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Role of endothelial BKCa channel activity in diminished vasoconstrictor reactivity following chronic hypoxia

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ROLE OF ENDOTHELIAL BKCa CHANNEL ACTIVITY IN DIMINISHED VASOCONSTRICTOR REACTIVITY FOLLOWING CHRONIC HYPOXIA

BY

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B.A., Buena Vista University, 2005

DISSERTATION

Submitted in Partial Fulfillment of the
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DEDICATION

In memory to all of those who fought and lost their battle with chronic obstructive pulmonary disease.

Through the years you provided strength and support to my endeavors; your spirit and love will continue to inspire my journey even though I lost you long ago. Let your memory live on through this work and may advances in the research you inspired me to follow aide in helping others experience life to the fullest.

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ROLE OF ENDOTHELIAL BKCa CHANNEL ACTIVITY IN DIMINISHED VASOCONSTRICTOR REACTIVITY FOLLOWING CHRONIC HYPOXIA

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ABSTRACT

Vasoconstrictor responsiveness of the systemic circulation are attenuated following prolonged exposure to hypoxia. Previous work from our laboratory has demonstrated vasoconstrictor reactivity is diminished due to hyperpolarization of the vascular wall in systemic arterioles. The hyperpolarization of the vascular wall was found to be dependent upon the endothelium, heme oxygenase (HO), and calcium-activated large conductance potassium channel (BK_{Ca}) as removal of the endothelium or blockade of HO or BK_{Ca} restored vasoreactivity. Interestingly, recent evidence for HO and BK_{Ca} association has emerged in carotid body glomus cells where HO and BK_{Ca} form oxygen sensing complexes. In these novel oxygen sensing complexes, HO-derived CO activate BK_{Ca} channels in localized domains that regulate carotid body membrane potential. The presence and function of HO and BK_{Ca} association has not been previously acessed in the systemic endothelium. The following studies investigated a novel HO-BK_{Ca} functional unit in systemic vascular endothelial cells. In whole cell patch clamp experiments it was demonstrated that HO-dependent activation of BK_{Ca} significantly increased

transmembrane currents in ECs following CH. Additionally, HO-dependent BK_{Ca} activity was unearthed following disruption of caveolin-1 function or potentially inhibited following incubation with the caveolin-1 scaffolding domain, demonstrating the inhibitory role of caveolin-1 on HO-BK_{Ca} complexes. Membrane potential recordings and isolated arteriole experiments further demonstrated EC BK_{Ca} regulates EC E_m and vasoconstrictor reactivity following CH through a loss in caveolin-1 inhibition. This study is the first to characterize HO-dependent BK_{Ca} activation regulation by caveolin-1 and demonstrate a functional role for endothelial BK_{Ca} in vascular tone.

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CHAPTER 1
INTRODUCTION

Chronic hypoxia (CH) is associated with pathological conditions that impair oxygenation or with prolonged residence at high altitude. Previous studies have demonstrated that vasoconstrictor responsiveness of the systemic circulation is attenuated following prolonged exposure to hypoxia (Auer & Ward, 1998;Doyle & Walker, 1991;Hu *et al.*, 1996;Toporsian & Ward, 1997). Diminished vasoconstrictor reactivity following CH is observed both systemically as a reduced total peripheral resistance response to vasoconstrictor agonists (Doyle & Walker, 1991) and in several individual vascular beds (O'Donaughy & Walker, 2000;Jernigan *et al.*, 2001;Auer & Ward, 1998;Caudill *et al.*, 1998) suggesting that it is a generalized response to this stimulus. Further, since diminished vasoconstrictor activity is maintained following acute return to normoxia (Doyle & Walker, 1991;Auer & Ward, 1998) and is largely unaffected by P_{O2} upon re-oxygenation (Doyle & Walker, 1991) this response to CH appears to be an adaptation, distinct from acute responses to hypoxia.

This impairment of vasoconstriction is endothelium-dependent. For example, reduced agonist-induced constriction following CH is reversed by endothelial disruption in both aorta (Caudill *et al.*, 1998;Doyle & Walker, 1991) and in resistance arteries (Earley *et al.*, 2002). A similar endothelium-dependence is observed in attenuated myogenic reactivity following CH in mesenteric arterioles (Earley & Walker, 2002). Furthermore, CH-induced blunted vasoconstriction in the mesenteric vascular bed is associated with an endothelium-dependent reduction in vascular smooth muscle (VSM)

calcium as well as VSM hyperpolarization (Earley & Walker, 2002;Earley *et al.*, 2002;Earley & Walker, 2003). Further, removal of the endothelium, inhibition of heme oxygenase (HO), (Earley *et al.*, 2002;Naik & Walker, 2003), CYP P450 products (Earley *et al.,* 2003), or BK_{Ca} channels (Naik & Walker, 2003;Hughes *et al.*, 2010) normalizes membrane potential and restores vasoreactivity in arterioles from CH rats.

Although the mechanisms responsible for altered responsiveness in the vasculature during CH are not fully defined, previous experiments suggest that endothelial nitric oxide (NO) (Earley & Walker, 2003), carbon monoxide (CO) (Jernigan *et al.*, 2001;Naik *et al.*, 2003;O'Donaughy & Walker, 2000;Gonzales & Walker, 2002;Caudill *et al.*, 1998) and epoxyeicosatrienoic acids (EETs) (Earley *et al.*, 2003) may be involved. In addition, VSM E_m and vasoreactivity are restored in mesenteric resistance arteries from CH rats following inhibition of large conductance calcium-activated K^+ (BK_{Ca}) channels (Earley *et al.*, 2003;Naik & Walker, 2003). Since NO, CO and EETs are all endogenous activators of these channels, a primary role of BK_{Ca} in altered vasoreactivity following CH is likely.

BK_{Ca} are important regulators of arterial tone via their hyperpolarizing influence on vascular smooth muscle (VSM). VSM BK_{Ca} channels are regulated by localized increases in calcium due to sparks from ryanodine-sensitive stores (Jaggar *et al.*, 2000). Although most studies concerning the role of BK channels in vascular control focus on the VSM, there is increasing evidence that endothelial cells (EC) also express these channels although both their expression and physiological significance in native cells have been questioned (Gauthier *et al.*, 2002). It has been suggested that although these channels may be quiescent in some vascular beds, their activity is unmasked in various

pathological conditions or in cell culture (Sandow & Grayson, 2009). The following sections will outline the development of vascular dysfunction associated with chronic hypoxia and chronic obstructive pulmonary disease, further discussing the role of the endothelium in modulating vascular tone in pathological conditions associated with CH; with a focus on the regulation and physiological role of BK_{Ca} channels in vascular function/dysfunction.

Clinical Definition of Hypoxia:

Hypoxia can be defined as "a deficiency in the amount of oxygen that reaches the tissues of body" ("Hypoxia" Merriam-Webster, 2011). This deficiency in oxygenation of the tissues may result from a variety of mechanisms, such as inadequate oxygenation of the arterial blood due to decreased atmospheric pressures for oxygen (PO_2) as experienced with increased altitude. Decreased oxygenation of tissues may also result from low hemoglobin concentrations, decreased cardiac output, or impaired unloading of oxygen from hemoglobin resulting in inadequate systemic oxygen delivery. Inadequate peripheral oxygen delivery, as in the case of right-to-left shunting in the lung, and diffusion impairment also result in hypoxemia (Pierson D.J., 2000). Thus, recognizing the mechanism(s) of hypoxemia is important for proper diagnosis and treatment of patients. The role of the efficiency of gas exchange on hypoxemia clinically is determined by calculating the alveolar-to-arterial PO_2 difference **($P_{(A-a)O2}$)**. Arterial blood is first obtained from patients to measure the partial pressures of oxygen and carbon dioxide in the arterial blood, P_{aO2} and P_{aCO2} respectively. Alveolar PO_2 (P_{AO2}) can then be calculated using the alveolar gas equation:

$$\mathbf{P_{AO2} = PI_{O2} - Pa_{CO2}/R}$$

Pa_{CO2} is the arterial carbon dioxide tension, R is the respiratory quotient, and PI_{O2} is calculated from barometric pressure (PB), the partial pressure of water vapor (P_{H2O}) at body temperature, and the FI_{O2}

$$\mathbf{PI_{O2} = (PB - P_{H2O}) \; X \; FI_{O2}}$$

When breathing air at sea level, PI_{O2} is: (760-47mmHg) X 0.21, or approximately 150 mmHg. The respiratory quotient (R), the overall ratio of CO_2 produced to O_2 consumed by the body, is about 0.8 (for most North American diets). Thus if the patient's Pa_{CO2} is 40mmHg:

$$\mathbf{P_{AO2} = 150 \; mmHg - 40 \; mmHg / \; 0.8 = 100 \; mmHg}$$

Calculated differences of $P_{(A- a)O2}$ less than 20 mmHg are considered normal, while values greater than 30 are abnormal (Pierson D.J., 2000). Administration of supplemental oxygen and determined $P_{(A-a)O2}$ values further aide in determining the patient's cause for hypoxemia. For example: normal values for $P_{(A-a)O2}$ with increased Pa_{CO2} (hypercapnia) would suggest decreased alveolar ventilation, while abnormal values for $P_{(A-a)O2}$, decreased P_{aCO2} with positive responses from the patient to supplemental oxygen would suggest ventilation-perfusion mismatching as seen in COPD patients. Understanding the root of the patient's hypoxemia enables appropriate measures to be taken to restore P_{aO2}. Acute hypoxia stemming from the use of barbiturates (respiratory depressants), or decreased hemoglobin content in the blood require different courses of treatments; and are not caused by impairment of lung function. In conditions that result in chronic hypoxia, P_{aO2} is not readily restored and may not be restored to normal ranges as long-

term exposure to the hypoxemia has resulted in chronic maladaptations that limit oxygen diffusion across tissues. Fick's law of diffusion states that the rate of gas transfer (V_{gas}) through a sheet of tissue is proportional to: the tissue area (A), the difference in gas partial pressure (P_1-P_2) between the two sides and inversely proportional to the tissue thickness (T). The rate of diffusion is proportional to the diffusion constant (D) which is defined as the quotient of the solubility and the square root of the molecular weight of the gas.

$$V_{gas} \propto \frac{A}{T} \cdot D \cdot (P_1\text{-}P_2)$$

$$\text{where, } D \propto \frac{Sol}{\sqrt{M.W.}}$$

Diseases such as COPD, where inflammation and occlusion of the airways occurs, result in defective tissue diffusion. Further discussion on COPD and the mal-adaptations to sustained hypoxia are discussed below.

Chronic Obstructive Pulmonary Disease

An estimated 6.9 billion people will inhabit the earth this year. Of the estimated 57 million annual deaths, 17 million will perish from cardiovascular disease ("COPD: World Mortality/ Morbidity Rates", Central Intelligence Agency Factbook, 2009). Worldwide, heart disease and stroke account for close to 13 million of the deaths from cardiovascular disease and remain the top 2 causes of death globally. Another emerging disease is COPD, which is currently the fourth leading cause of death worldwide ("COPD Facts" World Health Organization, 2004) and the third leading cause of death in the United States (Minino A.M. *et al.*, 2008). The World Health Organization reported in

2004 over 64 million people worldwide had been diagnosed and were living with COPD; of those 64 million over 3 million lost their lives ("COPD Facts" World Health Organization, 2004). The number of people with moderate to severe COPD continues to rise as current numbers indicate well over 80 million people now are diagnosed with the disease ("COPD Facts" World Health Organization, 2004). It is projected that by the year 2020 COPD will be the third leading cause of death in the world (Rabe K.F. *et al.*, 2007). This largely preventable disease continues to plague the some of the world's poorest countries as close to 90% of COPD deaths occur in low to middle income nations ("COPD: World Mortality/ Morbidity Rates", Central Intelligence Agency Factbook, 2009). Cigarette smoking and occupational and environmental exposure to toxic airborne pollutants include the main risk factors for developing COPD, with cigarette smoking as the leading risk factor.

It is interesting that relatively few risk factors connect the world's largest killers: the "chronic diseases" COPD, heart disease, and stroke. Smoking remains the number one risk factor for all three with dietary and lifestyle choices also aiding the development and progression of these diseases. Even though cigarette smoking rates have dropped in developed nations the past decade the projected number of deaths due to tobacco use continues to rise with an estimated 3 million deaths annually by 2025. In developing countries the use of tobacco and smoking is rising and an estimated 7 million deaths annually by 2025 is projected ("COPD Facts" World Health Organization, 2004). Epidemiologic evidence linking COPD to cardiovascular morbidity and mortality continues to increase. Patients with COPD have a two to three fold increase in the risk of cardiovascular events including death (Sin D.D. & Man S.F., 2005). Interestingly, COPD

is the only major chronic disease with an increasing death rate in sharp contrast to the recent dramatic decline in deaths from heart disease, stroke, and other cardiovascular disorders (Mannino D.M., 2003).

An Inflammatory Disease:

COPD is characterized by chronic airflow limitation that is associated with abnormal inflammatory responses of the lung (Rabe K.F. *et al.*, 2007). Patients may exhibit airway inflammation and mucus plugging as found in chronic bronchitis; or have remodeling and/or parenchymal destruction with losses of the elastic properties of the lung as found in emphysema. The severe inflammation and remodeling lead to airflow limitation and tissue specific adaptations to the decreased tissue oxygenation (Rabe K.F. *et al.*, 2007) and increased tissue hypercapnia. Airway inflammation develops in the early stages of COPD; inhaled irritants trigger abnormal inflammatory responses leading to recruitment and infiltration of inflammatory cells to the lung, resulting in airway inflammation and thickening (Devor & Crawford, 1984;Redline *et al.*, 1989). The recruitment of inflammatory cells and factors contributes to mucociliary dysfunction plugging conducting airways (Sethi S & Murphy T.F., 2001;Kardos P & Keenan J., 2006). Structural changes usually occur as a result of the chronic inflammation and include airway wall fibrosis, increases in bronchiolar smooth muscle, epithelial metaplasia, and destruction of lung parenchyma, and remodeling of the pulmonary vasculature (Jeffery P.K., 2001;Kardos P & Keenan J., 2006).

Secondary Pulmonary Arterial Hypertension (PAH):

The normally low resistance, low pressure pulmonary vasculature develops heightened tone and vasoconstriction resulting in increased pulmonary vascular resistance and pulmonary arterial pressure following long term exposure to hypoxia. The increased pulmonary vascular resistance in COPD patients can additionally lead to the development of secondary pulmonary arterial hypertension (PAH) (Burrows B *et al.*, 1972;Cermona G & Higenbottam, 2002). PAH is characterized by pulmonary arterial obstruction by vascular proliferation and remodeling, endothelial dysfunction, and an imbalance of vasoconstrictors to vasodilators promoting increased vascular tone and vasoconstriction. The hypothesized mechanisms behind the development of higher pulmonary arterial pressures will be presented in more detail below in "Cardiovascular Responses to Chronic Hypoxia", as chronic hypoxemia is the driving force behind the development of these mal-adaptations in a variety of lung diseases (Humbert *et al.*, 2004) or long term residence at high altitude, and are therefore not unique to secondary PAH.

Extrapulmonary Manifestations of COPD:

COPD extrapulmonary manifestations (systemic effects) stem from dyspnea and increased whole body inflammation. The increased inflammation, oxidative stress, and hypoxia contribute to the development of PAH, cor pulmonale, edema formation, muscle wasting, muscle fatigue, severe weight loss and malnutrition (Smith, 2009). Inflammatory mediators, such as TNF-α and interleukin-1β may aide in altering leptin metabolism, leading to a hypermetabolic state in patients with severe COPD (Smith, 2009). Muscle wasting and muscle fatigue are common in COPD patients (Eisner M.D *et al.*, 2008). Additionally, endothelial dysfunction from the excessive systemic inflammation places

patients at a greater risk for developing co-morbidities (Eisner M.D *et al.*, 2008) (Smith, 2009).

Cor pulmonale:

Increased pulmonary arterial pressure in PAH leads to the development of cor pulmonale (Lee-Chiong T.L. & Matthay R.A, 2002). Cor pulmonale is characterized by hypertrophy and dilation of the right ventricle and can range clinically from mild changes in right ventricle function to right heart failure (Lee-Chiong T.L. & Matthay R.A, 2002) (de Leeuw P.W. & Dess A, 2003). Cardiac output is initially maintained at normal in cor pulmonale, suggesting the disease is not just a cardiac disorder. It is postulated that volume overload stimulated from underfilling of the arterial system secondary to a fall in total peripheral resistance results in the development of pressure-induced hypertrophy of the right ventricle and eventual failure of the right heart (de Leeuw P.W. & Dess A, 2003). Patients with severe edema formation and cor pulmonale indeed exhibit normal cardiac outputs with low systemic blood pressure and vascular resistance (de Leeuw P.W. & Dess A, 2003;Anand I.S. *et al.*, 1992).

Systemic Edema:

Fluid accumulation and swelling in the legs and feet is common in patients with severe COPD and PAH. Edema formation can develop as stated above in cor pulmonale, however recent evidence indicates the development of cor pulmonale is not the only factor leading to the development of systemic edema in COPD. Hypoxemia can impair renal function by promoting renal arteriolar vasoconstriction and causing fluid to shift from intravascular to extravascular spaces. In addition to hypoxia, patient retention of CO_2 also provides a potent stimulus for edema formation with increased bicarbonate

reabsorption by the kidneys (Lee-Chiong T.L. & Matthay R.A, 2002;MacNee W, 1994). Atrial natriuretic peptide (ANP) levels may also contribute to COPD patient edema development. ANP, a 28-amino acid peptide is released in response to atrial stretch and sympathetic stimulation to decrease sodium reabsorption, blood volume, and blood pressure. A failure of ANP release is believed to contribute to salt and water retention and edema in patients with COPD/PAH cor pulmonale (Winter *et al.*, 1989). Normal and slightly elevated plasma levels of ANP have been recorded in patients (Lee-Chiong T.L. & Matthay R.A, 2002;Lee-Chiong T.L. & Matthay R.A, 2002;Winter *et al.*, 1989), suggesting that ANP insufficiency may contribute to the progression of edema. The increased pooling of blood in the veins of the lower extremeties and fluid leak from the increased vascular pressure results in patient discomfort and contributes to patient exercise intolerance.

Skeletal Muscle Function:

Systemic inflammation and hypoxia contribute to the development of skeletal muscle dysfunction in COPD patients. Systemic inflammation and increased reactive oxygen species has been demonstrated to lead to increased muscle protein damage and skeletal muscle catabolism (American Thoracic Society and European Respiratory Society, 1999). Chronic hypoxia, itself has an adverse effect on skeletal muscle by limiting muscle energy utilization and ATP production (American Thoracic Society and European Respiratory Society, 1999). In the exercising muscle, oxidative muscle metabolism is abnormal in patients with COPD. Working muscles depleted energy with higher free phosphates to phosphorylated creatine phosphate ratios (Payen *et al.*, 1993;Kutsuzawa *et al.*, 1995;Wuyam *et al.*, 1992) while oxidative phosphorylation was

found to be reduced in COPD patients (Mannix *et al.*, 1995) (American Thoracic Society and European Respiratory Society, 1999). Thus, with decreased ability for energy production and increased damage the health of and function of skeletal muscle in COPD patients is limited.

The debilitating disease is not strictly a pulmonary disorder, as more systemic effects of the disease are being recognized. For those who have known or currently are diagnosed with COPD, it is often the non pulmonary effects that are the most destructive to the patient's quality of life. Even with supplemental oxygen to aid the hypoxemia, patient mobility and ability to conduct everyday tasks are limited. Therefore, it is of vital importance to understand the underlying mechanisms in the development and progression of COPD as the disease climbs its way to being the world's third greatest killer by 2020. Current investigations utilizing animal and cellular models of COPD use a variety of stimuli to induce tissue/cellular adaptations similar to those seen in humans with COPD. One such model is long term exposure to chronic hypoxia. Many of the cardiovascular derangements found in COPD are observed in response to the chronic hypoxemia and are further discussed below.

Cardiovascular Responses to Hypoxia:

Acute Hypoxia:

Acute exposure to hypoxia results in increased heart rate, cardiac output, ventilation, catecholamine secretion, and blood pressure ("Hypoxia, polycythemia and cyanosis" Harrison's Principles of Internal Medicine. Braunwald *et. al.,* 1998). Stimulation of peripheral arterial chemoreceptors from the decreased P_{aO2} leads to depolarization and

release of catecholamines from type I glomus cells. Afferent fibers of the glossopharyngeal and vagus nerve are activated and the action potential is propagated to cardiorespiratory centers of the brain, which relay action potentials to efferent fibers of the vagus nerve decreasing parasympathetic stimulation of the heart leading to increased heart rate and sympathetic stimulation of the heart increasing stroke volume and heart rate to increase cardiac output ("Hypoxia, polycythemia and cyanosis" Harrison's Principles of Internal Medicine. Braunwald *et. al.,* 1998). Decreases in PO_2 are also sensed within the vasculature, as acute hypoxia causes hypoxic pulmonary vasoconstriction and systemic vasodilation.

Systemic Vasodilation:

In the systemic vasculature, exposure to acute hypoxia results in vasodilation. The innate response of the systemic vasculature to dilate to decreased PO_2 is important in maintaining constant blood flow and thus nutrient and oxygen delivery to delicate tissues. This especially is important in the cerebral circulation which consumes close to 20% of resting total-body oxygen and receives 14% of the body's cardiac output (Kandel *et al.,* 2000). Hypoxia-induced relaxation may occur directly from cellular sensing of the reduced PO_2 and/or from increased production of vasodilator metabolites from systemic tissues. Reductions in ATP production and increases in adenosine, lactic acid, and circulating potassium and hydrogen ions can increase vasodilation (Furchgot, R.F., 1966;Waypa & Schumacker, 2010;Taggart & Wray, 1998). The role of the endothelium in this response is varied, as many groups have demonstrated vessel dilation and smooth muscle relaxation in the absence of the endothelium (Smani *et al.*, 2002;Quayle *et al.*, 2006;Pinder *et al.*, 2009;Jackson, 2000). However in coronary arterioles hypoxic dilation

appears dependent on the endothelium (Lynch *et al.*, 2006;Jimenez *et al.*, 1996a;Liu & Flavahan, 1997) and endothelial production of nitric oxide (NO) (Jimenez *et al.*, 1996;Parent *et al.*, 1992;Liu & Flavahan, 1997;Lynch *et al.*, 2006). Direct responses to hypoxia within vascular smooth muscle may include the activation of K^+ channels (Quayle *et al.*, 2006) or result in decreased intracellular calcium and contraction through: 1. inhibition of calcium entry, 2. inhibition of intracellular calcium release, 3. accelerated uptake by the Sarco(endo)plasmic reticulum calcium-ATPase (SERCA) pump (Gupte & Wolin, 2006) (Waypa & Schumacker, 2010). The mechanisms responsible for decreased intracellular calcium following acute hypoxic exposure are still currently under investigation. Current hypotheses support a role for increased cellular reactive oxygen species (ROS), as it has been demonstrated that decreasing ROS and/or increasing the reduced status of smooth muscle cells attenuated hypoxic vasodilation (Waypa & Schumacker, 2010;Gupte & Wolin, 2006).

Hypoxic Pulmonary Vasoconstriction Response:

In contrast to vasodilation to hypoxia found in systemic arteries, the pulmonary circulation constricts in response to this stimulus. This unique response in the pulmonary circulation is biphasic with an acute phase followed by a slow sustained constriction. The acute phase occurs within seconds, does not require the endothelium (Zhang *et al.*, 1997;Waypa & Schumacker, 2010), is associated with increased release of intracellular calcium stores, closure of K_V channels, and an opening of voltage dependent calcium channels and non-selective cation channels (Archer *et al.*, 1998;Morio & McMurtry, 2002;Lu *et al.*, 2009;Waypa & Schumacker, 2010). The sustained or chronic phase

results in activation of myosin light chain kinase increasing actin-myosin cross-bridging and contraction (Waypa & Schumacker, 2010). Thus, acute hypoxia results in constriction of pulmonary arteries in areas of poor ventilation. Constriction of these arteries diverts blood flow to better ventilated areas, thereby matching ventilation to perfusion enabling more efficient oxygenation. Return to normoxic conditions rapidly reverses the cardiorespiratory changes to acute hypoxia. However in long term exposure to hypoxia, restoration to normoxic conditions does not readily reverse chronic adaptations of the cardiovascular system as discussed further below.

Chronic Hypoxia:

Long term exposure to hypoxia leads to chronic hyperventilation and compensatory metabolic acidosis produced by increased renal bicarbonate loss (Wyatt F, 2011). The sustained tissue hypoxia additionally increases red blood cell production, hemoglobin, and 2,3-bisphosphoglyceric acid production to increase the body's oxygen carrying capacity and thereby increase O_2 delivery to the peripheral tissues. The increase in red blood cells and decrease in plasma volume with chronic hypoxia lead to the development of polycythemia. Cardiac output, the product of stroke volume and heart rate, is normal at resting conditions as heart rate normalizes after long term exposure to hypoxia and only a slight decrease in stroke volume is observed in humans (Wyatt F, 2011). Additionally, hypoxic pulmonary vasoconstriction persists resulting in the development of increased pulmonary arterial pressure (Morrell *et al.*, 2009). This increase in pulmonary arterial resistance is associated with an increase in pulmonary arterial pressure.

Increases in Pulmonary Vascular Resistance:

Chronic hypoxia causes persistent hypoxic pulmonary vasoconstriction and an increase in the muscularization of pulmonary arteries resulting in the development of increased pulmonary arterial pressure and the development of pulmonary arterial hypertension (PAH) (Morrell *et al.*, 2009;Tuder *et al.*, 2009). The increased muscularization of the pulmonary arteries and the persistent vasoconstriction reduce arterial lumen diameter, resulting in an increase in vascular resistance as defined by Poiseuille's Law, which describes laminar flow of an incompressible uniform viscous liquid (Newtonian fluid) through cylindrical tubes. Through the assumption of laminar blood flow through cylindrical blood vessels we can estimate vascular resistance through the following equation:

$$\mathbf{R = 8\eta l / \pi r^4}$$

Where resistance R = is proportional to changes in viscosity (η) and length (l), and inversely proportional to changes in vessel radius (r). This estimate of vascular resistance illustrates the importance of blood viscosity, and especially vessel radius (diameter changes) on vascular resistance. Thus, increased pulmonary resistance following chronic hypoxia can be attributed to changes in arteriole diameter through enhanced vasoconstrictive properties or vascular remodeling of pulmonary arteries; and from increased viscosity of the blood through polycythemia.

Enhanced Vasoconstriction:

Chronic hypoxia has been demonstrated to increase vasoconstrictor reactivity of pulmonary arteries. The mechanisms responsible for the enhanced constrictor properties include increased smooth muscle sensitivity to calcium through RhoA signaling (Barman S.A., 2007;Hyvelin *et al.*, 2005;Jernigan *et al.*, 2004), increased smooth muscle calcium through cellular depolarization (Sweeney & Yuan, 2000;Naik *et al.*, 2005), and decreases in endothelial products that promote vasodilation (Fike *et al.*, 1998;Giaid & Saleh, 1995;Girgis *et al.*, 2005).

The small GTPase RhoA and its downstream effector Rho kinase (ROK) play an important role in the regulation of the contractile state of vascular smooth muscle by regulating phosphorylation of the myosin light chain by inhibiting myosin light chain phosphatase activity (Jernigan & Resta, 2004). Filament cross-bridge cycling and smooth muscle contraction are dependent upon the balance of the calcium dependent myosin light chain kinase and the calcium independent myosin light chain phosphatase. Thus, increased stimulation of RhoA results in enhanced calcium sensitivity of the contractile apparatus and inhibition of its downstream effector ROK would decrease calcium sensitivity. Following chronic hypoxia in models of PAH RhoA/ROK signaling is upregulated (Barman S.A., 2007;Hyvelin *et al.*, 2005;Jernigan & Resta, 2002) and inhibition of ROK acutely reverses the effects of chronic hypoxia induced PAH (Hyvelin *et al.*, 2001;Nagaoka *et al.*, 2004;Oka *et al.*, 2007), demonstrating that ROK-mediated vasoconstriction contributes greatly to increased pulmonary vascular resistance.

In addition to increased calcium sensitivity of the contractile apparatus, chronic hypoxia results in smooth muscle cell depolarization from decreased expression of

several isoforms of K_V channels (Platoshyn *et al.*, 2001). Smooth muscle depolarization following chronic hypoxia has been observed in cultured smooth muscle cells and small pulmonary arteries (Sweeney & Yuan, 2000;Naik *et al.*, 2005), in which the decreased expression of K_V channels increases calcium entry into smooth muscle cells from voltage dependent calcium channels and enhances constriction. The degree of tone or vasoconstriction is highly dependent upon smooth muscle membrane potential and intracellular calcium, however smooth muscle contraction and thus arteriole constriction can be influenced by the vascular endothelium. The vascular endothelium produces many important vasodilators that modulate vascular tone. Therefore, endothelial dysfunction and a loss in production of vasodilatory substances can also lead to an increase in vasoconstriction and vascular tone.

Pulmonary endothelial function following chronic hypoxia creates an improper balance between endothelium-derived vasoconstrictive and vasodilatory substances. For example, there is decreased synthesis of the potent vasodilators and anti-mitogens, NO and prostacyclin (Moro et al., 2008). Furthermore, hypoxia enhances endothelial synthesis and release of the endothelin-1 (ET-1) and thromboxane A_2 (TXA_2), two vasoconstrictive factors that promote vasoconstriction and VSM proliferation (Moro et al., 2008), leading to an imbalance of factors that influence vasoconstriction. To summarize, there are many contributing factors leading to the enhanced constrictor profile of pulmonary smooth muscle following prolonged exposure to hypoxia; all leading to the development of increased pulmonary vascular resistance and increased pulmonary arterial pressure.

Vascular Remodeling:

The loss of anti-proliferative factors such as NO and increased production of mitogenic factors such as ET-1 in chronic hypoxia induce vascular remodeling. ET-1 stimulation of ET_A receptors on the smooth muscle increases intracellular calcium and can activate calcium-sensitive transcription factors (Moro *et al.,* 2008). Decreased K_V channel expression also promotes cellular proliferation by increasing cytosolic calcium. A rapid increase in nuclear calcium occurs with the rise in cytosolic calcium; this increase in nuclear calcium accelerates cell proliferation by modulating the cell cycle (Park W.S. *et al.*, 2010). The decreased potassium efflux out of K_V channels also permits increased activity of caspases and nucleases, further facilitating vascular remodeling (Mandegar M & Yuan, 2011;Park W.S. *et al.*, 2010) and smooth muscle proliferation. There is also evidence for endothelial proliferation in the lumen of pulmonary arteries in patients with mild COPD (Barbera J.A. *et al.*, 1994). Together, enhanced vasoconstrictive properties and hyperproliferation of vascular smooth muscle and endothelial cells, promote increased vascular pressure in chronically hypoxemic patients through decreasing the arterial lumen and increasing vascular resistance. In addition to the effects of vascular remodeling and vasoconstriction, chronic hypoxia induced polycythemia may also promote increased vascular resistance.

Polycythemia:

Polycythemia is an increase in systemic hematocrit (Hct) above normal values. Congenital erythrocytoses from instrinsic genetic defects within bone marrow produce primary polycythemia (Messinezy M & Pearson TC, 1999). Secondary polycythemia has

two main etiologies; 1) acquired erythrocytoses associated with renal hypoxia, which is the consequence of low blood oxygen saturation, which triggers the production and release of ertythropoietin from the kidneys (Messinezy M & Pearson TC, 1999) and 2) myeloid cancers resulting in unexpected erythropoietin production. Patients with lung diseases, such as COPD and PAH, and in individuals who have resided at high altitude for extended periods may develop secondary polycythemia (Braunwald E *et al.*, 1998;Ersley AJ, 1995;Saldivar *et al.*, 2003). The increased Hct is believed to be an adaptation to increase the oxygen-carrying capacity. However the increased Hct may additionally have deleterious effects on hypoxic patients by increasing vascular resistance by increasing blood viscosity. Interestingly, in patients with myeloproliferative diseases the incidence of pulmonary hypertension appears to be common (Gupta R *et al.*, 2006), suggesting increased Hct and blood viscosity may be significant contributors to the development of increased vascular resistance and the development of PAH. However, exogenous application of erythropoietin and the development of polycythaemia in rats did not result in the development of PAH (Petit R.D. *et al.*, 1993; Walker *et al.,* 2000). In mouse and rat models of PAH the role of erythropoietin appears to be important in protecting the pulmonary vasculature from pulmonary vascular remodeling associated with PAH (Petit R.D. *et al.*, 1993;Satoh K *et al.*, 2006;Weissmann *et al.*, 2005) and development of the disease (Weissmann *et al.*, 2005). Thus the role of erythropoietin and polycythemia in increased vascular resistance in chronically hypoxic patients may result in an increased tendency to develop PAH, with increased Hct and erythryopoietin having beneficial as well as possible deleterious effects on the vasculature.

Systemic Vascular Reactivity in Chronic Hypoxia:

Acute hypoxia results in systemic vasodilation and chronic hypoxia decreases vascular tone. Chronic hypoxia significantly decreases vasoconstrictor responsiveness of the systemic circulation (Auer & Ward, 1998;Toporsian & Ward, 1997;Hu *et al.*, 1996;Doyle & Walker, 1991). For example, acute activation of the cardiopulmonary and arterial baroreceptors normally increases sympathetic responses resulting in increased peripheral vascular resistance and maintenance of blood pressure. Following CH, the effect of unloading cardiopulmonary and arterial baroreceptors fails to increase vascular resistance and the ability to maintain blood pressure is compromised (Wolfel E.E & Levine B.D., 2002). This has been observed systemically as a reduced total peripheral resistance response to vasoconstrictor agonists and in several individual vascular beds (O'Donaughy & Walker, 2000;Jernigan *et al.*, 2001;Auer & Ward, 1998;Caudill *et al.*, 1998) suggesting that it is a generalized response to this stimulus. Interesting, diminished vasoconstrictor reactivity of systemic arteries following CH can be restored by removal of the endothelium (Earley *et al.*, 2002;Naik & Walker, 2003), suggesting endothelial modulation of vascular tone is decreasing arteriolar tone and vasoconstriction.

Endothelial Modulation of Vascular Tone

The Endothelium:

In 1628, William Harvey described the role of blood vessels in the circulatory system and how they functioned in separating circulating blood and tissue. These “blood vessels” were later found to be lined with cells by von Reckingausen in the 1800s. As the

19th century came to an end, the endothelium was recognized as an inert cellophane like membrane that lined the vessels and functioned in maintaining vessel wall permeability. Starling's experiments and his law of capillary exchange in 1896 overshadowed early views by Heidenhahn (1891) which described the endothelium as an active secretory system. It was not until half way through the 20th century and the development of electronmicroscopes that it was recognized that the endothelium was more than a mere physical barrier (Fishman, 1982;Cines *et al.*, 1998). The vascular endothelium, is critical for survival as is modulates vascular permeability, maintains anticoagulant properties, and contributes to the physiological control of vasoregulation (Davies, 2009). The endothelial surface area in an adult human is composed of approximately 6 trillion cells, weighs 1kg, and covers a surface area of $7m^2$ (Cines *et al.*, 1998). Endothelial cells (ECs) line all vessels in every organ system and regulate the flow of blood, biologically active molecules, and nutrients. Blood flow is regulated in part by the antithrombotic surface of the endothelium, where increases in shear stress and inflammation trigger ECs to create prothrombotic and antifibrinolytic microenvironments which lead to the formation of clots, limiting blood flow. Blood flow is also regulated by endothelial uptake or release of vasoactive substances acting in a paracrine manner to constrict or dilate vessels (Cines *et al.*, 1998).

Adaptation to local environment:

The endothelium adapts to local hemodynamics acutely by release of vasoactive substances modulating local vasoconstriction/vasodilation l responses, and to sustained changes by altering gene and protein expression to promote adaptive structural remodeling of the artery (Davies, 2009). Acute changes in hemodynamics, such as

exercise, increase shear force upon endothelial cells resulting in a local increase in nitric oxide (NO), prostacyclin and cytochrome P450 products to induce vasodilation and increase blood flow to the working tissue. These short-term responses subside after the termination of exercise and hemodynamic conditions return. Long term alterations of hemodynamics may lead to phenotypic changes of the endothelium. For example in a porcine model of atherosclerosis, in areas of disturbed blood flow multidirectional forces in the aorta resulted in increased expression of coagulation factors such as plasminogen, von-Willebrand factor, and t-PA. Additionally, NFκB, interleukin-6, and macrophage chemoattractant proteins were upregulated resulting in a pro-artherosclerotic phenotype of the endothelium (Passerini A.G. *et al.*, 2004).

Flow-dependent Dilation:

Increased local blood flow sensed by the endothelium moderates local vasodilation. This response requires functional endothelium and is termed "flow-induced dilation". Flow-induced (or dependent) dilation was first observed in 1933 (Schretzenmayr A Uber kreislaufregulatorische Vorgange an den groBen Arterien bei der Musckelarbeit,1933). From early studies in isolated arteries and *in vivo* studies, it was demonstrated to be dependent upon activation of the endothelium as physical removal of endothelial cells eliminated dilation to increased flow (Pohl *et al.*, 1986;Holtz *et al.*, 1984;Lie *et al.*, 1970). Endothelial products hypothesized to be responsible for the dilation include NO, PGI_2, eicosatetraeonic acids (EETs), CO, and endothelium-derived hyperpolarizing factors (EDHFs). In addition to release of endothelial-derived relaxation factors from flow, acetylcholine and other intracellular calcium mobilizing agents, such as adenosine triphosphate, all result in endothelium-dependent relaxations and are used as

indicators of endothelial function within the vasculature. Thus, a lack or dampened response to infused acetylcholine as well as flow-dependent dilation would be indicative of impaired endothelial function.

Endothelial-dependent Dilation in COPD and Chronic Hypoxia:

Clinically, flow-induced dilation is often used to assess endothelial health and function. Flow-induced dilation reflects conduit artery function, whereas intrabrachial infusion of endothelium-dependent dilators, such as acetylcholine, reflects endothelial function in resistance arteries (Burghuber & Valipour, 2009;Maclay *et al.*, 2011;Lind *et al.*, 2009). COPD patients have been reported to be chronically vasodilated (Casiglia *et al.*, 1998); the chronic dilatory state may be due to augmented dilatory responses or decreased tone and vascular remodeling. Further investigations have demonstrated that endothelium-dependent dilation as assessed by acetylcholine infusion in systemic resistance arteries from COPD patients is decreased (Maclay *et al.*, 2011) as is flow-induced dilation (Moro *et al.*, 2008;Lind *et al.*, 2009). Increased arterial stiffening and derangements in vascular tone from arterial remodeling in larger conduit arteries may additionally contribute to the chronic vasodilated status of COPD patients (Maclay *et al.*, 2011). Endothelial dysfunction in these patients has been hypothesized to be largely due to exaggerated inflammatory responses and cigarette toxicity, which have been shown independently of COPD to decrease endothelial function (Edirisinge & Rahman, 2010;Heitzer & et.al, 1996).

Long term residence at high altitude has not been associated with a decrease in flow-dependent dilation (Gupta A.K. *et al.*, 1997); (Jansen *et al.*, 2007). Additionally, endothelium dependent dilation to hypoxia is maintained at high altitude (Gupta A.K. *et*

al., 1997;Jansen *et al.*, 2007). In laboratory models there are conflicting accounts on the effects of hypoxemia on endothelium-dependent dilation. Decreased endothelium-dependent dilation in aorta rings of chronically hypoxic rats has been reported (Toporsian & Ward, 1997). In contrast, multiple studies have demonstrated increased endothelium-dependent dilation. In isolated hearts from chronically hypoxic fetal guinea pigs endothelium-dependent dilation was increased (Thompson *et al.*, 2000). In Wistar rats exposed to 24 hours to 7 days of chronic hypoxia NO-dependent dilation or dilator responses to an acute hypoxic challenge were accentuated. The greatest increase in skeletal muscle arterioles endothelium-dependent dilator response occurred during the first 72 hours of hypoxic exposure and was greatly attenuated by the 7^{th} day. Even with the decreases in dilator responsiveness, endothelium-dependent relaxation was still significantly greater in CH treated rats than normoxic controls (Walsh & Marshall, 2006). Thus, depending on the length of exposure to hypoxia, the animal species, and the vascular bed under study, endothelium-dependent dilation may be enhanced following CH.

