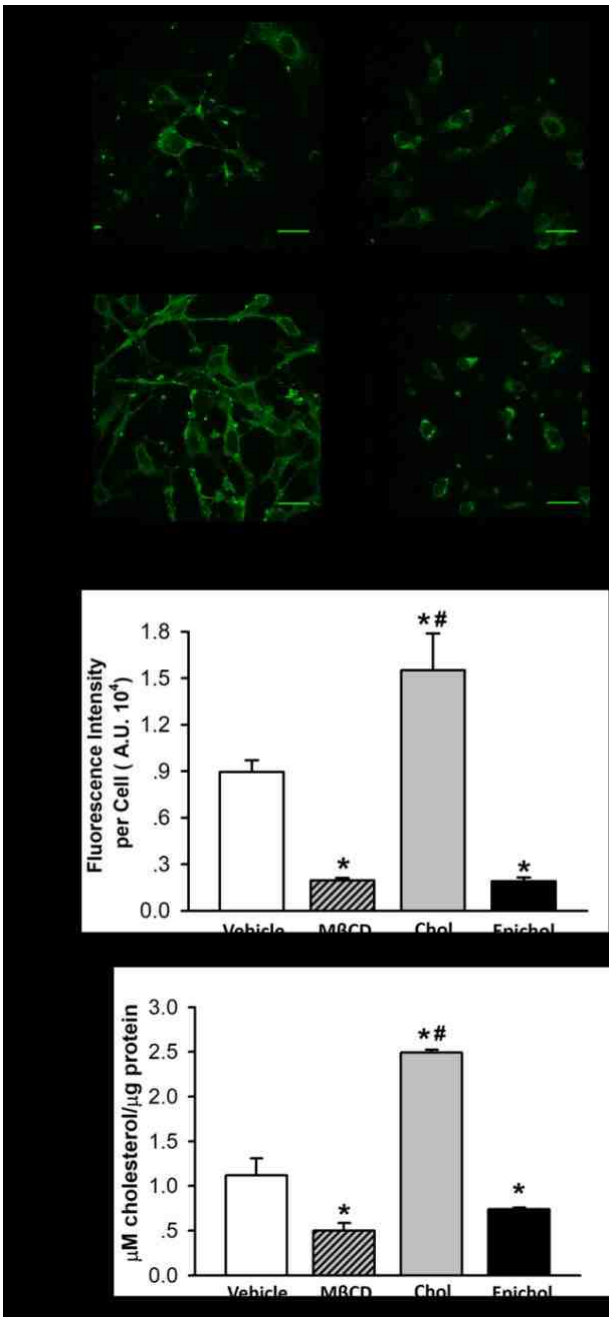

Figure 4. Epicholesterol reduces endogenous membrane cholesterol content of cultured PMVEC. **A)** Representative images of filipin fluorescence from PMVEC treated with vehicle, MβCD, cholesterol, or epicholesterol. **B)** Mean filipin fluorescence (arbitrary units; A.U.) in PMVEC from each group. Data were compared by the Kruskal-Wallis H test and Dunn's multiple comparison test. n = 5 (filipin fluorescence of 4-16 cells were measured per field of interest and averaged for an n=1). $^{*}P < 0.05$ vs. Vehicle; $^{\#}P < 0.05$ vs. MβCD and Epichol. **C)** Amplex Red cholesterol assay to detect cholesterol concentration in PMVEC. Data were compared by one-way ANOVA followed by the Student-Newman-Keuls test. $n = 3$. $^{*}P < 0.05$ vs. Vehicle; $^{\#}P < 0.05$ vs. MβCD and Epichol.

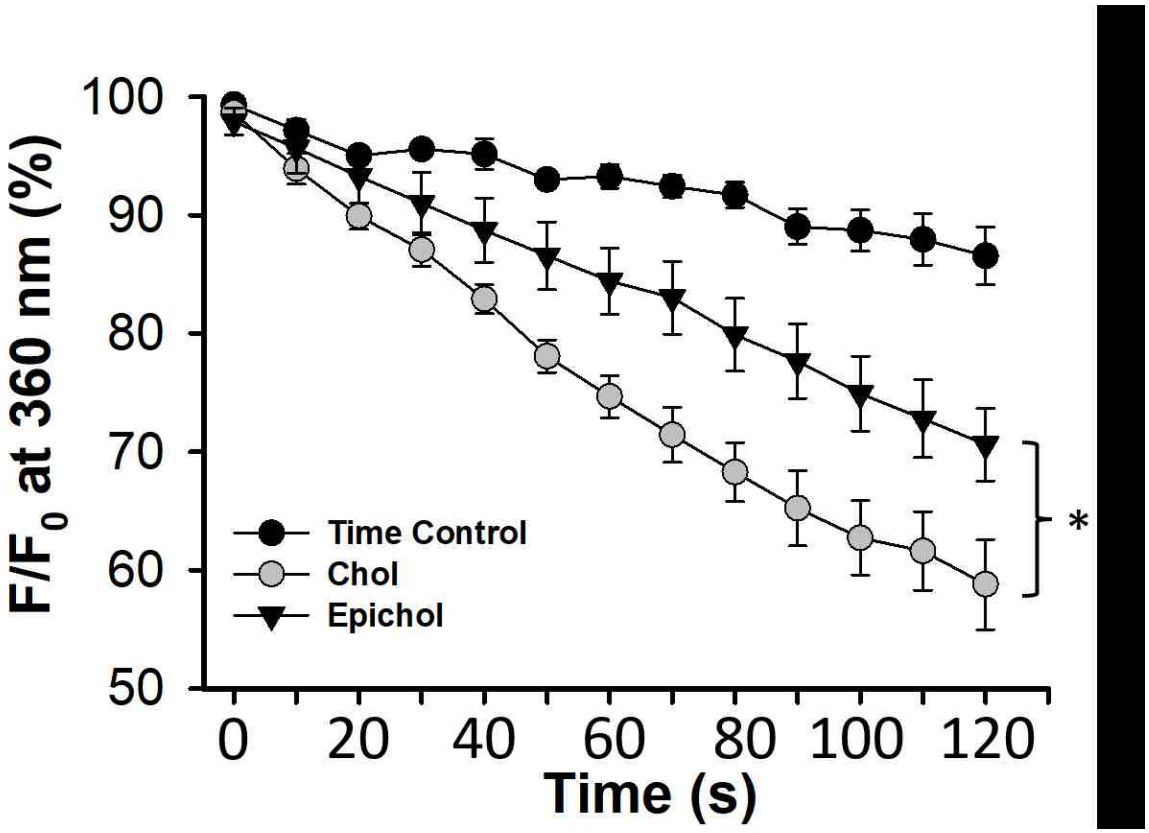

Figure 5. Epicholesterol substitution reduces SOCE in PMVEC pretreated with cholesterol. $n = 5$, Chol; $n = 5$, Epichol; $n = 3$, Time Control. *$P < 0.05$ vs. Chol over the range of 50 - 120 s.

