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# Pulmonary endothelial calcium entry following chronic hypoxia

Steven Menicucci

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**PULMONARY ENDOTHELIAL CALCIUM ENTRY FOLLOWING  
CHRONIC HYPOXIA**

**BY**

**STEVEN D. MENICUCCI**

B.S. Biology, New Mexico Institute of Mining and Technology, 2008

THESIS

Submitted in Partial Fulfillment of the Requirements for the Degree of

Master of Science  
Biomedical Sciences

The University of New Mexico  
Albuquerque, New Mexico

December, 2011

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Steven D. Menicucci

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## **Abstract**

**Background and Specific Aims:** Chronic hypoxia (CH) induced pulmonary hypertension is mediated in part by endothelial dysfunction, involving reduced intracellular Ca<sup>2+</sup> levels and decreased production of endothelium-derived vasodilators and anti-mitogenic compounds. Agonist-induced endothelial Ca<sup>2+</sup> entry is decreased following CH due to a derangement in T-type voltage-gated calcium channels (T-channels) and caveolin-1 containing membrane lipid domains that regulate calcium influx in these cells. Considering the importance of store-operated Ca<sup>2+</sup> entry (SOCE) to agonist-induced Ca<sup>2+</sup> entry, we *hypothesized* that CH impairs pulmonary endothelial SOCE through altered caveolin-1 regulation of T-channels. To test this hypothesis, we addressed the following specific aims:

Specific Aim 1: *Determine the role of T-channels in agonist-induced calcium entry and SOCE in pulmonary arterial endothelial cells (PAEC) from control and CH rats.*

Specific Aim 2: *Determine the contribution of caveolin-1 to SOCE in PAEC from control and CH rats.*

Experimental Approach: Fura-2 fluorescence microscopy was used to assess either ATP-induced  $\text{Ca}^{2+}$  influx or SOCE resulting from depletion of intracellular  $\text{Ca}^{2+}$  stores in freshly isolated PAEC from control and CH (4 wk at 0.5 atm) rats. Experiments were conducted in the presence or absence of the T-channel inhibitor mibefradil. In separate protocols, SOCE was assessed following pretreatment with either a peptide containing the scaffolding domain of caveolin-1 (AP-CAV) or a scrambled control peptide. Immunofluorescence microscopy was used to evaluate the distribution of endothelial T-channels and caveolin-1, while the density of caveolae was determined from electron micrographs of PAEC from each group.

Results: Both ATP-induced  $\text{Ca}^{2+}$  influx and SOCE were attenuated in PAEC from CH compared to control rats. Although T-channel inhibition selectively attenuated  $\text{Ca}^{2+}$  responses to ATP in cells from control rats and normalized responses between groups, mibefradil was without effect on SOCE in control cells. Furthermore, AP-CAV augmented SOCE in cells from CH rats while having no effect in controls. We observed similar immunofluorescent staining for T-channels and caveolin-1 between groups, and the incidence of endothelial caveolae was unchanged by CH.

Conclusions: CH impairs caveolin-1 regulation of SOCE in PAEC without affecting intracellular caveolin-1 distribution or caveolar density. However, decreased SOCE following CH appears to be independent of  $\text{Ca}^{2+}$  influx through T-channels.

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## **Introduction**

Chronic hypoxia (CH) resulting from residence at high altitude or chronic obstructive pulmonary disease leads to pulmonary hypertension (PH) through polycythemia, vascular remodeling, and vasoconstriction. Whereas the vasoconstrictor component of PH is mediated in part by acute effects of hypoxia to cause vascular smooth muscle (VSM) contraction, increases in VSM contractility and endothelial dysfunction are thought to be important contributing influences to this hypertensive response. Such endothelial derangements are characterized not only by increased production of endothelium-derived contractile factors, but also impaired synthesis or enhanced bioinactivation of endothelium-derived vasodilators that collectively favor vasoconstriction. Although mechanisms of endothelial dysfunction in PH are likely multifaceted, reduced endothelial  $[Ca^{2+}]_i$  following CH may contribute to dysregulation of these vasodilator pathways (Fig. 1). However, the causes of altered endothelial calcium regulation in the hypertensive pulmonary vasculature are not well understood. Therefore, the overall objective of this thesis is to understand mechanisms of impaired endothelial calcium entry in this setting.

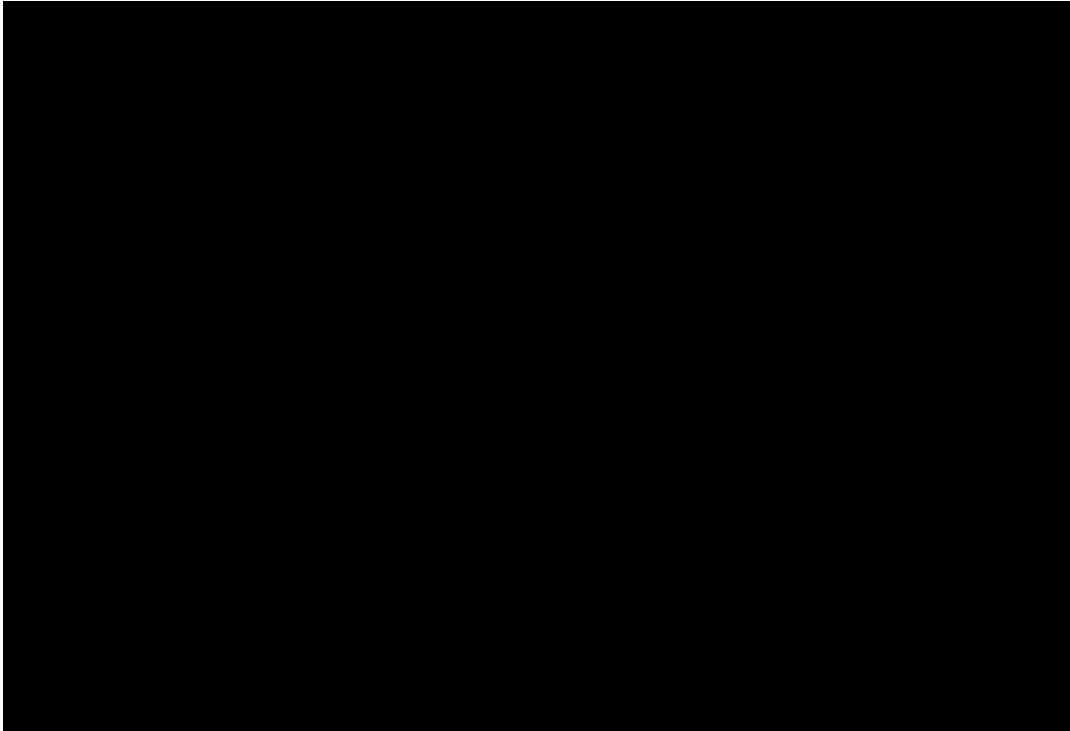


Figure 1. Diagram depicting the hypothesis for reduced endothelial cell  $\text{Ca}^{2+}$  leading to pulmonary hypertension following chronic hypoxia.

## **Endothelial Dysfunction Following Chronic Hypoxia**

### *Enhanced endothelium-dependent vasoconstriction*

CH is characterized by endothelial derangement that leads to increased production of vasoconstrictors and mitogenic compounds, including endothelin and reactive oxygen species (ROS). Endothelin is a potent vasoconstrictor with promitogenic characteristics. Endothelin has three isoforms endothelin-1(ET-1), endothelin-2 (ET-2), and endothelin-3 (ET-3). ET-1 is the most important in the vasculature and has been shown to be elevated in animal models of PH (26; 27). Endothelin-1 is a 21 amino acid peptide released from both the endothelium and smooth muscle and is a potent vasoconstrictor (22). Endothelin-1 is released from the endothelium in response to CH (24). Under hypoxic conditions ET-1 gene transcription is activated through a hypoxia inducible factor(HIF) dependent mechanism (51). ET-1 acts through endothelin receptors on the membrane of the smooth muscle and endothelium. Two isoforms of the endothelin receptor are known, endothelin receptor A (ET<sub>A</sub>) and endothelin receptor B (ET<sub>B</sub>). Activation of the ET<sub>A</sub> receptor on the smooth muscle leads to increased smooth muscle cell Ca<sup>2+</sup> and Ca<sup>2+</sup> sensitivity as well as smooth muscle cell proliferation which is the likely mechanism by which endothelin contributes to PH (22). ET<sub>B</sub> receptors located on the endothelium lead to increased NO production and vasodilation (52). However, in CH endothelial dysfunction may prevent the protective effect of these endothelial receptors. Inhibition of ET<sub>A</sub> receptors has been shown to prevent CH induced PH (7). In addition it has also been shown that inhibition of both ET<sub>A</sub> and ET<sub>B</sub> with bosentan prevents CH induced PH (8). These findings lead to endothelin receptor antagonists as a current therapeutic treatment

for PH (22). These results suggest that endothelin-1 is an important mediator of CH induced PH.

ROS are also an important component of endothelial derived vasoconstrictors. ROS production is increased in the endothelium with CH (17) and may directly contribute to contraction of the smooth muscle in this setting (20). ROS can also promote vasoconstriction indirectly by impairing endothelium dependent vasodilator pathways as (3; 25) discussed below in the third paragraph of the ‘Impairment of endothelium-derived vasodilator pathways’.

#### *Impairment of endothelium-derived vasodilator pathways*

Production of endothelium-derived vasodilators is decreased with endothelial dysfunction associated with CH and impaired handling of endothelial  $Ca^{2+}$  may contribute. The synthesis of several endothelium-derived vasodilators is  $Ca^{2+}$  dependent including NO, prostacyclin, and endothelium-derived hyperpolarizing factor (EDHF).

Endothelial nitric oxide synthase (eNOS) is the protein that produces NO. NO is responsible for vasodilation through activation of soluble guanylyl cyclase (sGC) in the smooth muscle and the subsequent activation of protein kinase G (PKG). eNOS is displaced from the inhibiting protein caveolin-1 in the presence of the  $Ca^{2+}$ -calmodulin complex and is activated to produce NO (21). Basal production of NO has been shown to be important in maintaining low vascular resistance in the pulmonary circulation (10). The role of NO in CH induced PH is complex due to reports showing increased eNOS (16; 34; 48; 60) expression levels but decreased endothelium dependent dilation and NO production in pulmonary arteries following CH (38). Murata et al. (38) demonstrated that eNOS phosphorylation of serine 1177, which is critical for efficient eNOS activity, is

impaired in pulmonary endothelial cells from rats exposed to CH. These authors also showed that eNOS interaction with the inhibitory protein caveolin-1 was increased with CH leading to decreased production of NO. Interestingly, in this paper (38) it was also shown that eNOS interaction with hsp90, a protein critical for proper eNOS function, is decreased following CH. These findings suggest that eNOS production of NO is decreased following CH.