Endothelial dependent dilation to agonists such as acetylcholine increases endothelial intracellular calcium levels and production of endothelial-derived vasodilators such as NO, PGI_2, CO, and epoxyeicosatrienoic acids (EETs). Each of these factors can directly or indirectly act upon smooth muscle K^+ channels and result in smooth muscle cell hyperpolarization and relaxation. One K^+ channel thought to largely contribute to membrane potential regulation and modulation of vascular tone, the large conductance calcium-activated K^+ channel (BK_{Ca}), can be activated by a large array of endothelial-

derived vasodilators. The roles of endothelial-derived NO, CO, EETs and EDHFs will be further discussed below in the context of BK_{Ca} regulation of vascular tone.

The Role of BK_{Ca} Channels in Vasoregulation

As previously mentioned, vascular smooth muscle (VSM) contractility is dependent upon intracellular Ca^{2+} and membrane potential (E_m). The balance of endothelial vasodilators to vasoconstrictors affects VSM E_m and/or Ca^{2+} and contractility. Many of the vasoactive substances produced by the endothelium work directly or indirectly on the actions of smooth muscle K^+ channels, which are primarily responsible for maintaining membrane potential (E_m) and intracellular Ca^{2+} levels. Since both intracellular Ca^{2+} and membrane voltage are determinants of BK_{Ca} activity, it is no surprise the channel acts as a regulator of E_m in smooth muscle cells. By fine tuning E_m and intracellular concentrations VSM BK_{Ca} channels regulate vascular tone (Tanaka Y *et al.*, ;Nelson & Quayle, 1995;Waldron & Cole, 1999).

VSM E_m:

VSM BK_{Ca} channels are thought to contribute to the maintenance of E_m in small myogenic vessels. The large efflux of K^+ that results from BK_{Ca} activation counteracts pressure or chemical/agonist-induced depolarization and vasoconstriction (Tanaka Y *et al.*, ;Waldron & Cole, 1999;Brayden & Nelson, 1992). VSM BK_{Ca} channels are localized to cellular caveolae. Caveolae are cellular invaginations that contain the structural proteins caveolins and are thought to be important signaling domains (Jaggar *et al.,* 2000). VSM BK_{Ca} channels activated by local increases in intracellular Ca^{2+} from

clusters of ryanodine receptors on the sacroplasmic reticulum (calcium sparks) (Nelson *et al.*, 1995). These calcium sparks increase channel activity and result in an efflux of K^+ that is sufficient to hyperpolarize the sarcolemma by 10-20mV (Jaggar *et al.*, 2000;Nelson *et al.*, 1995). Blockade of BK_{Ca} channels or ryanodine receptors causes membrane depolarization, elevations in smooth muscle intracellular Ca^{2+}, and results in vasoconstriction. In addition to agonists or vascular responses that increase VSM calcium sparks, endogenous endothelial derived vasodilators, such as NO, EETs, and CO additionally increase the activity of VSM BK_{Ca}, while endothelial derived products such as 20-hydroxyeicosatetraenoic acid (20 HETE) decrease BK_{Ca} activity.

Endogenous Products of the Endothelium That Stimulate VSM BK_{Ca}

Endothelial produced gaseo-transmitters such as NO (Bolotina *et al.*, 1994) , CO (Jaggar *et al.*, 2002), and hydrogen-sulfide (H_2S) (Jackson-Weaver *et al.*, 2010) may activate VSM BK_{Ca} channels within the systemic vasculature. The gaseo-transmitters NO and CO may directly act upon the channel to increase activity, Alternatively, indirect actions via the actions of cyclic guanosine-monophosphate (cGMP) and protein kinase G (PKG) or protein kinase A (PKA) phosphorylation of the channel may increase activity (Toro *et al.*, 1998). Additionally, the cytochrome P450 epoxygenase metabolites epoxyeicosatrienoic acids (EETs) activate VSM BK_{Ca} channels and dilate coronary arterioles (Zhang *et al.*, 2001). 11,12 EET increases BK_{Ca} activity in cell-attached and inside-out patches (Zhang *et al.*, 2001). Prostacyclin (PGI_2) is another endothelial derived relaxation factor that can increase BK_{Ca} activity indirectly through cyclic adenosine-monophosphate (cAMP) and PKA/PKG phosphorylation of the channel (Burnette &

White, 2006;Tanaka Y *et al.*, 2004). Channel modification by gaseotransmitters or phosphorylation increases channel open probability through a variety of structural and biochemical modifications that result in enhanced voltage or calcium sensitivity (Hou *et al.*, 2009). CO may act as a calcium surrogate (Hou *et al.*, 2008), and archidonic acid metabolites such as EETs may bind to the channel and alter channel structure to increase opening of the channel or to decrease channel inactivation, deactivation, or removal from the plasma membrane (Hou *et al.*, 2009). In addition to endothelial influence, BK_{Ca} channel activity has also been found to be sensitive to changes in the cellular environment. Increased cellular stretch and decreased PO_2 may increase and decrease BK_{Ca} activity respectively, and are further discussed below.

VSM BK_{Ca} Activation by Stretch; The Myogenic Response:

Stretch of the vascular wall by increases in vascular pressure is sensed by mechanosensitive ion channels that are able to produce intracellular signals in response to mechanical stimuli. BK_{Ca} channels have been demonstrated to be "stretch" sensitive ion channels and the mechanical sensitivity is hypothesized to be located in the channel's alpha subunit stress-axis regulated exon (STREX) a 59 amino acid insert in the C terminus (Naruse *et al.*, 2004;Naruse *et al.*, 2009;Tang Q.Y. *et al.*, 2005). In VSM cells from pulmonary (Kirber *et al.*, 2011), mesenteric (Dopico *et al.*, 1994), and coronary arteries (Wu & Davis, 2001) mechanical stretch increases BK_{Ca} channel opening. Activation of the channels is hypothesized to act as a "brake", thereby fine tuning stretch-induced depolarization of multiple stretch sensitive cation channels and thus limiting the magnitude of vasoconstriction (Wu & Davis, 2001;Hill *et al.*, 2010). Evidence also exists

for indirect activation of VSM BK_{Ca} channels from cell membrane extracellular matrix proteins, such as α5β1 integrin and fibronectin which activate the channels through a c-Src dependent mechanism (Hill *et al.*, 2010;Martinez-Lemus *et al.*, 2005;Wu *et al.*, 2008;Yang & et.al, 2009). Thus, voltage, Ca^{2+} and cellular stretch can activate BK_{Ca} channels to oppose vasoconstriction and promote vasodilation in arterioles.

BK_{Ca} as Oxygen Sensor:

BK_{Ca} channels have been demonstrated to be oxygen sensitive and act as oxygen sensors in carotid body glomus cells (Williams *et al.*, 2004) , pulmonary smooth muscle cells, chromaffin cells, central neurons (Haddad & Liu, 2000) and alveolar epithelial cells (Jovnovic *et al.*, 2003). Severe hypoxia has been shown to inhibit channel activity in a membrane-delimited manner in HEK-293 cells (Lewis *et al.*, 2002), whereas in neurons cytosolic factors are required for hypoxia to inhibit channel activity (Liu *et al.*, 1999). Current investigation into the mechanism responsible for oxygen sensing by BK_{Ca} channels has uncovered a role for heme and heme oxygenase (HO). BK_{Ca} channels are hemoproteins, and upon binding of heme are potentially inhibited (Yi *et al.,* 2010). In conditions in which free heme is degraded by HO, the channel remains active and can be stimulated by the HO product CO (Yi *et al.*, 2010). Recent co-immunoprecipitation experiments in HEK293 (Williams *et al.*, 2004) and Glomus type I cells have found BK_{Ca} and HO-2 can form "oxygen sensitive" complexes. Interestingly, knockdown of HO-2 significantly decreases channel activity (Williams *et al.*, 2004). The authors hypothesized that hypoxia inhibits HO production of CO and thus limits channel activity, suggesting that HO-2 was the "oxygen sensor" and CO levels regulated channel activity. However,

experiments that characterized the heme binding domain (HBD) of BK_{Ca} channels found that decreased oxygen and a reduced state of the HBD significantly increase the channels' affinity for heme and result in potent inhibition of the channel (Yi *et al.*, 2010). In contrast, in normoxic conditions channel affinity appears much higher for CO than heme and permits channel activity (Yi *et al.*, 2010). Thus, HO function and heme/CO binding appear to be very important regulators of BK_{Ca} activity and associate with the channel to form "oxygen sensitive" complexes. Whether these "oxygen sensitive" complexes exist in the vasculature is under current debate.

HO dependent hyperpolarization following CH: HO has two predominate isoforms found within the vasculature, HO-1 and HO-2. Following CH, whole artery HO-1 expression is significantly increased with no change in HO-2 (Jernigan *et al.,* 2001). Additionally, HO activity is significantly increased following CH (Jernigan *et al.,* 2001). Previously, we identified HO and BK_{Ca} channel dependent regulation of smooth muscle cell membrane potential and intracellular calcium concentration following CH in the rat (Earley *et al.*, 2002;Naik & Walker, 2003). Interestingly, the HO and BK_{Ca} dependent hyperpolarization is dependent upon the presence of the endothelium and is independent of ryanodine receptor stimulation (Earley *et al.*, 2002;Naik & Walker, 2003) suggesting the hyperpolarizing influence of BK_{Ca} activity may be localized to the endothelium.

Evidence for the Physiological Role of Endothelial BK_{Ca} Channels

BK_{Ca} channels are ubiquitously expressed throughout the body, and function in modulating E_m, intracellular Ca^{2+}, and intracellular K^+ to control neuronal excitability,

vascular tone, neurotransmitter release, cell volume regulation, and fluid secretion. Their presence in the endothelium has been recognized (Dong *et al.*, 2007;Wang *et al.*, 2005;Wrzosek, 2009;Sandow & Grayson, 2009). From cultured cell studies, endothelial BK_{Ca} channels were demonstrated to be inhibited by the scaffolding domain of caveolin-1 (Wang *et al.*, 2005) and localized to EC caveolae. Unitary conductance from EC BK_{Ca} channels are similar to reported values of BK_{Ca} in other cell types (Sandow & Grayson, 2009;Rusko *et al.*, 1992;Ling & O'Neill, 1992) and unlike VSM cellss, beta subunits do not appear to play a role in the channels calcium sensitivity and the channels are activated at more positive potentials (Eichhorn & Dobrev, 2007;Nimigean & Magleby, 1999;McManus *et al.*, 2011).

Defining the Physiological Role for Endothelial BK_{Ca}:

Similar to their functions in VSM cells BK_{Ca} activity has been hypothesized to regulate endothelial E_m; however recent studies conducted by Ledoux et. al suggest small and intermediate conductance calcium-activated potassium channels have a predominant role regulating endothelial E_m with no evidence for endothelial BK_{Ca} channels (Ledoux *et al.*, 2008). Defining the role for endothelial BK_{Ca} channels may prove to be a difficult task, as expression and activity vary widely throughout the vasculature and may additionally be altered by disease (Jovnovic *et al.*, 2003;Wrzosek, 2009;Sandow & Grayson, 2009). For example, in normoxic conditions, we have not found a role for endothelial BK_{Ca} activity. However, following CH exposure BK_{Ca} activity emerges and is the driving force behind vascular wall hyperpolarization following CH (Earley *et al.*, 2002;Naik & Walker, 2003). Thus, endothelial BK_{Ca} channels may contribute to

endothelial $E_m$following CH. In addition to contributing to endothelial E_m, endothelial BK_{Ca} channels have also been hypothesized contribute to flow-dependent dilation.

Flow Dependent Dilation

A role for endothelial BK_{Ca} channels in flow-dependent dilation was first characterized in rat skeletal muscle arterioles and mesenteric arterioles, where luminal administration of the BK_{Ca} selective inhibitor iberiotoxin (IBTX) eliminated dilation to flow (Sun *et al.*, 2001). Abluminal administration of IBTX was unable to block flow-induced dilation, demonstrating endothelial BK_{Ca} channels and not VSM BK_{Ca} were likely responsible for flow-induced dilation. Increases in shear stress from increases in hematocrit could potentially increase the role of these channels following disease, demonstrating an important physiological role for endothelial BK_{Ca} channels in signal transduction and dilation in health and disease. Thus endothelial BK_{Ca} channels are activated by calcium, voltage, and "mechanical stress" from shear stress of the vascular endothelium, but do they also function as an oxygen sensor?

Endothelial BK_{Ca} in Regulation of Vasoconstrictor Reactivity Following CH

With recent evidence indicating HO-BK_{Ca} complexes may function to regulate membrane potential and act as "oxygen sensors", the search to discover if HO-BK_{Ca} signaling complexes exist in the vasculature began. In pulmonary arterial smooth muscle cells, a weak association between HO and BK_{Ca} was found (Roth *et al.*, 2009). Studies of HO and BK_{Ca} complexes in the vascular endothelium have not been conducted. As previously mentioned, CH exposure results in persistent diminished vasoconstrictor reactivity that is dependent upon an intact endothelium, HO activity, and BK_{Ca}

hyperpolarization of the vascular wall (Earley *et al.*, 2002;Naik & Walker, 2003). Studies further delineating the pathway responsible for BK_{Ca} dependent hyperpolarization found that endogenous HO dependent BK_{Ca} activity occurred independently of ryanodine receptors, which are required for VSM BK_{Ca} activation, suggesting that BK_{Ca} channels responsible for the HO-dependent hyperpolarization may be localized to the endothelium. Therefore we focused our research to investigate a role for the endothelium in the HO-BK_{Ca} dependent loss of vasoconstrictor reactivity following CH. Endothelial BK_{Ca} channel activity is hypothesized to be negatively regulated by caveolin-1 (Cav-1) through its scaffolding domain. The activity of HO is additionally regulated by the scaffolding domain of Cav-1 (Kim *et al.*, 2004). Thus, decreases in Cav-1 function and/or expression following CH may enable increases in both channel and enzyme activity. Indeed, in pulmonary hypertension, Cav-1 expression in the pulmonary vasculature decreases (Achcar *et al.*, 2006;Cohen *et al.*, 2004;Jasmin *et al.*, 2004), thus it is possible derangements in Cav-1 may occur within the systemic circulation as well.

In the current study, we examined the role of Cav-1 and HO dependent activation of EC BK_{Ca} in freshly dispersed endothelial cells and gracilis arterioles from normoxic control and CH rats. We conducted isolated arteriole, electrophysiology, and fluorescence localization studies to test the hypothesis that ***CH exposure results in a loss in Cav-1 mediated inhibition of EC BK_{Ca} channels enabling increased channel activity by the endogenous activator CO resulting in endothelial hyperpolarization and a loss in vasoconstrictor reactivity***.

Proposed Mechanism for Diminished Vasoconstrictor Reactivity Following CH

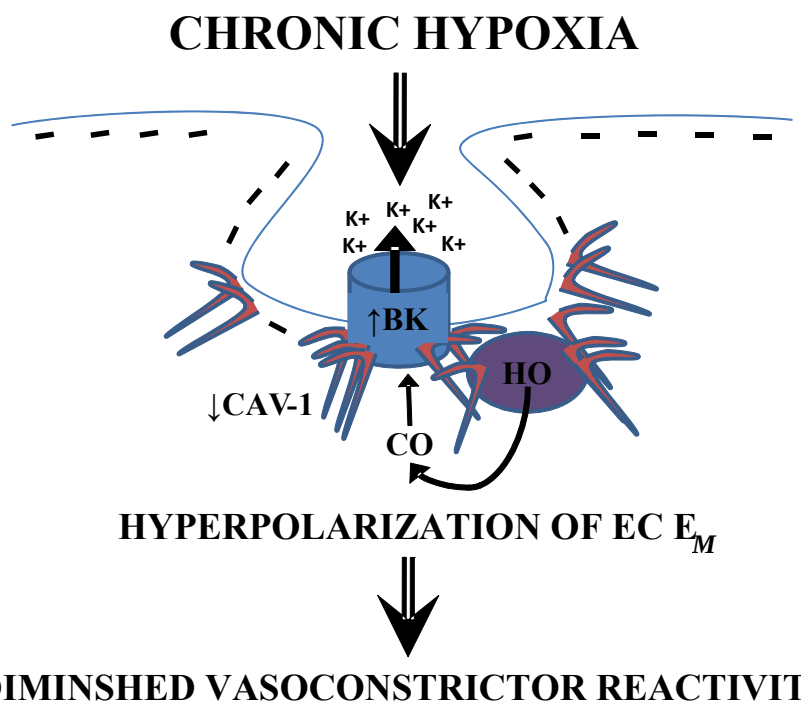

Figure 1: Schematic diagram representing the hypothesis that CH exposure results in a loss of caveolin-1 mediated inhibition of EC BK_{Ca} channels enabling increased channel activity by the endogenous activator CO resulting in endothelial hyperpolarization and a loss of vasoconstrictor reactivity.

Rationale and Specific Aims:

The systemic circulation following chronic exposure to hypoxia has a sustained decrease in vasoconstrictor reactivity that is dependent upon the presence of the endothelium (Doyle & Walker, 1991;Earley & Walker, 2002;Gonzales & Walker, 2002). Blockade of heme oxygenase or BK_{Ca} channels restores vascular reactivity of arteries from CH treated rats through restoration of vascular wall E_m (Earley *et al.*, 2002;Naik & Walker, 2003;Naik *et al.*, 2003;Naik & Walker, 2006). Moreover, recent studies suggest

that BK_{Ca} channels and HO in carotid body glomus cells function as regulators of membrane potential and as "oxygen-sensors" (Williams *et al.*, 2004). HO-BK_{Ca} regulation of cellular membrane potential and "oxygen sensing" in the vasculature are currently being explored, however their presence and function in the vascular endothelium have not been defined.

HO and BK_{Ca} channels are inhibited by the scaffolding domain of Cav-1. Derangements in Cav-1 localization, expression, and function and therefore likely mechanisms to influence the activity of BK_{Ca}. Interestingly, the role of endothelial BK_{Ca} channels has not been defined and is largely unknown. Their presence in normoxic conditions has been questioned, and channel activity may only emerge in the hypoxic setting. Therefore we studied gracilis resistance arterioles and freshly dispersed endothelial cells (ECs) from the aorta of control and CH rats to examine the role of endothelial BK_{Ca} in both conditions. Interestingly, initial studies demonstrated no role for endothelial BK_{Ca} activity in controls, but immunofluorescence localization studies provided evidence for the presence of the channel in both CH and control conditions. Because endothelial BK_{Ca} channels are inhibited by Cav-1, we additionally studied the effects of altering Cav-1 function through manipulation of cellular cholesterol and the addition of cell permeant peptides of the Cav-1 scaffolding domain in ECs from controls and CH animals to determine the role of Cav-1 in regulating BK_{Ca} activity following CH.

<u>Aim 1</u>: Determine the expression and activity of endothelial BK_{Ca} following CH

Hypothesis: Loss of vasoconstrictor reactivity following CH exposure results from increased endothelial BK_{Ca} channel activity and resultant membrane hyperpolarization.

Rationale: BK_{Ca} channels are likely involved in diminished vasoconstrictor reactivity following CH, however prior experiments show that ryanodine-sensitive stores are not involved in their regulation, suggesting VSM BK_{Ca} channels are not responsible for the diminished vasoconstrictor response. This observation lead us to believe the BK_{Ca} channels involved in the diminished vasoconstriction were localized to the endothelium. EC BK_{Ca} channels have been not been previously characterized in native tissues, therefore, experiments in this aim will isolate and characterize endothelial BK_{Ca} channels.

<u>OBJECTIVES</u>

1. Investigate EC BK_{Ca} activity following CH through whole cell patch clamp
2. Characterize EC BK_{Ca} channels through single channel records
3. Localize EC BK_{Ca} channel subunits through immunofluorescence
4. Characterize EC BK_{Ca} channels in cultured rat aortic endothelial cells (RAECs)

<u>Specific Aim 1.1</u> – Investigate EC BK_{Ca} activity following CH through whole cell patch clamp.

The presence and activity of endothelial BK_{Ca} channels is controversial.*The present study examined transmembrane currents using whole cell patch clamp to identify and characterize BK_{Ca} activity in freshly dispersed endothelial cells.* These experiments measured and characterized outward K^+ currents in freshly dispersed endothelial cells

from control and CH exposed rats and assessed current sensitivity to BK_{Ca} specific channel inhibitors and activators.

<u>Specific Aim 1.2</u> – Characterize endothelial BK_{Ca} channels through single channel records.

BK_{Ca} channels are classically identified by their unitary conductance, voltage and calcium sensitivity, and inhibition by the channel specific inhibitor, iberiotoxin (IBTX); thus we designed experiments to *determine the unitary conductance, voltage and calcium sensitivity of endothelial BK_{Ca}.* Endothelial BK_{Ca} unitary conductance was determined and compared to VSM BK_{Ca} unitary conductance to verify channel identity in whole cell-cell attached and inside-out patch clamp single channel recordings. Calcium and voltage sensitivity of endothelial BK_{Ca} were performed in inside-out patches and contrasted to VSM BK_{Ca} calcium and voltage sensitivity to determine if endothelial BK_{Ca} exhibited unique channel characteristics. Channel inhibition by IBTX was additionally verified.

<u>Specific Aim 1.3</u> – Endothelial BK_{Ca} channel subunit localization through immunofluorescent localization.

Determining the presence of EC BK_{Ca} channels has proven to be difficult as many previous studies have identified protein and/ or mRNA of the channel (Sandow & Grayson, 2010; Gauthier, K.M. *et al.,* 2002), *we designed these experiments to investigate BK_{Ca} subunit immunofluorescence in intact arteries and isolated endothelial cells.* Endothelial expression of BK_{Ca} α and β1 subunits was confirmed in resistance artery sections and in freshly isolated aortic endothelial cells using confocal immunoflourescence microscopy.

<u>Specific Aim 1.4</u> – Characterization of EC BK_{Ca} in Cultured Rat Aortic Endothelial Cells (RAECs).

Due to the limitations of sample purity and size in freshly dispersed ECs for western blot detection of BK_{Ca} protein, we utilized a cultured EC line from rat aorta (RAECs) and characterized the presence of BK_{Ca} in RAECs using immunofluorescence and western blot. RAEC expression of BK_{Ca} α was confirmed using confocal immunoflourescence microscopy. For western blot detection, RAECs were collected and lysed for protein and separated by SDS-PAGE and blotted for BK_{Ca} α.

<u>Aim 2:</u> Assess the role of the Cav-1 scaffolding domain in regulating endothelial BK_{Ca} channels following CH.

Hypothesis: CH exposure results in a loss of Cav-1 inhibition of EC BK_{Ca} enabling increased channel activity and a loss in vasoconstrictor reactivity.

Rationale: BK_{Ca} channels within ECs are hypothesized to be localized within caveolae and inhibited by the scaffolding domain of Cav-1. Loss of caveolar structure, Cav-1 presence, or the ability of Cav-1 to directly regulate the channel through its scaffolding domain following CH would result in increased channel activity and subsequent diminished vasoconstrictor reactivity. Experiments in this aim will address alterations to caveolae, Cav-1, and channel interaction with Cav-1 following CH exposure.

<u>OBJECTIVES</u>

1. Electrophysiological investigation of Cav-1 regulation of EC BK_{Ca}
2. Investigating the role of Cav-1 in EC hyperpolarization through membrane potential studies

3. Investigating the role of Cav-1 in diminished vasoconstrictor reactivity to agonists and intraluminal pressure (myogenic tone).
4. Investigate EC caveolar structure following CH
5. Immunolocalization of Cav-1 and BK_{Ca} in native tissue
6. Co-immunoprecipitation of EC BK_{Ca} and Cav-1 in cultured RAECs.

<u>Specific Aim 2.1</u>– Patch clamp investigation of Cav-1 regulation of endothelial BK_{Ca}

Since BK_{Ca} channels in cultured ECs are inhibited by association with Cav-1 and its scaffolding domain (Wang *et al.*, 2005) and are tightly regulated by lipid content of cellular membranes (Thompson & Begenisich, 2006;Weaver *et al.*, 2007), *we examined the effect of cholesterol depletion, cholesterol repletion, and the administration of a cell permeant peptide of the scaffolding domain of Cav-1 on BK_{Ca} currents in freshly dispersed cells from control and CH rats.* For cholesterol depletion experiments the drug methyl-β-cylcodextrin (MBCD) was administered and MBCD elicited currents were tested for sensitivity to IBTX or NS-1619 to test for the presence of EC BK_{Ca} currents. For cholesterol repletion experiments, MBCD and cholesterol (MBCD + ChL 8:1), shown to replete membrane cholesterol and restore caveolar function (Levitan *et al.*, 2000;Romanenko *et al.*, 2004), was administered and transmembrane currents were examined. To determine channel inhibition by the scaffolding domain of Cav-1, a synthesized cell permeant peptide of the scaffolding domain of Cav-1 (AP-CAV) or a scrambled control peptide administered via patch pipette and transmembrane currents were analyzed.

<u>Specific Aim 2.2</u> – Investigating the role of Cav-1 in EC hyperpolarization through membrane potential (E_m) studies.

Previous studies in our laboratory have demonstrated the vascular wall is hyperpolarized following CH and this hyperpolarization is sensitive to IBTX and the removal of the endothelium. Therefore *we designed these experiments to measure endothelial cell E_m in gracilis arteries from control and CH rats to test whether CH-induced changes in E_m could be inhibited by IBTX, AP-CAV, or mimicked by caveolar disruption with MBCD, s*upporting a role for EC BK_{Ca} in mediating the vascular wall hyperpolarization following CH.

<u>Specific Aim 2.3</u> – Investigating the role of Cav-1 in diminished vasoconstrictor reactivity to agonists and intraluminal pressure (myogenic tone).

Previous studies have demonstrated that following CH exposure systemic arterioles display diminished vasoconstrictor reactivity, in which myogenic tone and agonist-induced constriction are decreased compared to arterioles from normoxic control animals. Interestingly, removal of the endothelium or application of IBTX restored vasoconstrictor reactivity; *thus we designed experiments in isolated gracilis arterioles to assess the role of endothelial BK_{Ca} channels in mediating the decreased vasoconstrictor reactivity following CH.* Experiments were performed using cannulated, pressurized gracilis arterioles in which active and passive (Ca^{2+}-free) pressure–diameter relationships in the presence of AP-CAV or scrambled were determined to assess myogenic tone. Vasoconstrictor reactivity to the agonist, phenyelphrine (PE) was assessed over a concentration range of 10^{-9} μmol/L to 10^{-5} μmol/L in the presence of intraluminal AP-CAV or scrambled control peptide. Reversal of constriction with 10 μM ACh at the

termination of the PE concentration-response curve was used to verify endothelial integrity.

Specific Aim 2.4 - Investigate EC Caveolar Structure in Control, CH, and MBCD treatments.

Differences in caveolar structure have not been assessed in vascular endothelial cells following CH. Additionally, the mild MBCD treatments that arterioles are subjected to during our experimental procedures have not been assessed for alteration in caveolar structure. To address these questions, *we designed experiments to investigate caveolar structure through electron microscopy.* Gracilis arterioles and aorta from CH and control rats were fixed and processed, dehydrated with ethanol, and imbedded in Lowicryl K4M. Sections were observed using a Hitachi H7500 electron microscope and analyzed for changes in caveolar gross structure or number.

Specific Aim 2.5 - Immunolocalization of Cav-1 and BK_{Ca}.

EC BK_{Ca} channels are hypothesized to be localized to caveolar domains and associate with Cav-1 (Wang *et al.*, 2005). To determine if EC BK_{Ca} channels co-localize with Cav-1 in our model, *we designed immunofluorescence experiments in cross sections from gracilis arteries staining for BK α and Cav-1.* Cross sections from gracilis arteries from control and CH rats treated with primary antibodies for BK α and Cav-1 were analyzed for co-localization. Individual channel masks and the EC mask were combined to examine endothelial specific co-localization as defined by Manders correlation coefficient values (Manders *et al.*, 1992).

<u>Specific Aim 2.6</u> - Co-immunoprecipitation of EC BK_{Ca} and Cav-1 in cultured RAECs.

To determine if there is an association of BK_{Ca} and Cav-1 and support immunofluorescent co-localization findings, *we conduced co-immunoprecipitations of cultured RAECs*. Cultured cells were utilized to reduce animal usage as current techniques to separate endothelial cells from rat arteries do not provide samples void of arterial smooth muscle cells (which also contain BK_{Ca} and Cav-1) and do not provide large enough collections of cells for co-immunoprecipitations without pooling of cells from several animals. RAECs were collected and gently lysed for protein. BK_{Ca} or Cav-1 antibodies were immobilized to coupling resin or to control non-amine reactive resin for 4 hours at 4°C. Columns containing either the antibody linked resins or control non-amine reactive resins were washed and incubated with the lysates. Sample elutions were collected and analyzed by western blotting using antibodies for BK_{Ca} and Cav-1.

<u>Aim 3:</u> Investigate caveolin-1 regulation of heme oxygenase and HO-dependent BK_{Ca} activity following CH.

Hypothesis: CH treatment results in a loss of Cav-1 inhibition of both HO and BK_{Ca} resulting in EC hyperpolarization and diminished vasoconstriction.

Rationale: Whole cell patch clamp experiments and single channel records from Aim 1 will establish the effect of the HO product CO on EC BK_{Ca} channel activity. However, the question of whether BK_{Ca} activity is dependent upon HO activity has not been addressed. This aim will investigate the hypothesis that CH treatment results in a loss of

Cav-1 inhibition of both HO and BK_{Ca} resulting in EC hyperpolarization and diminished vasoconstriction.

OBJECTIVES

1. Patch clamp investigation of HO-dependent BK_{Ca} channel activity
2. Immunolocalization of HO-1, HO-2, Cav-1 and BK_{Ca}
3. Co-immunoprecipitation of HO-1, HO-2, BK_{Ca}, and Cav-1

Specific Aim 3.1 - Patch clamp investigation of HO-dependent BK_{Ca} channel activity.

Previous experiments in our laboratory have demonstrated the vascular wall hyperpolarization and diminished vasoreactivity is not only dependent upon the endothelium and BK_{Ca}, but also the activity of HO. The HO product, CO can activate VSM BK_{Ca} channels (Jaggar *et al.*, 2002) and HO-dependent activation of BK_{Ca} channels have been additionally found in carotid body glomus cells and act as an oxygen sensor (Williams *et al.*, 2004), *thus we designed experiments to address if there was HO-dependent activation of EC BK_{Ca} channels in our model through patch clamp electrophysiology.* HO-dependent activity of EC BK_{Ca} was assessed in freshly dispersed ECs through measurement of transmembrane currents in the presence and absence of HO-inhibitors and substrates. To corroborate findings in freshly dispersed cells we additionally performed experiments investigating HO-dependent BK_{Ca} activity in cultured RAECs.

Specific Aim 3.2 - Immunolocalization of HO-1, HO-2, Cav-1 and BK_{Ca}.

EC BK_{Ca} (Wang *et al.*, 2005), HO-1, and HO-2 (Kim *et al.*, 2004a) have been previously demonstrated to co-localize and be regulated by Cav-1. Additionally, HO-2 and BK_{Ca} (Williams *et al.*, 2004) have been shown to associate, thus we designed immunolocalization experiments in freshly dispersed ECs for CH and control rats to analyze the possible associations amongst HO-1, HO-2, Cav-1, and BK_{Ca}. Co-localization was determined and analyzed for differences in the degree of association following CH treatments.

Specific Aim 3.3 - Co-immunoprecipitation of HO-1, HO-2, BK_{Ca}, and Cav-1.

To corroborate findings of association amongst HO-1, HO-2, BK_{Ca}, and Cav-1 in freshly dispersed ECs *we designed co-immunoprecipitation experiments in cultured RAECs to investigate physical associations amongst HO-1 and BK_{Ca}, HO-2 and BK_{Ca}, HO-1 and Cav-1, and HO-2 and Cav-1.* RAECs were collected and gently lysed for protein. HO-1, HO-2, BK_{Ca} or Cav-1 antibodies were immobilized to coupling resin or to control non-amine reactive resin for 4 hours at 4°C. Columns containing either the antibody linked resins or control non-amine reactive resins were washed and incubated with the lysates. Sample elutions were collected and analyzed by western blotting using antibodies for HO-1, HO-2, BK_{Ca} and Cav-1.

Chapter 1:

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Chapter 2: General Methods for Dissertation

Experimental Groups

All protocols employed in this study were reviewed and approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Science Center (Albuquerque, NM). Experiments were performed on male Sprague-Dawley rats (Harlan). Rats exposed to CH were placed in a hypobaric chamber maintained at a barometric pressure of 380 mmHg for 48 hours. Normoxic control rats were housed in identical cages at ambient pressure (~630 mmHg).

Study of Isolated Resistance Arteries. Following exposure, rats were deeply anesthetized with sodium pentobarbital (50 mg ip). Hindlimbs were removed and placed in ice-cold physiological saline solution [PSS: 119 mmol/l NaCl, 4.7 mmol/l KCl, 25 mmol/l $NaHCO_3$, 1.18 mmol/l KH_2PO4, 1.17 mmol/l $MgSO_4$, 0.026 mmol/l K_2-EDTA, 5.5 mmol/l glucose and 2.5 mmol/l $CaCl_2$]. Gracilis arterioles (third-order branches from the femoral artery; passive inner diameter at 60 mmHg, 150-200 μm) were dissected free, cannulated on glass pipettes and mounted in an arteriograph. Arteries were slowly pressurized to 60 mmHg with PSS using a servo-controlled peristaltic pump (Living Systems) and superfused (10 ml/min) with warmed (37°C) PSS aerated with a normoxic gas mixture (21% O_2-6% CO_2-73% N_2). All experiments were performed under normoxic conditions to examine sustained alterations in vascular control rather than acute vasodilatory responses to hypoxia (Caudill *et al.*, 1998;Naik & Walker, 2003). Previous experiments have demonstrated that long term adaptations following CH exposure appear by 48 hours and are indistinguishable from effects of 4 week exposure (Jernigan *et al.*, 2001;O'Donaughy & Walker, 2000). The effects may be partially or fully reversed following 96

hours of normoxic exposure (Jackson, 2005). Arteriole exposure to normoxic conditions for 2.5-3 hours throughout the course of the experiment do not reverse sustained alterations elicited by CH exposure. Vessels were prepared for measurement of inner diameter (Earley & Walker, 2002;Earley *et al.*, 2002;Earley & Walker, 2003;Earley *et al.*, 2003), intracellular calcium (Earley & Walker, 2003) or endothelial cell E_m (Earley *et al.*, 2002;Paffett *et al.*, 2007) depending upon the protocol.

Measurement of Vascular Smooth Muscle Cell Calcium Concentration. Pressurized arteries were loaded abluminally with the cell-permeant, ratiometric, Ca^{2+}-sensitive fluorescent indicator fura-2 AM (Molecular Probes) as detailed previously (Earley & Walker, 2002). Immediately prior to loading, fura-2 AM (1 mM in anhydrous DMSO) was mixed 2:1 with a 20% solution of pluronic acid in DMSO, and this mixture diluted in PSS to yield a final concentration of 2 μM fura-2 AM and 0.05% pluronic acid. Arteries were incubated in this solution for 45 min at room temperature in the dark. The diluted fura-2 AM solution was equilibrated with the 21% O_2 gas mixture during this loading period. Vessels were rinsed for 20 min with aerated PSS (Dora *et al.*, 1997) following the loading period to wash out excess dye and to allow for hydrolysis of AM groups by intracellular esterases. Fura-2-loaded vessels were alternately excited at 340 and 380 nm at a frequency of 1 Hz with an IonOptix Hyperswitch dual excitation light source and the respective 510 nm emissions collected with a photomultiplier tube. Background-subtracted 340/380 emission ratios were calculated with IonOptix Ion Wizard software and recorded continuously throughout the experiment, with simultaneous measurement of inner diameter (i.d.) from red wavelength bright-field images as described above. VSM $[Ca^{2+}]_i$ is expressed as the mean F_{340}/F_{380} ratio from the background-subtracted 510 nm signal.

Measurement of Endothelial Cell E_m: Endothelial cell E_m was recorded from gracilis resistance arteries using sharp electrodes as detailed previously (Earley *et al.*, 2002;Naik & Walker, 2006). Endothelial E_m was measured in artery strips with the luminal surface exposed. Arteries were superfused (2 ml/min) with a HEPES buffered saline solution warmed to 37°C. Sharp electrodes (30-60 MΩ) were initially backfilled with Lucifer yellow (16.6 mg/ml in 1M LiCl) followed by 1M KCl, permitting *post hoc* epi-fluorescence dye identification of endothelial vs. VSM cells by the distinct cellular morphological and dye transfer characteristics of each cell type as previously described (Emerson & Segal, 2000).

Patch Clamp Studies of Isolated Endothelial Cells: Endothelial cells were freshly dispersed from the aorta for electrophysiological study. The aorta was chosen as the source of cells based on our earlier results showing parallel endothelium-dependent attenuation of vasoconstrictor reactivity following CH in aortic rings and resistance vessels that is similarly reversed by HO inhibition but not affected by NOS and COX blockade (Gonzales & Walker, 2002;Caudill *et al.*, 1998). Aortae were removed and placed in ice-cold HEPES buffered physiological saline solution (HBSS): mmol/l 150 NaCl, 6 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, 10 mmol/l HEPES, and 10 mmol/l glucose and adjusted to pH 7.4 with NaOH. Thoracic aortae were cut longitudinally and subsequently incubated for 2 hours in basal endothelial growth medium with 4% bovine serum albumin and 10 μg/ml of the endothelial specific probe, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine percholorate (Ac-LDL-Dil) at 37°C. Immediately following the endothelial labeling procedure previously described (Hogg *et al.*, 2002;Paffett *et al.*, 2007) aortae were cut into 2 mm strips and exposed to mild digestion solution containing 0.2 mg/ml dithiothreitol and 0.2 mg/ml papain in HBSS for 45 min at 37°C. Vessel strips were removed from the digestion solution and placed in 1 ml of HBSS containing 2 mg/ml

BSA. Single endothelial cells were released by gentle trituration with a small bore Pasteur pipette and stored at 4°C between experiments for up 5 hrs. One to two drops of the cell suspension were seeded on a glass coverslip mounted on an inverted fluorescence microscope (Olympus IX71) for 30 min prior to superfusion. Single endothelial cells were identified by the selective uptake of the fluorescently labeled acylated low density lipoprotein Ac-LDL-Dil with a rhodamine filter prior to each electrophysiological experiment. Freshly dispersed endothelial cells were superfused under constant flow (2 ml/min) at room temperature (22-23°C) in an extracellular solution (ECS: 141 mmol/l NaCl, 4.0 mM KCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 mmol/l HEPES, 10 mmol/l glucose and buffered to pH 7.4 with NaOH). Whole cell current data were generated with an Axopatch 200B amplifier (Axon Instruments) following a 5 min dialysis period with 4-6 MΩ patch electrodes filled with an intracellular solution (ICS: 140 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 5 mmol/l Mg_2ATP, 10 mmol/l HEPES, 1 mmol/l EGTA and adjusted to pH 7.2 with KOH]. $CaCl_2$ added to yield a free-Ca^{2+} concentration of 1 μM, as calculated using WinMAXC chelator software. Upon attainment of whole cell patch clamp configuration only ECs with series resistances < 15MΩ that maintained 1GΩ or greater seal resistances throughout the course of the experiment were kept for analysis. Whole cell currents were measured in response to voltage steps applied from -60 mV to +150 mV in 10 mV increments from a holding potential of -60 mV. For experiments utilizing the perforated patch technique, 4-6 MΩ patch electrodes were backfilled with amphotericin B. After gigaseals were obtained, the series resistance fell over a 10- to 15-min period to 15–20 MΩ and remained stable for up to 1 h. ECs with stable series resistances under 25 MΩ were used for experiments. Cell capacitance was monitored and transmembrane currents were expressed in terms of current density (pA/pF). There were no differences in cell capacitance between groups.