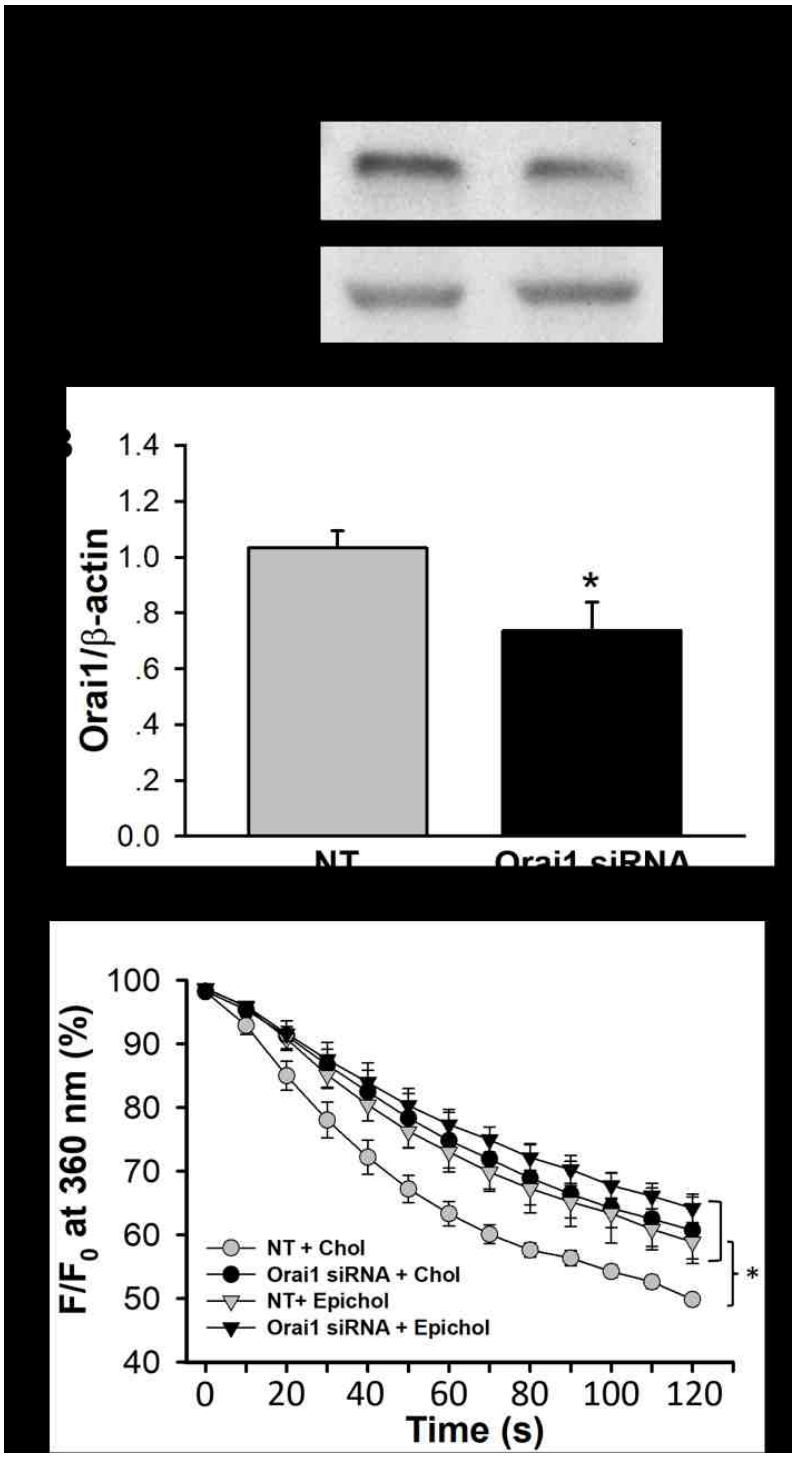

Figure 6. Orai1 siRNA knockdown inhibits membrane cholesterol-dependent SOCE in PMVEC. **A)** Representative western blot of Orai1 and β-actin protein bands in PMVEC transfected with Orai1 siRNA or non-targeting (NT) siRNA. **B)** Mean western blot data of Orai1 expression from NT and Orai1 siRNA treated PMCEC. Orai 1 levels are normalized to those of β-actin. $n = 4$ /group. *$P < 0.05$ vs. NT. **C)** SOCE in cultured PMVEC. $n = 3$-4 /group. *$P < 0.05$ vs. NT+Chol over the range of 60 - 120 s.