NO bioavailability may also be impaired by increased ROS production following CH. Jernigan et al. (19) have shown that ROS from the endothelium of isolated pulmonary arteries from CH rats impairs exogenous NO dilation. This finding suggests that reactive oxygen species from the endothelium suppress the effect of NO on the smooth muscle. This could be due to ROS reacting with NO to form peroxynitrite anion ( $\text{ONOO}^-$ ) (3). It has also been shown that CH increases ROS generation (39) and that  $\text{ONOO}^-$  leads to inhibition of eNOS (25). Indeed Jankov et al. (17) have shown increased protein nitrosylation in the endothelium following CH. These findings suggest that both proper eNOS function and endothelium dependent vasodilation are impaired in the pulmonary circulation following CH.

Prostacyclin is a vasodilator that is formed from arachidonic acid (AA). AA is produced by phospholipase  $\text{A}_2$  ( $\text{PLA}_2$ ) in a  $\text{Ca}^{2+}$  dependent manner. AA is converted into prostaglandin  $\text{H}_2$  by cyclooxygenase-1 (COX-1) which is converted into prostacyclin ( $\text{PGI}_2$ ) by prostacyclin synthase (PGIS).  $\text{PGI}_2$  activates  $\text{G}_s$ -coupled receptors on the smooth muscle membrane leading to vasodilation. COX inhibition has been shown to increase pulmonary vascular tone (57). Suggesting that  $\text{PGI}_2$  contributes to tone in the pulmonary circulation. In addition, mice over expressing PGIS show reduced arterial

wall hypertrophy in CH-induced-PH and some human forms of PH have shown a loss of PGIS (13; 13; 54). These findings suggest that prostacyclin could be important in diminished endothelium-dependent vasodilation following CH.

EDHF is a common term used to describe a number of different signaling pathways whereby the endothelium affects the smooth muscle to cause hyperpolarization of the muscle. One vasodilation mechanism classified as an EDHF is characterized by the  $\text{Ca}^{2+}$ -calmodulin complex (23; 59) activating small ( $\text{SK}_{\text{ca}}$ ) and medium ( $\text{IK}_{\text{ca}}$ ) conductance  $\text{Ca}^{2+}$  activated potassium channels which hyperpolarize the endothelial cell and then the hyperpolarization is conducted across to the smooth muscle cell through myoendothelial cell gap junctions. Hyperpolarization of the smooth muscle cells leads to a decrease in smooth muscle cell  $\text{Ca}^{2+}$  through inhibition of voltage gated L-type  $\text{Ca}^{2+}$  channels and relaxation (9). Another EDHF pathway is called the potassium cloud hypothesis. In this scenario the  $\text{Ca}^{2+}$ -calmodulin complex activates efflux of potassium through the  $\text{SK}_{\text{ca}}$  and  $\text{IK}_{\text{ca}}$  channels causing a small increase in  $[\text{K}^+]$  in the space between endothelial and smooth muscle cells. This increase in extracellular  $[\text{K}^+]$  activates vascular smooth muscle  $\text{Na}^+/\text{K}^+$ -ATPases and inwardly rectifying  $\text{K}^+$  channels leading to hyperpolarization (9). A third calcium sensitive EDHF pathway involves an alternative AA metabolite called epoxyeicosatrienoic acid (EET). EET's are formed through cytochrome (CYP) P450 metabolism of AA (6). The effect of EETs is to hyperpolarize the smooth muscle through activation of large conductance potassium channels ( $\text{BK}_{\text{Ca}}$ )(6). Morio et al. (30) showed that EDHF responses contribute to thapsigargin induced vasodilation in normotensive rat lungs and that these pathways change in the hypoxia induced hypertensive rat lung. They found that both gap junction dependent

EDHF and CYP P450 dependent EDHF pathways are lost in the hypoxia induced hypertensive lung from rats (30). Although this study illustrates that EDHF pathways are impaired following CH, the significance of this impairment to the development of PH remains to be established.

### **Dysregulation of Endothelial Cell Calcium Handling Following Chronic Hypoxia**

Intracellular  $\text{Ca}^{2+}$  is maintained at approximately  $10^{-7}$  M in endothelial cells under resting conditions (33).  $\text{Ca}^{2+}$  is extruded from the cytoplasm in an ATP dependent manner via the plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA) and the sarcoendoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA). A non ATP sensitive plasma membrane transporter, the  $\text{Na}^{+}$ - $\text{Ca}^{2+}$  exchanger, also contributes to resting  $\text{Ca}^{2+}$  levels.  $\text{Ca}^{2+}$  enters the cell through ion channels that temporarily open to allow  $\text{Ca}^{2+}$  to rapidly pass through the cell membrane and down the electrochemical gradient for  $\text{Ca}^{2+}$  (33). The regulation of  $\text{Ca}^{2+}$  channels is also important for controlling  $\text{Ca}^{2+}$  in endothelial cells. One important way  $\text{Ca}^{2+}$  enters the cell is when an agonist binds a receptor, leading to depletion of  $\text{Ca}^{2+}$  from the endoplasmic reticulum (ER) and activation of store operated  $\text{Ca}^{2+}$  entry (SOCE). Another pathway activated by an agonist binding a receptor is through the synthesis of diacylglycerol (DAG) and is termed receptor operated  $\text{Ca}^{2+}$  entry (ROCE). There are also  $\text{Ca}^{2+}$  channels that open in response to depolarization and allow for the influx of  $\text{Ca}^{2+}$ . Intracellular basal  $\text{Ca}^{2+}$  as well as  $\text{Ca}^{2+}$  entry is diminished in PAEC from CH rats (41; 43). These pathways could lead to decreased production of vasodilators and contribute to CH induced PH and will be discussed in the subsequent paragraphs.

*Effects of CH on store- and receptor-operated calcium entry*



The mechanism of decreased basal  $\text{Ca}^{2+}$  in the pulmonary endothelium is unknown but it could be dependent on decreased  $\text{Ca}^{2+}$  entry. Consistent with this possibility is evidence that agonist induced  $\text{Ca}^{2+}$  entry is diminished in PAEC following CH (38; 42). This response appears to be dependent on both reduced SOCE and ROCE (44). SOCE in PAECs is diminished following CH, as measured in endothelial cells in both intact arteries and isolated cell preparations (41). In contrast with these results, Fantozzi et al. (11) have shown that SOCE is increased in human endothelial cells following 72 hr hypoxic in cultured PAEC. However, these experiments were conducted in cell culture conditions and this could explain these discrepancies. In vivo the PAEC will be exposed to shear stress and other mechanical forces not present in cell culture. In vivo there is also other cell to cell communication and contacts that could be very important in the phenotype that develops in these PAEC. In addition, ROCE has also been measured in isolated PAEC and shown to be decreased following CH (44). Thus, impaired SOCE and ROCE following CH may account for decreased basal pulmonary endothelial  $\text{Ca}^{2+}$  following CH.

There are several potential mechanisms of decreased SOCE and ROCE in PAEC following CH. Impaired trafficking of the channels responsible for SOCE is one possible explanation for decreased SOCE in PAEC following CH. Two canonical transient receptor potential (TRPC) channels TRPC1 and TRPC4 have been implicated in SOCE and other animal models of PH have shown impairment of TRPC4 trafficking to the membrane in PAEC (36). A second potential mediator of impaired SOCE and ROCE in PAEC following CH is protein kinase C (PKC). Interestingly, non-specific PKC inhibition in PAEC from CH animals has been shown to restore SOCE (44). These

results indicate that PKC has an inhibitory effect on SOCE that is mediated by CH. In contrast PKC $\epsilon$  appears to augment ROCE in control cells and CH inhibits this ROCE that is in part mediated by PKC $\epsilon$  in control cells (44). A separate protein integrated with intracellular signaling, caveolin-1, has also been shown to be important in Ca<sup>2+</sup> entry in endothelial (36; 42) and caveolin-1 dysfunction could be contributing to impaired SOCE and ROCE following however this has not been explored. A fourth potential factor in decreased Ca<sup>2+</sup> entry is membrane cholesterol homeostasis. Interestingly it has also been shown that membrane cholesterol is decreased with CH and that when membrane cholesterol is restored, Ca<sup>2+</sup> entry is restored suggesting that decreased membrane cholesterol could contribute to impaired SOCE and ROCE following CH (42). This study will focus on potential mechanisms of decreased SOCE in PAEC following CH as well as elucidating which ion channels are involved and the contribution of caveolin-1 to this response.

#### *Chronic hypoxia inhibits depolarization-induced calcium entry*

Although endothelial cells are generally considered to be electrically unexcitable, endothelial T-type voltage-gated calcium channels (VGCC) have been described in some vascular beds including the brain, adrenal glands and lung (5; 56; 64). T-type VGCC are distinct from L-type VGCC in that they activate and de-activate rapidly over a large voltage range and they show smaller current and are activated at more hyperpolarized membrane potentials. T-channels are categorized by their inactivation properties as  $\alpha_{1G}$  (Ca<sub>v</sub>3.1),  $\alpha_{1H}$  (Ca<sub>v</sub> 3.2) and  $\alpha_{1I}$  (Ca<sub>v</sub> 3.3) (58). Zhou et al. (64) demonstrated that T-channel activation in pulmonary microvascular endothelial cells leads to Weibel-Palade body exocytosis. These studies showed that activation can be through G<sub>q</sub> linked

receptors in response to inflammatory cytokines or through depolarization using KCl. Furthermore, T-channels have been implicated in mediating depolarization-induced  $\text{Ca}^{2+}$  entry in isolated rat PAECs from control rats, as evidenced by an effect of the T-channel inhibitor, mibefradil, to attenuate this response. Therefore, pulmonary endothelial T-channels may play important functional roles in endothelium-dependent regulation of vascular tone in response to stimuli that depolarize the endothelium directly or indirectly through conducted depolarization from the vascular smooth muscle.