Single Channel Recordings. For single channel experiments, cell attached and inside-out patch clamp configurations were used. Patch pipettes had resistances ranging from 6 to 8 MΩ. Extracellular solutions (ECS) contained: 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 Glucose, 1.2 $CaCl_2$ (pH 7.30 with NaOH). Intracellular solutions (ICS) contained (in mM): 130KCl, 2 Na_2ATP, 3 $MgCl_2$, 10 HEPES, 1 EGTA, (pH 7.30 with KOH). For inside-out patch experiments ECS contained: 140 KCl, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$ (pH 7.3 with KOH). For experiments investigating the effect of Ca^{2+} concentration on single channel opening probability (N_{po}) on inside out patches EGTA and free Ca^{2+} ($CaCl_2$) were varied to provide 10 nM, 50 nM, 100 nM, 1 μM, and 10 μM free Ca^{2+} solutions. Patches were held at -60, 0, and 40 mV test potentials to for voltage sensitivity experiments. Single channel unitary conductance was determined from the slope of currents measured in response to voltage steps applied from -100 mV to 150 mV in 10 mV increments from a holding potential of -60 mV. EC BK_{Ca} unitary conductance in symmetrical K^+ and assymetrical K^+ were determined. All data were filtered at 5 kHz (-3dB) and digitized at 10 kHz.

Single Channel Recordings from VSM Cells. For single channel experiments on freshly isolated VSM cells, aortae were digested as stated above. Following gentle trituration freshly isolated VSM cells were placed on Sylgard coated cover slips. VSM cells were identified by spindle shape and an absence of DiI-Ac-LDL labeling. All experimental conditions were identical to EC recordings (methods stated above) and calcium sensitivity of VSM BK_{Ca} channels were compared to EC BK_{Ca} channels.

Immunofluorescence localization of BK α and BK β1 in artery sectionss: Arteries from control and CH rats were collected following transcardial perfusion with PSS containing 10 mg papaverin. The gracilis muscle was removed and frozen in OCT compound in liquid N_2 and

isopentane. 10 μm sections and thaw mounted to superfrost slides (Fisher) and fixed in 4% formaldehyde PBS at room temperature for 10 min. After fixation cells were permeabilized in 0.01% Triton-X PBS for 10 min and blocked in 4% donkey serum in PBS for 1 hr at room temperature. Cross sections were incubated with primary antibodies for BK α or BK β1 (Alamone). Some sections were additionally probed for with the endothelial marker, PECAM-1 (Santa Cruz) and BK α for endothelial specific localization. Primary antibodies for BK α or BK β1 were detected with Cy5-conjugated donkey, anti-rabbit secondary antibodies. PECAM-1 was detected with a Cy3-conjugated donkey, anti-mouse secondary antibody (all secondaries 1:500 dilution; Jackson Labs). The nuclear stain, Sytox (1:10,000 dilution; Molecular Probes) was then applied. Sections were visualized with a confocal laser microscope (LSM 510 Zeiss; 63x oil immersion lens).

Immunofluorescence localization of Caveolin-1 and BK α in Sectioned Arteries: Gracilis arteries from control and CH rats were collected following transcardial perfusion with PSS containing 10 mg papaverin. The gracilis muscle was removed and frozen in OCT compound in liquid N_2 and isopentane. 10 μm sections were adhered to superfrost slides (Fisher) and fixed in ice cold methanol (100%) for 10 min. After fixation cross sections were blocked in 4% donkey serum in PBS for 1 hr at room temperature. Cross sections were incubated with primary antibodies for BK α (Alamone 1:100) and Cav-1 (BD Biosciences 1:50). Primary antibodies for BK α were detected with Cy5-conjugated donkey, anti-rabbit secondary antibodies. Cav-1 was detected with a Cy3-conjugated donkey, anti-mouse secondary antibody (all secondaries 1:500 dilution; Jackson Labs). The nuclear stain, Sytox (1:10,000 dilution; Molecular Probes) was then applied. Confocal microscope images were obtained using a 63x oil immersion DIC objective (NA =1.4) at a resolution of 524 X 524 and an optical slice of 0.7 μm.

Immunofluorescence of Isolated Endothelial cells: Endothelial cells were freshly dispersed from the aorta (see detailed methods above) and used for immunofluorescent detection of BK α and BK β1 (Alamone 1:100), Caveolin-1 (BD Biosciences 1:50), Heme Oxyganse (HO-1) (Stressgen 1:100) or Heme Oxygenase (HO-2) (Stressgen 1:100). One to two drops of the cell suspension were seeded on a glass coverslip for 30 min prior to fixation in 4% formaldehyde PBS at room temperature for 15 min. After fixation cells were permeabilized in 0.01% Triton-X PBS for 10 min and blocked in 4% donkey serum in PBS for 1 hr. Cells were incubated with primary antibodies for BK α / β1 , Cav-1, HO-1, or HO-2 and detected with fluorescent protein conjugated secondary antibodies. Isolated cells were visualized with a confocal laser microscopy (LSM 510 Zeiss; 63x oil immersion lens).

Electron Microscopy of Endothelial Caveolae: Gracilis arterioles and aorta from CH and control rats (n = 4-6) were perfusion fixed, removed, and fixed in the same solution for an additional 15 min. Fixed samples were dehydrated with ethanol and imbedded in Lowicryl K4M. Sections were observed using a Hitachi H7500 electron microscope.

Culture of rat aortic endothelial cells: Frozen rat aortic endothelial cells (Cell Appllications) were thawed and seeded as directed by the manufacturer. Cells were grown on attachment factor coated plates in rat endothelial cell growth medium (Cell Applications) at 37°C at 5% CO_2 in a humidified incubator to confluency for western blot and co-immunoprecipitation experiments.

Western blot of cultured rat aortic endothelial cells: ECs passages 6-7 were washed twice in PBS then lysed in radio-immunoprecipitation assay buffer (RIPA) (Thermo Scientific) containing protease and phosphatase inhibitors (Thermo Scientific). Lysates were collected, centrifuged at 10,000 x g at 4°C for 5 minutes, and analyzed for protein content (Thermo - Scientific Pierce BCA Protein kit). Cell lysates (10 μg) were separated in 10% sodium dodecyl

sulfate polyacrylamide gels by electrophoresis and transferred to a nitrocellulose membrane. Blots were blocked in odyssey blocking buffer (LI-COR) 1 h at room temperature then incubated with primary antibodies for Cav-1 (1:500), BK_{Ca} (1:250), HO-1 (1:500), or HO-2 (1:200) in 0.01% Tween PBS overnight at 4°C (BK_{Ca}: Alamone, Cav-1: Santa Cruz, HO-1: Stressgen, HO-2: Stressgen). After washing (3× 10 min in 0.01% Tween PBS), blots were incubated 1 h at room temperature with dye conjugated secondary antibodies (LI-COR). Images were collected on an infrared imaging system (LI-COR).

Co-immunoprecipitation of BK_{Ca}, Cav-1, HO-1, and HO-2 in rat aortic endothelial cells: Rat aortic endothelial cells passages 6-7 were collected and lysed as described above. Co-immunoprecipitation (co-IP) was completed using Thermo Scientific Pierce co-IP kit as directed by the manufacturer. Briefly, the BK_{Ca}, caveolin-1, HO-1, or HO-2 antibodies were immobilized to the AminoLink Plus coupling resin or to control non-amine reactive resin for 4 hours at 4°C. Columns containing either the antibody linked resins or control non-amine reactive resins were washed and then loaded with arterial lysates and incubated overnight at 4°C. The next day columns were washed and protein eluted using the kit's provided elution buffer. Using the Thermo Scientific Pierce Micro BCA Protein Assay kit, washes were tested for protein content to verify successful washing before elution. Eluted samples were analyzed by Western blotting using antibodies for BK_{Ca}, Cav-1, HO-1, or HO-2 with infrared dye-conjugated secondary antibodies (LI-COR).

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CHAPTER 3: Novel Role of Endothelial BK_{Ca} Channels in Altered Vasoreactivity Following Hypoxia

Novel Role of Endothelial BK_{Ca} Channels in Altered Vasoreactivity Following Hypoxia

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Running Head: Endothelial BK channels in chronic hypoxia

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ABSTRACT

The systemic vasculature exhibits attenuated vasoconstriction following hypobaric chronic hypoxia (CH) that is associated with endothelium-dependent vascular smooth muscle (VSM) cell hyperpolarization. We hypothesized that increased activity of endothelial cell (EC) large conductance-calcium activated potassium channels (BK_{Ca}) contributes to this response. Gracilis resistance arteries from hypobaric CH (P_B = 380 mmHg for 48 hr) rats demonstrated reduced myogenic reactivity and hyperpolarized VSM membrane potential (E_m) compared to controls under normoxic *ex vivo* conditions. These differences were eliminated by endothelial disruption. In the presence of cyclooxygenase and nitric oxide synthase inhibition, combined intraluminal administration of the intermediate and small conductance calcium-activated K^+ channel blockers TRAM 34 and apamin was without effect on myogenic responsiveness and VSM E_m in both groups, however these variables were normalized in CH arteries by intraluminal administration of the BK_{Ca} inhibitor iberiotoxin (IBTX). Basal EC E_m was hyperpolarized in arteries from CH rats compared to controls and was restored by IBTX, but not by TRAM 34/apamin. K^+ channel blockers were without effect on EC basal E_m in controls. Similarly, IBTX blocked acetylcholine (ACh)-induced dilation in arteries from CH rats, but was without effect in controls, whereas TRAM 34/apamin eliminated dilation in controls. ACh-induced EC hyperpolarization and calcium responses were inhibited by IBTX in CH arteries and by TRAM 34/apamin in controls. Patch clamp experiments on freshly isolated ECs demonstrated greater K^+ current in cells from CH rats that was normalized by IBTX. IBTX was without effect on K^+ current in controls.

We conclude that hypobaric CH induces increased endothelial BK_{Ca} channel activity that contributes to reduced myogenic responsiveness and EC and VSM cell hyperpolarization.

Key Words: large conductance calcium-activated potassium channel; myogenic response; endothelium-dependent vasodilation; membrane potential; patch clamp

INTRODUCTION

Chronic hypoxia (CH) results from pathological conditions that impair oxygenation as well as from prolonged residence at high altitude. Previous studies have demonstrated that vasoconstrictor responsiveness of the systemic circulation is attenuated following prolonged exposure to hypoxia (Auer & Ward, 1998;Toporsian & Ward, 1997;Hu *et al.*, 1996;Doyle & Walker, 1991). Diminished vasoconstrictor reactivity following CH is observed both systemically as a reduced total peripheral resistance response to vasoconstrictor agonists (Doyle & Walker, 1991) and in several individual vascular beds (O'Donaughy & Walker, 2000;Jernigan *et al.*, 2001;Auer & Ward, 1998;Caudill *et al.*, 1998) suggesting that it is a generalized response to this stimulus. Further, since diminished vasoconstrictor activity is maintained following acute return to normoxia (Doyle & Walker, 1991;Auer & Ward, 1998) and is largely unaffected by P_{O2} (Doyle & Walker, 1991) this response to CH appears to be an adaptation, distinct from acute responses to hypoxia.

Blunting of both agonist-induced and myogenic vasoconstriction following CH appear to be endothelium-dependent. For example, reduced agonist-induced vasorelaxation is reversed following endothelial disruption in both aorta (Caudill *et al.*, 1998;Doyle & Walker, 1991) and resistance arteries (Earley *et al.*, 2002) from CH rats. A similar endothelium-dependence is observed in attenuated myogenic reactivity following CH in mesenteric arterioles (Doyle & Walker, 1991). Furthermore, CH-induced blunted vasoconstriction in the mesenteric vascular bed is associated with an endothelium-dependent reduction in vascular smooth muscle (VSM) calcium as well as VSM hyperpolarization (Earley & Walker, 2002;Earley *et al.*, 2002;Earley & Walker,

2003). Thus, the endothelium appears to play a central role in altered vasoreactivity following CH.

Although the mechanisms responsible for altered responsiveness in the vasculature during CH are not fully defined,, previous experiments suggest that endothelial nitric oxide (NO) (Earley & Walker, 2003), carbon monoxide (CO) (Jernigan *et al.*, 2001;Naik *et al.*, 2003;O'Donaughy & Walker, 2000;Gonzales & Walker, 2002;Caudill *et al.*, 1998) and epoxyeicosatrienoic acids (EETs) (Earley *et al.*, 2003) may be involved. In addition, VSM E_m and vasoreactivity are restored in mesenteric resistance arteries from CH rats following inhibition of large conductance calcium-activated K^+ (BK_{Ca}) channels (Earley *et al.*, 2003;Naik & Walker, 2003). Since NO, CO and EETs are all endogenous activators of these channels, a primary role of BK_{Ca} in altered vasoreactivity following CH is likely.

Calcium-activated potassium (K_{Ca}) channels are widely distributed in vascular tissue. They are classified into large (BK_{Ca}), intermediate (IK_{Ca}) and small (SK_{Ca}) conductance channels. K_{Ca} channels exert a powerful effect on E_m of both VSM and endothelial cells in resistance arteries and the level of K_{Ca} channel expression and activity is a fundamental determinant of vascular tone and blood pressure in both health and disease (Sobey, 2001). Activity of VSM BK_{Ca} channels is regulated by calcium sparks from ryanodine-sensitive stores and acts to promote VSM hyperpolarization and reduced calcium influx through voltage gated calcium channels (Jaggar *et al.*, 2000). Endothelial SK_{Ca} and IK_{Ca} channels appear to play a prominent role in agonist-induced endothelial hyperpolarization and consequent vasodilation (Gluais *et al.*, 2005;Eichler *et al.*, 2003;Hilgers *et al.*, 2006;Crane *et al.*, 2003;Hinton & Langton, 2003). However, the

physiological significance of endothelial BK_{Ca} channels has been questioned. Whereas several studies have demonstrated that BK_{Ca} channels are present in both freshly isolated and cultured endothelial cells (Brakemeier *et al.*, 2003;Wang *et al.*, 2005), a role for these channels in control of vascular tone is controversial (Jackson, 2005;Wang *et al.*, 2005;Gauthier *et al.*, 2002;Brakemeier *et al.*, 2003).

The present study was designed to test the hypothesis that enhanced activity of endothelial BK_{Ca} channels is responsible for blunted myogenic vasoconstrictor reactivity following CH. This hypothesis was based on several key observations, notably: 1) CH-induced attenuated vasoconstrictor reactivity is endothelium-dependent and can be reversed by blockade of endogenous activators of BK_{Ca} channels (NO,CO, EETs); 2) the response to CH is BK_{Ca}-dependent, however dilation in response to endogenously produced CO in CH arteries is not affected by ryanodine (Naik & Walker, 2003) which inhibits VSM calcium sparks; and 3) endothelial cell $[Ca^{2+}]_i$ is elevated following CH (Earley & Walker, 2003) suggestive of endothelial cell hyperpolarization. Together, these data suggest that endothelial BK_{Ca} channels may be involved in diminished vasoreactivity following CH. Although much of our previous work was performed in mesenteric arteries, we chose to examine gracilis resistance arteries in the present study to not only extend our observations to another vascular bed, but also due to the reported lack of myoendothelial electrical coupling in the hindlimb circulation (Sandow *et al.*, 2002). This characteristic enables unambiguous assessment of the effects of CH on endothelial vs. VSM E_m.

METHODS

Animals. Experiments were performed on male Sprague-Dawley rats (Harlan). All procedures were approved by the Institutional Animal Care and Use Committee of the University of New Mexico Health Sciences Center.

Hypoxic Exposure. CH rats were exposed to hypobaric hypoxia at a barometric pressure of 380 mmHg for 48 hours. Normoxic control rats were housed in identical cages at ambient pressure (~630 mmHg).

Study of Isolated Resistance Arteries. Following exposure, rats were deeply anesthetized with sodium pentobarbital (50 mg ip). Hindlimbs were removed and placed in ice-cold physiological saline solution [PSS: 119 mmol/l NaCl, 4.7 mmol/l KCl, 25 mmol/l $NaHCO_3$, 1.18 mmol/l KH_2PO4, 1.17 mmol/l $MgSO_4$, 0.026 mmol/l K_2-EDTA, 5.5 mmol/l glucose and 2.5 mmol/l $CaCl_2$]. Caudal femoral artery branches (third-order branches from the femoral artery; passive inner diameter at 100 mmHg, 150-200 μm) were dissected free, cannulated on glass pipettes and mounted in an arteriograph. Arteries were slowly pressurized to 100 mmHg with PSS using a servo-controlled peristaltic pump (Living Systems) and superfused (10 ml/min) with warmed (37°C) PSS aerated with a normoxic gas mixture (21% O_2-6% CO_2-73% N_2). All experiments were performed under normoxic conditions to examine sustained alterations in vascular control rather than acute vasodilatory responses to hypoxia (Caudill *et al.*, 1998;Naik & Walker, 2003). Previous experiments have demonstrated that long term adaptations following CH exposure appear by 48 hours and are indistinguishable from effects of 4 week exposure (Jernigan *et al.*, 2001;O'Donaughy & Walker, 2000). The effects may be partially or fully reversed following 96 hours of normoxic exposure (Jackson, 2005). Arteriole

exposure to normoxic conditions for 2.5-3 hours throughout the course of the experiment do not reverse sustained alterations elicited by CH exposure. For experiments involving endothelium disrupted arteries, a 1-ml air bubble was passed through the vessel lumen after cannulation. Endothelium-disruption was confirmed by the absence of a vasodilatory response to 10 μM acetylcholine (ACh). Vessels were prepared for measurement of inner diameter (Earley & Walker, 2002;Earley *et al.*, 2002;Earley & Walker, 2003;Earley *et al.*, 2003), $[Ca^{2+}]_i$ (Earley & Walker, 2003) or VSM or endothelial cell E_m (Earley *et al.*, 2002;Paffett *et al.*, 2007) depending upon the protocol.

Measurement of Vascular Smooth Muscle and Endothelial Cell E_m: Vascular smooth muscle (VSM) and endothelial cell E_m was recorded from gracilis resistance arteries using sharp electrodes as detailed previously (Earley *et al.*, 2002;Naik & Walker, 2006). VSM E_m recordings were performed on pressurized arteries prepared as above, whereas endothelial E_m was measured in artery strips with the luminal surface exposed. Arteries were superfused (2 ml/min) with a HEPES buffered saline solution warmed to 37°C. Sharp electrodes (30-60 MΩ) were initially backfilled with Lucifer yellow (16.6 mg/ml in 1M LiCl) followed by 1M KCl, permitting *post hoc* epi-fluorescence dye identification of endothelial vs VSM cells by the distinct cellular morphological and dye transfer characteristics of each cell type as previously described (Emerson & Segal, 2000).

Measurement of Endothelial cell $[Ca^{2+}]_i$. Endothelial cell $[Ca^{2+}]_i$ was measured as described previously (Knot *et al.*, 1999;Earley & Walker, 2003). Briefly, fura solution (0.05 μM fura 2-AM and 0.05% pluronic in PSS) was administered to the lumen of pressurized caudal femoral arteries using a servo-controlled peristaltic pump (Living Systems) in the dark. Administration of fura 2-AM to the luminal surface for a short time

has been previously shown to preferentially load endothelial cells (Kamouchi *et al.*, 1999). After a 1-min loading period, the lumen was perfused with PSS for 15 min to wash out excess fura solution and allow hydrolysis of AM groups by intracellular esterases. The vessel preparation was constantly superfused with warmed, aerated PSS. Fura-loaded vessels were alternatively excited at 340 and 380 nm at a frequency of 10 Hz and the respective 510 nm emissions were quantified using a photomultiplier tube and recorded with the use of IonWizard software (IonOptix, version 4.4). Photometric data were collected from the entire arterial segment under study. EC $[Ca^{2+}]_i$ was expressed as the mean F340/F380 ratio from the background-subtracted 510 nm signal. Ratiometric images of unstimulated vessels were collected for ~10 min, then phenylephrine (PE; 1 μM) was administered followed by 10 μM ACh. Lack of a change in EC calcium levels in response to PE demonstrated selective endothelial loading. ACh consistently induced an increase in endothelial cell $[Ca^{2+}]$, when administered to endothelium-intact vessels as shown by elevated F340/F380 ratio. To further demonstrate selective endothelial loading, arteries were denuded with an air bubble at the end of the protocol, allowed 10 minute recovery time, and PE and ACh tests were repeated. The lack of a change in F340/F380 in response to ACh after denudation was further evidence of selective endothelial loading.

Patch Clamp Studies of Isolated Endothelial Cells: Endothelial cells were freshly dispersed from the aorta for electrophysiological study. The aorta was chosen as the source of cells based on our earlier results showing parallel endothelium-dependent attenuation of vasoconstrictor reactivity following CH in aortic rings and resistance vessels that is similarly reversed by heme oxygenase inhibition but not affected by NOS

and COX blockade (Gonzales & Walker, 2002;Caudill *et al.*, 1998). Aortae were removed and placed in ice-cold HEPES buffered physiological saline solution (HBSS): mmol/l 150 NaCl, 6 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, 10 mmol/l HEPES, and 10 mmol/l glucose and adjusted to pH 7.4 with NaOH. Thoracic aortae were cut longitudinally and subsequently incubated for 2 hours in basal endothelial growth medium with 4% bovine serum albumin and 10 μg/ml of the endothelial specific probe, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine percholorate (Ac-LDL-Dil) at 37°C. Immediately following the endothelial labeling procedure previously described (Hogg *et al.*, 2002;Paffett *et al.*, 2007) aortae were cut into 2 mm strips and exposed to mild digestion solution containing 0.2 mg/ml dithiothreitol and 0.2 mg/ml papain in HBSS for 45 min at 37°C. Vessel strips were removed from the digestion solution and placed in 1 ml of HBSS containing 2 mg/ml BSA. Single endothelial cells were released by gentle trituration with a small bore Pasteur pipette and were stored at 4°C between experiments for up 5 hrs. One to two drops of the cell suspension were seeded on a glass coverslip mounted on an inverted fluorescence microscope (Olympus IX71) for 30 min prior to superfusion. Single endothelial cells were identified by the selective uptake of the fluorescently labeled acylated low density lipoprotein Ac-LDL-Dil with a rhodamine filter prior to each electrophysiological experiment. Freshly dispersed endothelial cells were superfused under constant flow (2 ml/min) at room temperature (22-23°C) in an extracellular solution (ECS: 141 mmol/l NaCl, 4.0 mM KCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 mmol/l HEPES, 10 mmol/l glucose and buffered to pH 7.4 with NaOH). Whole cell current data were generated with an Axopatch 200B amplifier (Axon Instruments) following a 5 min dialysis period with 4-6 MΩ patch electrodes filled with

an intracellular solution (ICS: 140 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 5 mmol/l Mg_2ATP, 10 mmol/l HEPES, 1 mmol/l EGTA and adjusted to pH 7.2 with KOH]. $CaCl_2$ added to yield a free-Ca^{2+} concentration of 1 μM, as calculated using WinMAXC chelator software. Upon attainment of whole cell patch clamp configuration only ECs with series resistances < 15MΩ that maintained 1GΩ or greater seal resistances throughout the course of the experiment were kept for analysis. Whole cell currents were measured in response to voltage steps applied from -60 mV to +150 mV in 10 mV increments from a holding potential of -60 mV. Cell capacitance was monitored and transmembrane currents were expressed in terms of current density (pA/pF). There were no differences in cell capacitance between groups.

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stain, Sytox (1:10,000 dilution; Molecular Probes) was then applied. Sections were visualized with a confocal laser microscope (LSM 510 Zeiss; 63x oil immersion lens).

Immunofluorescence of Isolated Endothelial cells: Endothelial cells were freshly dispersed from the aorta (see detailed methods above) and used for immunofluorescent detection of BK α and BK β1. One to two drops of the cell suspension were seeded on a glass coverslip for 30 min prior to fixation in 4% formaldehyde PBS at room temperature for 15 min. After fixation cells were permeabilized in 0.01% Triton-X PBS for 10 min and blocked in 4% donkey serum in PBS for 1 hr. Cells were incubated with primary antibodies for BK α / β1 (Alamone) and detected with Cy5-conjugated donkey, anti-rabbit secondary antibodies. Isolated cells were visualized with a confocal laser microscopy (LSM 510 Zeiss; 63x oil immersion lens).

Experimental Protocols:

Myogenic Responses. Experiments in this and subsequent protocols were performed under normoxic conditions to examine sustained alterations in vascular control rather than acute vasodilatory responses to hypoxia. Active and passive (Ca^{2+}-free) pressure–diameter relationships were determined, as described previously (Doyle & Walker, 1991) over intraluminal pressure steps between 40 and 140 mmHg. Vessel inner diameter was monitored using video microscopy and edge-detection software (IonOptix Sarclen). Initial diameters assessed in Ca^{2+}-replete superfusate at a pressure below the autoregulatory range (20 mmHg) did not differ between groups and ranged from 105 $\pm$ 3 to 115$\pm$ 3 μm. Endothelial integrity was assessed by constriction with 1 μM phenylephrine (PE) and dilation with 10 μM acetylcholine (ACh) prior to Ca^{2+}-free superfusion. Pressure-induced vasoconstrictor responses were determined for

endothelium-intact and endothelium-disrupted arteries as well as endothelium-intact arteries in the presence of combined NOS inhibition (L-NNA; 100 μM) and COX inhibition (indomethacin: 10 μM). In addition, VSM E_m was measured by sharp electrode at 40, 100 and 140 mmHg. Myogenic responsiveness was also determined during exposure to the following combinations of K^+ channel inhibitors: 1) charybdotoxin (CBTX – combined IK_{Ca} and BK_{Ca} blocker; 100 nM) plus apamin (SK_{Ca} blocker; 100 nM); 2) TRAM 34 (selective IK_{Ca} blocker; 1 μM) plus apamin (100 nM); and 3) IBTX (specific BK_{Ca} blocker; 100 nM) alone. All K^+ channel inhibitors were administered intraluminally in an effort to specifically target endothelial K^+ channels. VSM E_m was examined in separate experiments at 120 mmHg in the presence of all of the combinations of inhibitors except CBTX + apamin.

Effect of K^+ Channel Blockers on Endothelium-Dependent Vasodilatory Responses to ACh. Cumulative concentration-response curves to ACh (0.001-100 μM) were obtained in arteries pressurized at 100 mmHg. Vessels were pre-constricted to 30% of equilibrated lumen diameter with PE prior to ACh administration. Vasodilatory responses were determined in arteries treated with the various inhibitors outlined above. Parallel experiments were performed in fura 2 loaded vessels to examine the endothelial calcium response to ACh. Finally, endothelial E_m responses to 10 μM ACh were measured with sharp electrodes.

Effect of K^+ Channel Blockers on Basal Endothelial E_m. Endothelial cell E_m was measured using sharp electrodes in arteries treated with the various inhibitors or respective vehicles.

Effect of K^+ Channel Blockers on Isolated Endothelial Cell Transmembrane Currents. Transmembrane currents were measured in aortic ECs freshly dispersed from control and CH rats. After 5 min dialysis, whole cell currents were measured in response to voltage steps outlined above. After the initial recording, cells were superfused with the K^+ channel inhibitors tetraethylammonium (TEA; 10 mmol) and 4-aminopyridine (4-AP; 10 mM) to confirm measurement of K^+ currents. Voltage steps were applied and recorded after 5 min of superfusion with these inhibitors.

Effect of BK_{Ca} Channel Inhibition and Activation on Isolated Endothelial Cell Transmembrane Currents. After cell dialysis, recordings were taken before and 5 min after superfusion with the BK_{Ca} specific inhibitor IBTX (100 nM), or the BK_{Ca} activator NS1619 (1,3 dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one) (10 μM).

Effect of Cholesterol Depletion on Isolated Endothelial Cell Transmembrane Currents. Since BK_{Ca} channels in cultured endothelial cells are inhibited by association with caveolin-1 (Wang *et al.*, 2005), we examined the effect of cholesterol chelation on transmembrane currents in freshly dispersed cells from each group. After cell dialysis, the cholesterol depletion drug, methyl-β-cylcodextrin (MBCD) (100 μM) was superfused for 15 minutes before washout. Voltage step recordings were taken before and superfusion with MBCD. Following MBCD treatments cells were superfused with IBTX (100 nM) to test for the presence of EC BK_{Ca} currents. Electron microscopy of MBCD treated arteries demonstrated that this low concentration of the agent did not deplete the endothelia of caveolae (see Data Supplement Figure 4).

BK_{Ca} Subunit Immunofluorescence in Intact Arteries and Isolated Endothelial Cells. Endothelial expression of BK_{Ca} α and β1 subunits was confirmed in sectioned resistance arteries and in freshly isolated aortic endothelial cells using confocal immunoflourescence microscopy.

Calculations and Statistics. Data are expressed as means ± SE. Values of n refer to the number of animals in each group except for patch clamp studies where n represents the number of cells. Myogenic tone was calculated as the percent difference in inner diameter at each pressure when arteries were superfused with Ca^{2+}-containing vs. Ca^{2+}-replete physiological salt solution. Vasodilation was expressed as % reversal of PE-induced pre-constriction. Data were analyzed by repeated measures analysis of variance (RMANOVA) and by a Bonferroni modified unpaired Student's t-test for multiple comparisons when differences were indicated. Unpaired t-tests were used for single comparisons between groups. $P \leq 0.05$ was accepted as statistically significant.

RESULTS

Myogenic responsiveness and VSM cell E_m in pressurized arteries. Myogenic vasoconstriction of gracilis resistance arteries from control and CH rats were assessed in normoxic conditions to study persistent vascular adaptations following CH exposure and not acute mechanisms involved in responses to hypoxia. Myogenic vasoconstriction from CH rats was attenuated compared to normoxic controls (Fig. *1A*), confirming findings from the mesenteric vascular bed (Earley & Walker, 2002;Naik & Walker, 2006). Consistent with this finding, VSM cells in arteries from CH rats were hyperpolarized compared to controls at higher pressures (Fig. *1B*) again confirming earlier results (Earley & Walker, 2002;Earley *et al.*, 2002;Earley *et al.*, 2003). Blunted

myogenic responsiveness and VSM cell hyperpolarization of CH arteries compared to controls persisted in the presence of L-NNA and indomethacin (Fig. *1C/ 1D*). Myogenic responses and VSM E_m in the presence of vehicles for L-NNA and indomethacin were not different to those of untreated arteries (data not shown). Endothelial disruption restored myogenic reactivity and E_m of CH arteries to control levels (Fig. *1E/1F*) (Earley & Walker, 2002;Earley *et al.*, 2002). Effectiveness of endothelial disruption was verified by elimination of ACh-induced dilation. Lucifer yellow loading allowed visual identification of the cell type from which E_m recordings were obtained.

Effect of K^+ channel inhibitors on myogenic responsiveness and VSM E_m. The effects of K^+ channel inhibitors were assessed at a transmural pressure of 120 mmHg. CH arteries demonstrated reduced myogenic responsiveness at this pressure compared to arteries from controls, which persisted in the presence of L-NNA and indomethacin (Fig. *2A*). Luminal exposure to CBTX and apamin restored myogenic responsiveness of CH arteries at 120 mmHg to control levels. However, specific inhibition of endothelial IK_{Ca} and SK_{Ca} channels did not affect myogenic responsiveness in either group. In contrast, IBTX alone also restored myogenic responsiveness in CH arteries to control levels without affecting reactivity in the controls. This latter observation is supportive of an endothelial specific effect of intraluminal IBTX. A similar pattern of K^+ channel inhibitor effects were seen on VSM E_m. As discussed previously, VSM cells from CH arteries were hyperpolarized compared to controls at 120 mmHg and this hyperpolarization persisted in the presence of NOS and COX inhibitors, as well as in the presence of endothelial SK_{Ca} and IK_{Ca} inhibition (Fig. *2B*). However, IBTX restored VSM E_m to control levels when administered luminally (Fig. *2B*). VSM cells in control vessels treated with IBTX were

slightly depolarized compared .with untreated control arteries, although myogenic tone was unaffected. Myogenic responses and VSM E_m at 120 mmHg in the presence of vehicles were not different to those of untreated arteries (data not shown). To contrast luminal application of IBTX, a separate set of experiments employed IBTX applied to the superfusate to specifically target VSM. In contrast to luminal application, superfusate administration of IBTX resulted in a significant increase in spontaneous tone at 100 mmHg in control arteries (data not shown). These results suggest that luminal administration of IBTX selectively targets endothelial BK_{Ca} channels with minimal direct effect on the VSM.

Effect of K^+ Channel Blockers on Basal Endothelial E_m. Resting E_m in dye-identified ECs were hyperpolarized in arteries from CH rats compared with controls (Fig. *3*). This hyperpolarization persisted in the presence of L-NNA and indomethacin and after inhibition of IK_{Ca} and SK_{Ca} channels (Fig. *3*). In contrast, IBTX restored CH EC E_m values to control levels, but did not affect controls (Fig. *3*). E_m in the presence of vehicles was not different to those of untreated arteries (data not shown)

Effect of K^+ Channel Blockers on Endothelium-Dependent Vasodilatory Responses to ACh. ACh-induced vasodilation was observed following L-NNA and indomethacin treatment in arteries from both groups demonstrating an EDHF-type response. This response was abolished by combined intraluminal administration of SK_{Ca} and IK_{Ca} blockers in control arteries, but unaffected by BK_{Ca} inhibition (Fig. *4A*). In contrast, ACh dilation was eliminated by BK_{Ca} blockade with IBTX in arteries from CH rats (Fig. *4B*). Figure 5 compares the effect of the various inhibitors on vasodilation, E_m and EC $[Ca^{2+}]_i$ responses to 10 μM ACh. Vasodilatory responses were greater in

untreated arteries from CH rats compared to controls (Figure *5A*). As shown in the previous figure, arteries from both groups still demonstrated vasodilatory responses after NOS and COX inhibition (Fig. *5A*) although there was no longer a difference between groups. The normalization of vasodilatory responses between groups by combined L-NNA and indomethacin was the result of opposing tendencies between the two groups. ACh responsiveness tended to be slightly reduced in arteries from CH rats by this treatment, although this trend did not reach statistical significance. Similarly, vasodilation to ACh tended to increase with treatment. These data suggest that control vessels may possess a COX-dependent vasoconstrictor pathway that elicits VSM depolarization that is not present in arteries from CH rats. Following COX and NOS blockade, dilation to ACh was abolished in arteries of both groups exposed luminally to CBTX and apamin to inhibit endothelial SK_{Ca}, IK_{Ca} and BK_{Ca} (Fig. *5A*). In contrast, specific inhibition of endothelial SK_{Ca} and IK_{Ca} with TRAM 34 and apamin eliminated ACh-induced vasodilation only in arteries from control rats. Additionally, although treatment with the BK_{Ca} inhibitor IBTX alone had no effect on vasodilatory responses in controls, this agent abolished vasodilation in arteries from CH rats. In all cases, the effects of the blockers were similar at all concentrations of ACh studied. Endothelial disruption abolished ACh-induced vasodilation in both groups. Changes in endothelial cell E_m (Fig. *5B*) and EC $[Ca^{2+}]_i$ (Fig. *5C*) in response to ACh were also examined and the K^+ channel inhibitors elicited a profile similar to the vasodilatory responses. Although, the change in E_m in response to ACh in CH untreated arterial strips was not different from controls, basal E_m was hyperpolarized relative to controls (Fig. *3*) and thus the degree of hyperpolarization following ACh was greater in the CH group. ACh-

induced hyperpolarization was not affected by combined L-NNA and indomethacin in either group. Similar to the vasodilatory responses, combined treatment with TRAM 34 and apamin impaired hyperpolarization in response to ACh only in the controls, whereas IBTX blunted ACh-induced EC hyperpolarization only in arteries from CH rats (Fig. *5B*). Endothelial cell $[Ca^{2+}]_i$ responses to ACh closely mirrored the vasodilatory responses in each treatment group (Fig. *5C*).

Effect of K^+ Channel Blockers on Isolated EC Transmembrane Currents . Whole cell currents from freshly isolated ECs from CH and control animals were studied under normoxic conditions to assess long term adaptations to hypoxia and not acute hypoxic exposure effects. Whole cell currents in isolated ECs were greater over a wide range of voltages in cells from CH rats compared to controls (Fig. *6A*). Treatment with the K^+ channel inhibitors TEA and 4-AP nearly abolished currents in both groups indicative of K^+ conductance. Outward K^+ currents from the CH group and controls were significantly reduced following TEA and 4-AP blockade (Fig. *6D*). Residual current after TEA and 4-AP treatment was not different between the groups. To selectively block EC BK_{Ca} channels, current sensitivity to IBTX was tested. The difference in current between control and CH groups was eliminated by IBTX, whereas IBTX was without effect in control cells (Fig. *6C/7A/7C*). In addition, the BK_{Ca} activator NS1619 elicited a further increase in outward current in cells from CH rats, but was without effect in controls (Fig. *7B/ 7D*). Administration of a nitric oxide (NO) donor increased EC BK_{Ca} currents at depolarized potentials (Supplemental Fig. 5) but had no significant effect in controls.

Immunofluorescent localization of BK_{Ca}. Although these data suggest differential expression of BK_{Ca} channels between groups, we observed similar BK_{Ca}

α immunofluorescence in isolated aortic endothelial cells from control and CH rats (Fig. *8A/8B*) and in intact sections of gracilis resistance arteries (Fig. *8E/8F*). The β_1 regulatory subunit for the BK_{Ca} channel was also observed in endothelial cells from both groups (Fig. *8C/8D and 8G/8H*). Endothelial localization was confirmed with the endothelial specific marker PECAM-1 (Supplemental Data Figure 2). No staining for either subunit was observed when only secondary antibody was applied (Supplemental Data Figure 1). In addition, no staining was observed in rat T-cells that do not express BK_{Ca} channels (Supplemental Data Figure 3).

Effect of Cholesterol Depletion on Isolated EC Transmembrane Currents. Endothelial whole cell currents from control animals were significantly increased following cholesterol depletion with MBCD (Fig. *9A*), whereas currents from CH animals were unaffected (Fig. *9B*). The enhanced currents from controls were found to be sensitive to IBTX treatment (Fig. *9C/D*), supporting the immunofluorescence results that BK_{Ca} exist in both controls and CH ECs and are not differentially expressed.

DISCUSSION

The present study examined the contribution of endothelial BK_{Ca} channels to altered vasoreactivity following hypobaric CH. The major findings of this study are: 1) CH is associated with endothelium-dependent VSM hyperpolarization and associated blunted myogenic constriction and persistent VSM hyperpolarization; 2) endothelial cell E_m is also hyperpolarized after CH exposure and restored by BK_{Ca} channel inhibition, 3) similarly, selective endothelial cell BK_{Ca} blockade restores myogenic reactivity and VSM E_m to control levels and blocks endothelium-dependent vasodilatory responses to ACh, 4) endothelial cells isolated from CH rats demonstrate an IBTX-sensitive outward current

not present in cells from control animals, and 5) the IBTX-sensitive outward current is revealed in cells from control animals following cholesterol depletion. These results suggest that endothelial BK_{Ca} activity is increased by CH and is responsible for altered vasoreactivity.