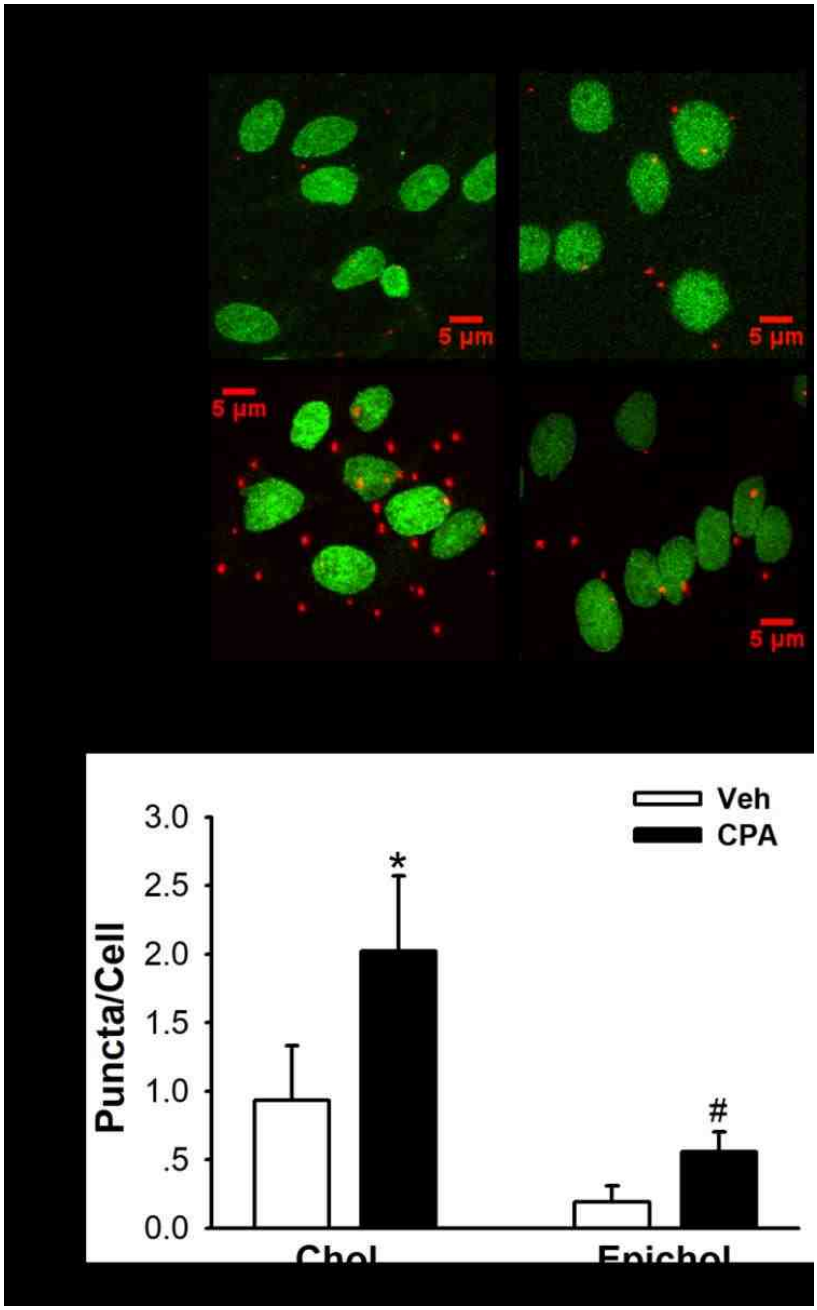

Figure 7. Epicholesterol substitution reduces STIM1-Orai1 interaction in PMVEC as assessed by *in situ* proximity ligation assay. **A)** Representative images of STIM1-Orai1 interactions (red puncta) in response to CPA in cultured PMVEC with manipulated membrane cholesterol. Nuclei area labeled with SYTOX (green). **B)** Summarized data of STIM1-Orai1 interaction expressed as average number of puncta/cell in cultured PMVEC. Cholesterol supplementation increased the CPA-induced STIM1-Orai1 interaction, whereas epicholesterol substitution significantly inhibited this response. Groups were compared by two-way ANOVA followed by multiple comparisons testing using the Student-Newman-Keuls test. n = 5/group. $^{*}P$ <0.05 vs. Chol Veh; $^{\#}P$ <0.05 vs. Chol CPA.

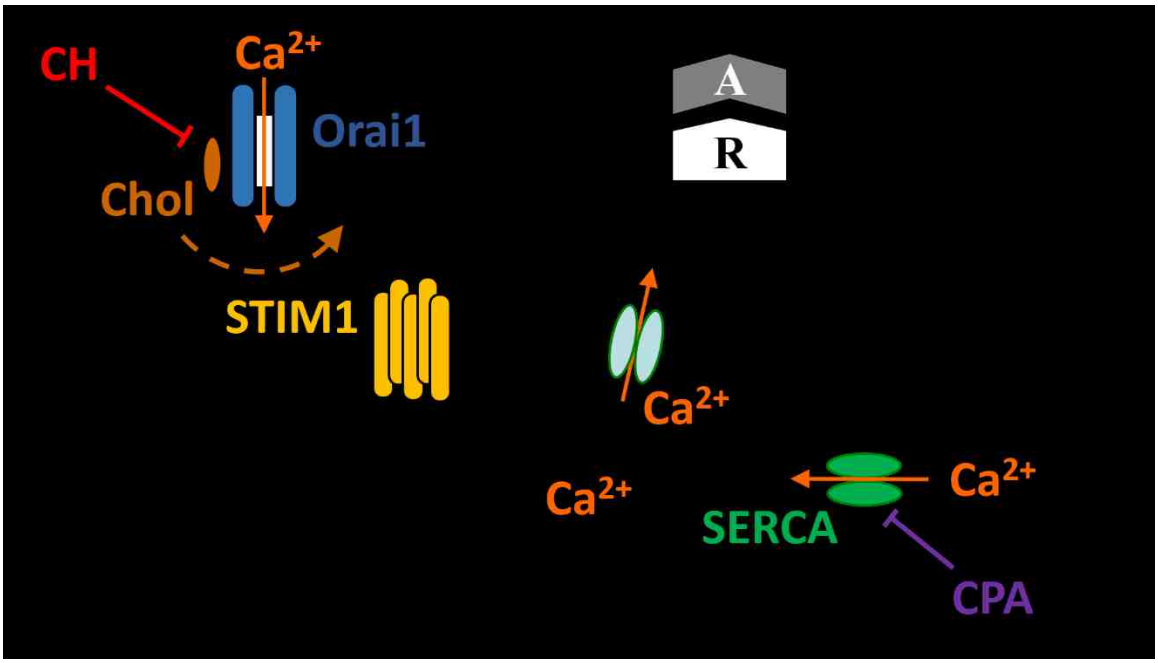

Figure 8. Proposed mechanism by which reduced cell membrane cholesterol (Chol) following CH attenuates SOCE in PAECs. Cholesterol facilitates the interaction of STIM1 and Orai1 in response to depletion of intracellular Ca^{2+} stores, *e.g.* by receptor (R)-mediated activation of inositol trisphosphate receptors (IP_3R), or inhibition of the sarco/endoplasmic reticulum Ca^{2+} -ATPase (SERCA). CH inhibits this mechanism by depletion of cell membrane cholesterol. Endoplasmic reticulum (ER), agonist (A).

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CHAPTER 4: DISCUSSION

The goal of this dissertation was to investigate the role of membrane cholesterol in pulmonary endothelial Ca^{2+} entry in CH-induced pulmonary hypertensive rats. In the current studies we tested the hypotheses that 1) *membrane cholesterol facilitates SOCE and depolarization-induced pulmonary endothelial Ca^{2+} entry*; 2) *membrane cholesterol regulates Orai1-mediated SOCE through facilitating STIM1-Orai1 interaction*, and 3) *reduced endothelial Ca^{2+} influx following CH is due to a loss of membrane cholesterol*. We identified the role of membrane cholesterol in regulating agonist-induced Ca^{2+} entry and its components in PAECs following CH through measurement of $[Ca^{2+}]_i$ using a ratiometric fluorescent Ca^{2+} indicator in live cells with cholesterol supplementation or substitution of cholesterol with its epimer, epicholesterol. The rationale behind these studies emerges from the observation in CH-induced PH that dysregulation of endothelium-derived mediators contributes to elevated vascular resistance in the pulmonary circulation. The production of many endothelium-derived vasodilators and anti-mitogenic substances like NO (18, 39, 89, 102) and prostacyclin (60, 144) are largely a function of endothelial $[Ca^{2+}]_i$. Therefore, it is possible that reduced endothelial $[Ca^{2+}]_i$ limits the production of NO, prostacyclin, and other endothelium-derived hyperpolarizing factors. Our laboratory previously reported that both basal $[Ca^{2+}]_i$ and agonist-induced Ca^{2+} influx are lower in PAECs from CH rats compared to their normoxic controls (95). Both endothelial SOCE and depolarization-induced Ca^{2+} entry through T-type VGCCs, two major components of ATP-induced Ca^{2+} influx, are also reduced following CH exposure (94, 96). Interestingly, membrane cholesterol levels along with the agonist-induced Ca^{2+} entry are decreased in PAECs of chronic hypoxic rats. This impaired Ca^{2+} influx is restored

simply by either membrane cholesterol supplementation or administration of caveolin-1 scaffolding domain peptide (95). Because membrane cholesterol and caveolin-1 are major structural components of caveolae, the diminished Ca^{2+} influx could be the result of decreased caveolae after CH. However, the number of caveolae are not altered in PAECs from CH rats compared to controls (95). These observations suggest a role of membrane cholesterol in mediating endothelial Ca^{2+} influx.