Similar to effects of CH to inhibit endothelial SOCE and ROCE, depolarization-induced  $\text{Ca}^{2+}$  influx is lower in PAEC from CH rats compared to those of control animals (44). Paffett et al. (43) have also shown the presence of T-channels in PAEC and that these channels contribute to depolarization-induced  $\text{Ca}^{2+}$  entry and ATP-induced ROCE. Following CH, this mibefradil sensitive component of ROCE is lost suggesting that reduced ROCE in CH is in part mediated through dysregulation of T-channels. PKC $\epsilon$  was shown to be required for T-channel function in ROCE in the control cells but CH impaired PKC $\epsilon$  activation of T-channels (43). This study implies that CH causes a loss of a PKC activated  $\text{Ca}^{2+}$  influx pathway that is dependent on mibefradil-sensitive channels. T-channels are important mediators of ROCE and depolarization induced  $\text{Ca}^{2+}$  entry in PAEC from control rats and their function is lost with CH which could be a contributing factor in the CH induced decrease in basal  $\text{Ca}^{2+}$  in PAEC.

## **Role of Endothelial Caveolin-1 in Calcium Homeostasis and Pulmonary**

### **Hypertension**

Caveolae are small invaginations in the plasma membrane first described by Yamada (61). Caveolae are most notably present in epithelial cells, endothelial cells, adipocytes and fibroblasts in the lung (4; 14; 50). Identification of the proteins that make up caveolae and the generation of knock-out mice for those genes has implicated caveolae in a large number of different physiological processes (15). Caveolin-1 is the most abundant protein in caveolae and is critical for caveolae formation (12). It is a membrane associated protein that is found in lipid dense regions of the plasma membrane. The scaffolding domain of caveolin-1 interacts with many different proteins (28) including  $\text{Ca}^{2+}$  conducting ion channels (36; 45). It has been shown that caveolin-1 is important in  $\text{Ca}^{2+}$  entry (42) trafficking of calcium channels and important in agonist induced  $\text{Ca}^{2+}$  entry (36).

Pulmonary hypertension is associated with caveolin-1 dysfunction and understanding the role of caveolin-1 in this pathology is important. Caveolin-1 knockout mice develop pulmonary hypertension as well as right ventricular hypertrophy and diminished agonist induced  $\text{Ca}^{2+}$  entry in PAEC (62). Monocrotaline treated rats develop pulmonary hypertension accompanied with a loss in caveolin-1 expression in the lung (18). Interestingly it has been shown that administering a cell permeable version of the scaffolding domain of caveolin-1 to monocrotaline treated rats alleviates the pulmonary hypertension and restores the expression of caveolin-1 (18). Several investigators have examined the mechanism of the resultant pulmonary hypertension following caveolin-1 dysfunction. Murata et al. (36) generated a mouse line that only expressed caveolin-1 in the endothelium by breeding a caveolin-1 global knock out mouse with a mouse over-expressing caveolin-1 in the endothelium resulting in a mouse that only expressed

caveolin-1 in the endothelium. These mice with rescued caveolin-1 expression showed normal pulmonary pressures with the full knock-out displaying pulmonary hypertension (35). These investigators went on to show that  $\text{Ca}^{2+}$  entry in the pulmonary endothelium of the knock out mouse was diminished in comparison to the pulmonary endothelial cells expressing caveolin-1 (37). Using silencing RNA these investigators were able to determine that calcium entry was diminished due to dysfunctional TRPC1 and TRPC4 cation channels in the endothelium. Using immunofluorescence it was shown that TRPC4 trafficking was dysfunctional in the cells not expressing caveolin-1 (36). This study shows that caveolin-1 is important for ion channel trafficking and  $\text{Ca}^{2+}$  entry in the pulmonary endothelium and that these functions are lost in pulmonary hypertension.

Caveolin-1 has also been shown to be important in the inhibition of eNOS activity (21). This has led other investigators to study the role of caveolin-1 in regulating eNOS in pulmonary hypertension. Caveolin-1 is primarily thought to be important in trafficking the eNOS protein to the membrane and bringing it in close proximity to essential proteins needed for the formation of nitric oxide as well as inhibiting eNOS activity until there is sufficient  $\text{Ca}^{2+}$  levels in the cell at which point the calmodulin- $\text{Ca}^{2+}$  complex displaces the caveolin-1 protein from eNOS allowing for the formation of NO (29). Mukhopadhyay et al. (31) demonstrated that eNOS trafficking is impaired in pulmonary vascular endothelial cells that are treated with monocrotaline or hypoxia. Their study illustrated that eNOS is trapped in the golgi following monocrotaline or hypoxic treatment and they hypothesized that the NO produced by the golgi trapped eNOS cannot reach the vascular smooth muscle to illicit vasorelaxation (32). Other investigators (63) have shown that if eNOS is genetically deleted along with caveolin-1

then pulmonary hypertension doesn't develop like it normally would in a caveolin-1 knock out mouse. These investigators show that the absence of caveolin-1 causes an increase in NO production leading to PKG nitrosylation and cellular dysfunction (63). These studies demonstrate that persistent eNOS activation, leading to detrimental protein nitrosylation, secondary to decreased expression of caveolin-1 is a contributing factor to PH. This illustrates the importance of caveolin-1 regulation of eNOS and how the derangement of this interaction contributes to pulmonary hypertension.

More recent studies suggest that caveolin-1 dysfunction contributes to decreased  $\text{Ca}^{2+}$  entry in PAEC from CH rats (42). In this paper the authors found that acute application of the caveolin-1 scaffolding domain restores the lost ATP-induced  $\text{Ca}^{2+}$  entry in PAEC from CH rats. Interestingly this study also found that decreased membrane cholesterol inhibits agonist induced  $\text{Ca}^{2+}$  entry in PAEC from control rats but has no effect on agonist induced  $\text{Ca}^{2+}$  entry in PAEC from CH rats. We also show that there is reduced membrane cholesterol in the PAEC from CH rats. These results indicate that there is both caveolin-1 dysfunction and alteration in cholesterol homeostasis contributing to reduced agonist induced  $\text{Ca}^{2+}$  entry in PAEC following CH. One possible explanation is the caveolin-1 dysfunction with CH also leads to impaired trafficking of cholesterol to the membrane by caveolin-1 (55). However, whether reduced membrane cholesterol mediates caveolin-1 dysfunction in this setting is a topic of future investigation.

## **Rationale and Specific Aims**

From the above discussion, it appears that CH inhibits ROCE mediated by T-channels and that agonist induced  $\text{Ca}^{2+}$  entry is diminished in CH due to caveolin-1 dysfunction. However whether decreased SOCE following CH is caused by impaired caveolin-1 regulation of ion channels and the contribution of T-channels to this response is currently unknown. Therefore, we tested the *hypothesis* that CH decreases pulmonary endothelial SOCE through altered caveolin-1 regulation of T-channels (Fig. 2).

**Specific Aim 1:** *Determine the role of T-channels in agonist-induced calcium entry and SOCE in PAEC from control and CH rats.*

Hypothesis: T-channel dysfunction inhibits pulmonary endothelial SOCE and agonist-dependent calcium entry following CH.

Rationale: Previous studies from our laboratory (41) have demonstrated decreased SOCE in PAEC from CH rats. This information in combination with evidence that CH impairs T-channel mediated  $\text{Ca}^{2+}$  influx in the pulmonary vascular endothelium led us to examine the role of T-channels in SOCE. In this scenario T-channel activation would be secondary to SOCE and would require the influx of  $\text{Na}^+$  and  $\text{Ca}^{2+}$  leading to the depolarization of the endothelial cell membrane and the opening of T-channels (depicted in Figure 2). T-channel function with CH could be impaired by altered membrane localization or interference with acute translocation of

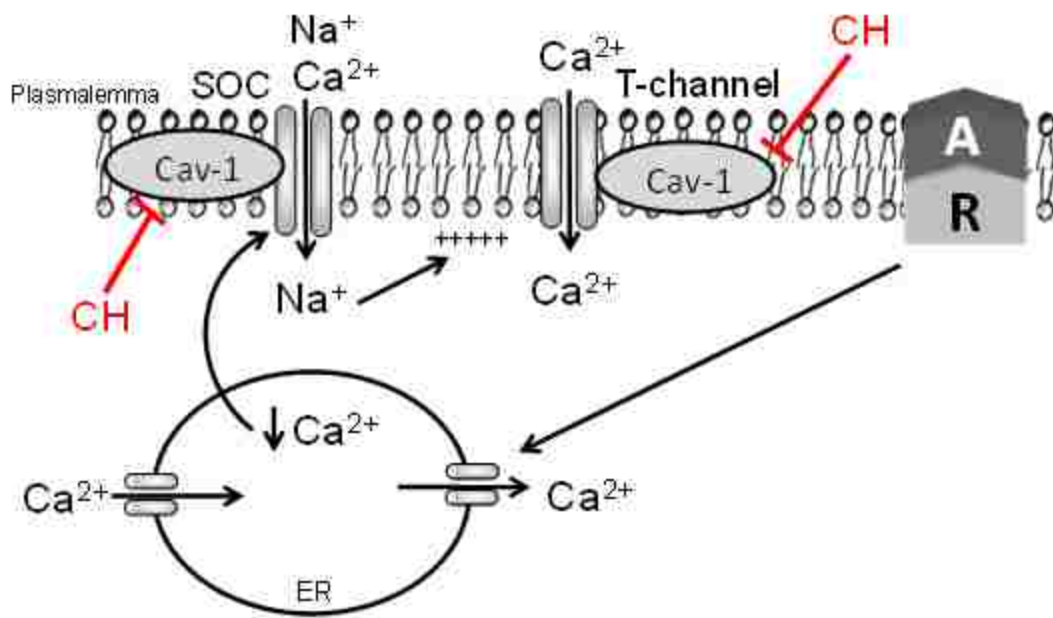


Figure 2. Schematic diagram depicting the hypothesis that CH impairs pulmonary endothelial SOCE through altered caveolin-1 regulation of T-channels. ER = Endoplasmic reticulum, A = Agonist, R = Receptor, Cav-1 = caveolin 1, SOC = Store operated cation channel, CH = Chronic hypoxia, Na<sup>+</sup> = sodium, Ca<sup>2+</sup> = Calcium



the channels to the membrane following agonist stimulation. Therefore, we wanted to determine if these channels are also important in agonist induced  $\text{Ca}^{2+}$  entry and SOCE and if they are dysfunctional following CH.