The blunted myogenic reactivity and VSM hyperpolarization observed in gracilis resistance arteries from CH rats are consistent with previous observations of diminished vasoconstrictor reactivity in mesenteric arterioles (Gonzales & Walker, 2002;Naik & Walker, 2006;Earley & Walker, 2002;Earley *et al.*, 2002;Earley & Walker, 2003;Earley *et al.*, 2003), diaphragmatic arteries (Siegl *et al.*, 2005) and aortic rings (Caudill *et al.*, 1998) after 48 hours of CH. Although the present study used a hypobaric model of CH, similar attenuation of vasoconstrictor reactivity is observed with normobaric CH of similar duration and P_{IO2} (Auer & Ward, 1998). Unlike the mesenteric circulation where myogenic tone developed at lower pressures (Doyle & Walker, 1991), gracilis arteries from control rats did not develop significant myogenic tone until pressures of 80 mmHg were reached. Consequently differences between control and CH arterial myogenic tone were not observed except at the higher end of the pressure-diameter relationship in gracilis arteries whereas in the mesenteric arteries this difference is apparent over most of the pressure-diameter relationship (Doyle & Walker, 1991). In further agreement with earlier studies (Earley & Walker, 2002;Naik & Walker, 2006), blunted myogenic reactivity and VSM hyperpolarization of CH arteries persisted in the presence of NOS and COX inhibition, but was reversed by endothelial disruption. Endothelial disruption could potentially have unintended direct effects on smooth muscle reactivity; however there was no significant difference in myogenic tone between control vessels with an

intact or disrupted endothelium. These results convincingly demonstrate a role of the endothelium in attenuated myogenic responsiveness of small gracilis arteries after CH that is not dependent upon release of NO or a COX product.

In addition to diminished myogenic reactivity, we observed that endothelial cell E_m in isolated gracilis arteries was relatively hyperpolarized following CH. We chose to study gracilis resistance arteries based on recent observations that this bed does not demonstrate myoendothelial gap junction communication (Sandow *et al.*, 2002;Zygmunt *et al.*, 1995;Wigg *et al.*, 2001). This characteristic allowed examination of endothelial cell E_m in a setting not influenced by responses conducted from the VSM. Consistent with Sandow et al. (Sandow *et al.*, 2002), we observed endothelial cell E_m that was much more depolarized than the VSM in this vascular bed, suggesting that myoendothelial coupling is not present. The potentials measured were considerably more depolarized than we have observed in endothelia from intact pulmonary arteries (Paffett *et al.*, 2007) and mesenteric arteries (-47 $\pm$ 3 mV; unpublished observation) using identical techniques. These observations coupled with the effectiveness of ACh to hyperpolarize the endothelium (Fig. *5B*) suggest that the relatively depolarized E_m observed under unstimulated conditions in this bed is not an artifact. Endothelial cell hyperpolarization in CH arteries persisted following NOS and COX inhibition and in the presence of SK_{Ca} and IK_{Ca} inhibitors. In contrast, although IBTX did not alter endothelial cell E_m values in arteries from control rats, it significantly depolarized CH endothelial cell E_m to normoxic control levels. These results suggest that there is tonic activity of endothelial BK_{Ca} channels following CH exposure not seen in controls that results in EC hyperpolarization.

To further investigate the role of endothelial BK_{Ca} channels in vascular control following CH, we compared the effects of intraluminal administration of the various K^+ channel inhibitors on vasodilatory, E_m and endothelial cell calcium responses to the endothelium-dependent dilator ACh between arteries from both groups. A hallmark of an agonist-induced EDHF-type response is its abolition by the combination of apamin and the nonselective K^+-channel blocker CBTX (Hilgers *et al.*, 2006;Crane *et al.*, 2003;Hinton & Langton, 2003). In the absence of myoendothelial gap junctions, VSM hyperpolarization can still occur due to either release of a diffusible EDHF such as EETs (Campbell *et al.*, 1996) or by elevations in intercellular K^+ levels (Edwards *et al.*, 1998). In the present study, combined apamin and CBTX completely inhibited vasodilation in response to ACh in both groups. However, since CBTX may block both IK_{Ca} and BK_{Ca} channels, we repeated these experiments with combined apamin plus the IK_{Ca} selective inhibitor TRAM 34 (Wulff *et al.*, 2000). Consistent with other reports (McSherry *et al.*, 2005;Hinton & Langton, 2003;Crane *et al.*, 2003;Hilgers *et al.*, 2006;Eichler *et al.*, 2003;Gluais *et al.*, 2005), this treatment abolished ACh-induced dilation in arteries from control rats suggesting a role of SK_{Ca} and IK_{Ca} channels in the EDHF-type response. In contrast, this treatment did not significantly inhibit dilation in the CH group, whereas BK_{Ca} channel inhibition with IBTX eliminated the response in this group. IBTX was without effect in control arteries as previously shown by others (Parkington *et al.*, 2002;Crane *et al.*, 2003;Hinton & Langton, 2003). These data again support the hypothesis that BK_{Ca} channels play an important role in endothelial cell physiology following CH not observed under control conditions.

In agreement with other studies (Sandow *et al.*, 2002;McSherry *et al.*, 2005), ACh stimulation elicited hyperpolarization in endothelial cells of both groups. Although the change in E_m in response to ACh did not differ between groups, peak ACh-stimulated E_m was more hyperpolarized in CH endothelial cells due to the shift in basal E_m in that group. Although ACh induced hyperpolarization in both groups, the response was SK_{Ca} / IK_{Ca} dependent in normoxic controls but BK_{Ca} dependent in arteries from CH rats. These findings in control arteries are in agreement with others who demonstrated that hyperpolarization in response to vasodilatory agonists is sensitive to SK_{Ca} (Siegl *et al.*, 2005;McSherry *et al.*, 2005;Weston *et al.*, 2005) and IK_{Ca} (Marrelli *et al.*, 2003;Weston *et al.*, 2005) inhibitors but not BK_{Ca} blockers (Siegl *et al.*, 2005). Thus, the reliance of ACh-induced endothelial cell hyperpolarization on BK_{Ca} channels following CH represents a switch from the normal involvement of IK/SK signaling in this response. This shift to a reliance on BK_{Ca} channels following CH was also observed when we examined the EC $[Ca^{2+}]_i$ response to ACh. Elevation of endothelial cell $[Ca^{2+}]_i$ following treatment with ACh occurs through multiple pathways. E_m may be a contributing influence on Ca^{2+} influx by affecting the electrochemical driving force through non-selective entry pathways (Kamouchi *et al.*, 1999). However, other studies suggest that intracellular Ca^{2+} may be independent of changes in membrane potential, as EC hyperpolarization *in situ* does not increase endothelial cell Ca^{2+} (Ungvari *et al.*, 2002;Ghisdal & Morel, 2001;Marrelli *et al.*, 2003;Takano *et al.*, 2004). Additionally, increased calcium influx following muscarinic stimulation has been found to occur independent of changes in membrane potential in freshly isolated endothelial cells (Cohen & Jackson, 2005).

Earlier studies showing that blunted myogenic responsiveness following CH is reversed by either endothelial removal or administration of IBTX (Earley *et al.*, 2003) made no attempt to differentiate between the roles of endothelial vs. VSM BK_{Ca} channels. In the present study, intraluminal administration of IBTX restored myogenic vasoconstriction and VSM E_m in arteries from CH rats to levels similar to controls. This treatment was without effect on myogenic reactivity in control arteries, although VSM E_m was slightly depolarized at 120 mmHg by IBTX in this group. Nevertheless, these experiments again suggest that endothelial hyperpolarization through BK_{Ca} channels is functionally important in regulating vascular tone following CH.

In support of studies in whole arteries, patch clamp experiments demonstrated greater outward current in cells freshly isolated from CH rats that was normalized by IBTX. These results closely correlate with *in situ* assessment of endothelial cell E_m in intact arteries and suggest that the difference in basal E_m following CH is due to enhanced tonic activity of BK_{Ca} channels. Interestingly, the BK_{Ca} channel activator NS1619 caused increased current only in cells from CH animals. This latter result could be evidence for differential expression of BK_{Ca} channels between groups, however we observed similar BK_{Ca} subunit immunofluorescence in endothelial cells from control and CH rats.

It has recently been documented that BK_{Ca} activity is negatively regulated by caveolin-1 in cultured endothelial cells (Wang *et al.*, 2005). Cholesterol depletion with MBCD to disrupt caveolin function resulted in greater IBTX-sensitive outward currents in cultured human umbilical vein endothelial cells (HUVECs) (Wang *et al.*, 2005). We hypothesized that CH exposure results in diminished caveolin-1 function thereby activating BK_{Ca}. Thus we treated ECs from control animals with the cholesterol depletion

agent MBCD possibly release EC BK_{Ca} channels from caveolin inhibition and mimic CH. We chose the concentration of 100 μM MBCD to affect caveolin function but not to eliminate caveolar structure. Previous studies using higher concentrations of MBCD in uterine myocytes ablated caveolar structure and additionally significantly decreased BK_{Ca} function (Shmygol *et al.*, 2007). Electron microscopy analysis of 100 μM MBCD treated cells revealed no significant changes in caveolar structure compared to untreated controls (Supplemental Figure 4) and CH (not shown). Interestingly, treatment with MBCD revealed an IBTX-sensitive outward current in ECs from control animals, thus supporting earlier evidence in cultured cells of negative regulation of endothelial BK_{Ca} by caveolin. This treatment did not further enhance currents in cells from CH rats. These data also support our observation of similar channel immunofluorescence in cells from control and CH rats. Thus, CH exposure may elicit channel activity through dissociation from caveolin-1. This is an interesting possibility since endothelial heme oxygenase-1 (HO-1) activity is similarly regulated by caveolin-1 (Kim *et al.*, 2004) and we have previously shown that HO inhibition restores reactivity in an IBTX-sensitive fashion in CH arteries (Naik & Walker, 2006;Naik & Walker, 2003). Thus, HO-derived CO may play a role in tonic activation of endothelial BK_{Ca} channels following CH. Future studies will focus on interactions between HO and caveolin-1 with endothelial BK_{Ca} channels as well as examination of the effects of CH on channel mRNA expression.

In summary, we have demonstrated a novel role of endothelial BK_{Ca} channels in vascular regulation following CH. Whereas these channels do not appear to be important in vasoreactivity in control arteries, CH exposure results in enhanced activity of

endothelial BK_{Ca} channels that may account for earlier observations of diminished constrictor reactivity in this clinically relevant setting.

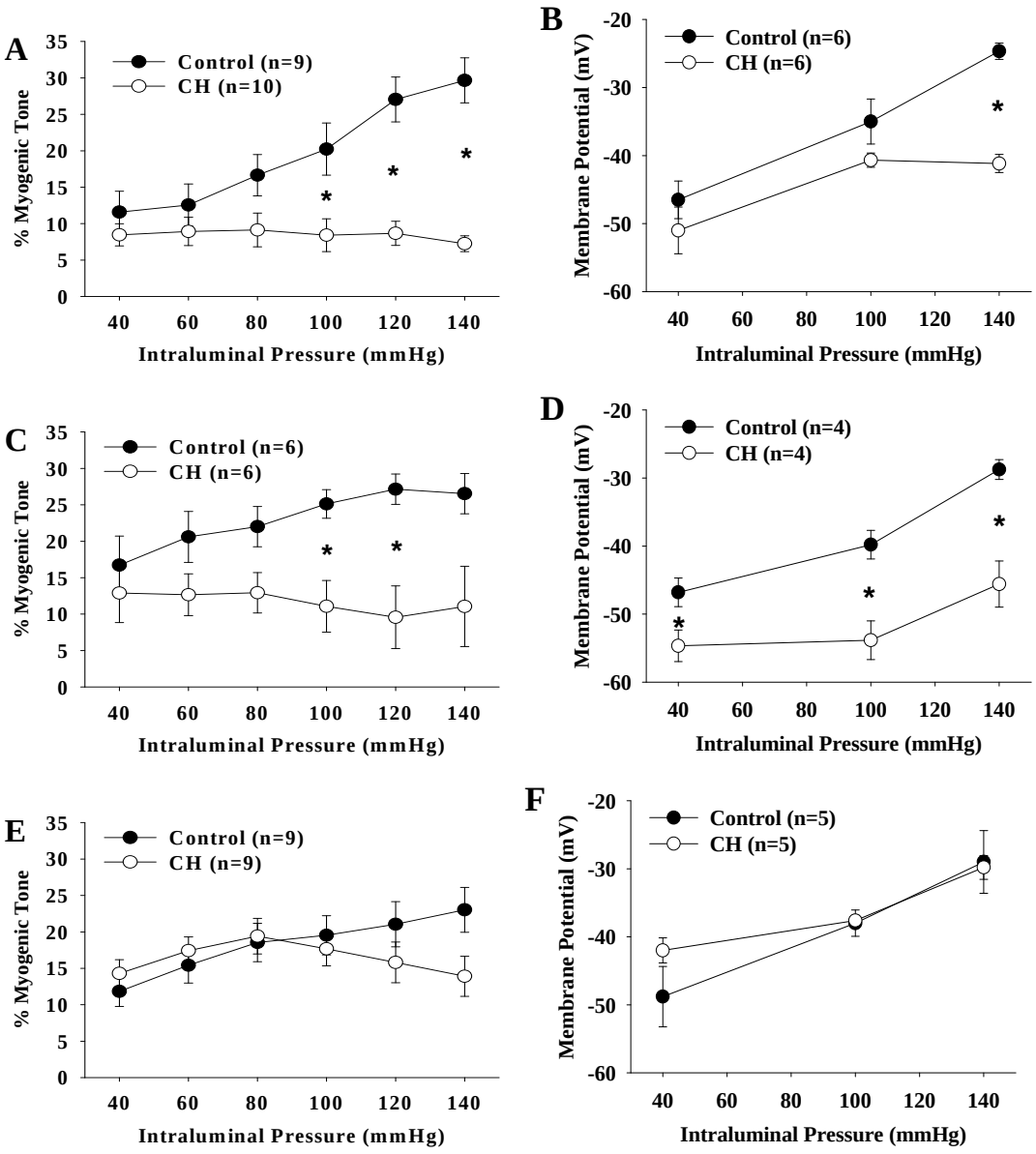


Figure 1: Myogenic tone (A, C, and E) and vascular smooth muscle (VSM) membrane potential (Em; B, D, and F) as a function of intraluminal pressure for endothelium-intact (A and B), NG-nitro-l-arginine (l-NNA) and indomethacin (Indo)-treated (C and D), and endothelium-disrupted (E and F) gracilis arteries isolated from normoxic control and chronic hypoxia (CH) rats.

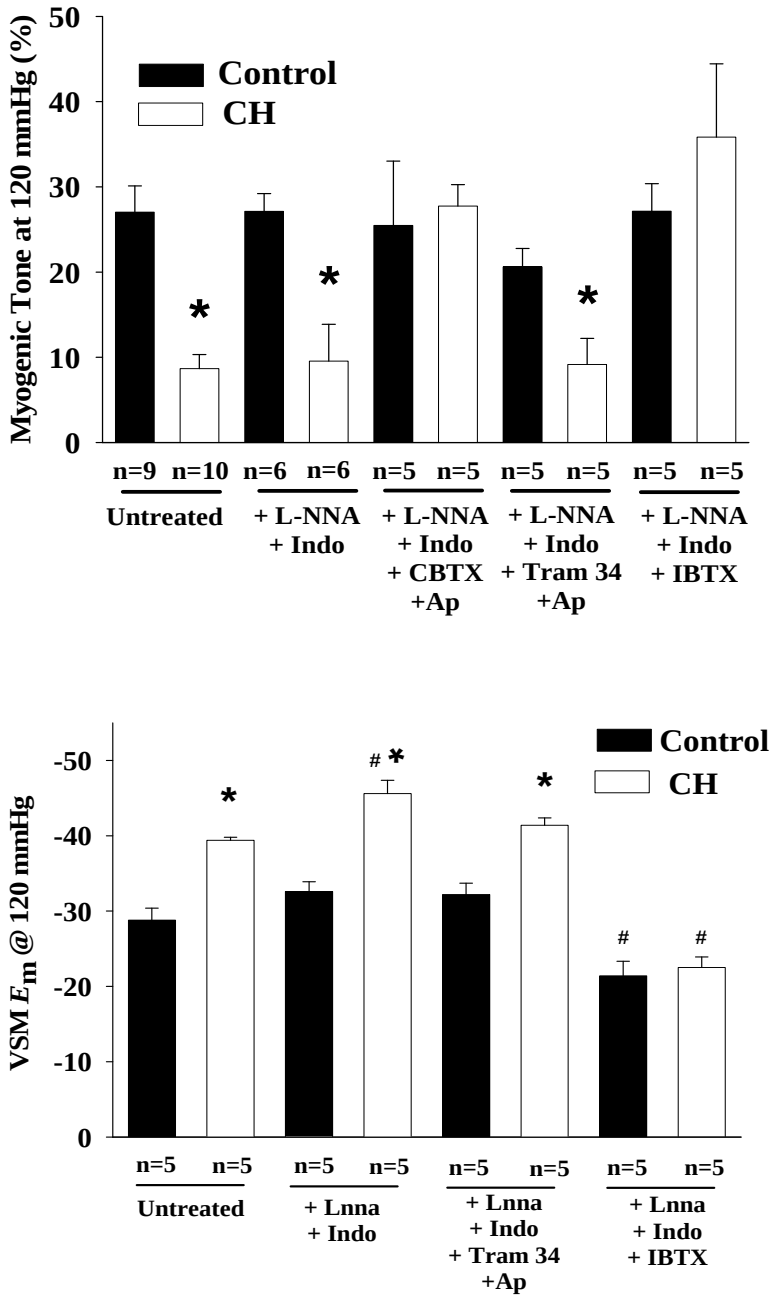


Figure 2: Myogenic tone (top) and VSM E_m (bottom) in endothelium-intact gracilis arteries isolated from normoxic control and CH rats at 120 mmHg from untreated, l-NNA and Indo-treated arteries, and arteries exposed to different combinations of intraluminal K^+ channel inhibitors.

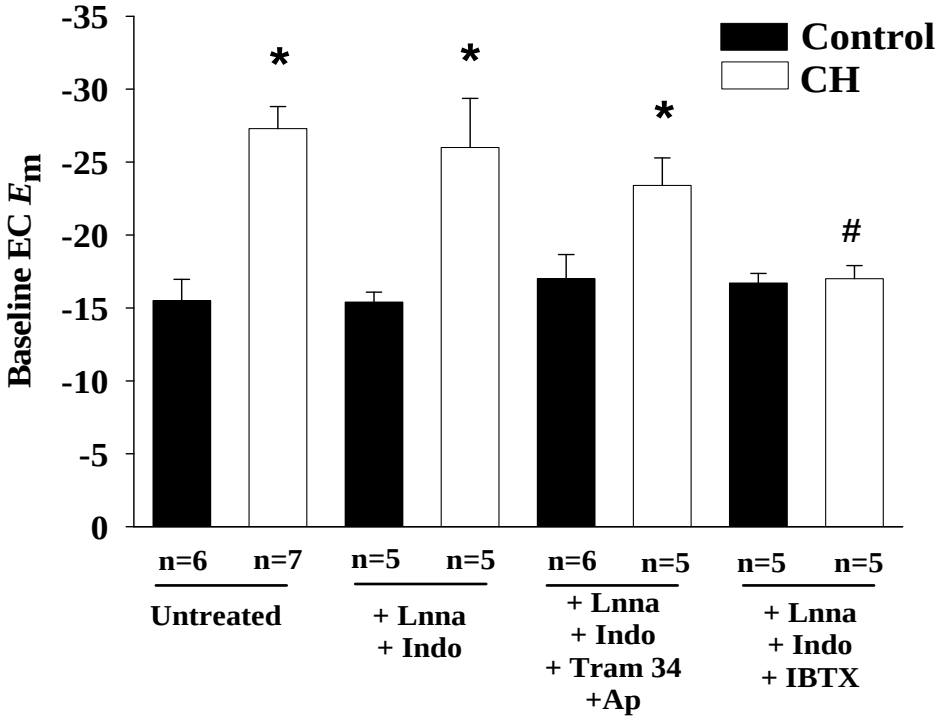


Figure 3: Endothelial cell (EC) E_m in gracilis artery strips isolated from normoxic control and CH rats. EC E_m was assessed in untreated, l-NNA and Indo-treated arterial strips, and arterial strips exposed to different combinations of K^+ channel inhibitors. Values are means ± SE; n, no. of rats. *P < 0.05, normoxic control vs. CH. #P < 0.05 for CH untreated vs. CH IBTX-treated strips.

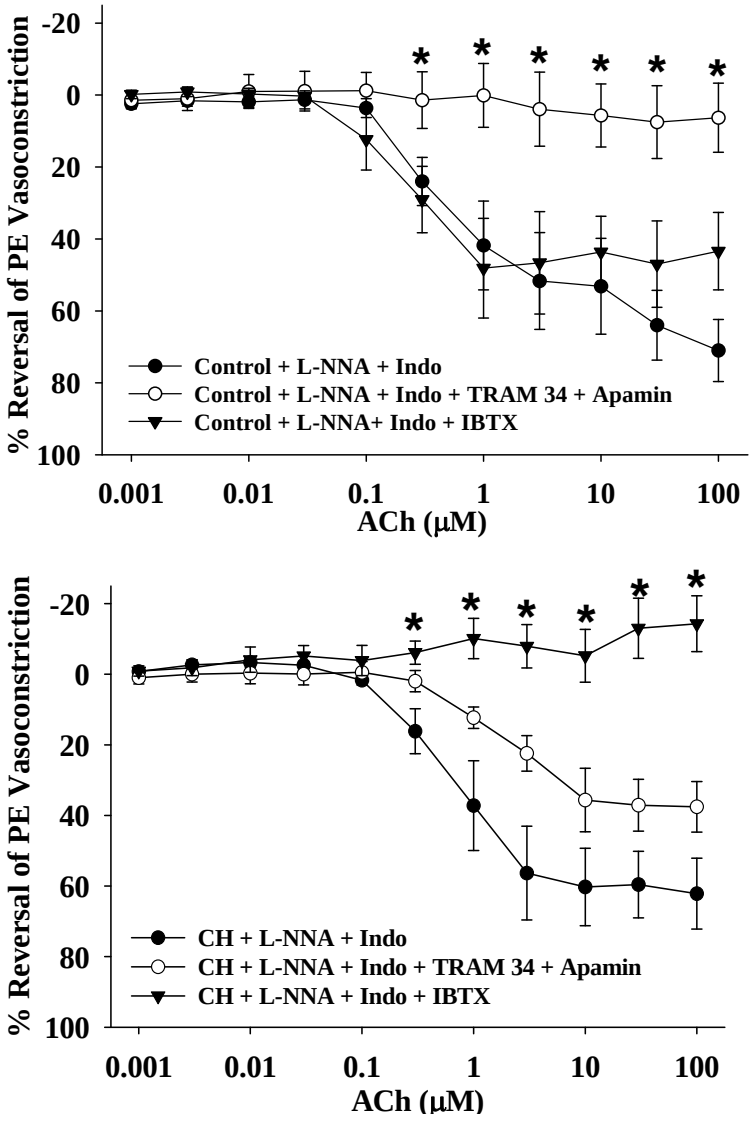


Figure 4: Concentration-response curves [% reversal of phenylephrine (PE) vasoconstriction] to acetylcholine (ACh) for control (A) and CH (B) gracilis arteries after treatment with l-NNA + Indo (n = 7 both groups), l-NNA + Indo + TRAM-34 + Ap (n = 4 control, n = 7 CH), or l-NNA + Indo + IBTX (n = 6 control, n = 5 CH). Values are means ± SE; n, no. of rats. *Differs from l-NNA + Indo for each group, $P < 0.05$.

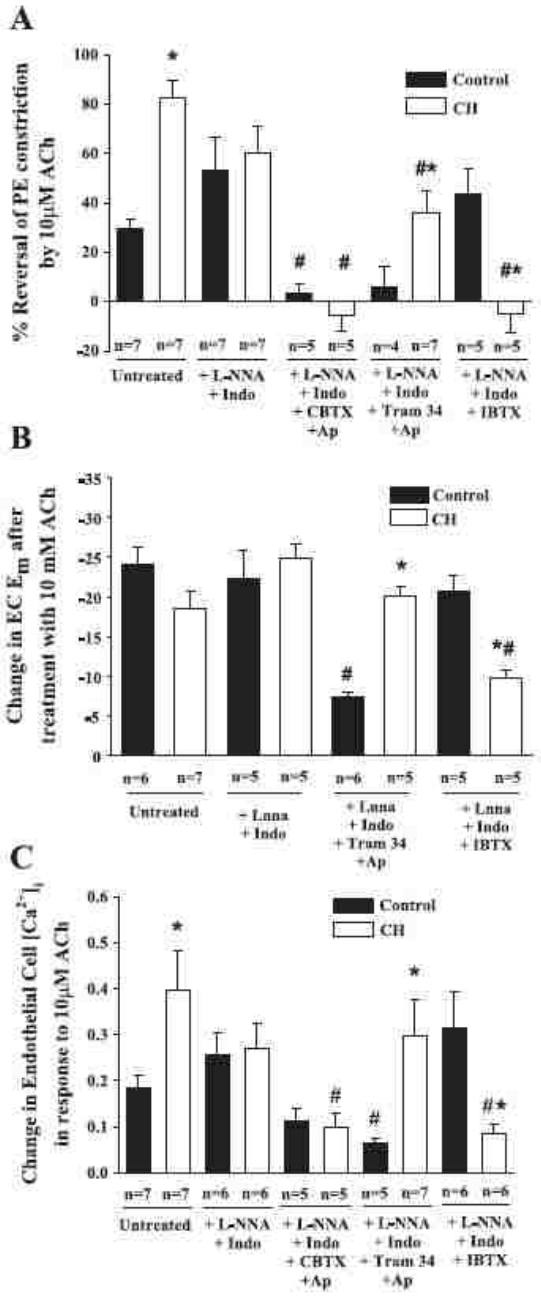


Figure 5: ACh-induced responses in gracilis arteries isolated from normoxic control and CH rats. Vasodilation responses (A), changes in E_m (B), and EC intracellular Ca^{2+} concentration ($[Ca^{2+}]i$; C) are shown in response to 10 μM ACh from untreated, l-NNA and Indo-treated arteries, and arteries exposed to different combinations of K^+ channel inhibitors. Values are means ± SE; n, no. of rats. *P < 0.05, normoxic control vs. CH. #P < 0.05, untreated vs. treated strips within groups.

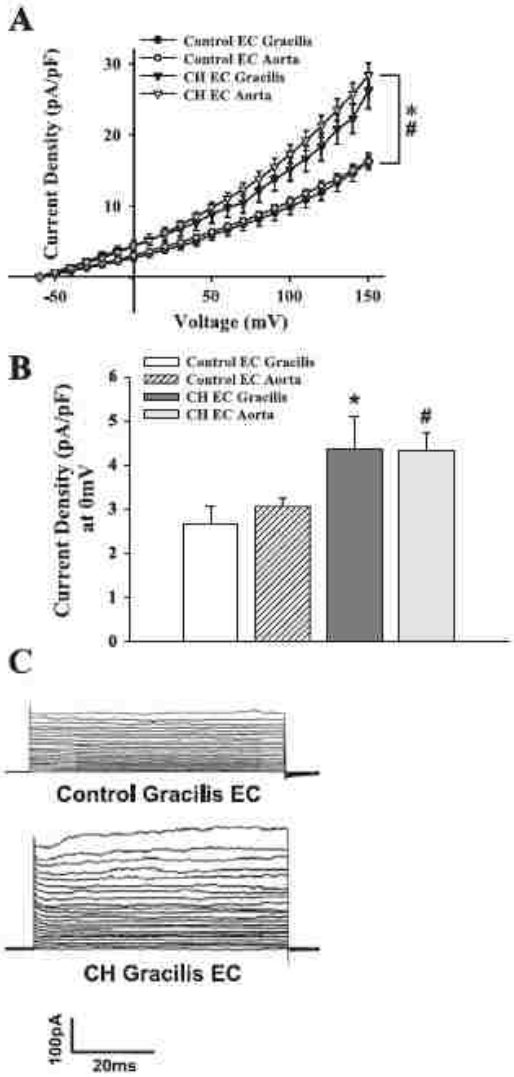


Figure 6: Whole cell K^+ currents from gracilis and aortic ECs from normoxic control (aortic: n = 10, gracilis: n = 9 cells) and CH rats (aortic: n = 9, gracilis: n = 5 cells). A: whole cell K^+ currents in both gracilis (*) and aortic (#) ECs from control rats were significantly less than CH at all voltage steps. B: currents at a physiologically relevant potential of 0 mV (*different from gracilis control; #different from aortic control). Currents were not different between aortic or gracilis ECs within groups (A and B). Values are means ± SE. C: gracilis EC voltage step traces from control and CH treatments.

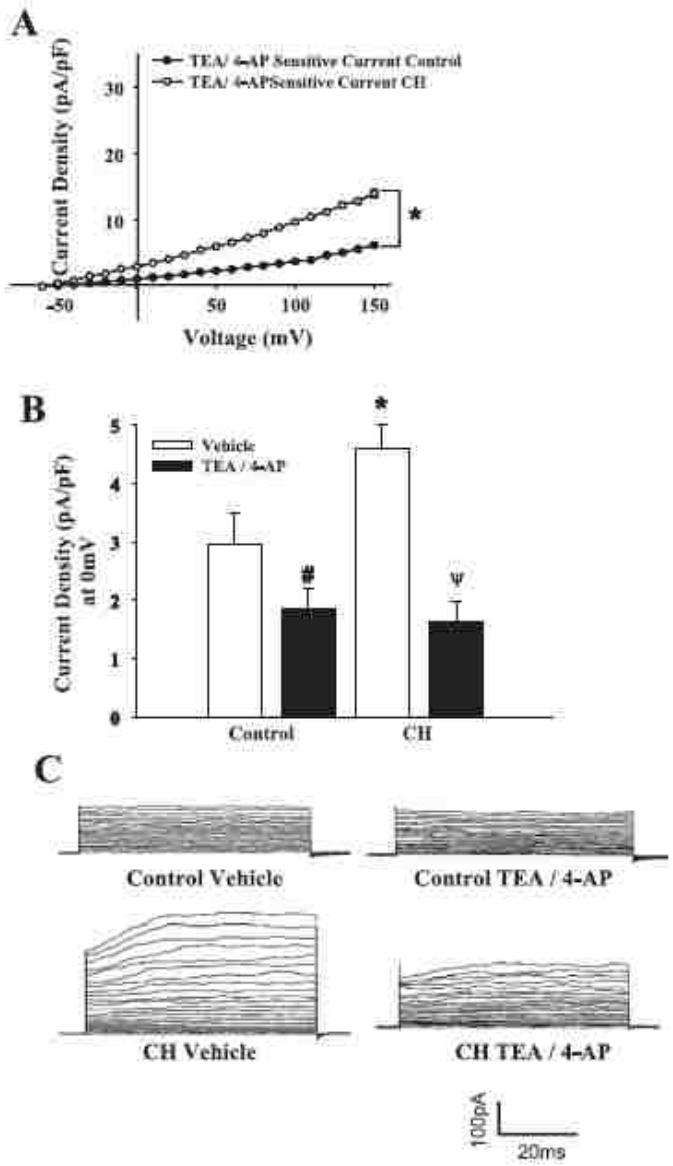


Figure 7: Administration of K^+ channel inhibitors tetraethylammonium (TEA; 10 mM) and 4-aminopyridine (4-AP; 10 mM) reduced total whole cell K^+ currents (n = 5 cells, controls and CH). A: current subtraction data showing that TEA/4-AP-sensitive transmembrane currents were greater in the CH group compared with control at all voltage steps. B: data at the test potential of 0 mV, where currents were normalized by TEA/4-AP between groups. Values are means ± SE. *P < 0.05, CH vs. normoxic control. #P < 0.05, control TEA/4-AP vs. control untreated. ψP < 0.05, CH TEA/4-AP vs. CH untreated. C: voltage step traces from control and CH groups with vehicle or TEA/4-AP.

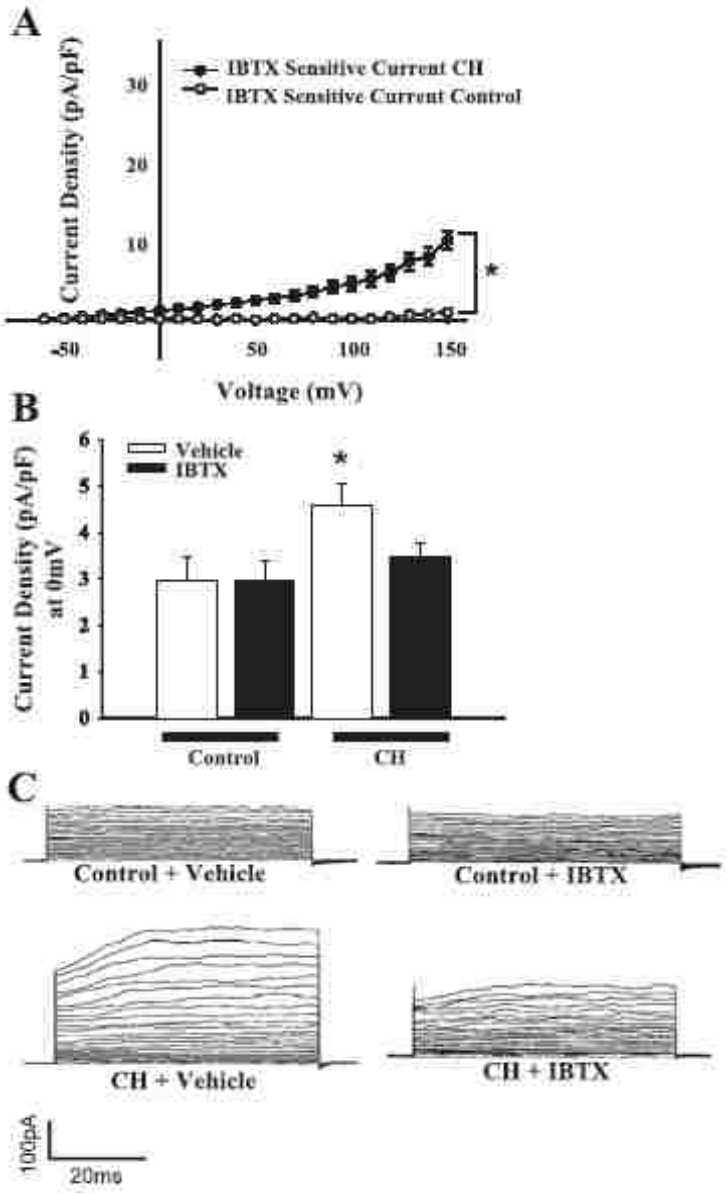


Figure 8: Effects of large-conductance, calcium-activated potassium (BK_{Ca}) channel inhibition in aortic ECs. IBTX (100 nM) diminished whole cell K^+ current in CH (n = 9) cells to control levels, but had no effect on control (n = 8) cells. A: IBTX-sensitive current over the entire range of voltage steps derived by current subtraction. B: data at the test potential of 0 mV. Values are means ± SE. *P < 0.05, CH vs. normoxic control. C: traces from voltage steps with vehicle and IBTX treatments.

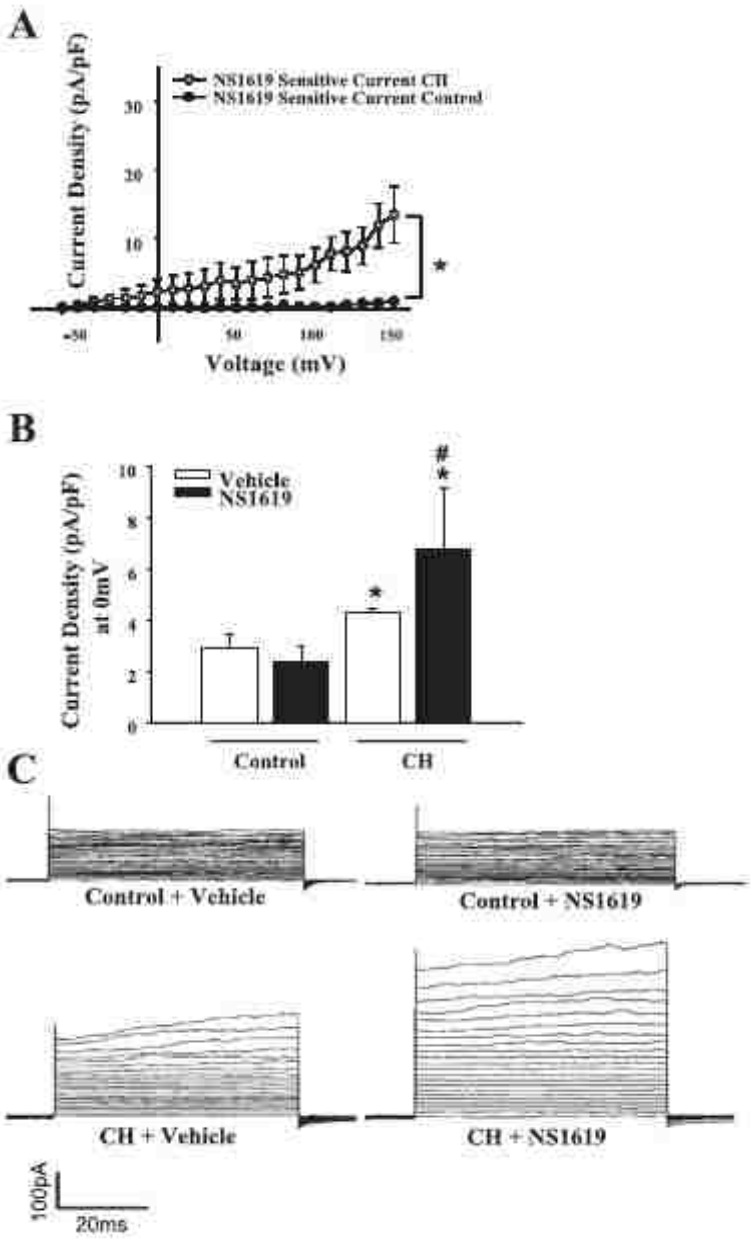


Figure 9: A: the BK_{Ca} activator NS1619 was without effect in control cells (n = 3), but increased outward current in cells from CH rats (n = 4). B: comparisons between groups were conducted at the testing potential of 0 mV. Values are means ± SE. *P < 0.05, CH vs. normoxic control. #P < 0.05, different from untreated CH. C: traces in the presence of vehicle or NS1619.