The major findings from the current studies are that substitution of endogenous membrane cholesterol with epicholesterol attenuates ATP-induced Ca^{2+} entry, SOCE and depolarization-induced Ca^{2+} entry in PAEC. In addition, decreased endothelial SOCE and depolarization-induced Ca^{2+} entry following CH are largely restored by cholesterol supplementation. Importantly, neither cholesterol supplementation nor epicholesterol substitution alters endothelial caveolar number. In addition, membrane cholesterol regulates Orai1-mediated SOCE in both native and cultured cells. Epicholesterol substitution greatly reduces SOCE and STIM1-Orai1 interaction in cultured PMVECs. These data suggest that membrane cholesterol directly regulates agonist-induced Ca^{2+} entry and its components. These observations also demonstrate that altered membrane cholesterol homeostasis may contribute to impaired endothelial Ca^{2+} entry following CH.

Effect of hypoxia on pulmonary endothelial $[Ca^{2+}]_i$

The mechanisms by which hypoxia affects endothelial $[Ca^{2+}]_i$ are not well-investigated. Studies investigating the effect of acute hypoxia on endothelial $[Ca^{2+}]_i$ *in vitro* show similar results in cells from the systemic and pulmonary vasculatures. In human umbilical vein endothelial cells (HUVEC) (6, 11), as well as in bovine (53) and porcine (58) PAECs, acute hypoxia increases endothelial $[Ca^{2+}]_i$. The significant increase of $[Ca^{2+}]_i$

in HUVECs is due to energy deficiency and subsequent influx of extracellular Ca^{2+} (6). Berna et al (11) later discovered that the Na^{+}/ Ca^{2+} exchanger is involved in this hypoxia-induced Ca^{2+} influx. By using an inhibitor of Na^{+}-glucose cotransport, they proposed that energy deficiency activates glycolysis and subsequent activation of Na^{+}-glucose cotransport, which leads to a series of actions including Na^{+} influx, Na^{+}/ Ca^{2+} exchanger activation, and Ca^{2+} influx. In PAECs, however, although acute hypoxia (5 min) causes a rapid and transient increase in $[Ca^{2+}]_i$ by releasing Ca^{2+} from ER stores (53), a longer duration of hypoxia (15 min) decreases $[Ca^{2+}]_i$ likely by causing membrane depolarization and reducing the electrochemical driving force for Ca^{2+} entry (124). While it is certainly possible that hypoxia has a dual-phase effect of releasing Ca^{2+} from internal stores and inhibiting Ca^{2+} influx in bovine PAECs, it should be noted that Hampl et al (53) used bovine cells of high passage (16-25), whereas Stevens et al (124) used primary bovine PAECs. The increased passage number of cells may cause changes in ion channel expression and activity (55, 70). In rat PAECs, for example, although T-type VGCCs are not found in cultured cells (145), their expression and function are described in freshly dispersed PAECs (96). This difference may lead to opposite effects of hypoxia on endothelial Ca^{2+} entry, as hypoxia-induced membrane depolarization may blunt Ca^{2+} influx driven by electrochemical gradient in cultured PAECs while increasing Ca^{2+} entry through T-type VGCCs in native PAECs.

In human PAEC, prolonged exposure to hypoxia (3% O_2, 60-72h) causes an increase in basal $[Ca^{2+}]_i$, which is due to enhanced expression of TRPC-encoded SOC channels and Ca^{2+} influx (53). However, it is possible that exposure of hypoxia to cultured cells may not mimic the *in vivo* effect of CH on PAECs. It is also likely that CH impairs

the regulation of pulmonary endothelial $[Ca^{2+}]_i$ as PH develops. Murata et al (87) showed that in 1 week CH-induced pulmonary hypertensive rats, agonist-induced Ca^{2+} influx in the pulmonary endothelium is significantly less compared to normoxic control rats. Consistently, in rats exposed to 4 weeks CH, our previous data demonstrate that, in PAECs, both basal $[Ca^{2+}]_i$ and agonist-induced Ca^{2+} influx with its major components, SOCE and depolarization-induced Ca^{2+} influx through T-type VGCCs, are lower compared to those of normoxic control animals (94–96). In addition, membrane cholesterol levels are also reduced in PAEC from rats exposed to CH. The most exciting discovery is that the impaired agonist-induced Ca^{2+} influx in PAEC can be restored by both membrane cholesterol supplementation and by administration of a caveolin-1 (Cav-1) scaffolding domain peptide (95). This finding suggests that the reduced endothelial agonist-induced Ca^{2+} influx following CH is not likely due to reduced expression of the relevant Ca^{2+} channels. In the current studies, we found that the diminished SOCE and depolarization-induced Ca^{2+} influx can also be restored by membrane cholesterol supplementation **(Figure 5 and 9: Chapter 3)**. These data suggest that cholesterol *per se* may affect Ca^{2+} entry in these cells. By using epicholesterol, we also determined that cholesterol directly modulates endothelial agonist-induced Ca^{2+} entry, SOCE and depolarization-induced Ca^{2+} entry independent of its structural role in the plasma membrane, which will be discussed later in "Membrane cholesterol: structural component vs. signaling mediator".

Although we did not examine the change of $[Ca^{2+}]_i$ in early stages of CH-induced PH, it is possible that hypoxia augments Ca^{2+} influx and $[Ca^{2+}]_i$ in PAEC during the early stages of hypoxic exposure (135). However, after prolonged hypoxia, endothelial Ca^{2+} entry may be gradually diminished due to altered cholesterol homeostasis, leading to

decreased $[Ca^{2+}]_i$ in PAECs. The finding that reduced endothelial Ca^{2+} entry in pulmonary arteries following CH was acutely restored by membrane cholesterol supplementation suggests that CH limits Ca^{2+} entry in PAECs by reducing membrane cholesterol rather than by decreasing ion channel expression. The physiological consequences of these changes in pulmonary endothelial Ca^{2+} influx and $[Ca^{2+}]_i$ will be discussed in detail later in "Physiological significance of current studies".

Pulmonary endothelium-mediated vasodilation following CH

The results of our current and previous studies (94) show that agonist-induced Ca^{2+} entry as well as basal $[Ca^{2+}]_i$ are reduced in PAECs following CH. Although we did not examine the physiological consequences of decreased pulmonary endothelial $[Ca^{2+}]_i$, the reduction of $[Ca^{2+}]_i$ may limit the synthesis and release of vasodilators such as NO, which would impair endothelium-dependent vasodilation in pulmonary arteries. However, opposite to this assumption, early studies from our and other laboratory demonstrated that CH enhances pulmonary artery endothelium-derived nitric oxide (EDNO)-dependent vasodilation (32, 59, 108, 115). In these studies using isolated perfused lungs, there was an increase in AVP/histamine/ET-1-induced EDNO-dependent vasodilation in rats exposed to 4 weeks of CH (32, 108). However, CH did not affect the vasodilation to an exogenous NO donor, suggesting that the change of agonist-induced vasodilatory responses after CH was due to reduced bioavailability of NO. Later studies also found that eNOS protein expression is elevated in CH rats compared to their normoxic controls (107). Thus, the apparent contradictory observation of impaired pulmonary endothelial $[Ca^{2+}]_i$ and augmented EDNO-dependent vasodilation following CH warrants discussion.