Approach: Fura-2 fluorescence microscopy was used to assess either ATP-induced  $\text{Ca}^{2+}$  influx or SOCE in freshly isolated PAEC from control and CH (4 wk at 0.5 atm) rats. The contribution of T-channels to these responses was assessed using the T-channel inhibitor mibefradil. In addition, immunofluorescence confocal microscopy was used to evaluate the cellular distribution of endothelial T-channels under basal conditions and following administration of ATP in cells from each group.

**Specific Aim 2:** *Determine the contribution of caveolin-1 to SOCE in PAEC from control and CH rats.*

Hypothesis: CH impairs caveolin-1 regulation of ion channels necessary for pulmonary endothelial SOCE.

Rationale: Previous studies from our laboratory (42) have shown that administration of the scaffolding domain of caveolin-1 restores diminished agonist induced  $\text{Ca}^{2+}$  entry that is normally observed in PAEC from CH rats suggesting that CH induced caveolin-1 dysfunction impairs agonist induced  $\text{Ca}^{2+}$  entry. However, whether caveolin-1 dysfunction contributes to impaired SOCE to mediate this response is unknown. Therefore we set out to address the question of whether the caveolin-1 scaffolding domain can restore SOCE in endothelia from CH rats independent of receptor stimulation. We also explored how CH is modulating caveolin-1 function, if it is through

trapping of caveolin-1 in microsomal compartments leading to decreased caveolae density.

Approach: SOCE was assessed in isolated PAEC from each group following pretreatment with either a peptide containing the scaffolding domain of caveolin-1 (AP-CAV) or a scrambled control peptide. Immunofluorescent staining of caveolin-1 was assessed by confocal microscopy, and the incidence of caveolae was determined from electron micrographs of PAEC from each group.

## **Methods**

All protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of New Mexico.

### *Experimental groups*

Male Sprague-Dawley rats between 200-250 grams were obtained from Harlan Industries. Rats exposed to CH were placed in a hypobaric chamber (~380 torr) for 4 weeks. Age-matched controls were kept in cages at ambient conditions (~630 torr). The hypobaric chamber was open 3 times/week to provide rat chow, clean water, and new bedding. Animals were maintained on 12:12 hr light:dark cycle.

### *Isolation of pulmonary arterial endothelial cells*

Rats were euthanized with sodium pentobarbital (32.5 mg i.p.) following a midline thoracotomy the heart and lungs were exposed. The left lung was excised and placed in ice cold HEPES buffered salt solution (HBSS) containing (in mM) 150 NaCl, 6 KCl, 1MgCl, 1.8CaCl<sub>2</sub>, 10 HEPES, and 10 glucose, titrated to pH 7.4 with NaOH (Sigma). The lung was pinned down in a Silastic coated dissection dish containing ice cold HBSS. Arteries (200-400 μm inner diameter) from the cranial section of the left lung were cleared of lung tissue and cut longitudinally. Arteries were placed in HBSS containing 0.2 mg/ml of dithiothreitol and 0.2 mg/ml papain at 37°C for 45 minutes. Vessels were removed and placed in 1 ml of HBSS containing 2 mg/ml BSA and placed on ice for 10 minutes. Endothelial cell sheets were released by gentle trituration with a small bore fire-polished Pasteur pipette and placed back on ice. Two hundred fifty ml of the cell suspension was placed on poly-L-lysine coated coverslips for 30 minutes following mounting on an inverted fluorescence microscope (Nikon Eclipse, TS100).

### *Assessment of Agonist-Induced and Store-Operated Ca<sup>2+</sup> entry*

Isolated endothelial cells were incubated with fura-2-AM (3 $\mu$ M and 0.05% pluronic acid; Invitrogen), a ratiometric fluorescent Ca<sup>2+</sup> indicator, for 5 minutes at room temperature and then washed with HBSS for 15 minutes at 37°C. Ratiometric changes in endothelial cell [Ca<sup>2+</sup>]<sub>i</sub> were determined by alternating a xenon arc lamp light source between 340 and 380 nm bandpass filters at 1 Hz (Ionoptix Hyperswitch) and the resultant fura-2 fluorescent emissions (F<sub>340</sub>/F<sub>380</sub>) were detected at 510 nm with a photomultiplier tube. To assess ATP induced Ca<sup>2+</sup> entry, the cells were superfused with Ca<sup>2+</sup> free HBSS containing the same contents as HBSS but CaCl<sub>2</sub> was substituted for equimolar MgCl<sub>2</sub>. Following stabilization of the fura-2 emission ratio ATP (20  $\mu$ M) was added to deplete intracellular Ca<sup>2+</sup> stores. Following stabilization of the ratio, Ca<sup>2+</sup> containing HBSS with ATP was added back to the superfusate and a sustained increase in fura-2 emission ratio was observed. This increased fura-2 emission ratio represents both SOCE and ROCE components of agonist-induced Ca<sup>2+</sup> influx (figure 3). To determine the magnitude of Ca<sup>2+</sup> response the area under the curve (AUC) was calculated and recorded in arbitrary units for exactly 4 minutes immediately following the initial increase in Ca<sup>2+</sup> (NCSS.LLC, 2007). To determine the role of T-channels in this response we used the T-channel inhibitor mibefradil (10  $\mu$ M; Sigma) which is the concentration previously reported in Paffett et al. (44) to inhibit depolarization induced Ca<sup>2+</sup> entry independent of L-channels and T-channel currents in neonatal cardiomyocytes. Mibefradil was added to the HBSS following fura 2-AM loading and maintained throughout the experiment.

In separate experiments examining SOCE, the endoplasmic reticulum  $\text{Ca}^{2+}$  ATPase inhibitor, cyclopiazonic acid (CPA; 10  $\mu\text{M}$ ; Calbiochem) was added during the  $\text{Ca}^{2+}$  free superfusion period.  $\text{Ca}^{2+}$  mobilization was observed and following stabilization of the  $\text{F}_{340}/\text{F}_{380}$ ,  $\text{Ca}^{2+}$  and CPA containing HBSS was added back and the  $\text{F}_{340}/\text{F}_{380}$  increased and plateaued. SOCE was assessed as the change in  $\text{F}_{340}/\text{F}_{380}$  after repletion of extracellular  $\text{Ca}^{2+}$ . To determine the role of caveolin-1 in SOCE following CH we used a peptide containing the scaffolding domain of caveolin-1 attached to a cell permeable peptide *antennapedia* (AP). Endothelial cells were incubated with 10  $\mu\text{M}$  of the caveolin-1 scaffolding domain peptide (AP-cav) for 30 minutes before fura 2-AM loading. In control experiments, a scrambled peptide conjugated to the AP sequence (AP-scam) was used to rule out potential non-specific effects of peptide incubation. For experiments examining the role of T-channels in SOCE, mibefradil (10  $\mu\text{M}$ ) was added to all solutions following the incubation with fura 2-AM.

#### *Immunofluorescence Microscopy for Caveolin-1 and T-type Calcium Channels*

Endothelial cells dissociated from small pulmonary arteries were allowed to adhere to 35 mm cover slips for 1 hour prior to fixation with 4% paraformaldehyde for 10 minutes. Cells were then permeabilized with 0.01% Triton-X for 10 minutes and then placed in 3% donkey serum for 1 hr. at room temperature. Following blocking and fixation, the cells were incubated overnight at 4°C with rabbit polyclonal caveolin-1 antibody (1:100, Santa Cruz, Santa Cruz CA, USA). Dylight 649 donkey anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Westgrove, PA, USA) was used to detect the primary antibody. Nuclei were labeled with Sytox (1:10,000) and samples were visualized with a confocal laser scanning microscope (LSM 510; Carl Zeiss

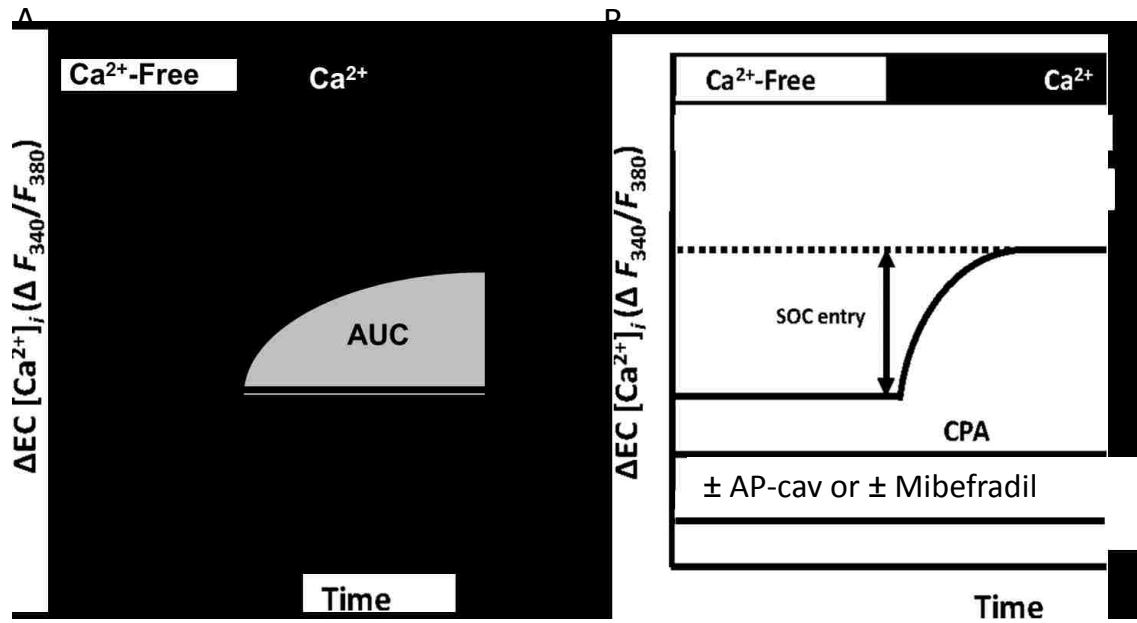


Figure 3. A) Experimental protocol depicting ATP-induced  $Ca^{2+}$  entry. ATP-induced  $Ca^{2+}$  entry was defined by measuring the AUC for the increase in  $F_{340}/F_{380}$  following substitution of  $Ca^{2+}$  containing HBSS for  $Ca^{2+}$ -free HBSS. The base line was set at the point where  $F_{340}/F_{380}$  was stabilized in  $Ca^{2+}$ -free HBSS containing ATP. B) SOCE was defined by the change in  $F_{340}/F_{380}$  from  $Ca^{2+}$ -free HBSS with CPA to  $Ca^{2+}$  containing HBSS in the presence of CPA.