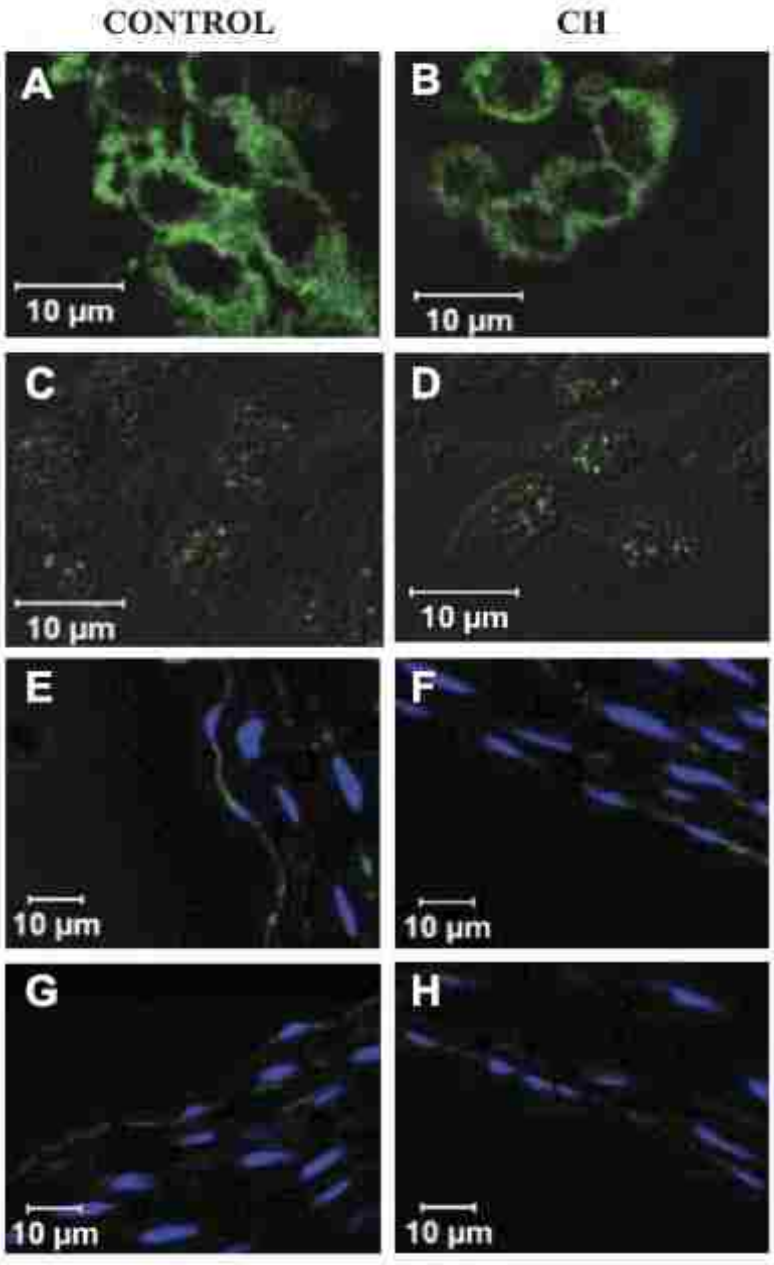


Figure 10: Positive immunofluorescence for BK_{Ca} α-subunit (BK α) and β1-subunit (BK β1) in freshly dispersed ECs and gracilis cross sections from control (left) and CH (right) rats. A–D: positive staining for BK α (A and B) and BK β1 (C and D) in aortic ECs from control and CH rats, respectively. E–H: positive staining for BK α (E and F) and BK-β1 (G and H) in gracilis cross sections from control and CH rats, respectively.

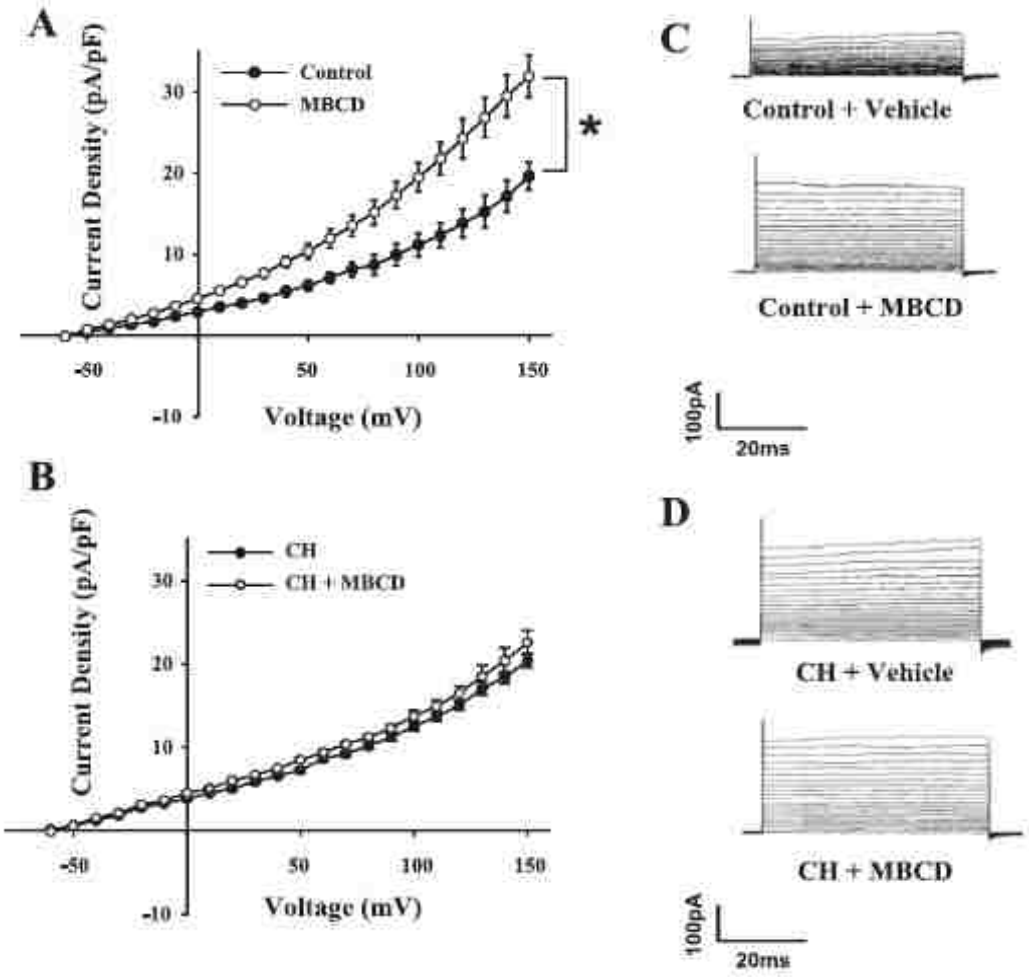


Figure 11: Cholesterol depletion with methyl β-cyclodextrin (MBCD) increased K^+ currents in ECs from control animals (A) with no effect on cells from CH rats (B). Values are means ± SE. C: traces from control cells before and after treatment with MBCD. D: traces from CH animal ECs before and after treatment with MBCD. *P < 0.05, MBCD vs. control.

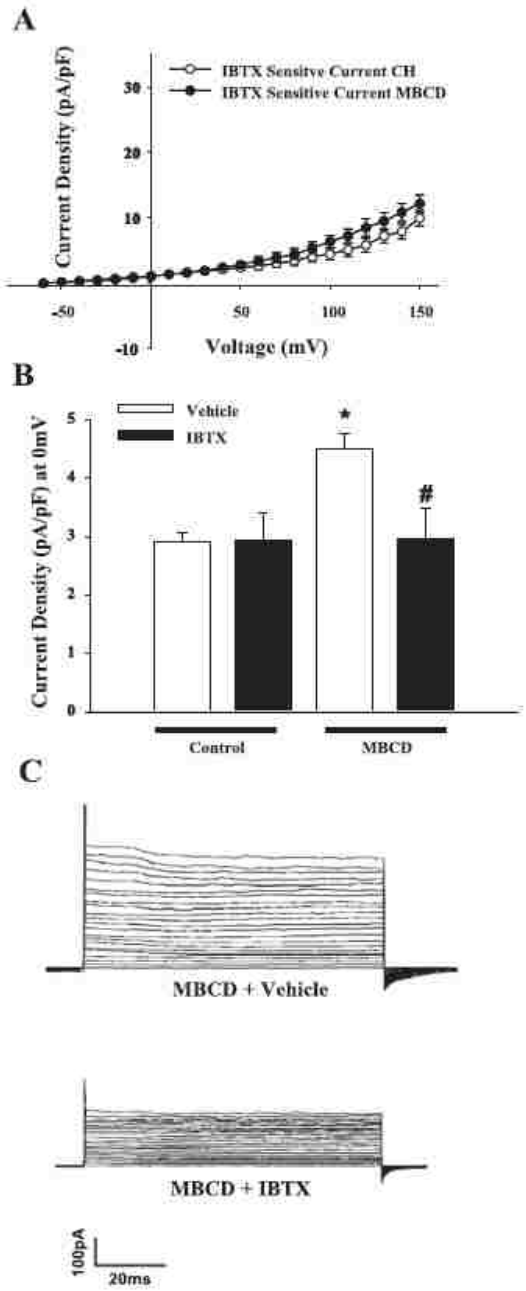


Figure 12: A: the increased current in MBCD-treated control ECs was sensitive to IBTX (100 nM) and demonstrated similar magnitude to IBTX-sensitive current observed in CH ECs. B: normalization of outward current by IBTX in MBCD-treated control cells at a physiologically relevant potential of 0 mV. Values are means ± SE. *P < 0.05, MBCD vs. control. #P < 0.05, MBCD IBTX vs. MBCD. C: traces from MBCD-treated cells in vehicle or IBTX.

SUPPLEMENT: Novel Role of Endothelial BK_{Ca} Channels In Altered Vasoreactivity Following Hypoxia

Supplemental Methods

T cell isolation and staining: Negative immunofluorescence controls were performed on cells that do not express BK_{Ca} channels. Whole blood was fixed in 16% formaldehyde and centrifuged. The supernatant was removed and the sample was re-suspended in 50% MeOH in PBS. Small aliquots of the sample were then dried on slides, permeabilized, and blocked in donkey serum. Samples were then treated with anti-CD3 (1:100); a T-cell marker, BK α (1:100) or BK β1 (1:100). Primary antibodies were detected with fluorescently labeled secondary antibodies and nuclei were labeled with Hoechst 33342. All images were collected with a confocal microscope using a 63x oil immersion objective.

Electron microscopy of rat aortic endothelial cells: To validate that the methyl-β-cycodextrin (MBCD) concentration (100 μM) chosen to mildly deplete membrane cholesterol did not drastically impact caveolar structure, electron microscopic images were obtained in control tissue and tissue subjected to cholesterol chelation. Aortic sections were fixed for 2 hours at room temperature in 2% formaldehyde + 3% glutaraldehyde in 0.1 M Na cacodylate, pH 7.4. Tissues were washed in 0.1 M cacodylate and treated for 1 hour at room temperature in 1% osmium-potassium ferrocyanide in 0.1 M cacodylate, followed by washes in 0.1M cacodylate and then in Barnstead H_2O. Tissues were then treated *en bloc* with 1% uranyl acetate (aq), for 1 hour at room

temperature and washed with Barnstead H_2O, dehydrated through a graded series of ethanol to 100%, infiltrated and embedded in Epon-Araldite resin (cured at 65ºC). Sections were cut 70-90 nm thick, mounted on Cu grids, stained with uranyl acetate and lead citrate, and examined on a Hitachi H7500 TEM at 80kV. Digital images were taken on a CCD camera (AMT XR60).

Effect of Nitric Oxide on BK_{Ca} Channel Activity in Isolated Endothelial Cells. As further confirmation of the activity of endothelial BK_{Ca} channels following CH, whole cell patch clamp experiments were performed in the presence and absence of a donor of nitric oxide (NO), a known activator of the channel (Bolotina *et al.*, 1994). After cell dialysis, recordings were taken before and after 5 min of superfusion with the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) (100 μM) in aortic endothelial cells from control and CH rats.

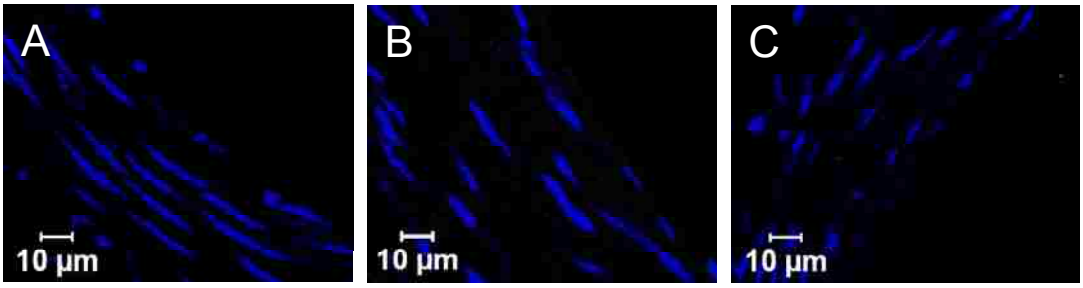
Supplemental Results

Validation of Specificity of BK_{Ca} Channel Immunofluorescence. Additional experiments validated the specificity of immunofluorescence for BK α and BK β1. Supplemental Figure 1A shows the lack of staining in the presence of only the secondary (no primary) antibody in gracilis arterioles. Furthermore, blocking peptides for each primary antibody prevented staining (Supplemental Figure 1 B, C). In addition, the endothelial cell localization of BK α was confirmed in sections of gracilis arterioles stained for both BK α and the endothelial cell specific marker PECAM-1 (Supplemental Figure 2) as described in the Methods section of the paper. As a final determination of specificity of the BK α and BK β1 primary antibodies, we examined staining in isolated rat T-cells that do not express the channel. Supplemental Figure 3 shows the lack of BK α or BK β1

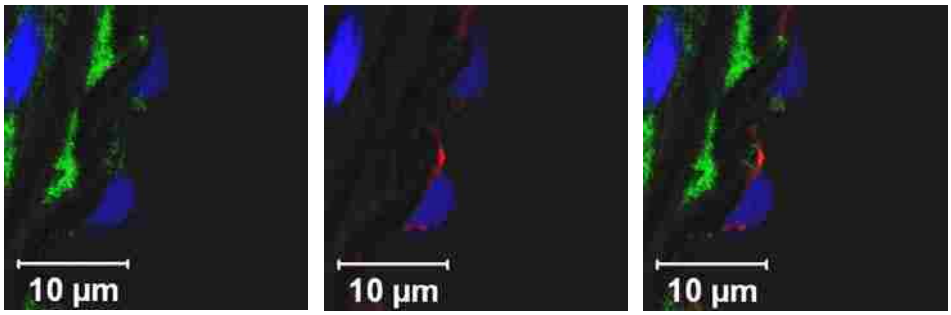
immunofluorescence in these cells, whereas they stain positive for the T-cell marker CD3. The same secondary antibody was used for all images.

Lack of Effect of MBCD on Gross Structure of Caveolae. A dose of MBCD (100 μM) was chosen that unmasked endothelial BK_{Ca} channels activity in control cells, but was much lower than the 10 mM concentration used by others in vascular preparations (Linder *et al.*, 2007;Linder *et al.*, 2005). Following administration of this lower dose of MBCD, no apparent differences were detectable in caveolae number or structure from untreated controls. CH treatment additionally did not result in an apparent reduction in caveolae number or structure. However, a 10mM concentration of MBCD eliminated EC caveolae (Supplemental Figure 4).

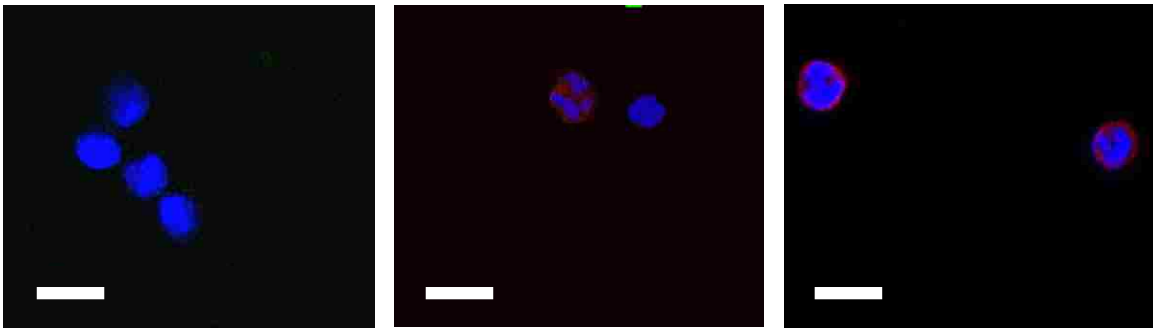
Effect of Nitric Oxide on BK_{Ca} Channel Activity in Isolated Endothelial Cells. Administration of the NO donor SNAP elicited a further increase in outward current in aortic endothelial cells from CH but not control rats (Supplemental Figure 5). NO has been identified as an activator of BK_{Ca} channels (Bolotina *et al.*, 1994) and thus this observation confirms their presence in endothelial cells from CH rats. Our other data showing that MBCD treatment unmasks channel activity in cells from control animals suggests that the channels are normally inhibited by likely association with caveolin-1 (Wang *et al.*, 2005) that is inhibition is removed following CH, thus permitting activation by NO.



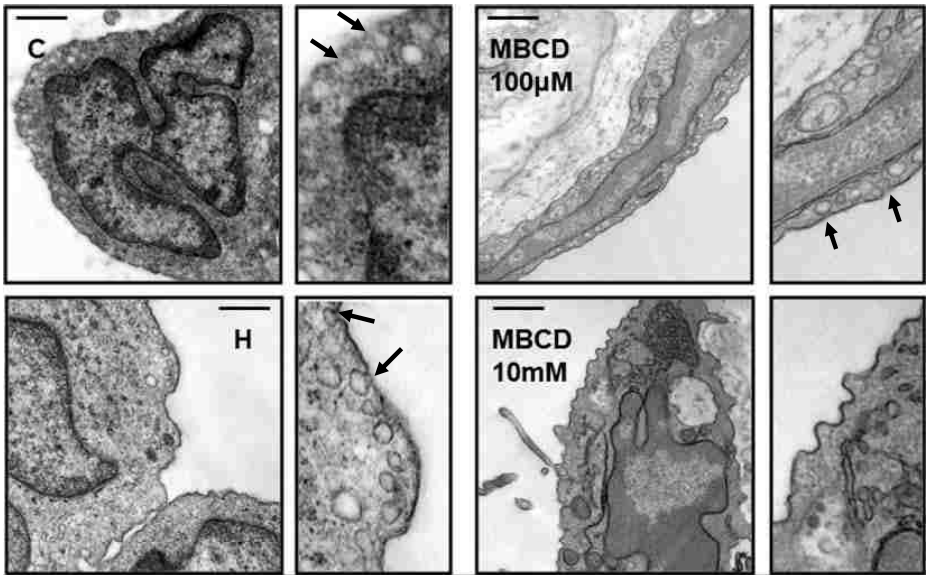
Supplemental Figure 1: Gracilis arteriole cross sections were treated with secondary antibodies and no primary antibodies to confirm lack of staining in the absence of primary antibody. (panel A). Additional cross sections were treated with BK α (panel B) or BK β1 (panel C) primary antibodies plus respective blocking peptides to confirm primary antibody specificity.

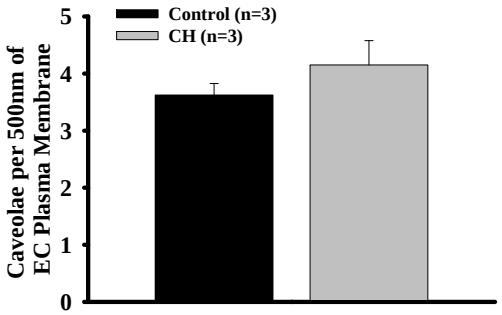


Supplemental Figure 2: BK α and PECAM-1 primary antibodies demonstrated endothelium specific staining. Cross sections were stained for BK α (green) and the endothelial marker PECAM-1 (1:100) (red). PECAM-1 staining appeared only in endothelial cells (middle and right panels), while BK α staining appeared in vascular smooth muscle cells and endothelial cells in the cross sections (left and right panels). Nuclei are blue.

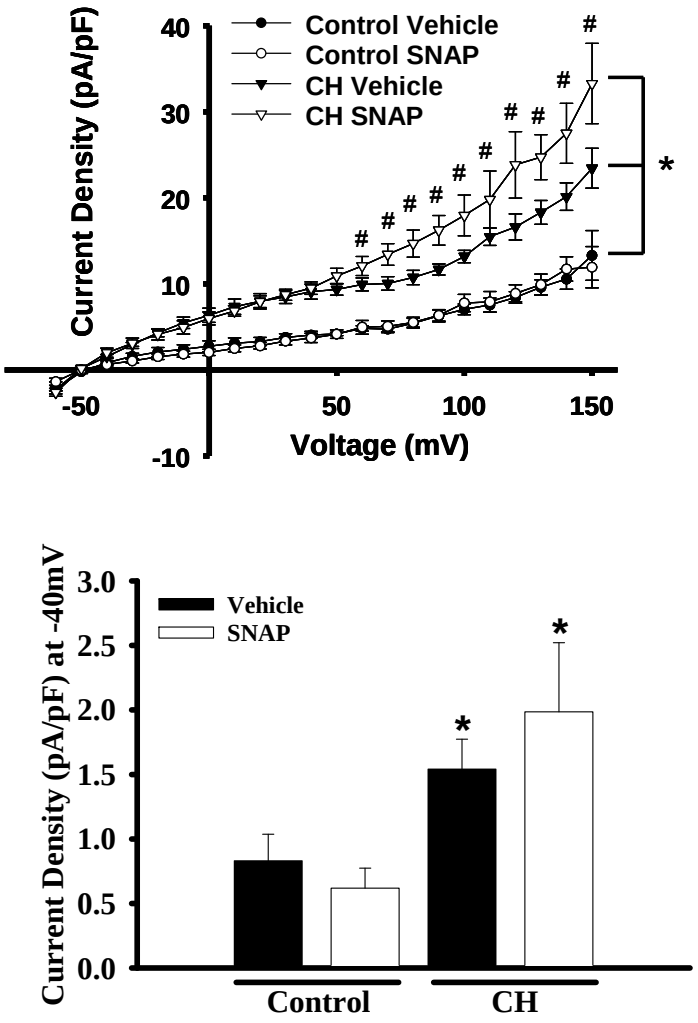


Supplemental Figure 3: Isolated leukocytes (T cells) from control rats were treated with primary antibodies to BK α as a negative control, since these cells do not express BK channels. Slides were stained for BK α (middle panel) or BKβ1 1:100 (right panel), the T-cell marker CD3 (red), and a nuclear stain (blue). No BK α or BK β1 staining appeared in leukocytes, in contrast to positive staining in vascular smooth muscle cells and endothelial cells in figure 8 and supplemental figure 2. Scale bar = 10 μm.





Supplemental Figure 4: Electron microscopy images of endothelial cells from control gracilis arterioles treated with vehicle, 100 μM or 10mM methyl β-cylcodextrin (MBCD). Treatment with MBCD did not eliminate caveolar structures at the 100 μM concentration (top panel), however the 10mM MBCD concentration eliminated caveolae. CH treatment did not alter caveolar presence or apparent structure compared to normoxic controls (bottom panel). The 100μM MBCD treatments elicited increased outward K^+ currents from control ECs (Fig. 11a) with no significant effect on outward currents in ECs from CH animals (Fig. 11b). Interestingly, putative reduction in cellular cholesterol by MBCD treatment without ablation of caveolae enabled EC BK_{Ca} channel activity in controls to a level similar to that found in CH animals (Figure 12A: IBTX-sensitive currents) Scale bar = 500 nM, images at 15000x.



Supplemental Figure 5: Exogenous nitric oxide (NO) increased outward K^+ currents in ECs from CH animals (n=6 cells) but had no effect on currents in controls (n=4 cells) (top panel). Current density at -40mV was significantly larger from ECs from CH animals (bottom panel) and exogenous NO from the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) 100μM increased outward current only in ECs from CH animals. *p<0.05 CH vs. control vehicle, #p<0.05 CH SNAP vs. CH vehicle (+70mV through +150mV).

ADDENDUM AIM 1: Determining the Expression and Activity of Endothelial BK_{Ca} Channels Following Chronic Hypoxia

Experiments detailed in "The Novel Role of Endothelial BK_{Ca} Channels in Altered Vasoreactivity Following Hypoxia" were designed to address objectives in Aim 1 of this project. Additional experiments that specifically address Specific Aims 1.1, 1.2, and 1.4 that were not included in the publication are detailed below.

Specific Aim 1.1: *Investigate EC BK_{Ca} activity following CH through whole cell patch clamp.*

Initial experiments in freshly dispersed ECs measured significantly greater outward K^+ currents in cells from CH animals versus controls. To identify the source of the increased currents, we treated ECs with TEA and 4-aminopyridine to block all K_{Ca} and K_V activity, respectively. Blockade of K_{Ca} currents significantly decreased outward currents and brought the two groups together, demonstrating the importance of K_{Ca} channels in total EC currents. Three K_{Ca} channels have been identified in the vascular endothelium; small (SK_{Ca}), intermediate (IK_{Ca}), and large calcium-activated potassium channels (BK_{Ca}). Due to the importance of SK_{Ca} and IK_{Ca} in ECs, which has suggested small and intermediate conductance calcium-activated potassium channels have a predominant role regulating EC E_m (Ledoux *et al.*, 2008) we examined total currents in the presence of the SK_{Ca} inhibitor, apamin and the IK_{Ca} inhibitor, TRAM-34 in freshly dispersed ECs from controls and CH animals.

Interestingly, administration of TRAM-34 and apamin did not normalize outward currents in CH ECs to levels of controls, (**Addendum Figure 1**) in contrast in TEA administration. These results led us to investigate if blockade of BK_{Ca} activity in CH ECs would normalize currents. It was determined that outward K^+ currents in freshly isolated ECs only from CH animals demonstrated sensitivity to IBTX, the BK_{Ca} specific inhibitor, which normalized currents. IBTX subtraction currents demonstrated non-ohmic increases in conductance, suggesting voltage sensitivity of the channel and further supporting our claim of the IBTX sensitive currents in ECs to be derived from BK_{Ca}. Another important validation of BK_{Ca} currents is investigation of the currents sensitivity to intracellular calcium. Conditions utilized in whole cell patch clamp contained 1μM free Ca^{2+}, an amount of calcium that is significantly higher than reported endogenous levels (Hu & Ziegelstein, 2000;Wickham *et al.*, 1998), that have been demonstrated to maximally activate BK_{Ca} channels. To determine if endogenous calcium levels were sufficient enough to activate EC BK_{Ca} channels we performed perforated patch clamp experiments in the freshly isolated rat aortic endothelial cells from normoxic and hypoxic animals and compared IBTX sensitive currents to studies conducted in whole cell patch clamp (**Addendum Figure 2**). To further characterize calcium sensitivity in EC BK_{Ca} channels, we designed patch clamp experiments with the xeno-estrogen derivative Tamoxifen, which increases BK_{Ca} activity with the presence of active β subunits, or decreases currents when only α subunits of the channel are functional (Dick *et al.*, 2001;Liu *et al.*, 2003;Duncan, 2005) (**Addendum Figure 3**) Calcium sensitivity in smooth muscle cells is largely dependent upon accessory β subunits, and their role in ECs has not been determined.

Patch Clamp Studies on Isolated Endothelial Cells: Endothelial cells were dispersed for electrophysiological study from control and CH rat aortae as previously described (Hughes *et al.*, 2010). For experiments utilizing conventional whole cell patch clamp configuration, biophysical criteria: (seal resistance > 1GΩ, series resistance <25MΩ) was checked following membrane rupture and monitored throughout the course of the experiment. Cells were held at -60mV and were dialyzed for 5 min with an intracellular solution (ICS: 140 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 5 mmol/l Mg_2ATP, 10 mmol/l HEPES, 1 mmol/l EGTA and adjusted to pH 7.2 with KOH). $CaCl_2$ was added to yield a free-Ca^{2+} concentration of 1 μM, as calculated using WinMAXC chelator software.

For experiments utilizing the perforated patch technique, 4-6 MΩ patch electrodes were backfilled with amphotericin B. After gigaseals were obtained, the series resistance fell over a 10- to 15-min period to 15–20 MΩ and remained stable for up to 1 h. ECs with stable series resistances under 25 MΩ were used for experiments.

Determination of the Contribution of SK_{Ca} and IK_{Ca} to EC Outward Currents

Currents in freshly dispersed ECs from control and CH animals were dialyzed for 5 minutes to wash out intracellular contents and total outward were measured in the presence of the vehicle, after 5 minutes of treatment with apamin, and lastly after 5 minutes with TRAM-34 and apamin. Total outward currents were compared for each group following treatments with K_{Ca} inhibitors.

Activity of EC BK_{Ca} in the Presence of Endogenous Intracellular Calcium or 1μM Free Calcium: Freshly dispersed ECs were dialyzed for 5 minutes to wash out intracellular contents and total outward currents were measured in the presence of vehicle, and after a

5 minute treatment with IBTX. ECs in perforated patch clamp mode currents were measured in vehicle conditions and following 5 minute treatment with IBTX. Outward currents were compared in each group between vehicle and IBTX treatments.

Determining the Role of β Subunits in EC BK_{Ca}: Currents in freshly dispersed ECs currents were measured following treatment with tamoxifen (1 μM) for 10 minutes. The effect of tamoxifen treatment in ECs was additionally compared to the actions of the drug in freshly isolated VSMCs, which have been demonstrated to have active β subunits.

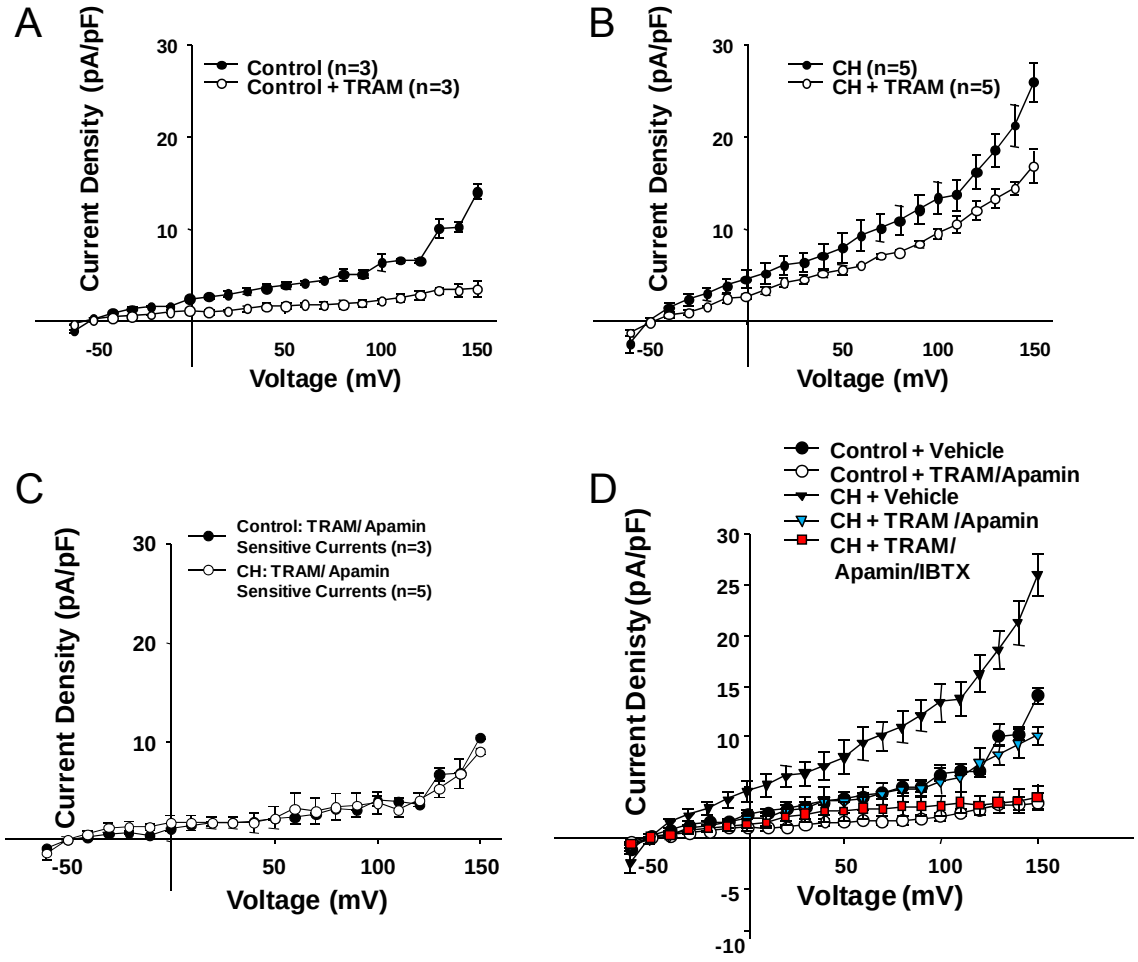
Results:

SK_{Ca} and IK_{Ca} Currents Contribute Largely to Total Outward Currents in ECs; Difference in CH vs. Control Currents Persists Following SK_{Ca} and IK_{Ca} Blockade: Blockade of SK_{Ca} and IK_{Ca} channels in ECs significantly decreased outward currents (**Addendum Figure 1**), however the residual current after SK/IK blockade was still significantly higher in CH than controls. Administration of IBTX to block EC BK_{Ca} had no effect in controls, but normalized currents to levels of controls in CH ECs. These results support a role for all K_{Ca} channels in EC function following CH, and a role only for SK/IK channels in normoxic conditions.

Channel Activity in the Presence of Endogenous Intracellular Calcium: Experiments in whole cell patch clamp and perforated patch clamp modes were not different in the magnitude of IBTX sensitive currents. Suggesting that endogenous levels of calcium are sufficient to drive channel activity in the absence of supra-physiologic calcium.

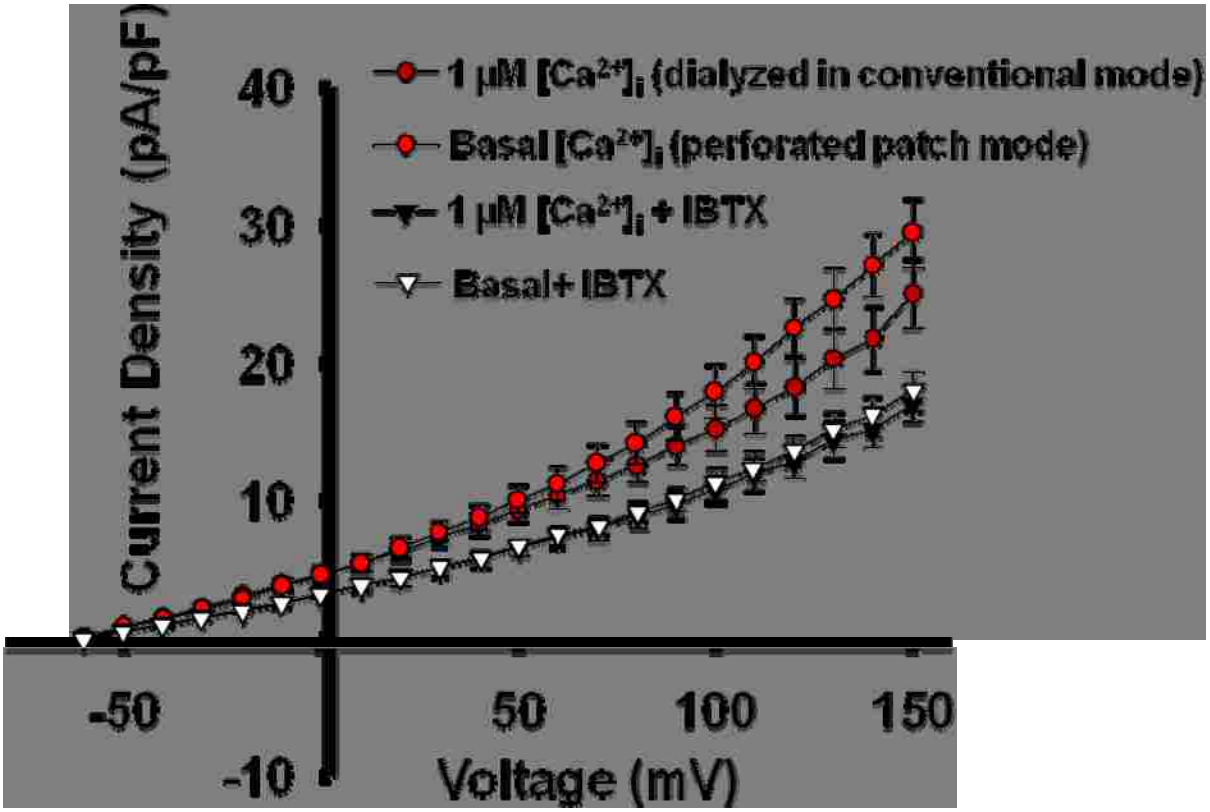
The Effect of Tamoxifen on ECs: Tamoxifen treatment decreased outward currents in CH ECs whilst increasing outward currents in VSMCs. These results suggest that β subunits, if present are not active in ECs. Immunofluorescence experiments localized the subunits to the endothelium, however these results demonstrate they are not important in calcium sensitivity in ECs in contrast to their important role in VSMCs.

CHAPTER 2 ADDENDUM: SPECIFIC AIM 1.1 – FIGURE 1



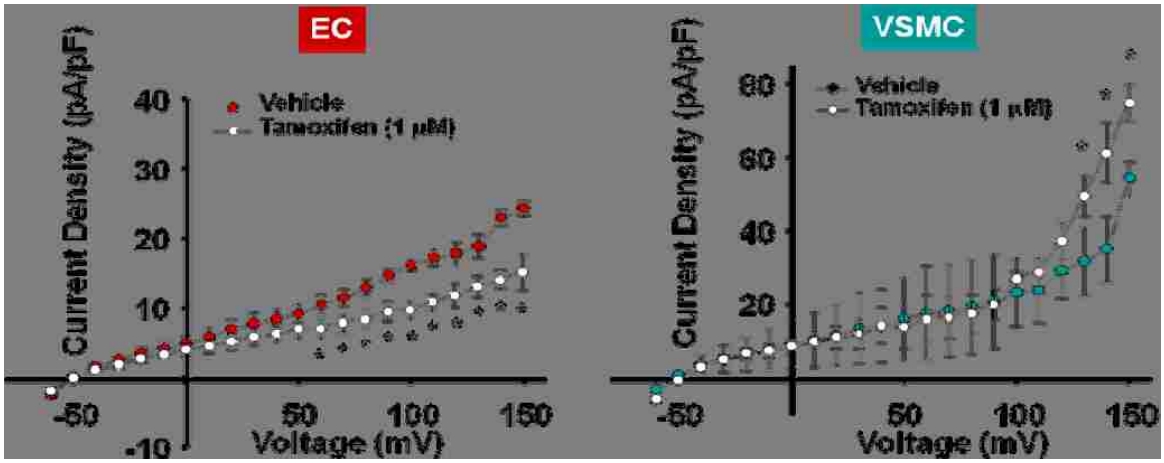
Addendum Figure 1: Administration of the IK_{Ca} inhibitor TRAM-34 (100µM) decreased currents in control ECs (**Panel A**) and CH ECs (**Panel B**). Results with the SK_{Ca} inhibitor, apamin had similar effects in both groups and subtraction currents from SK_{Ca} and IK_{Ca} were not different amongst groups (**Panel C**). Blockade of SK_{Ca} and IK_{Ca} almost eliminated outward currents in controls (**Panel D**). TRAM-34 and apamin (100nM) did not normalize current differences between CH and controls, however following the addition of IBTX to block BK_{Ca} to the cocktail of inhibitors currents were normalized (**Panel D**). These results demonstrate that SK/IK_{Ca} channels play a role in EC outward currents, with BK_{Ca} emerging following CH.

CHAPTER 2 ADDENDUM: SPECIFIC AIM 1.1 – FIGURE 2



Addendum Figure 2: K^+ currents in ECs dialyzed with 1 μM $[Ca^{2+}]_i$ or studied in perforated patch mode. BK inhibition with IBTX significantly reduced outward currents in both preparations. Data ± SEM. * $p < 0.05$ vs. untreated (all test potentials) n = 10-15 cells

CHAPTER 2 ADDENDUM: SPECIFIC AIM 1.1 – FIGURE 3



Addendum Figure 3: Whole cell K^+ current responses to tamoxifen (β1 agonist / BK α inhibitor) in VSMCs and ECs . Data ± SEM. * $p < 0.05$ vs. vehicle. (n = 5-7 cells)

Specific Aim 1.2: *Characterize EC BK_{Ca} channels through single channel records.*

Whole cell current experiments detailed above strongly support the presence and activity of EC K_{Ca} channels. To further support whole cell patch clamp data we performed experiments to determine unitary conductance for SK/IK/ EC BK_{Ca}. Channel frequency in isolated patches was measured for each channel in whole-cell cell attached and inside-out configurations. For BK_{Ca}, unitary conductance and channel opening (NPo) in the presence of varying levels of intracellular calcium and voltage were measured and contrasted to VSM BK_{Ca}. Changes in NPo following treatment withthe channel inhibitor, IBTX, were additionally conducted.