Because endothelium-derived NO is the result of multiple signaling cascades involving Ca^{2+}-dependent and Ca^{2+}-independent pathways, receptor stimulation by agonists may lead to NO production through different mechanisms. In addition, since the production of other endothelium-derived vasodilators like prostacyclin and EDHF are also closely regulated by endothelial $[Ca^{2+}]_i$ (56, 79, 117), NO may not fully responsible for agonist-induced endothelium-dependent vasodilation. The receptors responsible for AVP/histamine/ET-1 -induced EDNO are mainly V1-receptors (114)/H1 and H2-receptor (115, 126)/ETB receptors (33). V1-receptors, H1-receptors, and ETB receptors are Gq-protein coupled receptors (27, 69, 127, 128), the activation of which leads to Ca^{2+} entry though similar pathways as ATP-induced Ca^{2+} influx described in the introduction. V2- and H2-receptors are Gs-protein coupled receptors that may be involved in NO production through Ca^{2+}-independent mechanisms (65, 66). The stimulation of Gs-protein coupled receptors activates adenylyl cyclase and subsequently produces cyclic AMP (cAMP) from ATP. cAMP may phosphorylate eNOS-Ser (bovine eNOS-Ser^{1179} or human eNOS-Ser^{1177}) through AMP-activated protein kinase (22) or protein kinase B (PKB) (149) signaling pathways. Phosphorylation of eNOS-Ser increases the activity of eNOS by reducing the relative requirement of Ca^{2+} for its activation, which is achieved by increasing the rate of electron flux from the reductase to the oxygenase domain of the protein and reduces (82). Thus, with multiple signaling pathways leading to vasodilation, the physiological effect of reduced agonist-induced Ca^{2+} entry after CH may not be reflected when potential compensatory mechanisms are present. In fact, H2-receptor -mediated vasodilatory responses to histamine were augmented in the pulmonary circulation following CH (115). Besides, since EDHF also mediates histamine-induced vasodilation in the pulmonary

circulation (54), it is also possible that this vasodilatory pathway is augmented after CH, which may explain why non-EDNO-mediated vasodilation by histamine was increased after CH (108).

Despite the potential compensatory mechanisms by which CH increases agonist-induced NO production and vasodilation, Ca^{2+}-dependent EDNO production may still be increased as a result of enhanced expression of eNOS (107) and receptors for agonists (122) in PAECs from CH rats. Because endothelial ROS production is elevated following CH (62), enhanced Ca^{2+}-dependent EDNO production may be a compensatory mechanism to increase NO bioavailability. In addition, based on studies by Murata et al (87), in rats exposed to 1 week chronic hypoxia, agonist-induced Ca^{2+} entry and NO production are significantly reduced in pulmonary endothelium while no change of eNOS expression is detected. The reduced NO production could be the result of both decreased Ca^{2+}-dependent eNOS activation and impaired eNOS activity due to tight coupling of eNOS/caveolin-1. This evidence suggests that endothelial Ca^{2+} entry and associated NO production are impaired in the early stage of CH-induced PH, which may activate potential compensatory mechanisms that increase agonist-induced EDNO production.

Membrane cholesterol: structural component vs. signaling mediator

The role of membrane cholesterol in regulating ion channel function has received considerable attention during the last decade. It is now well-accepted that cholesterol is a crucial regulator of various ion channels and plays different roles depending on the type of ion channel. In general, membrane cholesterol may decrease the number of active channels or the activity of several K^+ channels, voltage-gated Na^+ and Ca^{2+} channels, and volume-

regulated anion channels (25, 74, 112, 130). However, other ion channels such as the nicotinic acetylcholine receptor, $GABA_A$ receptors, epithelial Na^+ channels and TRPC channels are inhibited by removal of membrane cholesterol (8–10, 71, 123). These dynamic roles of membrane cholesterol in regulating ion channel function are possibly achieved through two mechanisms: 1) indirect regulation by altering the properties of lipid microdomains; and 2) direct interaction with ion channels. The first mechanism was proposed by Lundbaek et al (78) that membrane cholesterol may affect ion channels by modulating bilayer stiffness and hydrophobic interactions between membrane proteins and the lipid bilayer. The stiffer membrane may lead to increased membrane deformation energy that affects the conformational change of ion channels when transitioning between open and closed states (78). A change in the lipid bilayer properties may also affect other signaling cascades within the lipid raft microdomains, which could indirectly modulate ion channel function. The concept of direct interaction between cholesterol and ion channels was first proposed by Marsh and Barrantes in a "lipid belt" model (78). The direct interaction between membrane cholesterol and ion channels was not demonstrated until the use of the enantiomer of the sterol, epicholesterol (111) and the identification of a cholesterol binding motif in several ion channels (30, 113, 120). Considering that direct and indirect effects of cholesterol on ion channels may exist simultaneously, discrimination between these two possibilities is challenging but important when exploring the mechanisms of cholesterol regulation of ion channels.

Many studies have shown that membrane cholesterol is important in regulating Ca^{2+} channels and that its effects on Ca^{2+} influx are diverse (14, 17, 104). In HSG and HEK293 cells, cholesterol depletion by MβCD blunts SOCE by suppression of STIM1 clustering

and activation of TRPC1 (97). In VSMCs, MβCD also reduces endothelin-1-induced Ca^{2+} entry through TRPC1 (10). Our previous and current findings showed that, in PAECs, agonist-induced Ca^{2+} influx along with membrane cholesterol levels are reduced following CH (95, 148). This impaired endothelial Ca^{2+} influx was restored by membrane cholesterol supplementation (95, 148). Our current studies also show that cholesterol directly modulates endothelial agonist-induced Ca^{2+} entry and its major components, SOCE and depolarization-induced Ca^{2+} entry. The direct functional effect of cholesterol was examined by replacing cholesterol with its enantiomer, epicholesterol, that has similar effects on membrane fluidity and lipid domain formation but differential effects on ion channel function. We found that substitution of endogenous membrane cholesterol with its epimer attenuates ATP-induced Ca^{2+} entry, SOCE and depolarization-induced Ca^{2+} entry in PAEC **(Figure 4, 5 and 9: Chapter 3)**. In addition, decreased endothelial SOCE and depolarization-induced Ca^{2+} entry following CH are largely restored by cholesterol supplementation **(Figure 5 and 9: Chapter 3)**. These data suggest that membrane cholesterol directly regulates agonist-induced Ca^{2+} entry and its components and further demonstrate that impaired endothelial Ca^{2+} entry following CH is due to altered membrane cholesterol homeostasis.

It should be noted that the rationale of using epicholesterol in this aim is based on the assumption that epicholesterol should have very similar effects to cholesterol on the physical properties of the plasma membrane but interact with Ca^{2+} channels differently. Our observation that neither cholesterol supplementation nor epicholesterol substitution alters endothelial caveolar number **(Figure 2: Chapter 3)** support this rationale. However, although other studies also showed that cholesterol and epicholesterol have similar effects

on membrane fluidity (46) and lipid domain formation (146), these two sterols may still have differential effects on the properties of plasma membrane (28, 29, 84). Parallel studies using the other enantiomer, *ent*-cholesterol that differs from cholesterol in the configuration of each of the eight stereocenters, might provide additional insight to how membrane cholesterol directly modulates pulmonary endothelial Ca^{2+} entry.