Inc. Thornwood, NY, USA) with a 63X oil immersion lens. Nonspecific binding of the secondary was determined by omitting primary antibody.

To determine if CH alters basal or agonist-stimulated T-channel distribution, similar methods were used to label T-channels in control and CH PAEC before and after agonist stimulation. However, prior to fixation, the cells were incubated with either ATP (20  $\mu$ M) or vehicle for 60 seconds at room temperature. Rabbit monoclonal antibody (1:100, Alomone Labs, Ltd., Jerusalem, Israel) for Ca<sub>v</sub>3.1 were used for the overnight incubation (4°C). The same secondary antibody used for caveolin staining was used and nonspecific binding of the secondary antibody was determined by omitting primary antibody. Five to 15 sheets from three separate rats in each group were observed.

#### *Transmission Electron Microscopy to Assess Caveolar Density*

The heart and lungs were exposed by midline thoracotomy in sodium pentobarbital (200 mg kg<sup>-1</sup> i.p.) anesthetized rats. Heparin (100U) was injected into the right ventricle and the pulmonary artery cannulated with a 13-gauge needle stub. The lungs were perfused at a pressure of 15 cmH<sub>2</sub>O with 100 ml of physiological saline solution (PSS) containing (in mM) 129.8 NaCl, 5.4 KCl, 0.83 MgSO<sub>4</sub>, 19 NaHCO<sub>3</sub>, 1.8 CaCl<sub>2</sub>, and 5.5 glucose, with 4% (wt/vol) albumin, 10<sup>-4</sup> M papaverine, and 1000 U of heparin. The lungs were then perfused at the same pressure with 100 ml of fixative (phosphate buffered saline containing 4% paraformaldehyde, 0.1% glutaraldehyde, and 10<sup>-4</sup> M papaverine). Samples of lung tissue were then fixed for transmission electron microscopy with 3% formaldehyde, 2% glutaraldehyde in 0.1M sodium cacodylate buffer, post-fixed in reduced osmium tetroxide (1% O<sub>3</sub>O<sub>4</sub> and 0.5% potassium ferrocyanide), *en bloc*, stained with 1% uranyl acetate, dehydrated and embedded in

epoxy resin. Caveolae between 60-100 nm diameter were counted at the membrane of endothelial cells in arterial sections and divided by the membrane length in  $\mu\text{m}$  using NIH Image J software. Vessels from 3 rats/group were analyzed. A total of 70 images encompassing 21 cells and 667  $\mu\text{m}$  of membrane were analyzed from arteries of control rats, whereas 84 images encompassing 25 cells and 758  $\mu\text{m}$  of membrane were analyzed in the CH group.

#### *Calculation and statistics*

Data are expressed as means  $\pm$  S.E. Values of  $n$  refer to the number of rats. A one-way or two-way analysis of variance was used where appropriate for comparison between groups. Individual groups were compared using the Student-Newman-Keuls test and a probability of  $\leq 0.05$  was accepted as statistically significant.



## **Results**

**Specific Aim 1:** *Determine the role of T-channels in agonist-induced calcium entry and SOCE in PAEC from control and CH rats.*

Hypothesis: T-channel dysfunction inhibits pulmonary endothelial SOCE and agonist-dependent calcium entry following CH.

*Effect of mibefradil on ATP-induced  $Ca^{2+}$  entry*

ATP induced  $Ca^{2+}$  entry was reduced in endothelial cells from CH rats compared to control rats as previously reported (42). The T-channel inhibitor mibefradil lowered ATP-induced calcium influx in controls cells without altering the response in cells from CH rats, resulting in similar  $Ca^{2+}$  entry between groups (Figure 4). We also saw similar results in endothelial cells from larger vessels (Appendix Figure 1) indicating that this response is not dependent on vessel size.

*T-channel immunofluorescence in PAEC*

To determine if diminished T-channel mediated  $Ca^{2+}$  entry following CH is associated with altered T-channel intracellular localization of these channels, T-channels were immunofluorescently labeled in isolated PAEC from control and CH rats. It is also possible that ATP induces a change in the localization of T-channels that confers  $Ca^{2+}$  entry that is not present in the PAEC from CH rats. However, T-channel immunofluorescence appeared similar between groups and between basal and ATP-stimulated conditions (Figure 5).

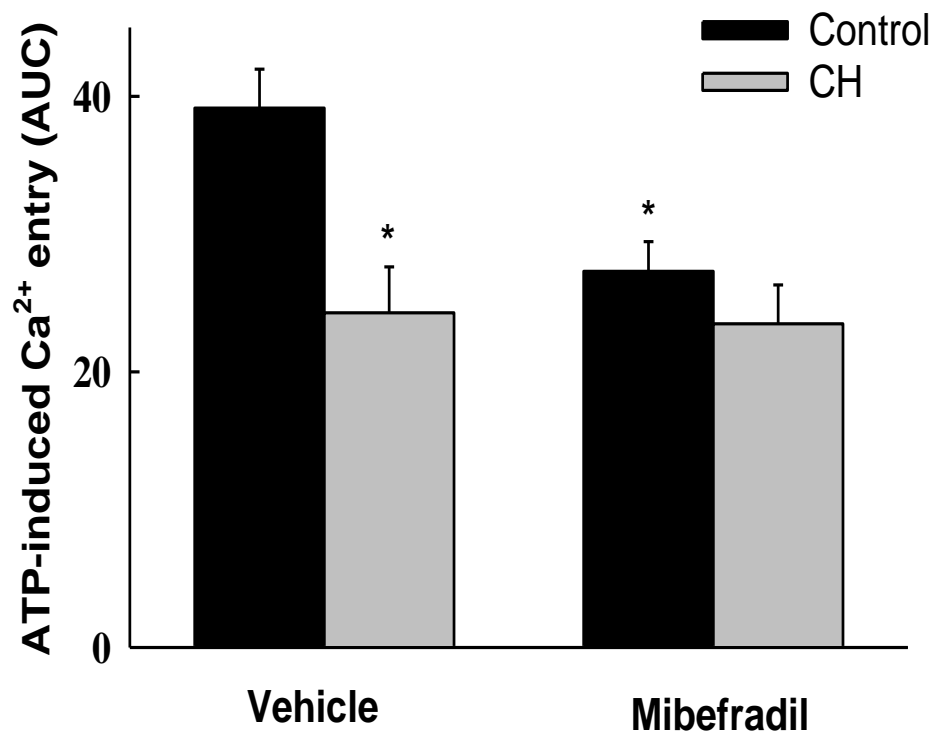


Figure 4. T-channel dependent Ca<sup>2+</sup> entry is lost in PAEC from CH rats. The T-channel inhibitor mibefradil (10 μM) diminished ATP-induced (20 μM) Ca<sup>2+</sup> entry in PAEC from control rats but is without effect in PAEC from CH rats (n=5-9 rats per group). \**P* ≤ 0.05 vs. control vehicle. Data are mean ± S.E.

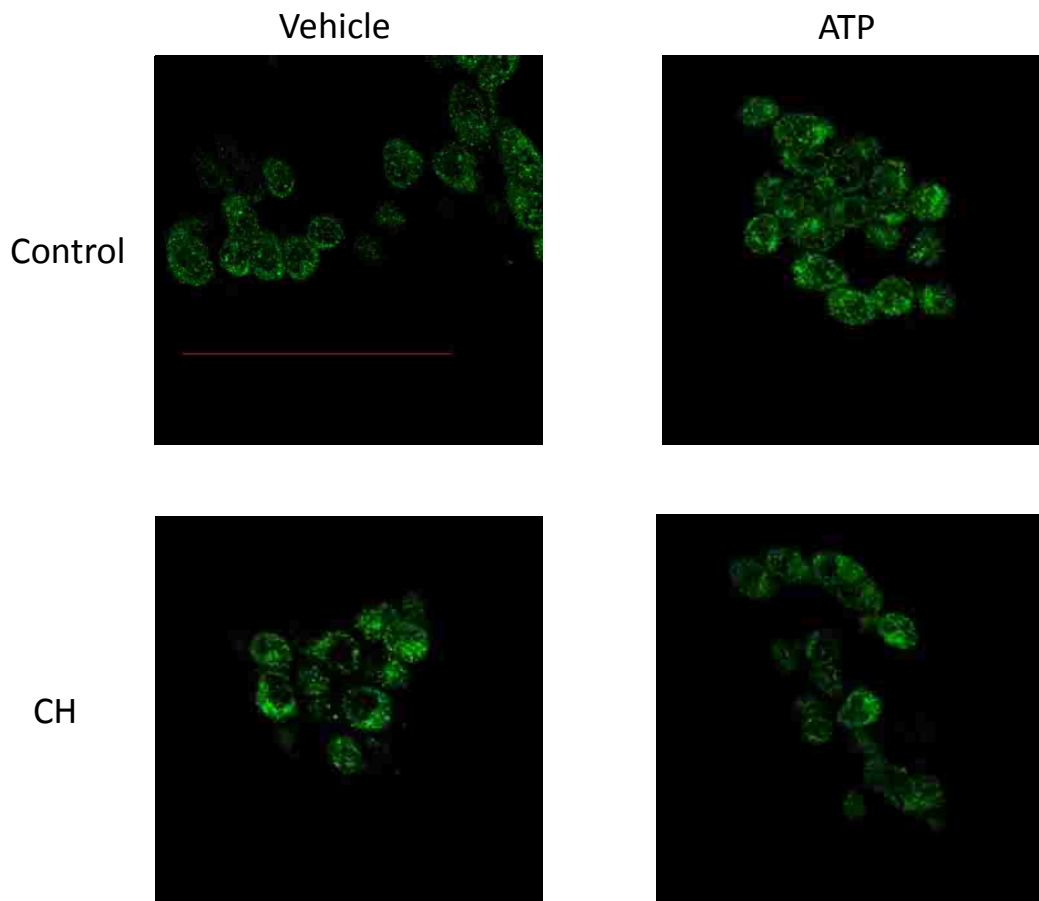


Figure 5. T-channel labeling in PAEC appears unaltered by ATP (20  $\mu$ M) or CH. PAEC labeled for T-channels (green) from control and CH animals. In the right panels cells were fixed 60 seconds after ATP (10  $\mu$ M) or vehicle administration. Scale bar (red) equals 50  $\mu$ m.