Single Channel Recordings. For single channel experiments, cell attached and inside-out patch clamp configurations were used. Patch pipettes had resistances ranging from 6 to 8 MΩ. Extracellular solutions (ECS) contained: 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 Glucose, 1.2 $CaCl_2$ (pH 7.30 with NaOH). Intracellular solutions (ICS) contained (in mM): 130KCl, 2 Na_2ATP, 3 $MgCl_2$, 10 HEPES, 1 EGTA, (pH 7.30 with KOH). For inside-out patch experiments ECS contained: 140 KCl, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$ (pH 7.3 with KOH). For experiments investigating the effect of calcium concentration on single channel opening probability (N_{po}) on inside out patches EGTA and free Ca^{2+} ($CaCl_2$) were varied to provide 10 nM, 50 nM, 100 nM, 1 μM, and 10 μM solutions. Patches were held at -60, 0, and 40 mV test potentials for voltage sensitivity experiments. Single channel unitary conductance was determined from the slope of currents measured in response to voltage steps applied from -100 mV to 150 mV in 10 mV increments from a holding potential of -60 mV. EC K_{Ca} unitary conductance in

symmetrical K^+ and assymetrical K^+ were determined. All data were filtered at 5 kHz (-3dB) and digitized at 10 kHz.

Whole cell currents were measured in response to voltage steps applied from -60 mV to +150 mV in 10 mV increments from a holding potential of -60 mV. Cell capacitance was monitored and transmembrane currents were expressed in terms of current density (pA/pF).

Single Channel Recordings from VSM Cells. For single channel experiments on freshly isolated smooth muscle cells, aortae were digested as stated above. Following gentle trituration freshly isolated VSMCs were placed on Sylgard coated cover slips. VSMCs were identified by spindle shape and an absence of DiI-Ac-LDL labeling. All experimental conditions were identical to EC recordings (methods stated above) and calcium sensitivity of VSM BK_{Ca} channels compared to that in EC BK_{Ca} channels.

Measurement of K_{Ca} channel unitary conductances: Whole cell-cell attached in assymetrical K^+ solutions or inside-out patches in symmetrical K^+ solutions were taken through a series of voltage steps from -100 to 150 mV in 10 mV increments and channel unitary conductance was calculated from the slope of the current to voltage curve. To avoid contamination of patches with other K_{Ca} channels, experiments determining SK_{Ca} unitary conductance contained TRAM-34 and IBTX, while experiments characterizing IK_{Ca} had apamin and IBTX, and BK_{Ca} with apamin and TRAM-34.

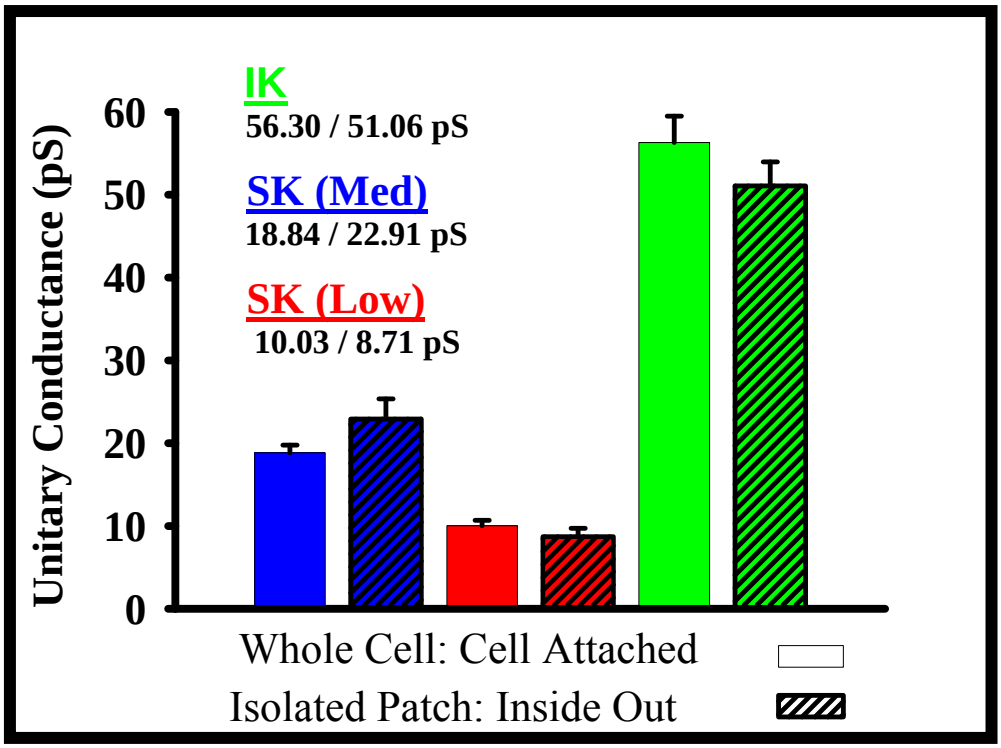
Results

Unitary conductances of four K_{Ca} channels in ECs: Four distinct K_{Ca} channels were identified in vascular endothelial cells, which CH ECs contained all four channels (two

SK_{Ca}, IK_{Ca}, and BK_{Ca}) and ECs from controls contained patches with two SK_{Ca} channels, and IK_{Ca} (**Addendum Figure 4**). Without the use of inhibitors, channel frequency in isolated patches were determined (**Addendum Figure 5**). Patches from control animals did not contain BK_{Ca} channels but contained similar frequencies of SK to IK_{Ca} channels in whole cell-cell attached (WC) configuration. CH patches contained significantly more BK_{Ca} channels and had similar frequency of SK to IK_{Ca} channels in WC configuration. In I/O configuration however there was a shift to having more SK_{Ca} channels in both CH and control ECs.

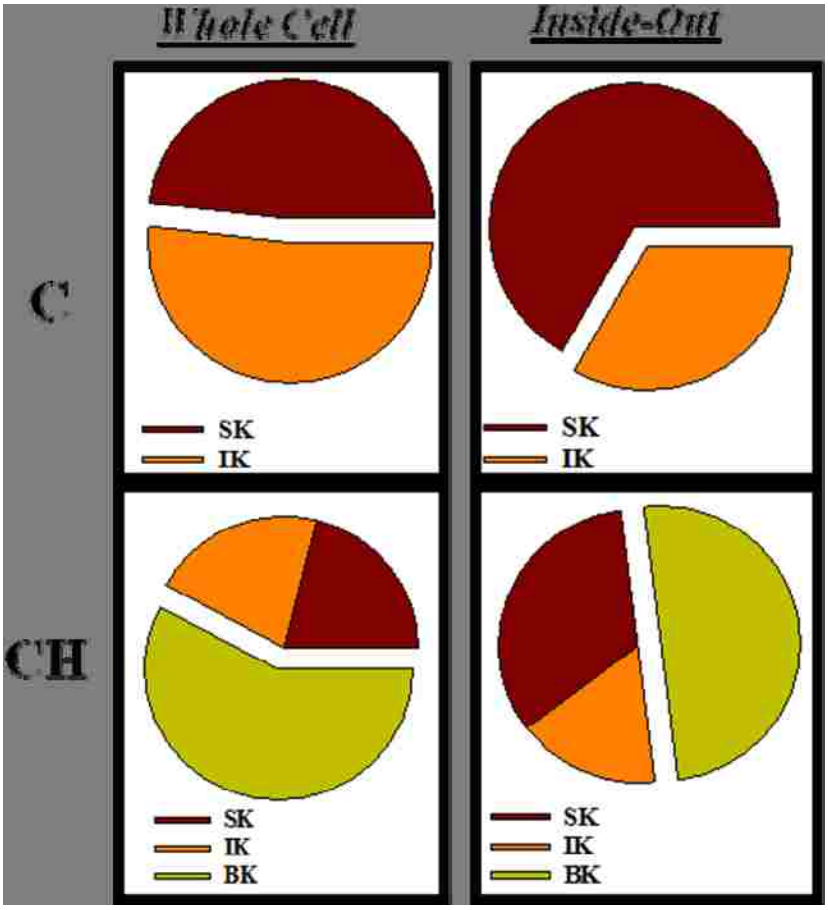
Comparison of EC BK_{Ca} and VSMC BK_{Ca} Unitary Conductances: Unitary conductances were not found to be different amongst EC and VSM BK_{Ca} channels (**Addendum Figure 5**).

CHAPTER 2 ADDENDUM: SPECIFIC AIM 1.2 – FIGURE 4



Addendum Figure 4: Unitary conductances of two distinct SK_{Ca} channels and IK_{Ca} in ECs . Data ± SEM. * $p < 0.05$ vs. vehicle. (n = 5-7 cells)

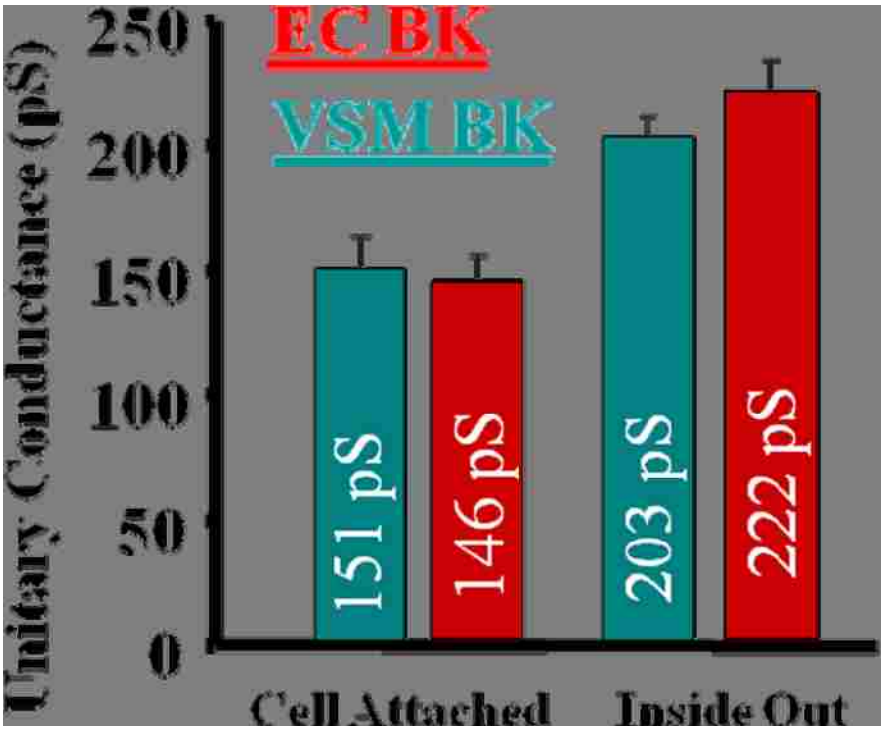
CHAPTER 2 ADDENDUM: SPECIFIC AIM 1.2 – FIGURE 5



Addendum Figure 5: Calculation of channel frequency in patches without inhibitors in vehicle conditions in inside-out (I/O) and whole cell - cell attached configurations (WC). Isolated patches on ECs from control animals did not demonstrate the presence of a large conductance channel. In control WC, 14 patches contained IK_{Ca} channels and 13 patches contained SK_{Ca} channels out of a total 27 patches studied for channel frequency in cells; while in CH WC 11 patches contained BK_{Ca} channels with only 4 patches identifying either SK_{Ca} or IK_{Ca} out of a total of 19 cells examined. For control I/O, 22 patches contained SK_{Ca} channels and 11 patches contained IK_{Ca} out of 33 studied patches; while

in CH I/O 9 patches contained BK_{Ca} channels with 6 patches identifying SK_{Ca} and 3 with IK_{Ca} out of the 18 patches studied.

CHAPTER 2 ADDENDUM: SPECIFIC AIM 1.2 – FIGURE 6



Addendum Figure 6: Unitary conductances of BK_{Ca} in VSMCs and ECs . Data ± SEM. * $p < 0.05$ vs. vehicle. (n = 5-7 cells)

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CHAPTER 4: Role of Caveolin-1 in Endothelial BK_{Ca} Regulation of Vasoreactivity

Role of Caveolin-1 in Endothelial BK_{Ca} Channel Regulation of Vasoreactivity

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Running Head: Riddle: Endothelial BK_{Ca} and vascular reactivity

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ABSTRACT

A novel vasodilatory influence of endothelial cell (EC) large conductance calcium activated potassium channels (BK_{Ca}) is present following *in vivo* exposure to chronic hypoxia (CH) and may exist in other pathological states. However, the mechanism of channel activation that results in altered vasoreactivity is unknown. We tested the hypothesis that CH removes an inhibitory effect of the scaffolding domain of caveolin-1 on EC BK_{Ca} channels to permit activation thereby affecting vasoreactivity. Experiments were performed on gracilis resistance arteries and ECs from control and CH (P_B = 380 mmHg for 48 hr) exposed rats. EC membrane potential was hyperpolarized in arteries from CH rats or arteries treated with the cholesterol depleting agent methyl-β-cyclodextrin (MBCD) compared to controls. Hyperpolarization was reversed by the BK_{Ca} antagonist iberiotoxin (IBTX) or by a scaffolding domain peptide of caveolin-1 (AP-CAV). Patch clamp documented an IBTX-sensitive current in ECs from CH rats and in MBCD treated cells not present in controls. This current was enhanced by the BK_{Ca} activator NS1619 and blocked by either AP-CAV or cholesterol supplementation. EC BK_{Ca} displayed similar unitary conductance but greater calcium sensitivity than BK_{Ca} from vascular smooth muscle. Immunofluorescence imaging demonstrated greater association of BK_{Ca} α subunits with caveolin-1 in control arteries than CH. Finally, AP-CAV restored both myogenic and phenylephrine-induced constriction in arteries from CH rats without affecting controls. We conclude that CH unmasks EC BK_{Ca} activity by

removal of an inhibitory action of the caveolin-1 scaffolding domain that may depend upon cellular cholesterol levels.

Key words: hyperpolarization; large conductance calcium activated potassium channel; scaffolding domain

INTRODUCTION

Large conductance calcium activated potassium channels (BK_{Ca}) are important regulators of arterial tone via their hyperpolarizing influence on vascular smooth muscle (VSM). VSM BK_{Ca} channels are regulated by localized increases in calcium due to sparks from ryanodine-sensitive stores (Jaggar *et al.*, 2000). Although most studies concerning the role of BK channels in vascular control focus on the VSM, there is increasing evidence that endothelial cells (EC) also express these channels although both their expression and physiological significance in native cells have been questioned (Gauthier *et al.*, 2002). It has been suggested that although these channels may be quiescent in some vascular beds, their activity is unmasked in various pathological conditions or in cell culture (Sandow & Grayson, 2009). We have recently demonstrated that endothelial BK_{Ca} channels are active following *in vivo* exposure to chronic hypoxia (CH) and that they participate in regulation of vasoreactivity (Hughes *et al.*, 2010x).

Systemic hypoxemia occurs in pathological conditions that impair pulmonary gas exchange or as a result of residence at high altitude. Patients subjected to CH demonstrate systemic vasodilation (Casiglia *et al.*, 1998) and blunted reflex vasoconstriction to lower body negative pressure challenges (Heistad *et al.*, 1972). Diminished vasoconstrictor reactivity is not reversed by restoration of normoxia in these patients (Casiglia *et al.*, 1998) or in experimental animals subjected to prolonged hypoxia (Auer & Ward, 1998;Doyle & Walker, 1991) suggesting that CH elicits long term adaptations in vascular function.

CH causes a generalized reduction in vasoconstrictor reactivity evidenced by similar blunting of both myogenic and agonist-induced reactivity (Earley & Walker, 2002;Earley *et al.*, 2002;Jernigan *et al.*, 2001;O'Donaughy & Walker, 2000). This impairment of vasoconstriction is endothelium-dependent and is associated with VSM hyperpolarization (Earley *et al.*, 2002;Hughes *et al.*, 2010w). Inhibition of either heme oxygenase (HO) (Earley *et al.*, 2002;Naik & Walker, 2003) or BK_{Ca} channels (Naik & Walker, 2003;Hughes *et al.*, 2010v) normalizes membrane potential and restores vasoreactivity in arterioles from CH rats. Interestingly, although HO-mediated VSM hyperpolarization and relaxation is BK_{Ca}-dependent in these arteries, it is unaffected by inhibition of pathways linked to VSM BK_{Ca} channels such as ryanodine receptors and soluble guanylate cyclase activity (Naik & Walker, 2003), supporting a role for endothelial BK_{Ca} channels. This possibility was recently confirmed in studies specifically targeting endothelial BK_{Ca} channels with luminal administration of the specific inhibitor iberiotoxin (IBTX) in arteries from CH rats that restored vasoconstrictor responsiveness and normalized EC and VSM membrane potential to control levels (Hughes *et al.*, 2010u). Whole cell patch clamp experiments demonstrated an IBTX-sensitive current in ECs from CH rats that was not observed in controls. Interestingly, BK_{Ca} channels were detected by immunofluorescence in ECs from both control and CH rats and BK_{Ca} current could be elicited in control cells with mild cholesterol depletion (Hughes *et al.*, 2010t). These results suggest that although endothelial BK_{Ca} channels are expressed in both groups, their activity is normally inhibited in a cholesterol-dependent fashion and that CH exposure allows their activation.

Caveolae are cholesterol rich plasmalemmal microdomains characterized by their flask-like structure and by the presence of the scaffolding protein caveolin-1 (Cav-1). Cav-1 has a high affinity for cholesterol and possesses an intracellular binding domain that regulates the activity of various signaling molecules. Importantly, Cav-1 inhibits BK_{Ca} activity in cultured ECs (Wang *et al.*, 2005) through its scaffolding domain (amino acids 82-101: DGIWKASFTTFTVTKYWFYR), however the role of Cav-1 on BK_{Ca} channel function in native ECs has not been examined and Cav-1 regulates BK_{Ca} channels differently in other cell types. Cav-1 knockout mice (*cav-1 -/-*) develop cardiac hypertrophy, pulmonary hypertension, and systemic hypotension (Murata *et al.*, 2007). Additionally *cav-1-/-* mice demonstrate augmented endothelium-dependent relaxation (Drab *et al.*, 2001a;Yu *et al.*, 2006) and decreased myogenic (Adebiyi *et al.*, 2007) and agonist-induced vasoconstriction (Drab *et al.*, 2001b) that parallel observations in CH rats (Earley & Walker, 2002;Earley *et al.*, 2003;Hughes *et al.*, 2010s). Thus, it is possible that altered vascular control in settings such as CH may be related to dysfunction of endothelial Cav-1.

The goal of the present study was to test the hypothesis that CH enhances EC BK_{Ca} channel activity and alters vasoreactivity via loss of an inhibitory effect of Cav-1. To examine this hypothesis in the most physiologically relevant manner possible, experiments were performed on intact arteries or on cells freshly dispersed from rats exposed to either CH or control conditions.

METHODS

Detailed descriptions of the methods employed are presented in an accompanying online supplement.

Animals. Experiments were performed on male Sprague-Dawley rats (Harlan Industries). All procedures were approved by the Institutional Animals Care and Use Committee of the University of New Mexico Health Sciences Center.

Hypoxic Exposure. CH rats were exposed to hypobaric hypoxia at a barometric pressure of 380 mmHg for 48 hours, whereas normoxic control rats were housed in identical cages at ambient pressure (barometric pressure, ~630 mmHg). In previous studies, we have determined that 48 hour CH exposure results in vasoconstrictor hyporeactivity indistinguishable from more prolonged (4 week) exposure and that the effects of CH do not reverse for at least 96 hours post-hypoxia (Jernigan *et al.*, 2001;O'Donaughy & Walker, 2000).

Endothelial Membrane Potential in Intact Arteries. Rats were anesthetized with sodium pentobarbital (50 mg ip) and hindlimbs removed and placed in ice-cold HEPES buffered physiological saline solution (HBSS): mmol/l 150 NaCl, 6 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, 10 mmol/l HEPES, and 10 mmol/l glucose and adjusted to pH 7.4 with NaOH. Gracilis artery branches (passive inner diameter at 100 mmHg, 150-200 μm) were carefully dissected, cut into vascular strips, and superfused (10 ml/min) with warmed (37°C) HBSS. All experiments were performed under normoxic conditions to examine sustained alterations in vascular control rather than acute responses to hypoxia. Vessel strip endothelial cell membrane potential (E_m) was measured by impalement with sharp

electrodes backfilled with Lucifer yellow for subsequent confirmation of endothelial identity (Earley *et al.*, 2004;Naik *et al.*, 2005;Hughes *et al.*, 2010r).

Patch Clamp Studies on Isolated Endothelial Cells: Endothelial cells were dispersed for electrophysiological study from control and CH rat aortae as previously described (Hughes *et al.*, 2010q). Previous studies demonstrated that endothelial cells from aorta display the same characteristics as cells from gracilis arteries in terms of their response to CH (Hughes *et al.*, 2010p). One to two drops of the resulting cell suspension were seeded on a glass cover slip mounted on an inverted fluorescence microscope (Olympus IX71) for 30 min prior to superfusion. Single endothelial cells were identified by the selective uptake of the fluorescently labeled acylated low density lipoprotein Ac-LDL-Dil with a rhodamine filter (Paffett *et al.*, 2007) prior to each electrophysiological experiment. Freshly dispersed endothelial cells were superfused under constant flow (2 ml/min) at room temperature (22-23°C) in an extracellular solution (ECS: 141 mmol/l NaCl, 4.0 mM KCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 mmol/l HEPES, 10 mmol/l glucose and buffered to pH 7.4 with NaOH). Whole cell current data were generated using an Axopatch 200B amplifier (Axon Instruments). For experiments utilizing conventional whole cell patch clamp configuration, biophysical criteria: (Seal resistance > 1GΩ, series resistance <25MΩ) was checked following membrane rupture and monitored throughout the course of the experiment. Cells were held at -60mV and were dialyzed for 5 min with an intracellular solution (ICS: 140 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 5 mmol/l Mg_2ATP, 10 mmol/l HEPES, 1 mmol/l EGTA and adjusted to pH 7.2 with KOH). $CaCl_2$ was added to yield a free-Ca^{2+} concentration of 1 μM, as calculated using WinMAXC chelator software. For experiments utilizing the perforated patch

technique, 4-6 MΩ patch electrodes were backfilled with amphotericin B. After gigaseals were obtained, the series resistance fell over a 10- to 15-min period to 15–20 MΩ and remained stable for up to 1 h. ECs with stable series resistances under 25 MΩ were used for experiments.

Single Channel Recordings. For single channel experiments, cell attached and inside-out patch clamp configurations were used. Patch pipettes had resistances ranging from 6 to 8 MΩ. Extracellular solutions (ECS) contained: 130 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES, 10 Glucose, 1.2 $CaCl_2$ (pH 7.30 with NaOH). Intracellular solutions (ICS) contained (in mM): 130KCl, 2 Na_2ATP, 3 $MgCl_2$, 10 HEPES, 1 EGTA, (pH 7.30 with KOH). For inside-out patch experiments ECS contained: 140 KCl, 10 HEPES, 1 $CaCl_2$, 1 $MgCl_2$ (pH 7.3 with KOH). For experiments investigating the effect of calcium concentration on single channel opening probability (N_{po}) on inside out patches EGTA and free calcium ($CaCl_2$) were varied to provide 10 nM, 50 nM, 100 nM, 1 μM, and 10 μM free calcium solutions. Patches were held at -60, 0, and 40 mV test potentials to for voltage sensitivity experiments. Single channel unitary conductance was determined from the slope of currents measured in response to voltage steps applied from -100 mV to 150 mV in 10 mV increments from a holding potential of -60 mV. EC BK_{Ca} unitary conductance in symmetrical K^+ and assymetrical K^+ were determined. All data were filtered at 5 kHz (-3dB) and digitized at 10 kHz.

Whole cell currents were measured in response to voltage steps applied from -60 mV to +150 mV in 10 mV increments from a holding potential of -60 mV. Cell capacitance was monitored and transmembrane currents were expressed in terms of current density (pA/pF).

Single Channel Recordings from VSM Cells. For single channel experiments on freshly isolated smooth muscle cells, aortae were digested as stated above. Following gentle trituration freshly isolated VSMCs were placed on Sylgard coated cover slips. VSMCs were identified by spindle shape and an absence of DiI-Ac-LDL labeling. All experimental conditions were identical to EC recordings (methods stated above) and calcium sensitivity of VSM BK_{Ca} channels were compared to EC BK_{Ca} channels.

Immunofluorescence in Sectioned Arteries: Gracilis arteries from control and CH rats were collected following transcardial perfusion with PSS containing 10 mg papaverin. The gracilis muscle was removed and frozen in OCT compound in liquid N_2 and isopentane. 10 μm sections were adhered to superfrost slides (Fisher) and fixed in ice cold methanol (100%) for 10 min. After fixation cross sections were blocked in 4% donkey serum in PBS for 1 hr at room temperature. Cross sections were incubated with primary antibodies for BKα (Alamone 1:100) and Cav-1 (BD Biosciences 1:50). Primary antibodies for BKα were detected with Cy5-conjugated donkey, anti-rabbit secondary antibodies. Cav-1 was detected with a Cy3-conjugated donkey, anti-mouse secondary antibody (all secondaries 1:500 dilution; Jackson Labs). The nuclear stain, Sytox (1:10,000 dilution; Molecular Probes) was then applied. Confocal microscope images were obtained using a 63x oil immersion DIC objective (NA =1.4) at a resolution of 524 X 524 and an optical slice of 0.7 μm. For co-localization analysis, z stacks (10 sections, 1 μm) were analyzed for fluorescence overlap of BK_{Ca} α and Cav-1. Images underwent nearest-neighbor deconvolution and individual channels were thresholded to normalize intensity between channels. Thresholded channels were then made into masks. Endothelial cells were then traced within the lumen of arteriolar cross sections made into

a mask for analysis. Individual channel masks and the EC mask were combined to examine endothelial specific co-localization as defined by Mander's correlation coefficient values (Manders *et al.*, 1992). Values range from 0 to 1, with values close to 0 indicating non-overlapping images and values close to 1 reflecting co-localization.

Study of Isolated Resistance Arteries. Hindlimbs from control or CH animals were removed and placed in ice-cold physiological saline solution [PSS: 119 mmol/l NaCl, 4.7 mmol/l KCl, 25 mmol/l $NaHCO_3$, 1.18 mmol/l KH_2PO4, 1.17 mmol/l $MgSO_4$, 0.026 mmol/l K_2-EDTA, 5.5 mmol/l glucose and 2.5 mmol/l $CaCl_2$]. Gracilis arterioles (passive inner diameter at 60 mmHg, 140-200 μm) were dissected free, cannulated on glass pipettes and mounted in an arteriograph (Living Systems, CH-1). The proximal end of the artery was cannulated with a tapered glass pipette, secured in place with a single strand of silk ligature and gently flushed to remove blood from the lumen. The distal end of the artery was then cannulated and the vessel stretched longitudinally to approximate its *in situ* length. Arteries were slowly pressurized to 60 mmHg with PSS using a servo-controlled peristaltic pump (Living Systems) and superfused (10 ml/min) with warmed (37°C) PSS aerated with a normoxic gas mixture (21% O_2-6% CO_2-73% N_2). Arteries were required to hold a steady pressure upon switching off the servo-control function to confirm the absence of a leak. Any vessels with apparent leaks were discarded. The vessel chamber was then transferred to the stage of a Nikon Eclipse TS100 microscope and the preparation superfused with PSS. Bright-field images of vessels were obtained with an IonOptix CCD100M camera and dimensional analysis performed by IonOptix Sarclen software to measure inner diameter as described in previous studies from our laboratory (Earley & Walker, 2002). All experiments were performed under normoxic

conditions to examine sustained alterations in vascular control rather than acute vasodilatory responses to hypoxia. Arteriole exposure to normoxic conditions for 2.5-3 hours throughout the course of the experiment do not reverse sustained alterations elicited by CH exposure

Measurement of Vascular Smooth Muscle Cell Calcium. Pressurized arteries were loaded abluminally with the cell-permeant, ratiometric, Ca^{2+}-sensitive fluorescent indicator fura-2 AM (Molecular Probes) as detailed previously (Earley & Walker, 2002). Immediately prior to loading, fura-2 AM (1 mM in anhydrous DMSO) was mixed 2:1 with a 20% solution of pluronic acid in DMSO, and this mixture diluted in PSS to yield a final concentration of 2 □M fura-2 AM and 0.05% pluronic acid. Arteries were incubated in this solution for 45 min at room temperature in the dark. The diluted fura-2 AM solution was equilibrated with the 21% O_2 gas mixture during this loading period. Vessels were rinsed for 20 min with aerated PSS (37 °C) following the loading period to wash out excess dye and to allow for hydrolysis of AM groups by intracellular esterases. Fura-2-loaded vessels were alternately excited at 340 and 380 nm at a frequency of 1 Hz with an IonOptix Hyperswitch dual excitation light source and the respective 510 nm emissions collected with a photomultiplier tube. Background-subtracted 340/380 emission ratios were calculated with IonOptix Ion Wizard software and recorded continuously throughout the experiment, with simultaneous measurement of i.d. from red wavelength bright-field images as described above. VSM $[Ca^{2+}]_i$ is expressed as the mean F_{340}/F_{380} ratio from the background-subtracted 510 nm signal.

Experimental Protocols:

Due to the sustained nature of the effect of CH exposure, all experiments on tissue from CH and control rats were performed under normoxic conditions.

Effect of CH or Cholesterol Depletion on Basal Endothelial E_m. Endothelial cell E_m was measured using sharp electrodes in gracilis arteries from control and CH rats. To test whether CH-induced changes in E_m could be mimicked by caveolar disruption, some arteries from control animals were treated with 100 μmol/L of the cholesterol depleting agent methyl-β-cyclodextrin (MBCD; Sigma). This concentration of MBCD is 100 fold less than that commonly used in vascular preparations and we have previously shown that it does not affect caveolae structure or number in this preparation (Hughes *et al.*, 2010o).

Iberiotoxin (IBTX) Sensitivity of E_m. To examine the role of BK_{Ca} channels on endothelial E_m, sharp electrode measurements were made in control, CH and MBCD-treated gracilis arteries in the presence 100 nmol/L of the BK_{Ca} specific inhibitor IBTX (Sigma) or its vehicle.

Effect of Caveolin-1 Scaffolding Domain Peptide on Basal Endothelial E_m. Endothelial cell E_m was measured in arteries from control and CH rats and control arteries treated with MBCD in the presence of 10 μmol/L of the Cav-1 scaffolding domain peptide (AP-CAV; Twentyfirst Century Biochemicals) or a scrambled control peptide.

Effect of CH or Cholesterol Depletion on Isolated Endothelial Cell Transmembrane Currents. Currents were measured in aortic ECs freshly dispersed from control and CH

rats. Previous studies demonstrated that ECs from aorta display identical electrophysiological characteristics as cells from gracilis arteries in terms of their response to CH and to cholesterol depletion (Hughes *et al.*, 2010n). In perforated patch clamp mode, whole cell currents were measured in response to a series of voltage steps. After initial recording, one group of cells from control rats was superfused with MBCD for 15 minutes before voltage steps were applied and currents re-examined.

Effect of BK_{Ca} Channel Inhibition and Activation on Isolated Endothelial Cell Transmembrane Currents. In perforated patch clamp mode, recordings were taken before and 5 min after superfusion with IBTX (100 nmol/L), or the BK_{Ca} activator NS1619 (1,3 dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one) (Sigma) (10 μmol/L) in cells from control and CH rats. Additional measurements were taken in a group of control cells after treatment with 100 μmol/L MBCD.

Effect of Cholesterol Repletion on Endothelial Cell BK_{Ca} Currents. Whole cell currents were first measured in cells from control rats in perforated patch clamp mode following incubation with vehicle. Following the initial recording, cells were superfused with MBCD for 15 minutes before voltage steps were applied and currents recorded, whereas cells from CH animals remained in vehicle conditions for 15 minutes. A mixture of MBCD and cholesterol (MBCD + ChL 8:1) shown to replete membrane cholesterol and restore caveolar function (Levitan *et al.*, 2000;Romanenko *et al.*, 2004) was then administered to each group and transmembrane currents were re-examined.

Effect of AP-CAV on Whole Cell Currents. Currents were assessed using conventional whole cell patch clamp mode in aortic ECs from control and CH rats. One group of control cells was also treated with MBCD as above. Measurements were first made following cellular rupture in vehicle conditions. Next, AP-CAV or the scrambled control peptide were backfilled into patch pipettes and dialyzed into the clamped cells for 5 minutes. Voltage steps were again applied and currents recorded following dialysis of AP-CAV or scrambled peptide.

Determination of Unitary Conductance of EC BK_{Ca} Channels. Unitary conductance was determined in symmetrical K^+ in inside-out patches of aortic ECs from CH animals and control ECs pre-treated with MBCD. Assessments were also made in whole cell-cell attached mode to determine conductance in asymmetrical K^+ relevant to the *in situ* environment.

Determination of EC BK_{Ca} Channel Calcium and Voltage Sensitivity. Inside-out patches from CH rats were held at varying test potentials and intracellular calcium levels to investigate channel opening probability (NP_o). Parallel experiments were performed on freshly isolated VSM to compare calcium sensitivity of BK_{Ca} channels between the two cell types.

Sensitivity of EC BK_{Ca} Channels to Iberiotoxin (IBTX). Inside-out patches from CH aortic ECs were held at varying test potentials in 1μmol/L free Ca^{2+}– solution. NP_o was assessed in the presence or absence of intrapipette IBTX.

Myogenic Responses. Experiments were performed on cannulated, pressurized gracilis arterioles. Active and passive (Ca^{2+}-free) pressure–diameter relationships were

determined, as described previously (Hughes *et al.*, 2010m;Earley & Walker, 2002) over intraluminal pressure steps between 20 and 160 mmHg. Vessel inner diameter was monitored using video microscopy and edge-detection software (IonOptix Sarclen). VSM was selectively loaded with the ratiometric calcium indicator fura 2-AM and $[Ca^{2+}]_i$ monitored as previously described (Earley & Walker, 2002;Earley *et al.*, 2002). Endothelial integrity was assessed by dilation with 10 μmol/L acetylcholine (ACh) prior to Ca^{2+}-free superfusion. Pressure-induced vasoconstrictor and calcium responses were determined in the presence of AP-CAV or scrambled control peptide (Scrambled). AP-CAV or Scrambled were administered intraluminally in an effort to specifically target the endothelium.

Vasoconstrictive Reactivity to Phenylephrine (PE). Arteriole inner diameter and VSM $[Ca^{2+}]_i$ were assessed in response to increasing PE concentrations (10^{-9} μmol/L to 10^{-5} μmol/L) in the presence of intraluminal AP-CAV or scrambled control peptide. Reversal of constriction with 10 μM ACh at the termination of the PE concentration-response curve was used to verify endothelial integrity.

Calculations and Statistics. Data are expressed as means ± SE. Values of n refer to the number of animals in each group except for patch clamp studies where n represents the number of cells studied. Data were analyzed by repeated measures analysis of variance (RMANOVA) and by a Bonferroni modified unpaired Student's t-test for multiple comparisons when differences were indicated. Unpaired t-tests were used for single comparisons between groups. $P \leq 0.05$ was accepted as indicating a statistically significant difference.

RESULTS

Effect of CH or Cholesterol Depletion on Basal Endothelial E_m. Consistent with our earlier report (Hughes *et al.*, 2010l), endothelial cell E_m was hyperpolarized in gracilis arteries from CH rats compared to controls (Figure 1A). Mild cholesterol depletion with 100 μmol/L MBCD elicited a similar degree of hyperpolarization (Figure 1A). As observed earlier (Sandow *et al.*, 2002;Hughes *et al.*, 2010k), resting E_m was more depolarized in these arteries than in some other beds, likely due to the lack of myoendothelial gap junctions (Sandow *et al.*, 2002). We have observed significantly more polarized E_m in other beds using techniques identical to those used in the present study (Paffett *et al.*, 2007).

Iberiotoxin (IBTX) Sensitivity of E_m. IBTX significantly depolarized membrane potential to baseline vehicle treated control level in EC cells from CH rats and in MBCD treated arteries, but was without effect in controls (Figure 1B). These results support the hypothesis that BK_{Ca} channel activity causes endothelial hyperpolarization following MBCD and CH treatments.

Effect of Caveolin-1 Scaffolding Domain Peptide on Basal Endothelial E_m. The Cav-1 scaffolding domain peptide AP-CAV had no effect on endothelial E_m in arteries from control rats compared to arteries receiving the scrambled peptide (Figure 1C). In contrast, AP-CAV depolarized the endothelium of CH arteries and arteries treated with MBCD to control (scrambled) levels (Figure 1C). These results suggest that the

depolarizing influences of CH and cholesterol depletion are caused by loss of an inhibitory effect of the Cav-1 scaffolding domain on basal BK_{Ca} channel activity.

Effect of CH or Cholesterol Depletion on Endothelial Cell Transmembrane Currents. Whole cell outward currents were greater in ECs from CH rats and MBCD treated cells compared to untreated controls at each test potential from -50 mV to +150 mV (Figure 1D). Cholesterol depletion and CH resulted in similar outward currents that were not different (Figure 1D/E).

Measurement of EC BK_{Ca} Unitary Conductance and Channel Sensitivity to Voltage and Ca^{2+}. EC BK_{Ca} unitary conductance in patches from CH rats (Figure 2A) was 221 ± 12 pS in symmetrical K^+. Conductance measured in asymmetrical K^+ in cell attached whole cell mode was 145 ±9 pS. Unitary conductances did not vary between cells from CH rats and control cells treated with MBCD (Figure 2A). EC BK_{Ca} channels displayed enhanced calcium sensitivity compared to VSM channels (Figure 2B) consistent with our evidence for tonic activity of these channels at basal $[Ca^{2+}]_i$ in EC from CH rats. EC channels assessed from CH cells also displayed voltage sensitivity (Figure 2C).

Effect of BK_{Ca} Channel Inhibition and Activation on Endothelial Cell Transmembrane Currents. IBTX significantly reduced whole cell currents from CH ECs (at test potentials from -50 to +150 mV; Figure 3B and D), but had no effect in controls (Figure 3A and D) as previously described in conventional whole cell studies with much higher $[Ca^{2+}]_i$ (Hughes *et al.*, 2010j) than in the present perforated patch experiments. Similarly, IBTX normalized outward current in cholesterol depleted cells to levels of controls (at test potentials from -40 to +150 mV; Figure 3C and D), demonstrating active BK_{Ca} channels

following caveolar disruption with MBCD. Consistent with these whole cell measurements, IBTX applied via the pipette to inside-out patches effectively inhibited channel opening (Figure 3E).

The BK_{Ca} activator NS1619 had no effect in cells from control rats (Figure 4A and D), but further increased outward currents in CH cells at test potentials between -40 and +150 mV (Figure 4B and D). NS1619 also effectively enhanced current in cholesterol depleted cells (from -30 to +150 mV) (Figure 4C and D).

Effect of Cholesterol Repletion on Whole Cell Currents. Administration of MBCD-ChL to restore Cav-1 function significantly reduced outward currents in cells from CH rats (Figure 5B and D) and cells treated with MBCD (Figure 5B and D), but had no effect in controls (Figure 5A and D). These results demonstrate the dynamic regulation of EC BK_{Ca} by cellular cholesterol and presumably Cav-1.

Effect of AP-CAV on Whole Cell Currents. AP-CAV potently inhibited BK_{Ca}-dependent current in ECs from CH rats (Figure 6B and D) and MBCD treated ECs from control rats (Figure 6C and D), demonstrating an inhibitory effect of the Cav-1 scaffolding domain on channel activity in these settings. AP-CAV treatment had no effect on current in untreated controls (Figure 6A and D). Currents observed with the scrambled peptide did not differ from vehicle in any of the groups (data not shown).