Caveolae, caveolin-1, and membrane cholesterol regulation of Ca^{2+} channels

Caveolae are small (50-100 nm) invaginations of the plasma membrane that are rich in cholesterol and the structural protein Cav-1. They are one type of lipid raft with dynamic assemblies of proteins, ion channels, and high levels of cholesterol and glycosphingolipids. Due to their unique characteristics as a platform for various signaling molecules, caveolae regulate many signaling pathways, including Ca^{2+} entry. In fact, Ca^{2+} signaling components such as G-protein coupled receptors, PLC, IP_3R, and SOC are found in lipid raft domains/caveolae (15, 43, 86, 99). Both TRPC1 and 4, as SOCs, are not only located in caveolae, but also interact with Cav-1, which is crucial for SOCE (15, 86).

Cav-1, as one of three isoforms of caveolin, is abundantly expressed in endothelial cells (116). Cav-1 is localized to caveolae and serves as one of the basic structural components of caveolae (36, 106). Besides its structural role in caveolar formation, Cav-1 also plays a regulatory role in mediating Ca^{2+} influx, as suggested by studies using mutated Cav-1 in cultured cells or Cav-1 knockout mice indicating that Cav-1 is required for SOCE by interacting with Ca^{2+} channels (15, 86). Thus, similar to membrane cholesterol, Cav-1 may regulate endothelial Ca^{2+} influx through its structural modulation of caveolae and its functional interaction with ion channels. What makes the interpretation of how Cav-1

affects endothelial Ca^{2+} influx more complicated is that Cav-1 may also regulate cholesterol homeostasis via cholesterol influx/efflux and intracellular trafficking (38, 40, 151). As reported in our previous studies (95), agonist-induced Ca^{2+} entry is diminished in PAECs of CH rats and can be restored to levels of normoxic controls by either cholesterol supplementation or administration of the Cav-1 scaffolding domain peptide, AP-Cav. Because there is no change of caveolae number in pulmonary endothelium after CH exposure (95), any structural changes in caveolae may have minimal effects on endothelial Ca^{2+} channel function. Based on the findings of our current studies, membrane cholesterol may directly regulate Ca^{2+} channels or signaling pathways leading to channel activation. Because indirect measurements of Cav-1 expression by immunofluorescence labeling show no obvious changes in Cav-1 levels after CH (95), AP-Cav may restore the diminished endothelial Ca^{2+} influx in CH rats by facilitating cholesterol trafficking from ER lipid droplets to the plasma membrane. This potential mechanism of ion channel regulation is supported by studies of Toselli et al (130). They found that Cav-1-transfected NG108-15 cells have increased membrane cholesterol content and significantly reduced N-type Ca^{2+} current compared to wild-type control. This effect was mimicked in wild-type cells by cholesterol supplementation, suggesting that membrane cholesterol, not Cav-1, is required for N-type Ca^{2+} channel inhibition.

Nevertheless, with evidence that Cav-1 may interact with large conductance, Ca^{2+}-activated K^+ channels (BK) (136), we cannot exclude the possibility that Cav-1 may also directly modulate certain Ca^{2+} channels function in PAEC. Besides, membrane cholesterol not only regulates Cav-1 expression (52) but also affects Cav-1 trafficking between the plasma membrane and Golgi apparatus (101, 121). Membrane cholesterol may also help

stabilize Cav-1 on the plasma membrane (41). Thus, membrane cholesterol may indirectly affect endothelial Ca^{2+} influx through regulation of Cav-1 expression and trafficking. This regulation of Cav-1 by membrane cholesterol may be observed in PASMC, in which both SOCE and ROCE, as well as agonist-induced contraction in pulmonary artery rings are augmented due to increased Cav-1 following CH (63). These Ca^{2+} entry pathways appear to be independent of membrane cholesterol as cholesterol supplementation does not increase these responses. The disruption of caveolae by MβCD, however, inhibits all Ca^{2+} responses, potentially through reduction of cholesterol-mediated surface expression of Cav-1. Considering the regulatory effect of membrane cholesterol and Cav-1 on each other, it is very likely that both components of caveolae are involved in the changes of pulmonary endothelial Ca^{2+} homeostasis during CH exposure.

Cholesterol regulation of STIM1, Orai1, and TRPC.

The essential role of membrane cholesterol in regulating SOCE has been shown in many cell types as membrane cholesterol depletion by MβCD attenuates SOCE (31, 45, 51, 64, 97). In the present studies, we substituted endogenous cholesterol with epicholesterol, and found that cholesterol is required for Orai1-mediated SOCE in both isolated PAEC and cultured PMVEC. We also showed that epicholesterol substitution significantly reduced the STIM1-Orai1 interaction, suggesting that membrane cholesterol regulates endothelial SOCE by facilitating this interaction that is required for Orai1 activation. Interestingly, Derler et al (30) and Pacheco et al (93) reported cholesterol-binding sites on Orai1 and STIM1, respectively, and demonstrated an inhibitory effect of membrane cholesterol on SOCE in cultured human embryonic kidney 293 (HEK293) cells and rat basophilic

leukemia 2H3 cells. Both studies further showed that the cholesterol-Orai1 and cholesterol-STIM1 interaction was attenuated by mutating the cholesterol-binding site of the target protein. The reason for the conflicting observations of how membrane cholesterol regulates SOCE in our current studies and those of other groups (30, 93) is not clear, but may be due to heterogeneity between either different cell types or between transfected/cultured cells and native endothelial cells. Additionally, since SOCE is a complex multi-step signaling pathway, it is also possible that membrane cholesterol affects components of SOCE differently. Galan et al (45) showed that membrane cholesterol depletion by MβCD attenuates SOCE and STIM1-Orai1 association in HEK293 cells. However, if stores are depleted prior to MβCD treatment, SOCE is enhanced while STIM1-Orai1 association remain unaffected by cholesterol depletion. Pani et al (97) found that MβCD treatment attenuates store depletion-induced STIM1 clustering and SOCE. Together, these findings suggest that plasma membrane cholesterol is required for STIM1 oligmerization and subsequent interaction with and activation of Orai1. Whether the cholesterol regulation of STIM1 clustering is achieved through maintaining caveolar structure or direct modulation of trafficking is not clear. However, since the silencing of Cav-1, which reduces the number of caveolae (26), does not affect STIM puncta formation in response to ER store depletion (98), it is possible that cholesterol on the plasma membrane or ER membrane directly affects STIM1 oligmerization. After the STIM1-Orai1 complex is formed, however, cholesterol may inhibit SOCE through direct interaction with Orai1 and STIM1 (30, 45, 93).

TRPC channels are also activated by stimuli that lead to PIP_2 hydrolysis, and are identified as components of SOCE. Our current findings do not preclude a potential role

for cholesterol to regulate TRPC channels independent of Orai1 and STIM1. Despite a lack of evidence for direct regulatory cholesterol binding sites on TRPC channel sequences, the membrane cholesterol sensitivity of TRPC channels has been reported in several cell types (1, 10, 73, 86, 140). For example, the extraction of membrane cholesterol by cyclodextrins impairs TRPC1 signaling processes (10, 77). In addition, both TRPC1 and TRPC4 are expressed in murine lung endothelial cells (86). In these cells, Cav-1 regulates the subcellular distribution of TRPC1 and 4 to a low-density cholesterol-enriched membrane fraction. Cav-1 has also been shown to modulate surface expression of TRPC1 in cultured HSG cells (98). In endothelial cells from endothelium-specific Cav-1 knockout mice, SOCE is significantly blunted, which is restored in cells from Cav-1 reconstructed mice (86). Considering the regulatory role of Cav-1 on cholesterol homeostasis, although cholesterol may play a pivotal regulatory role in TRPC1-mediated SOCE by regulating membrane structure, direct effects of cholesterol on TRPC trafficking and functions are possible.