### *Role of T-channels in SOCE*

Attenuated ATP-induced  $\text{Ca}^{2+}$  responses following CH appear to involve loss of T-channel dependent  $\text{Ca}^{2+}$  influx (Figure 4), however, whether this effect of CH reflects a reduced role for T-channels in the SOCE component of agonist-induced  $\text{Ca}^{2+}$  mobilization is not clear. To examine this possibility, we initially set out to evaluate a potential contribution of T-channels to SOCE in PAEC from control rats. However, we found that mibefradil was without effect on SOCE in these cells (Figure 6), thus arguing against a role for T-channel in SOCE following CH.

**Specific Aim 2:** *Determine the contribution of caveolin-1 to SOCE in PAEC from control and CH rats.*

Hypothesis: CH impairs caveolin-1 regulation of ion channels necessary for pulmonary endothelial SOCE.

### *Caveolin-1 scaffolding peptide augments SOCE following CH*

SOCE was diminished in the CH group as described previously (41). Although administration of the caveolin-1 scaffolding domain (AP-Cav) was without effect on SOCE in control cells, AP-cav augmented SOCE in cells from CH rats compared to those treated with the scrambled control peptide. This effect of AP-cav in CH cells resulted in greater SOCE compared to similarly treated control cells (Figure 7). This effect was also observed in endothelial cells from the main pulmonary artery (Appendix Figure 2).

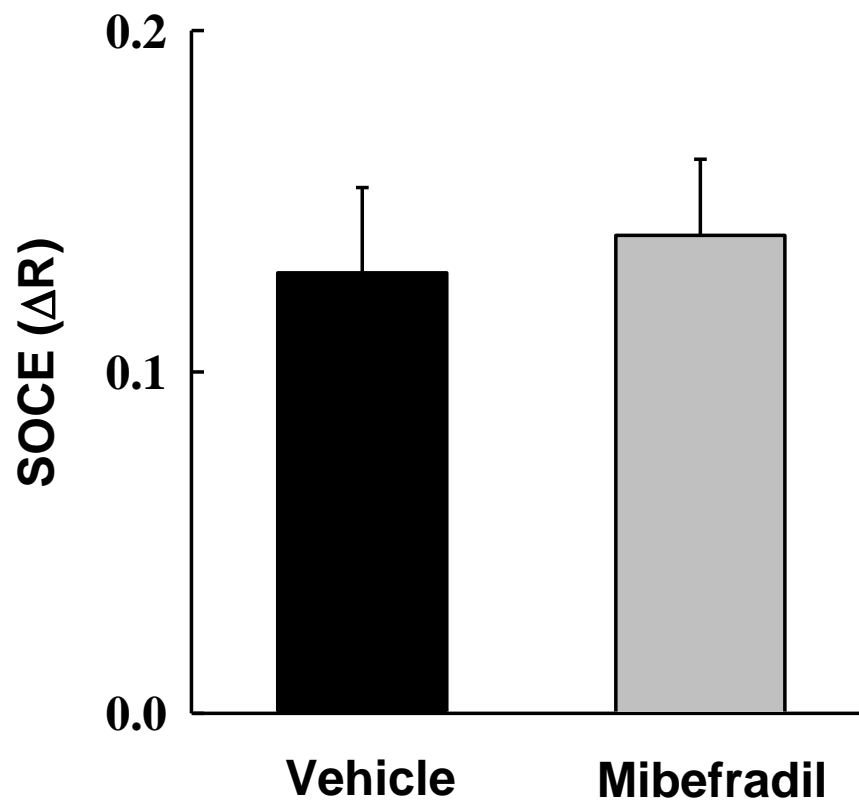


Figure 6. T-channels do not contribute to SOCE in PAEC from control rats. SOCE is defined as the measured change in  $F_{340}/F_{380}$  ( $\Delta R$ ) fluorescence upon repletion of extracellular  $\text{Ca}^{2+}$  (1.8 mM) in the presence or absence of mibefradil (n=5 per group). Data are mean  $\pm$  S.E.

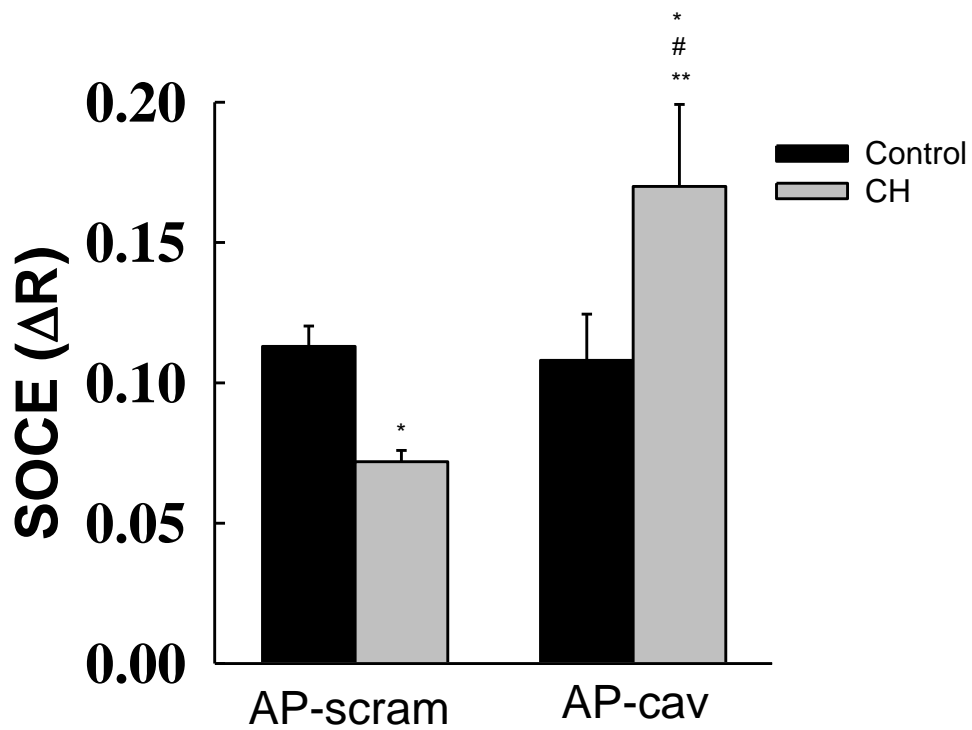


Figure 7. Administration of the scaffolding domain of caveolin-1, AP-cav (10  $\mu$ M) enhances SOCE in PAEC from CH rats. AP-cav is without effect in control PAEC. \* =  $P \leq 0.05$  vs. AP-scram control. # =  $P \leq 0.05$  vs. AP-cav control. \*\* =  $P \leq 0.05$  vs AP-scram CH. (n=5-7 per group). Data are mean  $\pm$  S.E.

*Caveolin-1 immunofluorescence and caveolar density*

Cells freshly isolated from control and CH rats demonstrated similar immunofluorescent staining for caveolin-1 (Figure 8). No staining was observed in sections treated only with secondary antibody. In addition, quantification of caveolar density from transmission electron micrographs revealed no difference between groups (Figure 9 and 10).

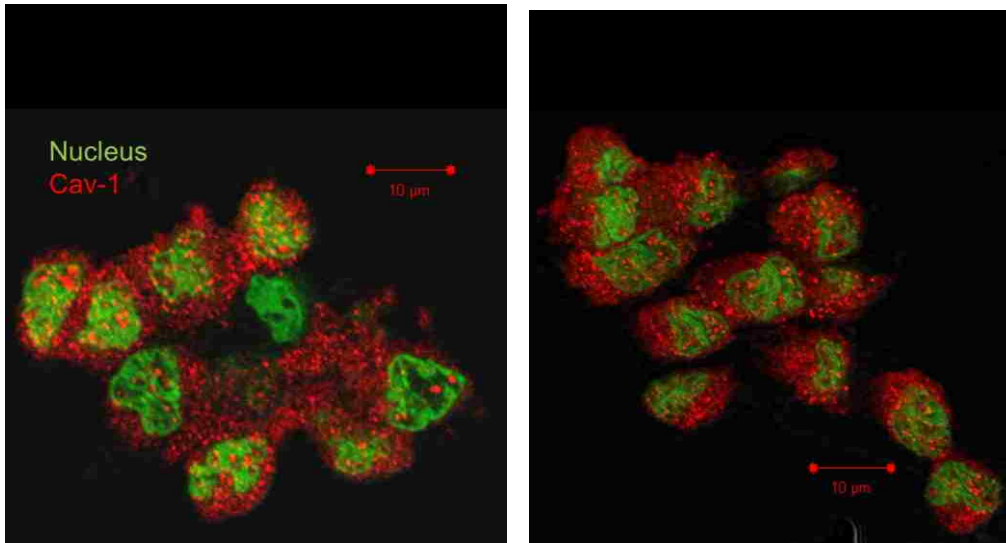


Figure 8. Immunofluorescence images of caveolin-1 (red) in freshly dispersed pulmonary arterial endothelial sheets from control (left) and chronically hypoxic (CH; right) rats. Nuclei are labeled green.