Co-localization of Cav-1 and BK_{Ca} α Subunit in Gracilis Arterioles. Staining for BK_{Ca} α (Alamone, 1:100, shown in green) was positive in VSM and ECs (Figures 7A) in both control and CH groups. Cav-1 (BD Transduction 1:50, shown in red) was also found in VSM and EC (Figure 7A) in control and CH groups. However, overlap of Cav-1 and

$BK_{Ca}\alpha$ was significantly less in arteries from CH rats compared to controls as assessed by Manders correlation coefficient (Figure 7B).

Effect of AP-CAV on Myogenic- and PE-induced Tone in Gracilis Arteries from Control and CH Rats. In gracilis arteries receiving scrambled peptide, myogenic-induced tone and VSM $[Ca^{2+}]_i$ responses were less in arteries from CH rats compared to controls (Figure 8A/B) consistent with earlier reports from untreated vessels (Hughes *et al.*, 2010i). Luminal application of AP-CAV to target and inhibit EC BK_{Ca} restored myogenic tone and VSM $[Ca^{2+}]_i$ in CH arteries similar to luminal IBTX or endothelial disruption in earlier studies (Earley & Walker, 2002;Earley *et al.*, 2002;Hughes *et al.*, 2010h), but was without effect in controls. AP-CAV similarly restored PE-induced vasoconstrictor and $[Ca^{2+}]_i$ responses in arteries from CH rats to levels of scrambled controls without affecting arteries from normoxic rats (Figure 8 C/D).

DISCUSSION

The major findings of the present study are 1) CH or cholesterol depletion removes Cav-1 inhibition of BK_{Ca} channels leading to persistent channel activity and hyperpolarization of ECs; 2) cholesterol repletion or introduction of the scaffolding domain of Cav-1 potently inhibits EC BK_{Ca} activity and normalizes outward K^+ currents to levels of controls; 3) unitary conductance of EC BK_{Ca} channels are similar to those seen in VSM, but exhibit greater Ca^{2+} sensitivity; 4) co-localization of endothelial BK_{Ca} and Cav-1 is reduced following CH exposure; and 5) diminished myogenic and agonist induced tone following CH exposure can be restored by endothelial introduction of the

scaffolding domain of Cav-1. These results provide evidence for a novel mode of vascular control elicited by CH involving loss of an inhibitory effect of Cav-1 on EC BK_{Ca} channels.

Consistent with an earlier report (Hughes *et al.*, 2010g), we observed EC hyperpolarization in arteries from CH rats compared to controls. Interestingly, mild cholesterol depletion with MBCD elicited a similar degree of hyperpolarization in control arteries. Neither treatment results in discernable alteration in gross structure or number of endothelial caveolae (Hughes *et al.*, 2010f), suggesting that modest interference of Cav-1 function or impaired cholesterol homeostasis have functional consequences on EC membrane potential. Hyperpolarization of the endothelium can lead to hyperpolarization of the underlying VSM through several postulated mechanisms to promote vasodilation. VSM hyperpolarization can occur by conduction of charge through low resistance myoendothelial gap junctions (Griffith, 2004), however the femoral circulation does not appear to have these structures (Sandow *et al.*, 2002). This is evident from the relatively depolarized EC E_m that we and others observe in this bed that contrasts with the mesenteric circulation that possesses direct myoendothelial communication (Sandow *et al.*, 2002;Hughes *et al.*, 2010e). Another possible mechanism of VSM hyperpolarization is through elevated extracellular [K^+] due to activation of EC K^+ channels that in turn activate VSM inwardly rectifying K^+ channels (K_{ir}) and $Na^+/K^{+/}$ATPase (Jackson, 2005). Regardless of the initiating event, VSM hyperpolarization results in inhibition of voltage gated Ca^{2+} channels and hence dilation. Persistent hyperpolarization of the vascular wall as seen with CH (Hughes *et al.*, 2010d;Earley & Walker, 2002;Earley *et al.*, 2002) opposes vasoconstriction and thus dampens vasoconstrictor reactivity.

Interestingly, EC hyperpolarization following CH or MBCD treatment appears to be due to tonic activity of EC BK_{Ca} channels not seen in controls. We have previously shown that this basal hyperpolarization leads to more profound hyperpolarization responses to agonists like acetylcholine (ACh), thereby amplifying endothelium-dependent vasodilation (Hughes *et al.*, 2010c). However, BK_{Ca} channels in VSM require high local $[Ca^{2+}]$ from sparks to elicit activity (Jaggar *et al.*, 2000), which led us to hypothesize that EC channels may exhibit enhanced Ca^{2+} sensitivity. Previous experiments from our laboratory examined IBTX sensitive currents in conventional whole cell configuration with intracellular Ca^{2+} levels maintained at 1 μmol/L (Hughes *et al.*, 2010b). This $[Ca^{2+}]$ maximally activates the channel and thus provides little insight into channel activity at endogenous $[Ca^{2+}]_i$. In the present study, we employed perforated patch clamp configuration to maintain more physiological $[Ca^{2+}]_i$ and still observed BK_{Ca} currents in cells from CH rats and cells treated with MBCD. Although EC BK_{Ca} channels exhibited unitary conductances consistent with those from VSM, the Ca^{2+} sensitivity of EC channels was enhanced as evidenced by significantly greater channel opening at 10 and 100 nmol/L $[Ca^{2+}]$ than seen in VSM patches. Thus, within the endothelium, basal Ca^{2+} levels seem sufficient to maintain BK_{Ca} channel activity. This enhanced Ca^{2+} sensitivity does not appear to be related to activity of the β1 accessory subunit of the BK_{Ca} channel, since the β1 activator tamoxifen (Dick *et al.*, 2001) inhibited rather than enhanced outward current in EC from CH rats, whereas tamoxifen predictably stimulated current in VSM (data not shown).

Previous studies on cultured ECs suggest that the scaffolding domain of Cav-1 exerts an inhibitory effect on BK_{Ca} channels that can be eliminated by cholesterol

depletion (Wang *et al.*, 2005). Our data support this inhibitory role of Cav-1 and for the first time demonstrate that an *in vivo* stimulus can tonically activate endothelial BK_{Ca} by affecting this mode of regulation. In addition, consistent with cultured cell studies, cholesterol depletion with MBCD unmasked BK_{Ca} currents in ECs from control animals. The presence of BK_{Ca} in EC from both control and CH rats was confirmed by immunofluorescence (Figure 7), however the apparent association between channels and Cav-1 was less in arteries from CH rats compared to controls as assessed by pixel overlap analysis. The importance of the scaffolding domain of Cav-1 in regulating BK_{Ca} channel activity was demonstrated by the effectiveness of AP-CAV to inhibit BK_{Ca} currents in cells from CH rats and cholesterol-depleted control ECs and it effect to reverse EC hyperpolarization in intact arteries from these groups. Importantly, both myogenic and PE-induced vasoconstrictor responsiveness were also restored by luminal AP-CAV without affecting control arteries, in a pattern identical to the effects of intraluminal IBTX or endothelial disruption (Earley & Walker, 2002;Earley *et al.*, 2002;Hughes *et al.*, 2010a). VSM calcium responses to both pressure and PE were also restored by AP-CAV consistent with earlier studies documenting a similar effect of endothelial removal (Earley & Walker, 2002;Earley *et al.*, 2002) and consistent with reversal of VSM hyperpolarization and restoration of calcium influx through voltage-gated calcium channels. These results clearly demonstrate the central role of altered EC Cav-1 function in altered vascular control following CH; however these alterations could reflect differences in cholesterol homeostasis between groups rather than demonstrable differences in Cav-1 expression or number of EC caveolae.

In the present studies, cholesterol supplementation inhibited BK_{Ca} currents both in cells from CH rats and MBCD treated ECs, suggesting a key role of cholesterol in channel regulation. Cholesterol directly binds to Cav-1 both *in vitro* (Murata *et al.*, 1995) and *in vivo* (Thiele *et al.*, 2000). Cav-1 localization at the plasma membrane is closely associated with the presence of free cholesterol (Pol *et al.*, 2005), where decreases in cellular cholesterol levels significantly reduce Cav-1 transport and membrane association. Interestingly, CH exposure reduces *de novo* cholesterol synthesis by inhibiting HMG-CoA-reductase activity in cultured cells (Nguyen *et al.*, 2007). HMG-CoA-reductase is the rate limiting enzyme in *de novo* synthesized cellular cholesterol, thus CH exposure could limit cholesterol production in the present model. It is noteworthy that cultured rat aortic ECs exposed to hypoxia demonstrate reduced cellular cholesterol and cholesterol esters (Arai *et al.*, 1996). In addition, inhibition of *de novo* cholesterol with cerivastatin enhances EC BK_{Ca} activity and results in significant membrane hyperpolarization in human umbilical vein ECs (Kuhlmann *et al.*, 2004b). These actions of the HMG-CoA-reductase inhibitor on EC BK_{Ca} activity were rapidly reversed by the addition of mevalonate, suggesting that endothelial BK_{Ca} channel activity is regulated by this agent and other non-steriodal isoprenoids (Kuhlmann *et al.*, 2004a). Isoprenoids such as geranyl pyrophosphate and farnesyl pyrophosphate are required for membrane insertion, caveolar targeting and localization of multiple proteins (Gelosa *et al.*, 2007). Thus, changes in EC free cholesterol following hypoxemia could possibly underlie the changes in vascular function seen in the CH model and have functional implication to other pathologies associated with altered EC cholesterol homeostasis.

In conclusion, the present experiments describe a novel mode of regulation of vascular tone associated with Cav-1 regulation of EC BK_{Ca} channels. Although these studies center on a model of hypoxemic disorders, *i.e.* CH, the establishment of the central role of cholesterol in regulating this pathway suggests that these observations may be relevant in a variety of pathophysiological and therapeutic settings.

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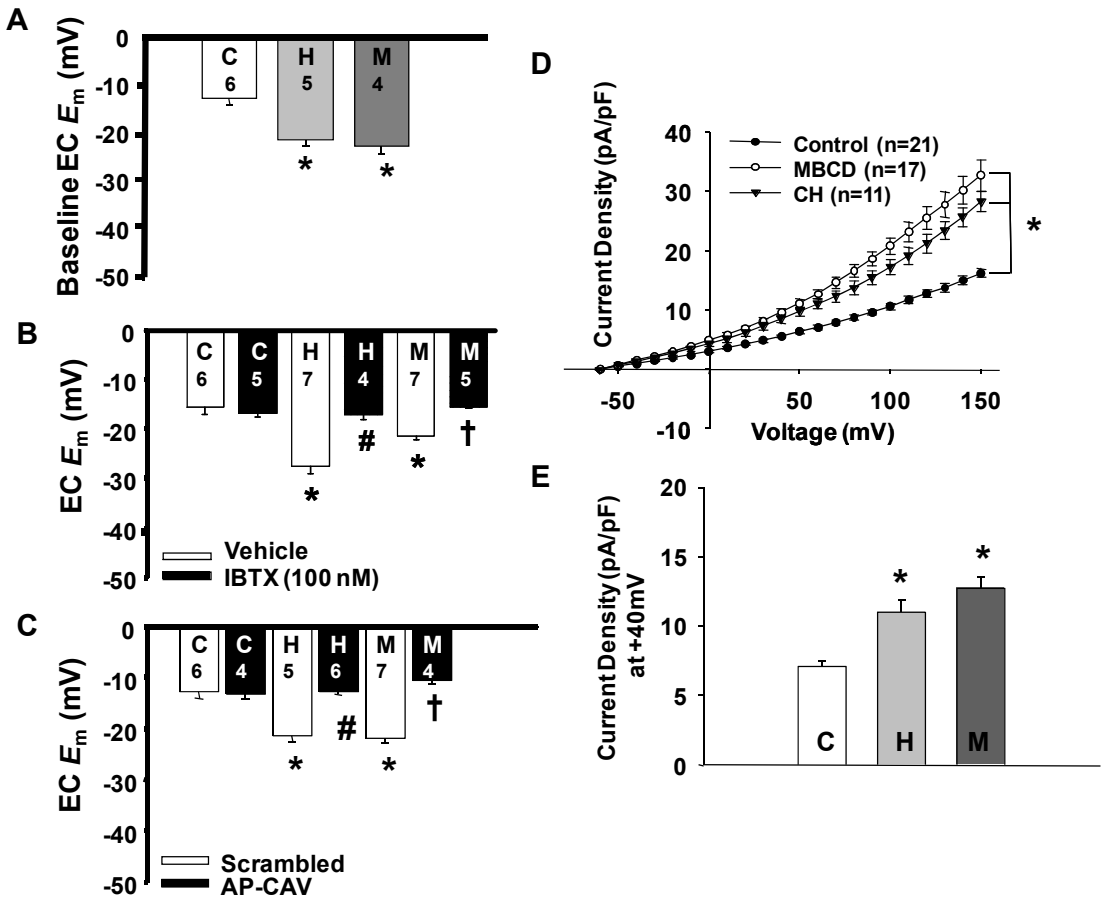


Figure 1. Endothelial membrane potential (E_m) and outward K^+ current. **Panel A:** E_m in arterial strips from control (C) and chronically hypoxic (H) rats and control strips treated with MBCD (M); **Panel B:** effect of IBTX to reverse hyperpolarization in CH and MBCD-treated vessels; **Panel C:** reversal of hyperpolarization by AP-CAV; **Panel D:** outward current measured in perforated patch configuration in cells from each group; **Panel E:** mean current density at +60 mV for each group. CH and MBCD-treated cells outward currents were significantly greater than controls (at -40 to +150 mV test potentials). n indicated for each group within the figure. * $p \leq 0.05$ vs. control; # $p \leq 0.05$ vs. CH vehicle or scrambled; † $p \leq 0.05$ vs. MBCD vehicle or scrambled.

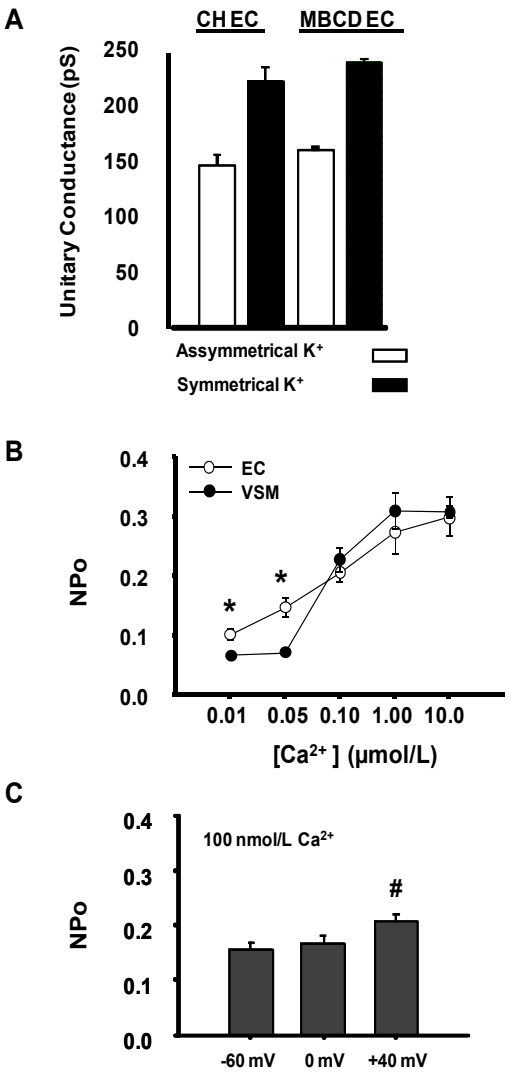


Figure 2. Endothelial BK_{Ca} channel characteristics in cell attached and inside-out patches. **Panel A:** Unitary conductance in symmetrical (inside-out) and asymmetrical (cell attached) K^+ in cells from CH rats and controls treated with MBCD (n=7-14 patches per group). There were no differences between treatments. **Panel B:** Ca^{2+} sensitivity of open probability (NPo) in inside-out patches of ECs (n=12) and VSM cells (n=8) from CH rats. * $p \leq 0.05$ vs. VSM. **Panel C:** Voltage sensitivity of NPo at 100 nmol/L $[Ca^{2+}]_i$ in inside-out patches from CH rats (n=11). # $p \leq 0.05$ vs. -60 mV.

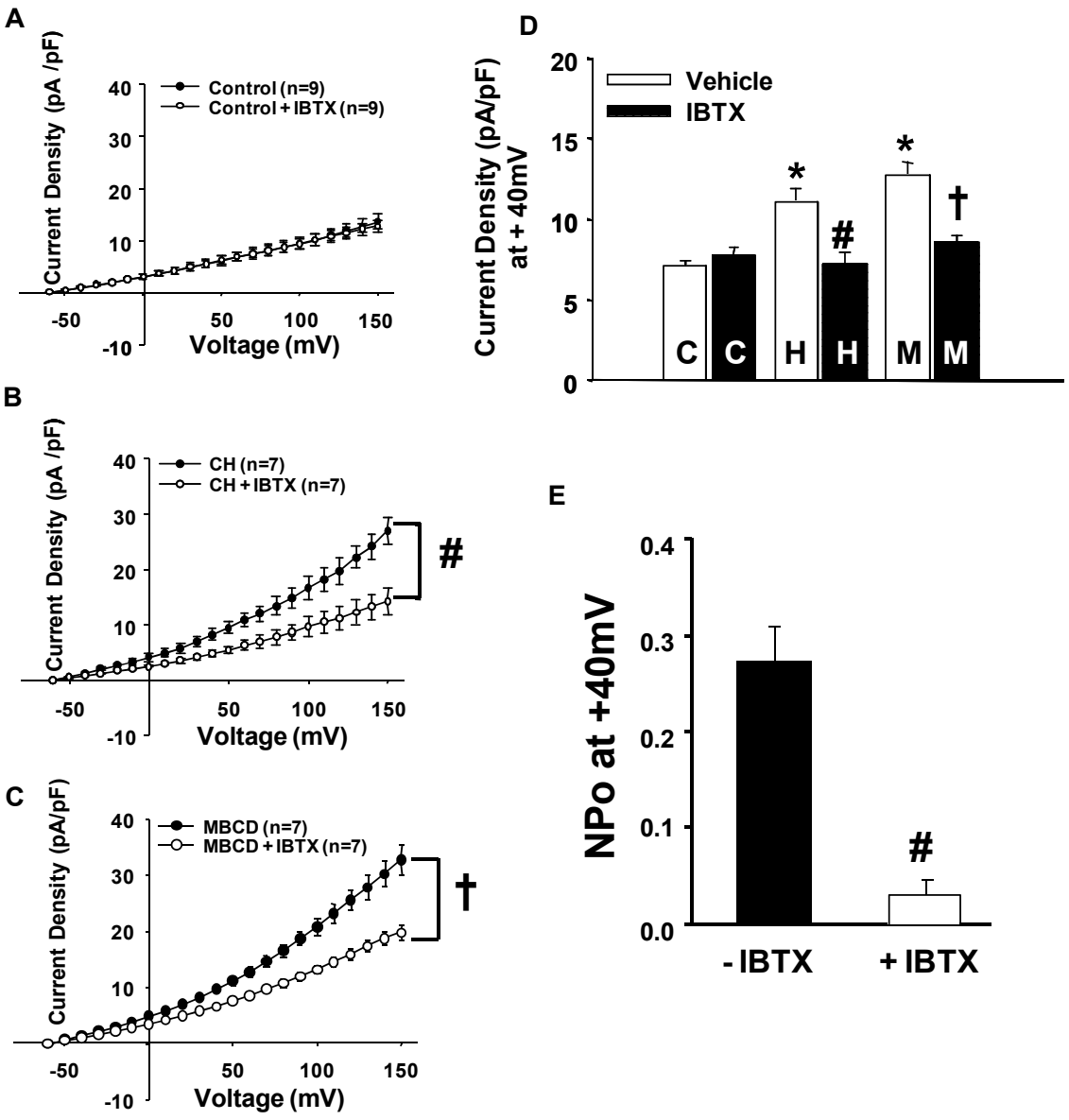


Figure 3. Iberiotoxin (IBTX) sensitivity of currents. **Panels A-C** show transmembrane currents in the presence of IBTX or its vehicle. IBTX-sensitive currents were observed in the CH and MBCD groups, but not controls. **Panel D:** mean current density at +40 mV. **Panel E:** effect of IBTX on NPo in inside-out patches from CH cells. * $p \leq 0.05$ vs. vehicle control; # $p \leq 0.05$ vs. CH vehicle; † $p \leq 0.05$ vs. MBCD + vehicle.

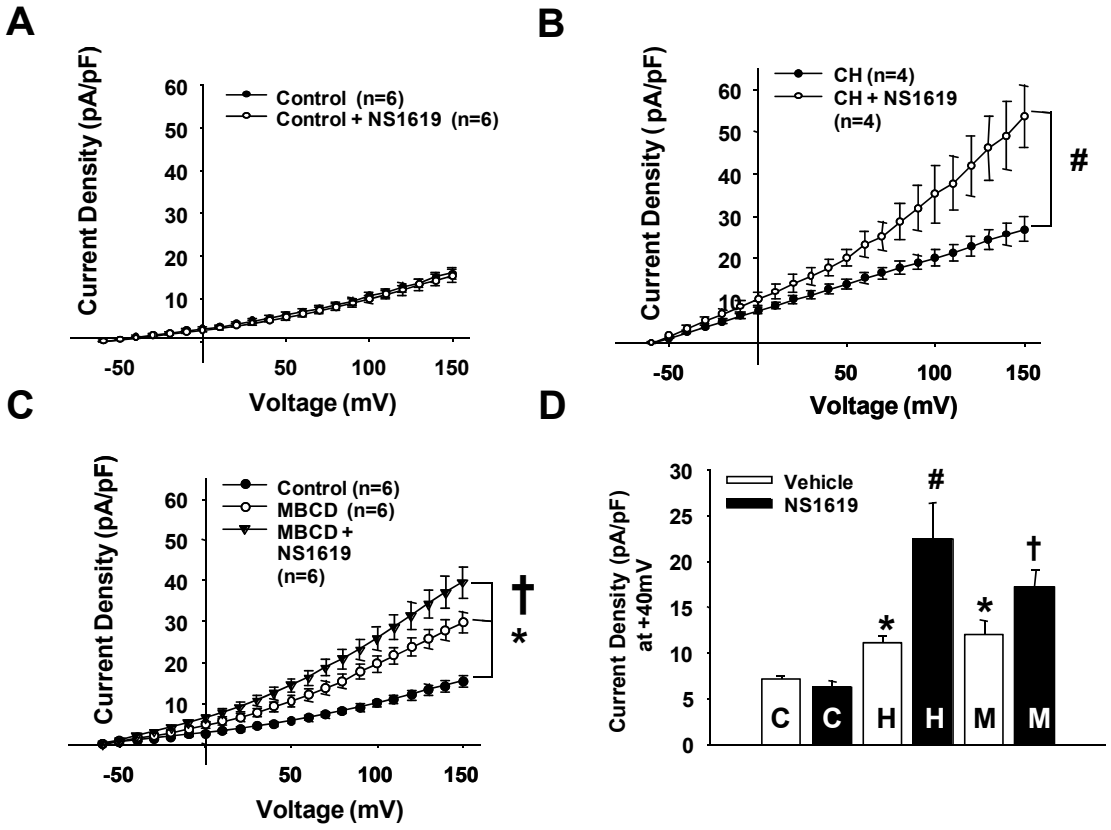


Figure 4. Effect of the BK_{Ca} activator NS1619 on transmembrane currents in each group. NS1619 significantly enhanced current in CH (-40 to +150 mV) **(Panel B)** and MBCD (-30 to +150 mV) **(Panel C)** cells, but did not affect controls **(panel A)**. * $p \leq 0.05$ vs. control vehicle; # $p \leq 0.05$ vs. CH vehicle; † $p \leq 0.05$ vs. MBCD.

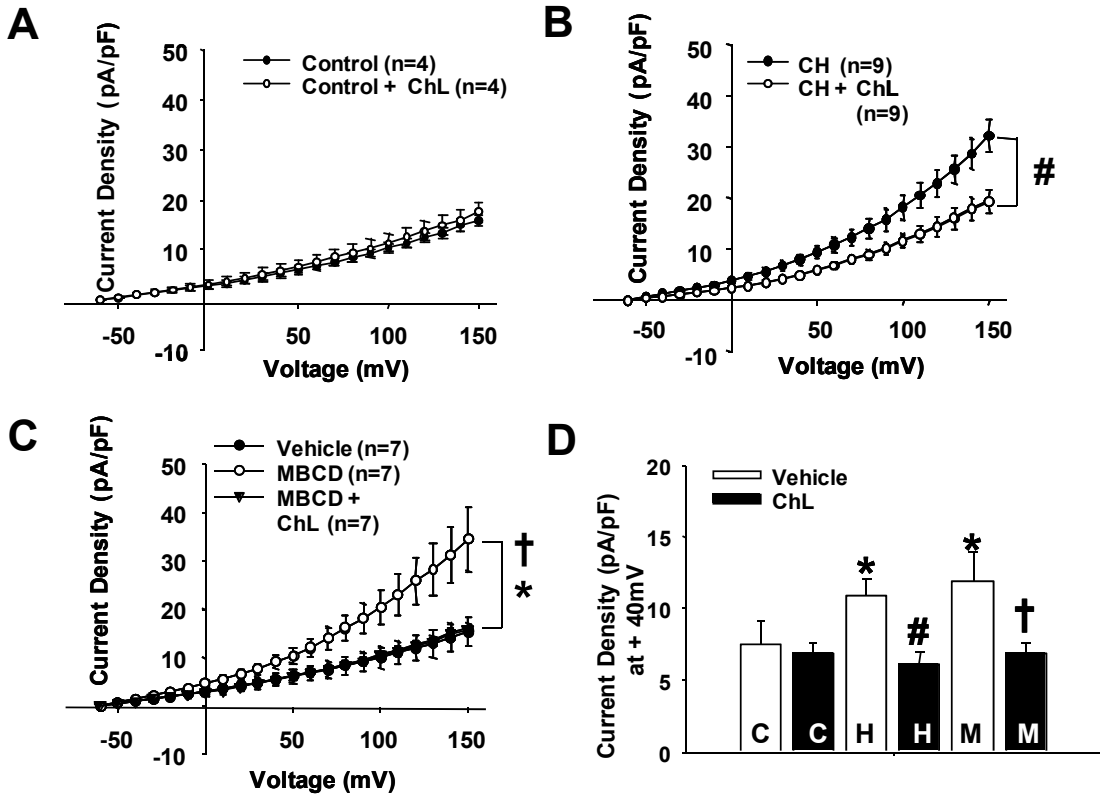


Figure 5. Effect of cholesterol repletion (Chl) on transmembrane currents. Chl diminished outward current in CH (-40 to +150 mV) **(Panel B)** and MBCD-treated (-30 to +150 mV) **(Panel C)** cells but did not affect controls **(Panel A)**. * $p \leq 0.05$ vs. control vehicle; # $p \leq 0.05$ vs. CH vehicle; † $p \leq 0.05$ vs. MBCD.

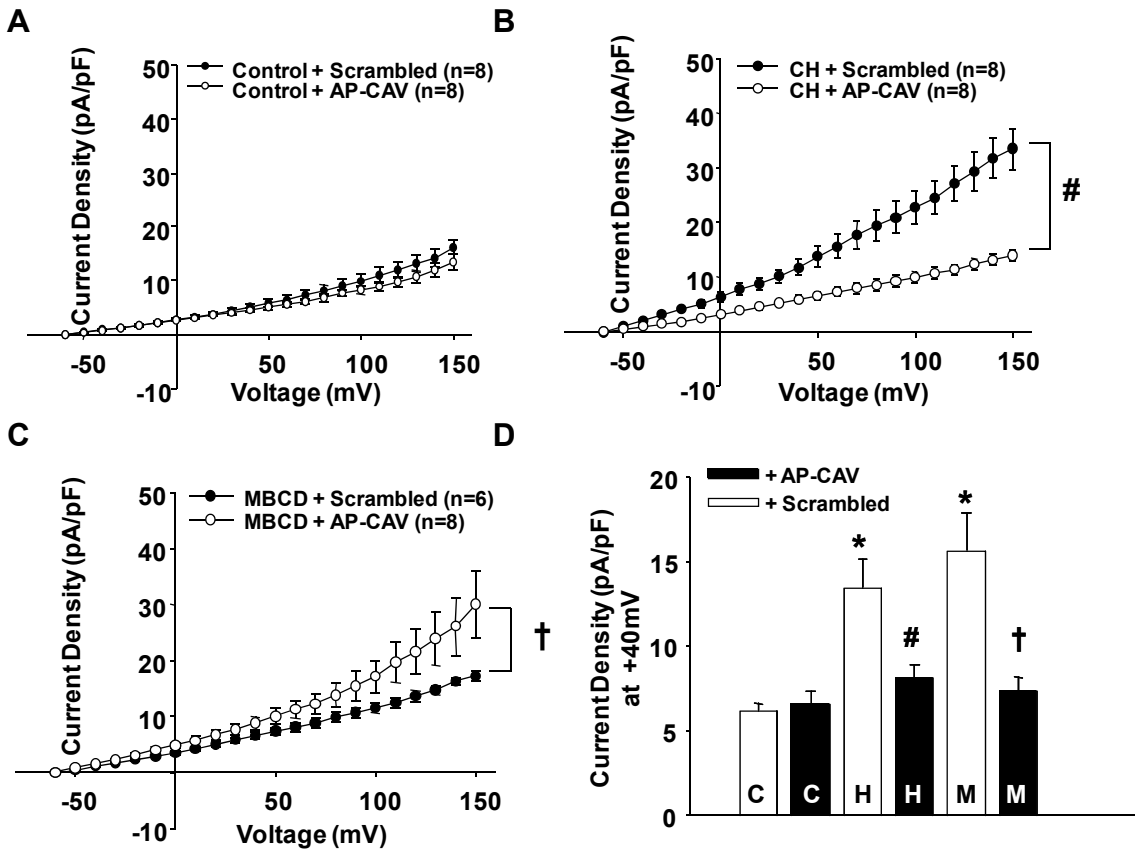
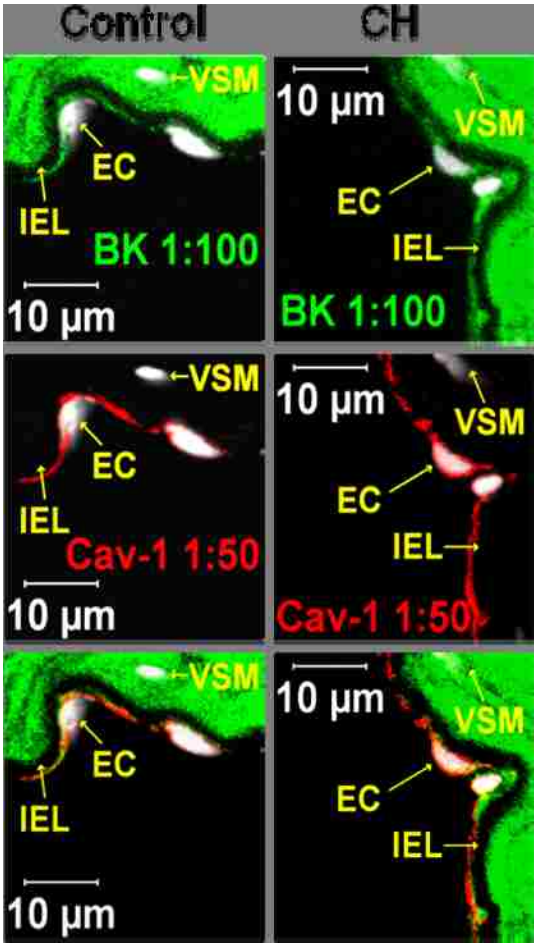


Figure 6. Effect of the cav-1 scaffolding domain peptide (AP-CAV) on transmembrane currents. AP-CAV reduced current to the level of scrambled control in CH (-50 to +150 mV) **(Panel B)** and MBCD-treated (-40 to + 150 mV) **(Panel C)** cells but did not affect controls **(Panel A)**. * $p \leq 0.05$ vs. control vehicle; # $p \leq 0.05$ vs. CH vehicle; † $p \leq 0.05$ vs. MBCD.

A



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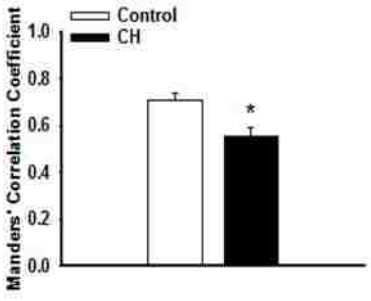


Figure 7. Co-localization of BKα (green) and caveolin-1 (red) in the endothelium (EC) and vascular smooth muscle (VSM) of gracilis resistance arterioles from control (C) and CH rats **(Panel A)**. Nuclei are stained white in all sections. Overlap of BKα and caveolin-1 was significantly less in CH ECs compared to control as determined by Manders correlation coefficient **(Panel B)**.

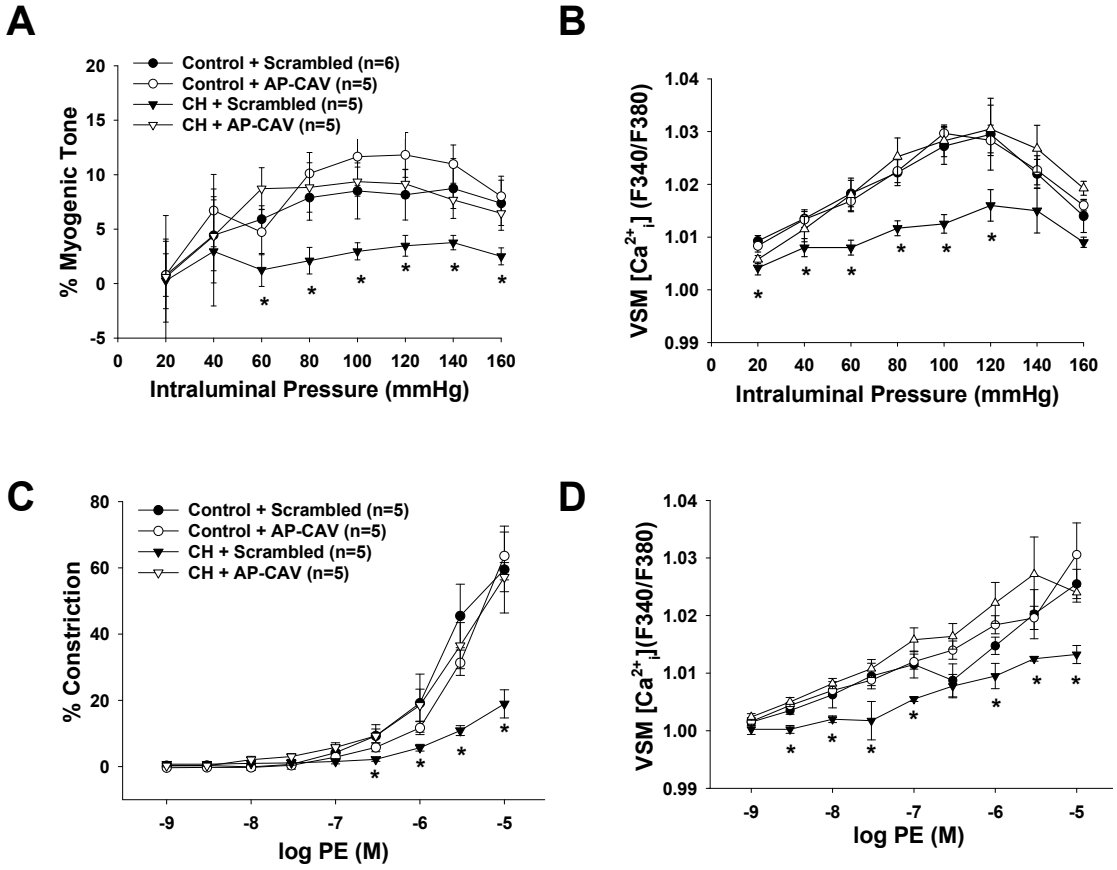


Figure 8. AP-CAV restores vasoconstrictor reactivity in arteries from CH rats to levels of scrambled controls. **Panels A and B** show the pressure-induced (myogenic) constrictor responses in arteries treated with AP-CAV or scrambled peptide and the parallel smooth muscle $[Ca^{2+}]_i$ responses. **Panels C and D** show responses to phenylephrine (PE). * $p \leq 0.05$ vs. control scrambled. Starting vessel diameters were 145 ± 7 μm and 151 ± 6 μm for myogenic and PE experiments and did not differ between experimental groups.

SUPPLEMENT: Role of Caveolin-1 in Endothelial BK_{Ca} Regulation of Vasoreactivity

Supplemental Methods:

Culture of rat aortic endothelial cells: Frozen rat aortic endothelial cells (Cell Appllications) were thawed and seeded as directed by the manufacturer. Cells were grown on attachment factor coated plates in rat endothelial cell growth medium (Cell Applications) at 37°C at 5% CO_2 in a humidified incubator to confluency for western blot and co-immunoprecipitation experiments.

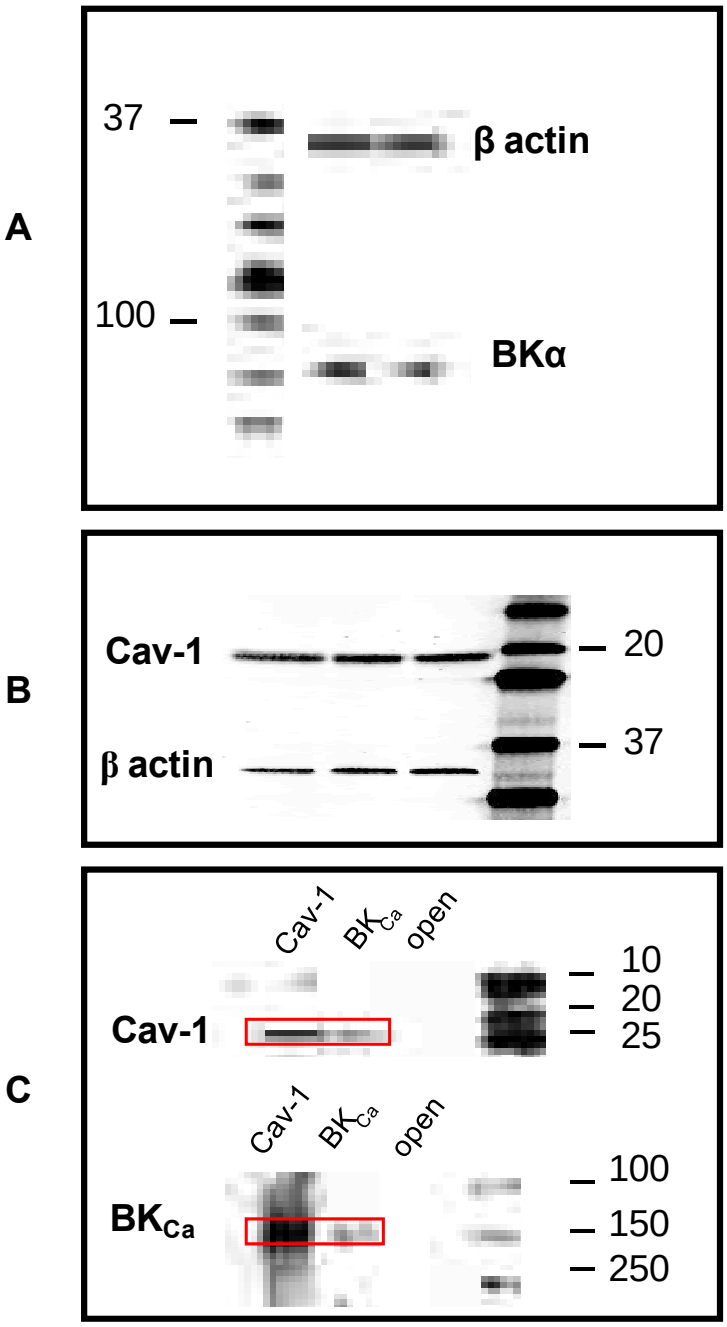
Western blot of cultured rat aortic endothelial cells: ECs passages 6-7 were washed twice in PBS then lysed in radio-immunoprecipitation assay buffer (RIPA) (Thermo Scientific) containing protease and phosphatase inhibitors (Thermo Scientific). Lysates were collected, centrifuged at 10,000 x g at 4°C for 5 minutes, and analyzed for protein content (Thermo - Scientific Pierce BCA Protein kit). Cell lysates (10 μg) were separated in 10% sodium dodecyl sulfate polyacrylamide gels by electrophoresis and transferred to a nitrocellulose membrane. Blots were blocked in odyssey blocking buffer (LI-COR) 1 h at room temperature then incubated with primary antibodies for Cav-1 (1:500) or BK_{Ca} (1:250) in 0.01% Tween PBS overnight at 4°C (BK_{Ca}: Alamone, Cav-1: Santa Cruz). After washing (3× 10 min in 0.01% Tween PBS), blots were incubated 1 h at room temperature with dye conjugated secondary antibodies (LI-COR). Images were collected on an infrared imaging system (LI-COR).