Cholesterol may regulate surface expression of many membrane-bound proteins and ion channels. It has been shown that endocytosis (13, 138), proteins distribution in membrane microdomains (129), and endosomal mobility (20) are closely associated with membrane cholesterol levels. In rat atrial myocytes, lowering of membrane cholesterol by MβCD enhances outward potassium current by recruiting Kv1.5 channels from recycling endosomes (7). In addition, the role of membrane cholesterol in ion channel surface expression may vary depending on the channel isoform (44). In our previous and current studies, CH significantly reduced membrane cholesterol in PAECs (95, 148). It is not clear if chronic hypoxia changes the surface expression of certain Ca^{2+} channels through

membrane cholesterol-regulated pathways. Current literature does, however, demonstrate the inhibitory effect of hypoxia on K^+ channel trafficking to the plasma membrane (23, 90). With the possibility that cholesterol modulates trafficking of cation channels, whether the effect of cholesterol supplementation of restoring diminished Ca^{2+} entry in PAECs is achieved through facilitating Ca^{2+} channel surface expression remains to be determined.

Potential Mechanisms of diminished membrane cholesterol by CH

Dietary uptake of cholesterol and endogenous biosynthesis are two major sources of cholesterol for mammalian cells. Cholesterol is synthesized in the ER and cytoplasm from acetyl coenzyme A (acetyl-CoA) to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) in the initial steps (49). Then HMG-CoA is converted to mevalonic acid by the rate-limiting enzyme, HMG-CoA reductase, which is the target of cholesterol lowering drugs like statins (61). Mevalonic acid is converted to squalene, and then to lanosterol through multiple steps. An additional 19 steps are required before cholesterol is synthesized from lanosterol. After its synthesis, cholesterol is either transferred to the plasma membrane or is converted to cholesterol esters, which prevent over-accumulation of intracellular free cholesterol (19). Excessive cholesterol is also prevented by negative feedback control mechanisms, one of which involves rapid degradation of HMG-CoA reductase through ER membrane proteins known as insulin-induced gene (Insig) -1 and -2 (118). An accumulation of sterol in the ER membrane causes binding of HMG-CoA reductase to Insigs and subsequent ubiquitination of reductase to degradation (118). Two potential mechanisms of decreased membrane cholesterol following CH include: 1) inhibition of *de novo* cholesterol biosynthesis; and 2) membrane cholesterol oxidation. Mukodani *et al*. first reported that

hypoxia induces lipid accumulation and impairs cholesterol synthesis in cultured rabbit skin fibroblasts (83). The mechanism by which hypoxia affects cholesterol synthesis was later explored by Nguyen et al. (92), who reported that hypoxia induces both accumulation of cholesterol biosynthetic intermediates and activation of HIF1-α-mediated induction of Insigs. These two signaling pathways lead to rapid degradation of HMG-CoA reductase and subsequently limit synthesis of cholesterol (92). In addition, CH can also increase the production of reactive oxygen species (ROS) (42, 75), which may facilitate membrane cholesterol oxidation. Because filipin cannot be used to label oxidized cholesterol (103), it is possible that reduced filipin staining in PAECs of CH group is the result of membrane cholesterol oxidation by hypoxia-induced ROS. Cholesterol oxidation not only has potential to disrupt the interaction between cholesterol and ion channels and many regulatory proteins, but may also inhibit *de novo* cholesterol synthesis (88). In addition, cholesterol oxidation causes Cav-1 accumulation in the Golgi apparatus (121), which may contribute to impaired Ca^{2+} channel function. With very limited evidence of how cholesterol oxidation by ROS may lead to endothelial dysfunction in pulmonary arteries, future studies are required to evaluate the potential contributions of this mechanism to reduced PAEC membrane cholesterol and Ca^{2+} entry following CH.

Membrane cholesterol and hypercholesterolemia

In addition to effects on serum cholesterol, hypercholesterolemia also leads to the increase of membrane choleterol levels of vascular endothelial cells (34), smooth muscle cells (21), and erythrocytes (131). Studies suggest that hypercholesterolemia increases $[Ca^{2+}]_i$ of VSMCs and augments contraction (24, 142). Increased $[Ca^{2+}]_i$ of VSMCs could

be the result of excessive membrane cholesterol in hypercholesterolemia, as suggested by studies looking into the effect of cholesterol enrichment on basal and agonist-induced Ca^{2+} entry in these cells (12, 48). Augmented sensitivity of VSMCs to contractile stimuli may also be associated with ROS produced during hypercholesterolemia (109, 125). Meanwhile, hypercholesterolemia is also reported to impair endothelium-dependent relaxation (119, 147), which may also be caused by excessive membrane cholesterol added during hyperchoelsterolemia that reduces SOCE, eNOS phosphorylation, and NO production (4).

However, our current observation that cholesterol supplementation rescues blunted endothelial Ca^{2+} entry in PAEC from CH rats suggests a different role of cholesterol in regulating Ca^{2+} entry pathways. Although it is reported that CH does not affect total cholesterol levels in blood of pulmonary hypertensive rats (85), our studies do show the reduction of both serum cholesterol (unpublished data) and membrane cholesterol in PAECs after CH exposure (95, 148). This observation generates an interesting question of whether inhibition of cholesterol synthesis by HMG-CoA reductase inhibitors may mimic the effect of CH in reducing Ca^{2+} entry in PAECs. Rather, studies from other groups using statins, HMG-CoA reductase inhibitors to treat hypercholesterolemia, demonstrate a beneficial effect of this cholesterol-lowering drug in attenuating the development of CH-induced PH (47, 85). Statins could promote endothelial function through several mechanisms (80). For example, statin treatment may restore the reduced endothelial NO through multiple mechanisms including increasing eNOS expression (72), enhancing eNOS activity (37, 85), or antioxidant effects (134, 139). Studies also suggests that, by decreasing cholesterol synthesis and Cav-1 expression, statins reduce the inhibitory effect

of caveolin on eNOS and enhance NO release (37). Since chronic treatment of statins does not reduce NO production and endothelium-dependent vasodilatory responses to agonist under normoxic conditions (85) and they have pharmacological actions other than just inhibiting cholesterol synthesis, statins may not serve as a useful tool to investigate the mechanism by which cholesterol regulate Ca^{2+} entry in PAECs. However, the antioxidant effects of statins that increase NO bioavailability in pulmonary endothelium during CH-induced PH suggest their potential role in preventing cholesterol oxidation (76, 88, 100). Considering the fact that ROS are also associated with the development of atherosclerosis, it is possible that oxidized cholesterol may contribute to impaired endothelial Ca^{2+} entry in these diseases.