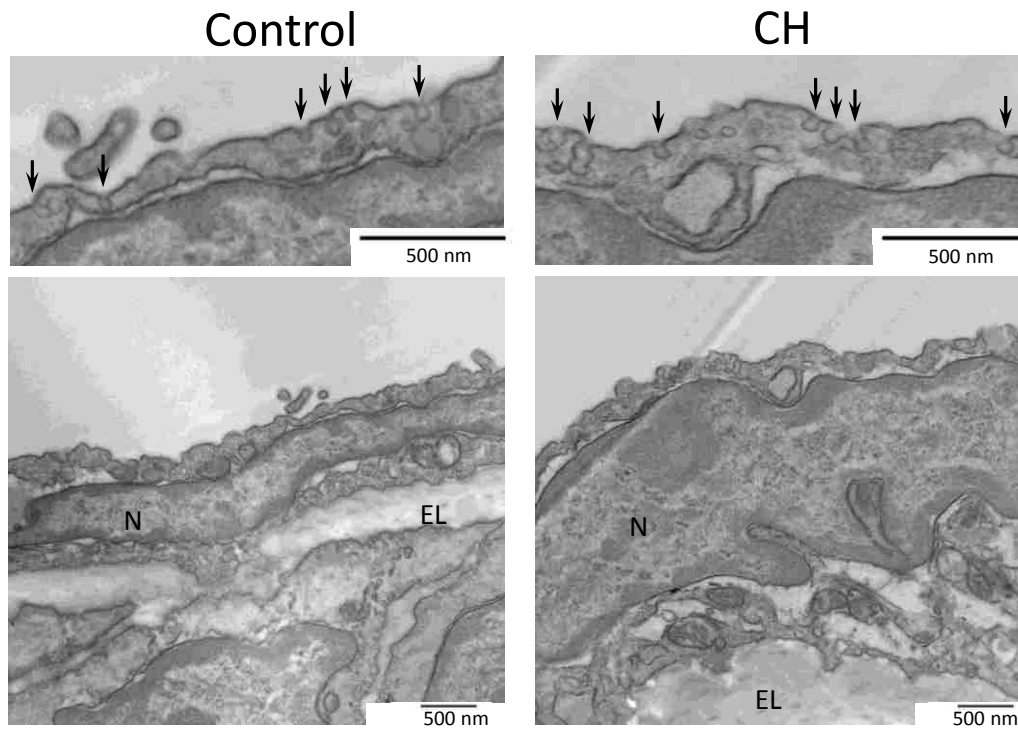


Figure 9. Transmission electron microscopy was used to determine the number of caveolae in the endothelium of pulmonary arteries from control and CH rats. Representative electron micrographs at low and high magnification of caveolae (denoted by arrows) in arteries from control (left) and CH (right) rats. N, nucleus; EL, elastic lamina.

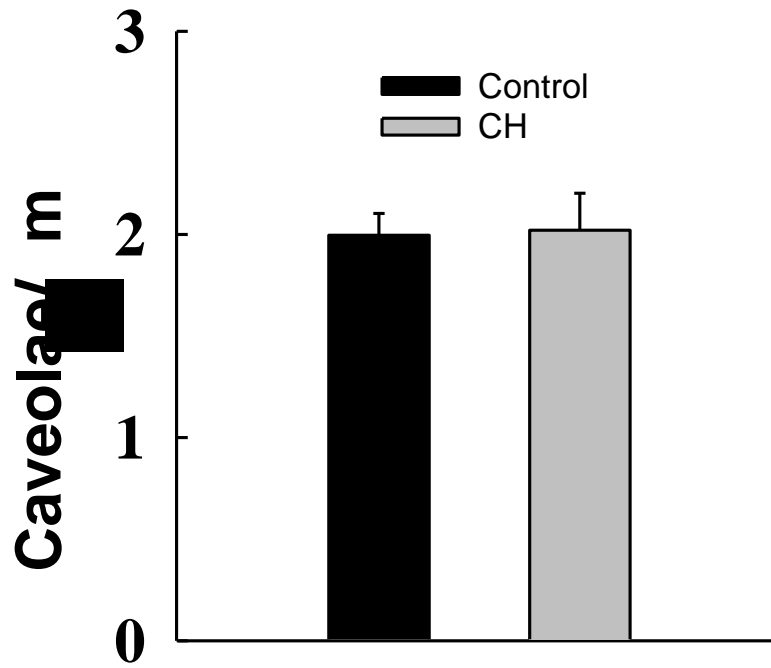


Figure 10. Caveolar density is expressed as the number of caveolae per  $\mu\text{m}$  length of membrane ( $n = 3$  rats/group). There were no significant differences.

## **Discussion**

The overall objective of this thesis was to examine the potentially altered role of T-channel and caveolin-1 function in attenuated pulmonary endothelial SOCE following CH. The major findings of this project are: 1) ATP-mediated PAEC  $\text{Ca}^{2+}$  entry was attenuated following CH; 2) mibefradil inhibited this response to ATP in PAEC from control but not CH rats, thus supporting a role for T-channel dysfunction in reduced agonist-dependent  $\text{Ca}^{2+}$  influx following CH; 3) in contrast, SOCE was unaltered by mibefradil in PAEC from control rats; 4) the scaffolding domain of caveolin-1 restored SOCE in PAEC from CH rats, while having no effect in control cells; 5) however, CH did not alter the intracellular distribution of caveolin-1 or incidence of caveolae in PAEC. We conclude that reduced SOCE in endothelial cells from pulmonary hypertensive arteries is unrelated to T-channel dysfunction, but rather involves an effect of CH to impair caveolin-1 dependent regulation of other ion channels required for SOCE.

Previous studies from our laboratory have shown that SOCE, ROCE and depolarization induced  $\text{Ca}^{2+}$  entry are attenuated in PAEC following CH (41; 43). Consistent with these previous findings we presently observed that ATP-induced  $\text{Ca}^{2+}$  entry is diminished in PAEC from CH rats, which likely involves both SOCE and ROCE components. Although the mechanism by which CH attenuates agonist induced  $\text{Ca}^{2+}$  entry and the specific ion channels involved are not fully understood, a recent study from our group found that the ROCE component of this response is impaired following CH due to a reduced contribution of T-channel mediated  $\text{Ca}^{2+}$  influx (43). Consistent with these observations, ATP mediated  $\text{Ca}^{2+}$  entry was diminished in the presence of mibefradil, a T-channel inhibitor, in PAEC from control rats but not in PAEC from CH

rats. This infers that T-channel dysfunction contributes to the diminished ATP-induced  $\text{Ca}^{2+}$  entry in PAEC from CH rats. Potential mechanisms by which T-channel dysfunction is impaired following CH include diminished T-channel expression or reduced cell membrane expression. However, our qualitative assessment of T-channel immunofluorescence did not reveal apparent differences in staining between groups, nor did we observe any differences in localization following ATP stimulation in cells from control and CH groups. These findings suggest that other modes of regulation are involved in T-channel dysfunction following CH.

T-channel mediated  $\text{Ca}^{2+}$  influx in the endothelium could be important in the development of PH. It is possible that vascular smooth muscle cell depolarization caused by acute hypoxia or other stimuli could be conducted by myoendothelial gap junctions to the endothelium, leads to an increase in intracellular  $\text{Ca}^{2+}$  and production of vasodilators and antimitogenic factors. This could serve to buffer the constrictive response to acute hypoxia. However, CH leads to a loss of proper T-channel function and this could exacerbate the development of PH in CH.

It is unknown if T-channels play a role in the diminished SOCE component of this response. It is possible that T-channels contribute to SOCE secondary to membrane depolarization following cation influx through non-selective cation channels activated during the SOCE response; although such a role for voltage gated  $\text{Ca}^{2+}$  channels has not been previously described. However, we did not observe an effect of mibefradil on SOCE in PAEC from control cells indicating that T-channels do not contribute to SOCE in control conditions. These results suggest that CH alters other ion channels involved in the SOCE response, most likely members of the TRPC family including TRPC1 and

TRPC4 that have previously been implicated in mediating SOCE in endothelial cells (47; 53).

Recent studies from our laboratory suggest that attenuated agonist-induced pulmonary endothelial  $\text{Ca}^{2+}$  entry following CH results from a derangement of caveolin-1 containing cholesterol-enriched membrane domains (42). For example, we found that ATP-induced  $\text{Ca}^{2+}$  entry and inward cation currents are diminished in PAEC from CH rats and that this  $\text{Ca}^{2+}$  entry can be restored with the administration of a cell permeable caveolin-1 scaffolding domain peptide. However, this earlier study did not address the role of caveolin-1 in diminished SOCE following CH, which is why we decided to look at the potential role of caveolin-1 in SOCE. We presently found that caveolin-1 dysfunction in PAEC from CH rats may lead to diminished SOCE. These studies suggest that CH decreases agonist-induced  $\text{Ca}^{2+}$  in part by impairing caveolin-1 dependent regulation of the ion channels required for SOCE. However, whether caveolin-1 dysfunction similarly accounts for diminished ROCE and depolarization induced  $\text{Ca}^{2+}$  entry following CH remains to be determined.

There are several potential mechanisms that may account for diminished caveolin-1 regulated SOCE in PAEC from CH rats. For example, it is possible that CH decreases caveolin-1 expression and/or impairs caveolin-1 dependent trafficking of ion channels to the membrane. Indeed, Jasmin et al. (18) showed that caveolin-1 expression is down regulated in monocrotaline treated rats that develop pulmonary hypertension. Furthermore, Murata et al. (36) observed impaired trafficking of ion channels to the membrane in PAEC from caveolin-1 knock out animals. Although we did not observe apparent alterations in either PAEC membrane localization or expression of cav-1 after

CH exposure, these findings do not exclude the possibility that attenuated SOCE in PAEC from CH rats results from impaired trafficking of ion channels to the cell membrane.

It is also possible that caveolin-1 dysfunction following CH leads to altered membrane micro-domains that bring critical SOCE components within close proximity to each other. Another possibility is that caveolin-1 dysfunction leads to a decrease in the number of caveolae leading to an un-organized distribution of the critical proteins needed for SOCE such as STIM-1 and TRPC1 (46) and impaired SOCE function. Indeed, caveolin-1 deficient mice lack caveolae (62) and monocrotaline treated rats displaying pulmonary hypertension show a reduction in the number of caveolae in the endothelium (49). However, we did not see a difference in caveolar density between the PAEC from the control and CH groups. Alternatively, our recent paper shows that this effect of CH may be attributed to altered membrane cholesterol content (42). In this paper it was observed that PAEC from CH rats have diminished membrane cholesterol. Consistent with CH causing a decrease in membrane cholesterol Nguyen et al. (40) have shown that hypoxia limits cholesterol synthesis in a hypoxia inducible factor dependent manner. Paffett et al. (42) also showed that agonist induced  $\text{Ca}^{2+}$  entry is diminished when cholesterol is depleted from PAEC from control rats and that restoring membrane cholesterol in PAEC from CH rats restores agonist induced  $\text{Ca}^{2+}$  entry. Reduced membrane cholesterol could therefore be a contributing factor to the decreased SOCE we see in PAEC from CH rats.