Physiological significance of current studies

The current studies advance our mechanistic understanding of impaired Ca^{2+} entry in PAECs as a key feature of endothelial dysfunction during CH-induced PH. Since agonist-induced Ca^{2+} entry, SOCE, and depolarization-induced Ca^{2+} entry are important in mediating pulmonary endothelial $[Ca^{2+}]_i$, restoring Ca^{2+} influx in PAECs may help increase endothelial Ca^{2+}-dependent vasodilation in pulmonary arteries following CH. The discovery that membrane cholesterol is important in mediating these Ca^{2+} entry pathways thus provides membrane cholesterol as a potential new therapeutic target in treating CH-induced PH.

Our current studies also add potential insights in understanding the mechanisms of acute hypoxic pulmonary vasoconstriction (HPV). Pulmonary arteries constrict in response to acute hypoxia, which is responsible for limiting the ventilation-perfusion ratio mismatch

during localized alveolar hypoxia. One of the hypothesized mechanisms suggests that HPV is achieved primarily through activation of voltage-dependent L-type Ca^{2+} channels in VSMCs in response to membrane depolarization as a result of inhibition of K_V channels by hypoxia (5, 81, 141). However, studies using isolated perfused lungs and isolated pulmonary arteries show that HPV is not very sensitive to blockers of L-type Ca^{2+} channels (110, 137). In addition, because VSMCs are located in pulmonary arterioles and arteries that are rather remote from the capillaries adjacent to alveoli, the connection between oxygen sensing in areas of gas exchange and vasoconstriction remained undefined. Studies from Kuebler group in recent years filled this gap between oxygen sensor and effector in HPV (50, 135). In their proposed mechanism of HPV, alveolar capillaries sense hypoxia and conducts the depolarization response retrogradely along the endothelial cell layer through connexin-40 gap junctions to upstream arterioles and large arteries. Membrane depolarization activates T-type VGCCs and increases production of arachidonic acid (AA) metabolites epoxyeicosatrienoic acids (EETs) in a Ca^{2+}-dependent manner. EETs then diffuse to VSMC and cause vasoconstriction potentially through activation of TRPC6 and TPRV4. In CH-induced PH, HPV is greatly reduced (132, 143, 150). Given the importance of endothelial T-type VGCCs in HPV, our observations that CH impairs T-type channel-mediated Ca^{2+} entry by reducing membrane cholesterol suggest that the loss of T-type channel function may be one of causes to diminished HPV in CH-induced PH (148).

In addition to hypoxia, other physiological stimulus may also cause membrane depolarization and activate T-type channel. Shear stress, for example, induces the production of ATP in endothelial cells (3, 67), which may serve as an autocrine factor that depolarizes endothelial cells. In addition, shear stress may initiate SOCE though increased

IP_3 production (105). Since store depletion leads to Ca^{2+} entry through T-type channels (145) in PMVECs, shear stress could potentially activate T-type channels indirectly. Another source of endothelial depolarizing stimuli is the membrane depolarization of VSMC. This is achieved by direct electrical communication between these two type of cells through gap junctions (2). In pulmonary arteries, depolarized VSMC may activate T-type channels on endothelial cells and Ca^{2+}-induced endothelium-dependent vasorelaxation to relieve the tone. However, in spite of increased shear stress (133) and PASMC depolarization (16) after CH, these potential mechanisms of vasodilation could be impaired in pulmonary arteries due to reduced cholesterol-mediated T-type channel function.

Cholesterol is rarely considered "good" in many diseases (35, 91), especially in atherosclerosis (57). Adding cholesterol to endothelial cells from aorta reduces both ATP-induced Ca^{2+} entry and SOCE, along with flow-induced NO production (4), which is the opposite of what we found in PAECs. Our findings suggest that the contribution of membrane cholesterol to endothelial dysfunction during development of various vascular diseases may be different between the pulmonary circulation and systemic vasculature.

Limitations of studies

In our current studies, isolated native PAECs and cultured PMVECs were investigated. PAECs were isolated in normoxic conditions, which removed the hypoxic stimulus to cells from CH animals and enables us to study effects of CH independent of acute hypoxic influences. Although these changes may not affect expression or synthesis of ion channels, proteins, and lipids, they do have potential impact to trafficking and compartmentation of

these components involved in the endothelial Ca^{2+} entry. It should also be noted that by studying cells, the effect of many physiological stimuli, such as stretch of pulmonary arteries associated with lung movement during breathing and vasoactive compounds released by erythrocytes, were not included, which may also influence the endothelial Ca^{2+} influx.

We used fura-2-AM as a fluorescent Ca^{2+} indicator to measure endothelial Ca^{2+} influx. The ratiometric measurement of fura-2 only provides information in global change of $[Ca^{2+}]_i$ without indication of localized Ca^{2+} events, which may be important in regulating the production of EDHF through activation of small and intermediate conductance Ca^{2+}-activated K^+ channels (68). Thus, how a change of membrane cholesterol would affect Ca^{2+} responses in caveolae or areas close to lipid rafts is unknown. Although Mn^{2+} quenching technique is a clean approach in measuring Ca^{2+} entry, limited information of kinetics of stimulated Ca^{2+} influx was acquired. Additionally, all cells were stimulated in the absence of extracellular Ca^{2+} for a period of time, and Ca^{2+} responses were examined afterwards. This approach certainly did not mimic native agonist-induced Ca^{2+} influx, which happens immediately upon stimulation and store depletion. Overall, these limitations of approaches should be considered when interpreting findings of current studies.

Summary

To summarize, membrane cholesterol contributes to the normal function of many Ca^{2+} influx pathways in PAECs. The stimulation with an agonist like ATP not only initiates SOCE by activating Orai1 through clustered STIM1 in response to ER Ca^{2+} store depletion, but also causes membrane depolarization and subsequent Ca^{2+} entry via T-type VGCCs.

These agonist-induced Ca^{2+} influx components become inactive following CH due to reduced membrane cholesterol levels and the loss of functional regulation of ion channels. Impaired cholesterol-dependent endothelial Ca^{2+} influx may contribute to decreased production and release of endothelium-derived vasodilators and anti-mitogenic factors following CH. These features of endothelial dysfunction may additionally contribute to increased vasoconstrictor reactivity, arterial smooth muscle mitogenesis, and development of PH.

Challenges of future studies are to identify the potential contribution of ROS to decreased endothelial cholesterol following CH, either through direct cholesterol oxidation or impaired sterol trafficking, and the potential impact of these responses to trafficking and surface expression of Cav-1 and Ca^{2+} channels including TRPC1 and Orai1. Additionally, it is important to explore the effect of CH on ER STIM1 clustering in pulmonary artery endothelium. Finally, the physiological effect of cholesterol supplementation on NO production and endothelium-mediated vasodilation in pulmonary arteries after CH exposure remains to be identified.

To conclude, elevated cellular membrane cholesterol has been linked to many diseases (35, 57, 91). In contrast, CH-induced PH appears to be associated with diminished membrane cholesterol in PAECs. Our current studies clearly establish a prominent role of membrane cholesterol in the regulation of agonist-induced Ca^{2+} entry in PAECs. These studies also support a role for reduced membrane cholesterol in impaired Orai1-mediated SOCE following CH. These findings may provide a mechanistic basis to explain CH-induced diminution of endothelial Ca^{2+} influx and associated endothelial dysfunction that is central to the pathogenesis of pulmonary hypertension.

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