Limitations of this study include problems associated with isolating PAEC from rats because by doing this we removed the effects of shear stress as well as other

mechanical forces. Shear stress is an important physiological stimulus for  $\text{Ca}^{2+}$  entry in endothelial cells (1) and could be an important mediator of eNOS activity possibly through regulation of basal  $\text{Ca}^{2+}$ . Indeed if shear-induced  $\text{Ca}^{2+}$  entry is attenuated following CH, this could lead to further decreases in basal  $\text{Ca}^{2+}$  and impaired synthesis of vasodilator and antimitogenic factors. An additional limitation is the use of pharmacological agents to induce SOCE. Inhibition of proper ER function and submitting cells to a  $\text{Ca}^{2+}$  free extracellular solution does not take place *in vivo*. Additionally, dissociating the endothelial cells from the vessel could alter function. However, previous studies from our laboratory have demonstrated both reduced endothelial basal  $\text{Ca}^{2+}$  and reduced SOCE in intact isolated arteries from CH rats. Previous studies from our laboratory have also documented that vascular smooth muscle cells are depolarized following CH and this depolarization could be conducted to the endothelial layer. The isolation of these cells leads to a loss of the cell to cell connections that these cells have *in vivo*. It is also important to note that there is changing pressure and radial stretch with normal respiration in the lung which also exerts mechanical forces on the vascular endothelium which could also influence  $\text{Ca}^{2+}$  entry. Another limitation was our interpretation of immunofluorescence data because we were unable to quantify fluorescent intensity in these images. Future studies are needed to determine gene and protein levels and one possible technique for doing this could be labeling endothelial cells and sorting them using flow cytometry which would allow us to have a pure endothelial isolate with which we could conduct quantitative PCR or western blotting. Another limitation is the use fura-2 to measure a change in global intracellular  $\text{Ca}^{2+}$  which does not detect the effect that local changes of  $\text{Ca}^{2+}$  might have. These localised

changes could have profound effects if they are localized in the same region as eNOS for instance because there might be much larger differences in  $\text{Ca}^{2+}$  levels between PAEC from control and CH animals in eNOS localized regions that we are not detecting. This could greatly affect the production of vasodilators and lead to a severe decrease in vasodilation and antimitogenic factor production in CH. We could address this issue in future studies by using a faster  $\text{Ca}^{2+}$  indicator like fluo-4 and using a spinning disc confocal microscope to detect these changes. Another potential limitation is the lack of specificity of mibefradil for inhibition of T-channels. However Paffet et al. (44) have shown that the concentration employed in this study inhibits T-channel currents in neonatal cardiomyocytes and inhibits depolarization induced  $\text{Ca}^{2+}$  influx in PAEC independent of L-channel inhibition. Our animal model of CH induced PH is limited in its dissimilarities with human forms of pulmonary hypertension. However, this model is pretty good and in fact many treatments for PH have been developed from studies using this model.

Preventing the deleterious effects of endothelial dysfunction is a hallmark in the treatment of PH. Numerous treatments exist to try and offset the effect of endothelial dysfunction including administration of NO, prostacyclin analogs, endothelin receptor antagonist and phosphodiesterase inhibitors (2) and this study as well as others suggest that the scaffolding domain of caveolin-1 could be a potential treatment for endothelial dysfunction. One side effect of the therapeutic application of AP-cav is that its effect is not limited to the pulmonary circulation. Interestingly recent studies from our lab have shown that the scaffolding domain of caveolin-1 restores systemic vasoreactivity following CH (unpublished results). The results in this project and our recent paper (42)

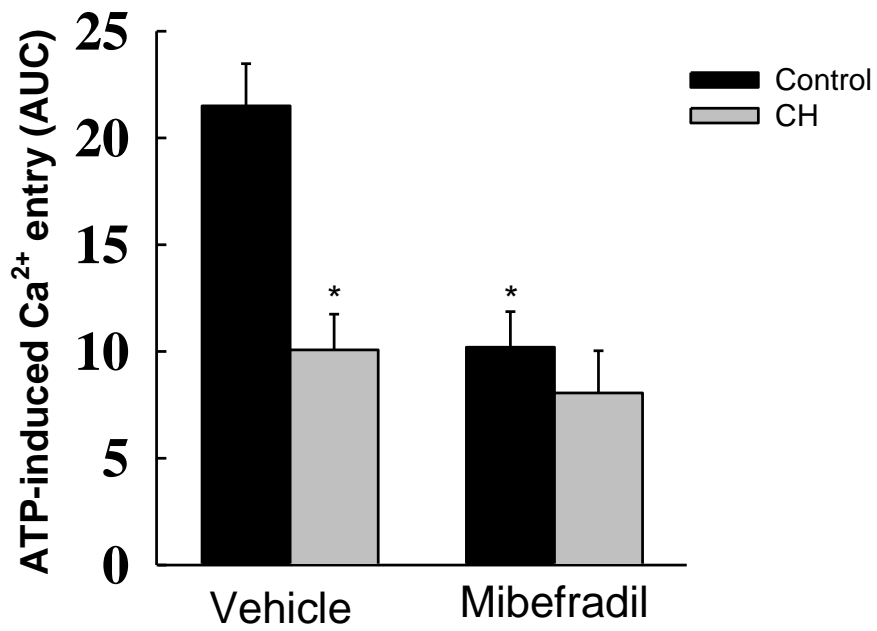


suggest that application of the caveolin-1 scaffolding domain could reduce the deleterious effect of decreased  $\text{Ca}^{2+}$  entry in PAEC in pulmonary hypertensive patients.

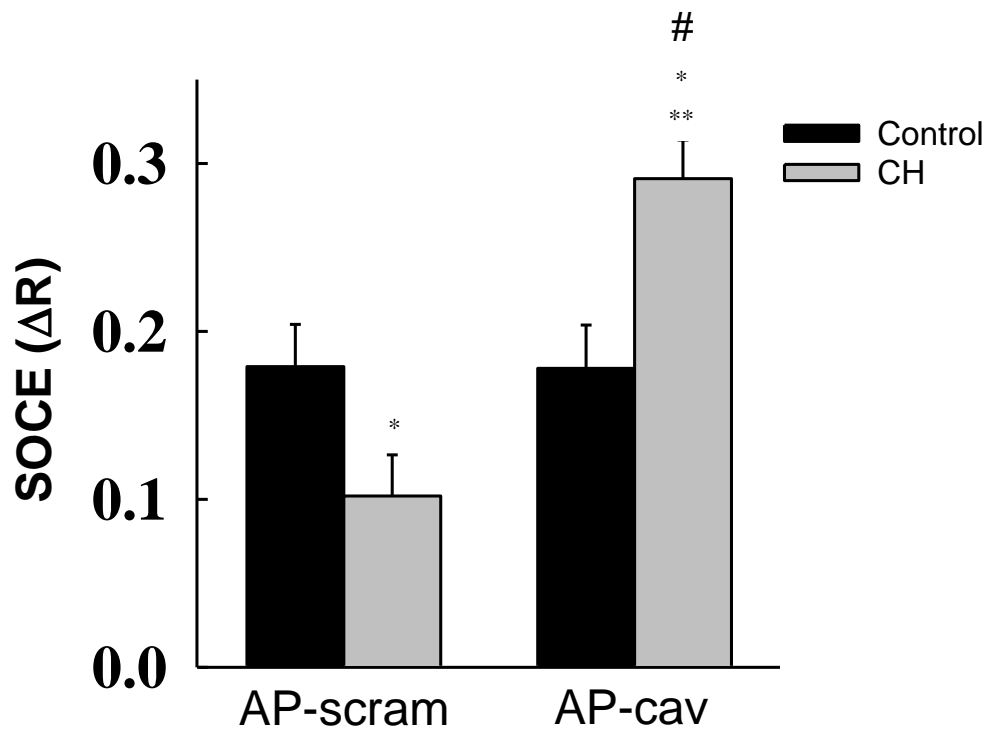
In conclusion, our present findings show decreased agonist induced  $\text{Ca}^{2+}$  entry and decreased SOCE which is mediated by dysfunctional caveolin-1. Future studies should determine if caveolin-1 levels are similar between groups. The identity of the channels normally involved in SOCE is still unknown which should be a critical goal for future studies. Another important future direction would be determining how caveolin-1 interacts with these channels and what the mechanism of caveolin dysfunction is in CH. There also is a need for future studies to address the role of altered cholesterol synthesis leading to decreased agonist induced  $\text{Ca}^{2+}$  entry following CH in PAEC. Future studies might determine whether chronic administration of AP-cav can alleviate the symptoms of CH induced PH and whether or not this can restore basal  $\text{Ca}^{2+}$  and  $\text{Ca}^{2+}$  entry in endothelial cells. It would also be interesting to study the effects of cholesterol repletion/depletion *in vivo* perhaps through the use of statins to inhibit cholesterol synthesis. The results of this study suggest caveolin-1 dysfunction is an important area of study in order to understand the physiological mechanisms contributing to pulmonary hypertension.

## Appendix

The protocols are the same for these studies as the ones listed in the methods section. The only difference is the mean size of the vessels. For the ATP induced  $\text{Ca}^{2+}$  entry experiments the largest intrapulmonary artery running longitudinally down the side of the left lung was dissected and endothelial cells were isolated. For the SOCE experiments the main pulmonary artery was dissected and utilized for endothelial cell isolation.



Appendix figure 1. ATP-induced Ca<sup>2+</sup> entry is diminished in large intrapulmonary artery endothelial cells (LIPAEC) from CH rats in comparison to controls. The T-channel inhibitor mibefradil (10  $\mu$ M) inhibited Ca<sup>2+</sup> entry in LIPAEC from CH rats but not controls implying that T-channel function is lost in LIPAEC from CH rats (n=5-8 rats per group). \* $P \leq 0.05$  vs. control. Data are mean  $\pm$  S.E.



Appendix Figure 2. Caveolin-1 dysfunction contributes to diminished SOCE in main PAEC (MPAEC) from CH rats. The scaffolding domain of caveolin-1 restores and enhances SOCE in MPAEC from CH rats but has no effect in controls (n=5-7 rats per group). \*  $P \leq 0.05$  vs. AP-scram control, #  $P \leq 0.05$  vs. AP-cav control, \*\*  $P \leq 0.05$  vs. AP-scram CH. Data are mean  $\pm$  S.E.

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