Co-immunoprecipitation of BK_{Ca} and Cav-1 in rat aortic endothelial cells: Rat aortic endothelial cells (RAECs) passages 6-7 were collected and lysed as described above. Co-immunoprecipitation (co-IP) was completed using Thermo Scientific Pierce co-IP kit as directed by the manufacturer. Briefly, the BK_{Ca} or Cav-1 antibodies were immobilized to the AminoLink Plus coupling resin or to control non-amine reactive resin for 4 hours at 4°C. Columns containing either the antibody linked resins or control non-amine reactive resins were washed and incubated overnight at 4°C with arterial lysates. The next day columns were washed and protein eluted using the kit's provided elution buffer. Using the Thermo Scientific Pierce Micro BCA Protein Assay kit, washes were tested for protein content to verify successful washing before elution. Eluted samples were analyzed by Western blotting using antibodies for BK_{Ca} or Cav-1 with infrared dye-conjugated secondary antibodies (LI-COR).

Supplemental Results

Western Blot detection of BK_{Ca} in cultured rat aortic ECs. Western blot experiments (Figure 1A) demonstrate the presence of BK_{Ca} channels and Cav-1 (Figure 1B) in cutltured rat aortic endothelial cells, supporting our findings in native tissue.

Co-Immunoprecipitation of BK_{Ca} and Cav-1 in cultured rat aortic ECs. A BK_{Ca} antibody was able to co-immunoprecipitate BK_{Ca} and Cav-1 and a Cav-1 antibody was able to co-immunoprecipitate Cav-1 and BK_{Ca} (Figure 1C). These results support our previous findings in native tissues with Cav-1 and BK_{Ca} association through immunofluorescence.



Supplemental Figure 1. Western blots for BK_{Ca} and Cav-1 in cultured rat aortic endothelial cells. Blots probed for BK_{Ca} displayed a single band around 120 kD (**Panel A**). Blots probed for Cav-1 had dense staining at 22kD (**Panel B**). Co-Immunoprecipitation experiments for BK_{Ca} and Cav-1. BK_{Ca} co-immunoprecipitated with Cav-1 and BK_{Ca} (**Panel C**) Cav-1 co-immunoprecipitated with BK_{Ca} (**Panel C**).

Addendum Chapter 3

Chapter 3 Addendum Methods:

Immunofluorescence in gracilis arterioles: Gracilis arteries from control and CH rats were collected following transcardial perfusion with PSS containing 10 mg papaverin. The gracilis muscle was removed and frozen in OCT compound in liquid N_2 and isopentane. 10 μm sections were adhered to superfrost slides (Fisher) and fixed in ice cold methanol (100%) for 10 min. After fixation sections were blocked in 4% donkey serum in PBS for 1 hr at room temperature. Sections were incubated with primary antibodies for Cav-1 (BD Biosciences 1:50). Primary antibodies for Cav-1 was detected with a Cy3-conjugated donkey, anti-mouse secondary antibody (1:500 dilution; Jackson Labs). The nuclear stain, Sytox (1:10,000 dilution; Molecular Probes) was then applied. Confocal microscope images were obtained using a 63x oil immersion DIC objective (NA =1.4). The individual channel (Cy3 for Cav-1) was thresholded and then made into a mask. A second mask was then created by tracing the endothelial cells within the lumen of arteriolar cross sections. Only Cav-1 staining within the EC mask was used for study. Relative fluorescence for Cav-1 in CH and control animals was determined and normalized to fluorescent standards for Cy3.

Immunofluorescence in RAECs: RAECs passages 4-6 were grown to confluency on attachment factor coated glass bottom dishes for imaging. Cells were fixed in 4% paraformaldehyde PBS for 10 min, permeabilized in 0.01% Triton-X PBS, and blocked in 4% donkey serum in PBS for 1 hr at room temperature. Cells were then incubated with primary antibodies for BK_{Ca} α (Alamone 1:100). Primary antibodies for BK_{Ca} α were

detected with Cy5-conjugated donkey, anti-rabbit secondary antibodies (secondary 1:500 dilution; Jackson Labs).

Patch Clamp Studies on Isolated Endothelial Cells: Endothelial cells were dispersed for electrophysiological study from control and CH rat aortae as previously described (16). One to two drops of the resulting cell suspension were seeded on a glass cover slip mounted on an inverted fluorescence microscope (Olympus IX71) for 30 min prior to superfusion. Single endothelial cells were identified by the selective uptake of the fluorescently labeled acylated low density lipoprotein Ac-LDL-Dil with a rhodamine filter (29) prior to each electrophysiological experiment. Freshly dispersed endothelial cells were superfused under constant flow (2 ml/min) at room temperature (22-23°C) in an extracellular solution (ECS: 141 mmol/l NaCl, 4.0 mM KCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 mmol/l HEPES, 10 mmol/l glucose and buffered to pH 7.4 with NaOH). Whole cell current data were generated using an Axopatch 200B amplifier (Axon Instruments). In conventional whole cell patch clamp configuration, biophysical criteria: (Seal resistance > 1GΩ, series resistance <25MΩ) was checked following membrane rupture and monitored throughout the course of the experiment. Cells were held at -60mV and were dialyzed for 5 min with an intracellular solution (ICS: 140 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 5 mmol/l Mg_2ATP, 10 mmol/l HEPES, 1 mmol/l EGTA and adjusted to pH 7.2 with KOH). $CaCl_2$ was added to yield a free-Ca^{2+} concentration of 1 μM, as calculated using WinMAXC chelator software.

Experimental Protocols:

Effect of Cholesterol Saturation of AP-CAV on Endothelial Cell BK_{Ca} Currents. AP-CAV (10μM) or scrambled was mixed into MBCD + Cholesterol (MBCD + ChL 8:1) and administered via patch pipette to control ECs. In whole cell patch clamp mode, currents were measured. Following dialyzation with AP-CAV or scrambled (MBCD + ChL), voltage steps were applied and currents were recorded. Cells were then superfused with MBCD for 15 minutes; voltage steps were applied and currents were then recorded and compared to the dialyzed cells in vehicle.

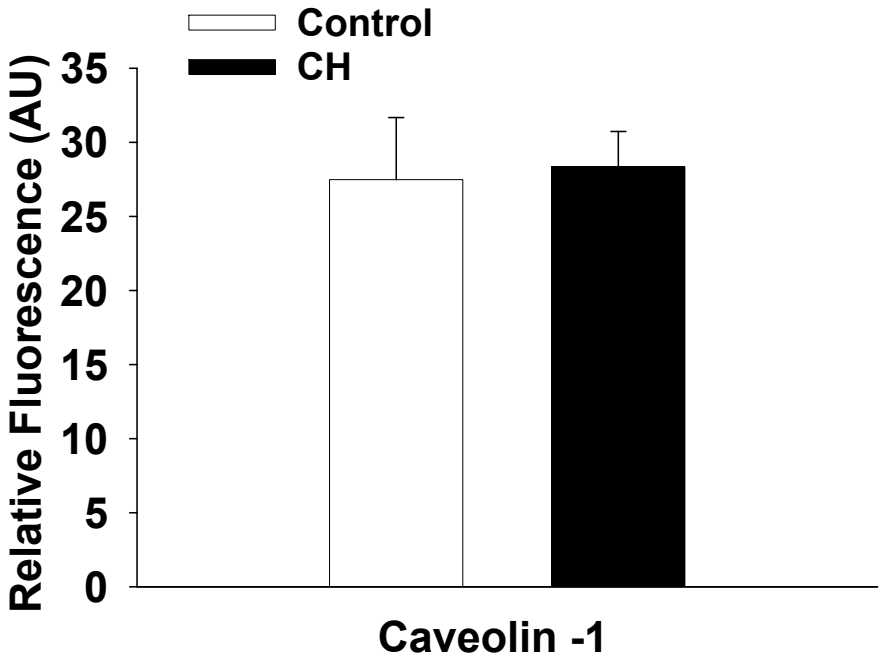
Results:

Relative Expression of Cav-1 in Gracilis Arteriole Cross Sections: Relative expression of EC Cav-1 was determined to not be different amongst control and CH animals within gracilis arterioles (**Chapter 3 Addendum Figure 1**). These results suggest there is no apparent change in Cav-1 expression following CH.

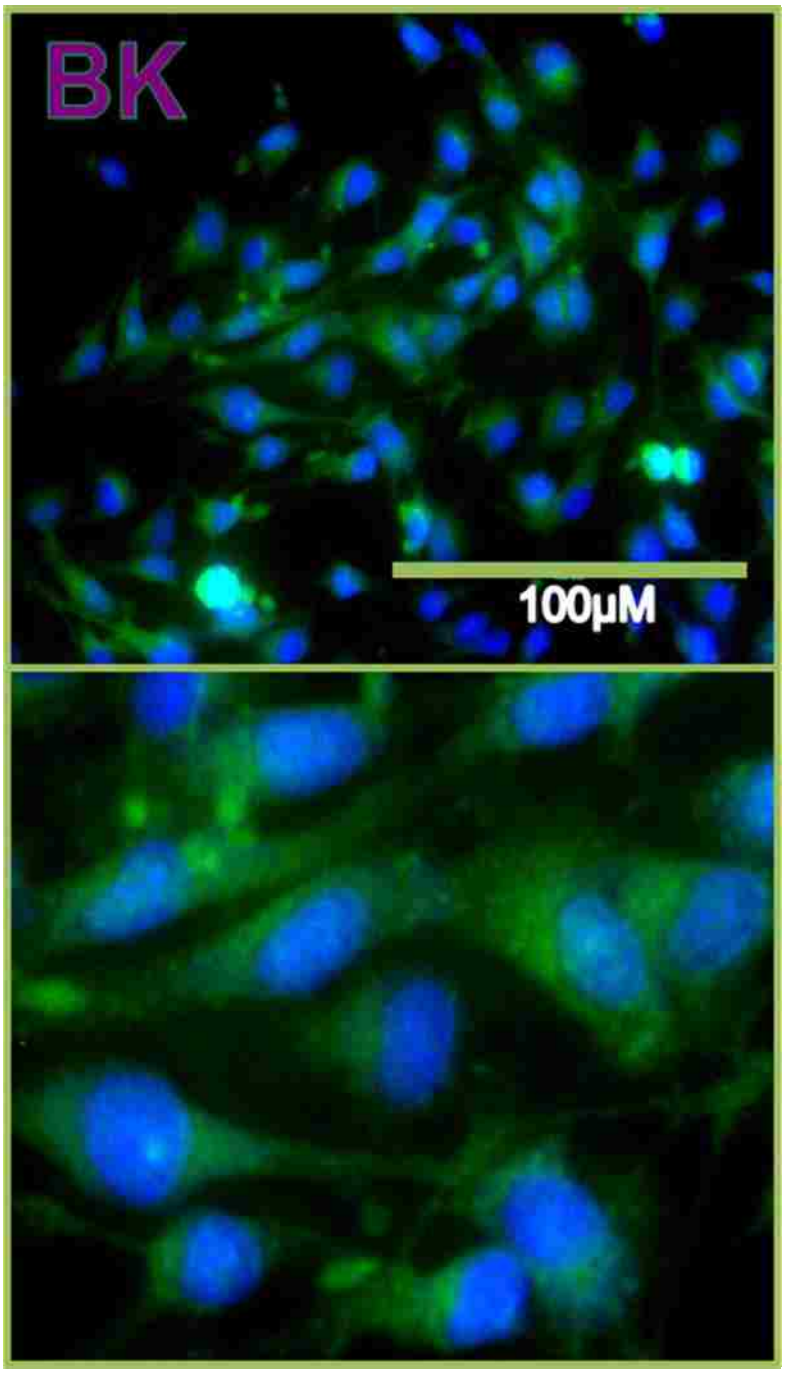
Immunolocalization of BK_{Ca} in RAECs: RAECs passages 4-6 stained positive for BK_{Ca} (**Chapter 3 Addendum Figure 2**). These results support our immunolocalization studies previously conducted in native ECs from rat aorta (Hughes *et al.,* 2010) and gracilis (**Figure 7**). These results are also in accordance with western blot studies conducted in these same cells (**Supplement Figure 1**).

Effect of Cholesterol Supplementation of AP-CAV on Endothelial Cell BK_{Ca} Currents: To further demonstrate that the effects of AP-CAV were independent of effects of cholesterol, control cells were subjected to AP-CAV/ MBCD + ChL intracellular solutions and treated with MBCD. AP-CAV / MBCD + ChL inhibited MBCD elicited

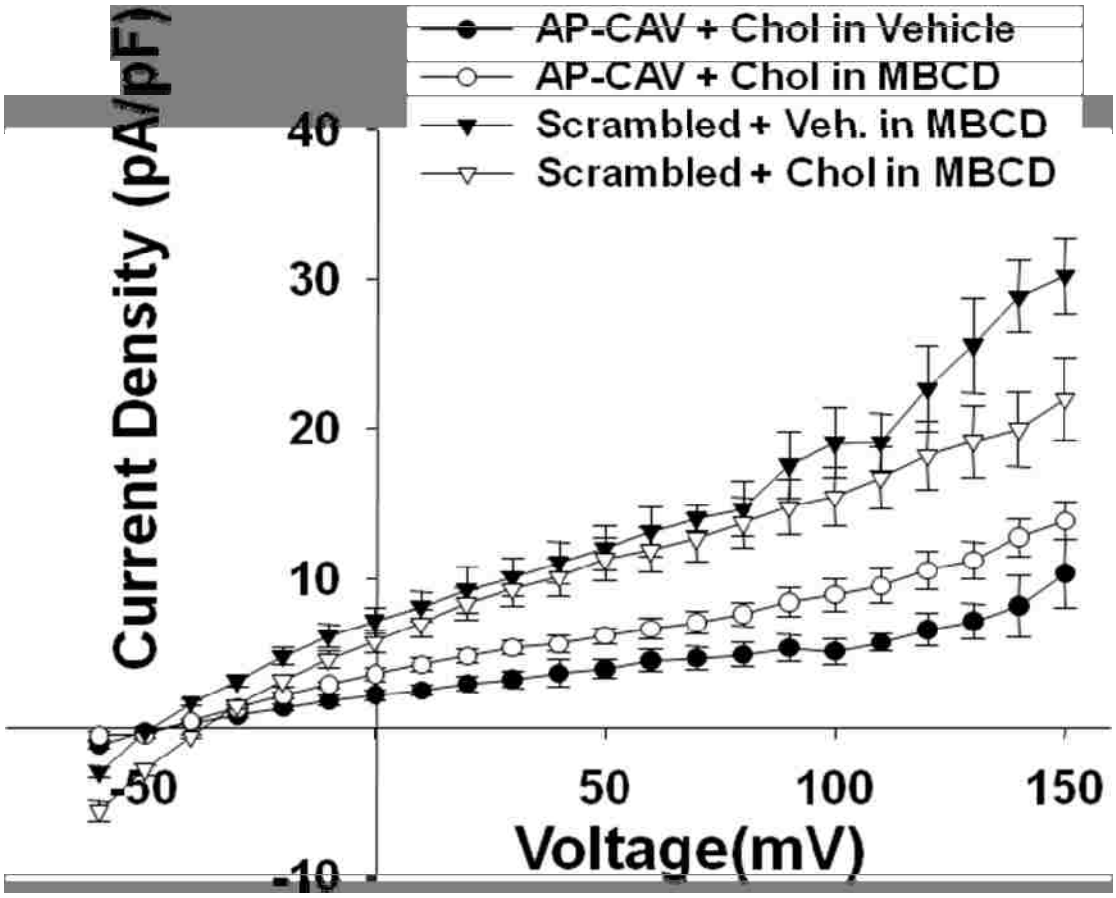
outward currents (**Chapter 3 Addendum Figure 3**), while treatment with scrambled / MBCD + ChL did not. Treatment with MBCD did not release AP-CAV/ MBCD + ChL inhibition, however in scrambled/ MBCD + ChL currents did increase following cholesterol depletion. Thus, AP-CAV and the actions of Cav-1 through its' scaffolding domain can inhibit EC BK_{Ca} independently of cholesterol.



Addendum Chapter 4 Figure 1: Gracilis arteriole cross sections from control and CH rats did not demonstrate changes in relative Cav-1 fluorescence. n = 12 images from 4 animals in each group.



Addendum Chapter 4 Figure 2: RAECs stained positive for BK_{Ca} α (pseudo-colored green) (1:100 Alamone).



Addendum Chapter 4 Figure 3: Outward currents from freshly isolated ECs dialyzed with scrambled / MBCD + ChL following cholesterol depletion with MBCD (100μM) were increased. In contrast, AP-CAV/ MBCD + ChL treated cells did not demonstrate increases to MBCD. Scrambled + MBCD/ChL vehicle is shown following MBCD treatments for comparison of scrambled / MBCD + ChL treatments. (n=4).

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CHAPTER 5: Regulation of Endothelial BK_{Ca} Channels by Heme Oxygenase-Derived Carbon Monoxide and Caveolin-1

Regulation of Endothelial BK_{Ca} Channels by Heme Oxygenase-Derived Carbon Monoxide and Caveolin-1

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Running Head: Heme Oxygenase, Caveolin-1, and Endothelial BK_{Ca}

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ABSTRACT

A novel vasodilatory influence of endothelial cell (EC) large conductance calcium activated potassium channels (BK) is present following in vivo exposure to chronic hypoxia (CH) and may exist in other pathological states. However, the mechanism of channel activation that results in altered vasoreactivity is unknown. Previously we demonstrated that inhibition of either BK channels or heme oxygenase (HO) restores vasoconstrictor reactivity following CH. Additionally, administration of the scaffolding domain of caveolin-1 (cav-1) inhibits EC BK activity and restores vasoconstrictor reactivity in this setting. These results led us to hypothesize that CH exposure results in a loss in cav-1 inhibition of EC BK channels, resulting in their activation by HO-derived carbon monoxide (CO). Experiments were conducted on freshly dispersed rat aortic ECs from control and CH (P_B = 380 mmHg for 48 hr) exposed rats and on cultured rat aortic endothelial cells (RAECs). In electrophysiology experiments, outward currents were greater in cells from CH rats as well as from cells from control rats and cultured cells treated with the cholesterol depleting agent methyl-β-cyclodextrin (MBCD). These enhanced currents were reduced to control by HO inhibition. Channel activity could be restored by the CO donor CORM-2 during HO inhibition. Administration of the scaffolding domain eliminated BK currents in cells from CH rats and current was not restored by addition of CORM-2. Co-localization experiments in freshly dispersed ECs from control and CH rats demonstrated association between HO-2, Cav-1 and BK. Additionally, co-immunoprecipitation experiments in RAECs corroborated the presence

of HO-2/BK complexes associated with cav-1. We conclude that CH unmasks EC HO-dependent BK activity by removal of an inhibitory action of cav-1 on the channel.

Key words: electrophysiology, methyl-β cyclodextrin, scaffolding domain

INTRODUCTION

Chronic hypoxia (CH) results from pathological conditions or prolonged residence at high altitude that impair oxygenation. Patients with hypoxemia resulting from obstructive lung diseases have lower calf vascular resistance (Casiglia *et al.*, 1998) and blunted reflex vasoconstriction in response to a challenge of lower body negative pressure (Heistad *et al.*, 1972). In experimental models of chronic hypoxemia, systemic vasoconstrictor responsiveness is attenuated following prolonged exposure to both normobaric or hypobaric hypoxia (Auer & Ward, 1998;Doyle & Walker, 1991). In addition, CH reduces total peripheral resistance responses to vasoconstrictor agonists (Doyle & Walker, 1991) and diminishes vasoconstrictor reactivity within several systemic vascular beds (Caudill *et al.*, 1998;Jernigan *et al.*, 2001;O'Donaughy & Walker, 2000). Upon return to normoxic conditions the blunted vasoconstrictor reactivity persists (Doyle & Walker, 1991) and remains for up to 96 hours (Jernigan *et al.*, 2001). This response is associated with vascular smooth muscle (VSM) and endothelial cell (EC) membrane potential hyperpolarization that involves activation of endothelial large conductance calcium-activated K^+ channels (BK) (Earley *et al.*, 2000;Hughes JM *et al.*, 2010;Naik & Walker, 2003). Consistent with these observations, removal of the endothelium restores agonist-induced and myogenic vasoconstrictor reactivity within the mesenteric and gracilis circulations following CH (Earley *et al.*, 2002;Earley & Walker, 2002;Hughes JM *et al.*, 2010).

Activity of endothelial BK channels is inhibited by the scaffolding domain of caveolin-1 (cav-1) (Hughes JM *et al.*, 2010;Wang *et al.*, 2005) (Riddle *et. al.,* 2011) and may be enhanced by a variety of endothelial vasoactive substances. Indeed, nitric oxide (NO), carbon monoxide (CO), and the cytochrome P450 product 11,12 epoxyeicosatrienoic acid (EET) all activate BK channels (Baron *et al.*, 1997;Bolotina *et al.*, 1994;Burnette & White, 2006;Dong *et al.*, 2007;Jackson, 2005;Xi *et al.*, 2004). Previous work from our laboratory has demonstrated a role of heme oxygenase (HO) (Gonzales & Walker, 2002) as a hyperpolarizing influence following CH that diminishes agonist-induced vasoconstrictor reactivity (Gonzales & Walker, 2002). Additionally, HO inhibition or administration of the BK channel blocker iberiotoxin similarly restores VSM membrane potential (Hughes JM *et al.*, 2010;Naik & Walker, 2003) in arteries from CH rats. There is also evidence in other cell types of association between HO and BK channels (Kooli *et al.*, 2008;Williams *et al.*, 2004;Yi *et al.*, 2010) and the HO product CO activates VSM BK channels (Jaggar *et al.*, 2002;Wang *et al.*, 1997;Wang *et al.*, 1997;Wang, 1998;Wang & Wu, 1997). Since EC BK channels appear to be tonically active following CH, we hypothesized that HO-derived CO serves as an endogenous stimulus under conditions in which cav-1 inhibition of the channel is impaired. This novel mode of endothelium-dependent vascular regulation would explain the HO- and BK-dependency of diminished vasoconstrictor reactivity in this setting.

METHODS

Animals: Experiments were performed on male Sprague-Dawley rats (Harlan). All procedures were approved by the Institutional Animals Care and Use Committee of the University of New Mexico Health Sciences Center.

Hypoxic Exposure: CH rats were exposed to hypobaric hypoxia at a barometric pressure of 380 mmHg for 48 hours. Normoxic control rats were housed in identical cages at ambient pressure (~630 mmHg).

Isolation of Endothelial Cells: Freshly dispersed aortic endothelial cells (ECs) were used for patch clamp and immunofluorescence imaging experiments. The aorta was chosen as the source of cells based on earlier results showing parallel endothelium-dependent attenuation of vasoconstrictor reactivity following CH in aortic rings and resistance vessels that is similarly reversed by heme oxygenase (HO) inhibition (Caudill *et al.*, 1998;Gonzales & Walker, 2002). Further, cells from the aorta and gracilis resistance arteries demonstrate identical effects of CH to unmask functional BK channels (Hughes JM *et al.*, 2010). Aortae were removed and placed in ice-cold HEPES buffered physiological saline solution (HBSS): mmol/l 150 NaCl, 6 mmol/l KCl, 1 mmol/l $MgCl_2$, 1.5 mmol/l $CaCl_2$, 10 mmol/l HEPES, and 10 mmol/l glucose and adjusted to pH 7.4 with NaOH. Thoracic aortae were cut longitudinally and subsequently incubated for 2 hours in basal endothelial growth medium with 4% bovine serum albumin and 10 μg/ml of the endothelial specific probe, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine percholorate (Ac-LDL-Dil) at 37°C. Immediately following the endothelial labeling procedure previously described (Hogg *et al.*, 2002;Hughes JM *et al.*, 2010;Paffett *et al.*, 2007) aortae were cut into 2 mm strips and exposed to mild digestion solution containing 0.2 mg/ml dithiothreitol and 0.2 mg/ml papain in HBSS for 45 min at 37°C. Vessel strips were removed from the digestion solution and placed in 1 ml of HBSS containing 2 mg/ml BSA. Single endothelial cells were released by gentle trituration with a small bore Pasteur pipette and were stored at 4°C between experiments for up 5 hrs. One to two

drops of the cell suspension were seeded on a glass coverslip mounted on an inverted fluorescence microscope (Olympus IX71) for 30 min prior to superfusion. Single endothelial cells were identified by the selective uptake of the fluorescently labeled acylated low density lipoprotein Ac-LDL-Dil with a rhodamine filter prior to each electrophysiological experiment.

Culture of Rat Aortic Endothelial Cells (RAECs): Due to limitations in the number of freshly dispersed cells attainable from the aortae, western blot co-immunoprecipitation studies were performed on commercially available cutured RAECs (Cell Appllications). Cells were seeded on plates coated with attachment factor solution and grown in rat endothelial cell growth medium (Cell Applications) at 37°C at 5% CO_2 in a humidified incubator. Cells were grown to confluency on attachment factor coated plates for western blot and co-immunoprecipitation experiments. In addition, patch clamp experiments were performed on these cells to demonstrate functional presence of BK channels.

Patch Clamp Studies: Freshly dispersed endothelial cells were superfused under constant flow (2 ml/min) at room temperature (22-23°C) in an extracellular solution (ECS: 141 mmol/l NaCl, 4.0 mM KCl, 1 mmol/l $MgCl_2$, 1 mmol/l $CaCl_2$, 10 mmol/l HEPES, 10 mmol/l glucose and buffered to pH 7.4 with NaOH). Whole cell current data were generated with an Axopatch 200B amplifier (Axon Instruments) following a 5 min dialysis period using 4-6 MΩ patch electrodes filled with an intracellular solution (ICS: 140 mmol/l KCl, 0.5 mmol/l $MgCl_2$, 5 mmol/l Mg_2ATP, 10 mmol/l HEPES, 1 mmol/l EGTA and adjusted to pH 7.2 with KOH]. $CaCl_2$ added to yield a free-Ca^{2+} concentration of 1 μM, as calculated using WinMAXC chelator software. Whole cell currents were measured in response to voltage steps applied from -60 mV to +150 mV

in 10 mV increments from a holding potential of -60 mV. For experiments utilizing conventional whole cell patch clamp configuration, biophysical criteria: (Seal resistance > 1GΩ, series resistance <25MΩ) was checked following membrane rupture and monitored throughout the course of the experiment.

Cultured RAECs were grown to confluency before trypsinizing and plating on glass bottom dishes for study. Cultured cells were tested for the ability to uptake Ac-LDL-Dil for endothelial verification prior to experimentation.

Protocols:

Role of HO-Derived CO in EC BK Activity: Experiments were performed on freshly dispersed ECs from control and CH rats. Whole cell macroscopic currents were recorded before and after superfusion with the HO inhibitors zinc protoporphryin (ZnPPIX; 500 nM) or chromium mesoporphyrin (CrMP; 100 μM). Additional experiments were conducted testing the effect of the HO substrate hemin (100 μM) on transmembrane currents as well as the effect of the CO donor CORM-2 (100 μM). The presence of BK currents was confirmed in some studies by use of the specific inhibitor iberiotoxin (IBTX; 100 nM) or the BK activator NS-1619 (100 μM).

Another series of experiments were performed in whole cell mode on RAECs to confirm the existence of BK currents. Macroscopic currents were observed with vehicle treatment and in the presence of CrMP or CrMP plus IBTX.

Role of Caveolin-1 and Cholesterol in EC BK Activity: Experiments were performed on freshly dispersed cells from control and CH rats. Cells from control animals were superfused with the cholesterol depleting agent methyl-β-cyclodextrin (MBCD; 100 μM) to disrupt cav-1 microdomains and unmask BK currents as we have previously reported

(Hughes JM *et. al*., 2010; Riddle *et. al.,* 2011). Following this treatment, cells were superfused with ZnPPIX to assess the role of HO on channel activity. Cells from CH rats were first dialyzed with the cell permeant cav-1 scaffolding domain peptide (AP-CAV; 10 mM) to inhibit channel activity as previously reported (Riddle *et. al.,* 2011) and then superfused with the CO donor CORM-2 as above to test if exogenous CO restores current.

***Immunofluorescence of Isolated Endothelial Cells*:** Endothelial cells were freshly dispersed from the aorta (see detailed methods above) and used for immunofluorescent detection of BK α, HO-1, HO-2, and cav-1. One to two drops of the cell suspension were seeded on a glass coverslip for 30 min prior to fixation in 4% formaldehyde PBS at room temperature for 15 min. After fixation cells were permeabilized in 0.01% Triton-X PBS for 10 min and blocked in 4% donkey serum in PBS for 1 hr. Cells were incubated with primary antibodies for HO-1 (Stressgen) or HO-2 (Stressgen), and cav-1 (BD Biosciences) for association of HO and cav-1. Other sections were treated with primary antibodies for BKα, (Alamone) and HO-1 or HO-2. All primary antibodies were detected with secondary antibodies conjugated to fluorescent dyes. Isolated cells were visualized with a confocal laser microscopy (LSM 510 Zeiss; 63x oil immersion lens). Co-localization of HO-1 and BK or HO-2 and BK was analyzed by calculating the Manders correlation coefficient. Co-localization (pixel overlap between channels) was defined by Manders correlation coefficient values (Manders *et al.*, 1992) ranging from 0 to 1, with values close to 0 indicating non-overlapping images and values close to 1 reflecting co-localization. Similar analysis was performed to assess the association of cav-1 with HO-1 and HO-.2.

Western blot of cultured rat aortic endothelial cells: ECs passages 6-7 were washed twice in PBS then lysed in radio-immunoprecipitation assay buffer (RIPA) (Thermo Scientific) containing protease and phosphatase inhibitors (Thermo Scientific). Lysates were collected, centrifuged at 10,000 x g at 4°C for 5 minutes, and analyzed for protein content (Thermo - Scientific Pierce BCA Protein kit). Cell lysates (10 μg) were separated in 10% sodium dodecyl sulfate polyacrylamide gels by electrophoresis and transferred to a nitrocellulose membrane. Blots were blocked in odyssey blocking buffer (LI-COR) 1 h at room temperature then incubated with primary antibodies for HO-1 (1:500) or HO-2 (1:200) in 0.01% Tween PBS overnight at 4°C (HO-1: Stressgen, HO-2: Stressgen). After washing (3× 10 min in 0.01% Tween PBS), blots were incubated 1 h at room temperature with dye conjugated secondary antibodies (LI-COR). Images were collected on an infrared imaging system (LI-COR).

Co-immunoprecipitation of BK_{Ca}, Cav-1, HO-1, and HO-2 in rat aortic endothelial cells: Rat aortic endothelial cells, passages 6-7, were collected and lysed as described above. Co-immunoprecipitation (co-IP) was completed using Thermo Scientific Pierce co-IP kit as directed by the manufacturer. Briefly, the BK (Alamone 1:250), caveolin-1 (Santa Cruz 1:500), HO-1, or HO-2 antibodies were immobilized to the AminoLink Plus coupling resin or to control non-amine reactive resin for 4 hours at 4°C. Columns containing either the antibody linked resins or control non-amine reactive resins were washed and incubated overnight at 4°C with arterial lysates. The next day columns were washed and protein eluted using the kit's provided elution buffer. Using the Thermo Scientific Pierce Micro BCA Protein Assay kit, washes were tested for protein content to verify successful washing before elution. Eluted samples were analyzed by Western

blotting using antibodies for BK, cav-1, HO-1, or HO-2 with infrared dye-conjugated secondary antibodies (LI-COR).

RESULTS

Role of HO-Derived CO in EC BK Activity. Endothelial transmembrane currents in cells from CH rats were significantly larger than controls as previously reported (Hughes JM *et. al.,* 2010). This enhanced current in cells from CH rats has been previously shown to be blocked by IBTX (Hughes JM *et. al.,* 2010), as seen in the present studies (Figure 1C). Interestingly, blockade of HO activity in ECs from CH animals also normalized currents to controls similar to IBTX (Figure 1B and C), without effect in control cells (Figure 1A). Inhibition of BK activity by HO blockade was reversed by the BK channel opener NS-1619 (Fig. 1D/ 1E). We have previously shown that NS-1619 is without effect in control cells (Hughes JM *et. al.,* 2010). Thus, tonic BK channel activity is dependent upon an HO product following CH, however functional channels are still present in these cells and can be activated by other stimuli.

We also observed that addition of excess substrate for the HO enzyme caused further enhancement of outward current only in cells from CH rats. Figure 2 shows this differential effect of hemin in cells from the two groups of animals, however this augmentation was only observed at very positive potentials and may not be relevant at physiological membrane potentials. Nevertheless, these data suggest that HO activity may be mildly substrate limited in this preparation and that further stimulation of HO is associated with greater channel activity.

Role of CO in BK Channel Activation. CO is a product of HO and has been shown to activate VSM BK channels (Jaggar *et. al.,* 2002). We performed experiments to test whether administration of a CO donor could reverse the effects of HO inhibition. Figure 3A and B show the effect of CrMP to inhibit outward current and the efficacy of the CO donor CORM-2 to reverse this effect in cells from CH rats. Although CORM-2 reversed the effects of CrMP, it was without further effect in the absence of HO blockade in cells from either control or CH rats (Figure 3 C-E). These data support the hypothesis that HO-derived CO is responsible for tonic activation of EC BK channels in cells from CH animals.

HO-Dependence of BK Currents in Cholesterol Depleted EC. We have previously demonstrated that cholesterol depletion with MBCD (100 μM) unmasks BK currents in ECs from control animals due to a loss in cav-1 inhibition of the channel (Hughes JM *et. al.,* 2010, Riddle *et. al.,* 2011). Thus we hypothesized that disruption of cav-1 inhibition of BK channels by cholesterol depletion would elicit a HO-dependent activation of the channel similar to results observed in cells from CH animals. Consistent with this hypothesis, MBCD treatment of control ECs elicited HO-dependent currents (Fig 4A/B). Thus, like in CH cells, tonic BK channel activity is dependent upon HO when caveolar domains are disrupted. These results suggest a tight coupling of HO with BK channels in these cells within lipid-rich microdomains.

Role of the Scaffolding Domain of Caveolin-1 on HO-dependent BK Currents. Similar to our previous results (Hughes JM *et. al.,* 2010; Riddle *et. al.,* 2011), AP-CAV treatment significantly decreased outward currents from CH ECs (Figure 5 A and B). In contrast to the HO inhibition experiments above however, exogenous CO had no effect in the

presence of AP-CAV. These results support the hypothesis that cav-1 inhibits BK activity rendering the channels insensitive to activators such as NS-1619 and CO.

Co-localization of HO-1, HO-2, with Cav-1; BK α with HO-2.. Immunofluorescence imaging of freshly dispersed ECs demonstrated co-localization of HO-2 with BK α in aortic ECs from CH and control rats. However, the Mander's correlation coefficient representative of pixel overlap between fluorescence channels was not different between CH and controls (Figure 6A). In contrast to HO-2, HO-1 demonstrated little co-localization with BK in cells from either group of rats.

Caveolin-1 demonstrated similar co-localization with both HO-1 and HO-2 in cells from each group (Figure 6B). Thus, both isoforms of HO appear to be present in caveolin-containing domains, but only HO-2 might be associated with the BK channel (Kim *et al.*, 2004).

HO-dependent BK_{Ca} currents in RAECs. Due to limitations in collecting sufficient endothelial cell specific samples from native tissue, cultured RAECs were used for this and subsequent western blot protocols. As a validation for use of these cells, a limited number of patch clamp experiments were performed to confirm the presence of BK currents and their regulation by HO in RAECs. We found that blockade of HO activity with CrMP significantly decreased outward currents in RAECs (Figure 7). The combination of IBTX and the HO inhibitor CrMP had no further inhibitory effect. These results demonstrate that HO-dependent BK complexes are likely present in cultured RAECs.

Western Blot detection of HO-1, and HO-2 in RAECs. Single bands for HO-1 or HO-2 appeared at 32 kD and 36kD respectively, (Figure 8) within the reported molecular

weights for each isoform. Previous studies had identified BK and cav-1 in RAECs (Riddle *et. al.,* 2011).

HO-2 and BK α immunoprecipitated in RAECs. Caveolin-1 immunoprecipitated with HO-1 and HO-2 (Figure 8B), and with BK α (Figure 9C) supporting previous work demonstrating association between BK and cav-1 (Riddle *et. al.,* 2011; Wang *et al.*, 2005) in cultured ECs; and cav-1 and HO-1 / cav-1 and HO-2 in pulmonary artery endothelial cells (Kim *et al.*, 2004). BK α immunoprecipitated with cav-1 and HO-2 (Fig. 9A); whereas HO-2 immunoprecipitated with BK α and cav-1 (Figure 9B). HO-1 immunoprecipitated with cav-1 only. These results support the co-localization data in freshly dispersed ECs and suggest that HO-2 / BK complexes exist in ECs in association with cav-1.

DISCUSSION

The major findings from this study are: 1) endothelial BK channels exhibit tonic HO-dependent activation following *in vivo* exposure to CH or *ex vivo* cholesterol depletion; 2) cultured RAECs demonstrate similar HO-dependent BK channel activity ; 3) BK channel activity can be restored following HO inhibition in cells from CH rats by the HO product CO or by NS-1619; 4) association of BK channels with the scaffolding domain of cav-1 prevents activation by HO or the HO product CO; and 5) EC BK channels are associated with the HO-2 isoform, but not HO-1 in caveolin enriched domains. These findings suggest that BK channels and HO-2 form a functional unit within caveolae that is regulated by the scaffolding domain of cav-1.

Our findings of a functional association between HO-2 and BK channels in endothelial cells are consistent with prior observations in other cell types. Recent co-immunoprecipitation experiments in HEK293 and glomus type I cells (Williams *et al.*, 2004) found that BK and HO-2 form oxygen sensitive complexes. Interestingly, knockdown of HO-2 significantly decreased channel activity (Williams *et al.*, 2004). The authors hypothesized that acute hypoxia inhibits HO production of CO and thus limits channel activity, suggesting that HO-2 is the "oxygen sensor" that regulates glomus cell membrane potential and hence neurotransmitter release in the carotid body. BK channels are hemoproteins, and upon binding of heme are potently inhibited. In conditions in which free heme can be degraded by HO, the channel remains active and can be stimulated by the HO product CO (Yi *et al.*, 2010). However, experiments characterizing the heme binding domain (HBD) of BK channels found that decreased oxygen and a reduced state of the HBD significantly increased the channel's affinity for heme and resulted in potent inhibition (Yi *et al.*, 2010). In contrast, under normoxic conditions channel affinity was much higher for CO than heme and permitted channel activity (Yi *et al.*, 2010). Thus, HO function and heme/CO binding appear to be very important regulators of BK activity and associate with the channel to form "oxygen sensitive" complexes. In the present studies, all experiments were performed under normoxic conditions to eliminate any complicating effects of acute hypoxia. Thus, *in vivo* during CH, the regulation of EC BK channels could be affected by the influence of acute hypoxia on HO activity. However, the degree of hypoxia used *ex vivo* to inhibit HO activity is likely much more severe than that observed in the intact vessel wall during CH. The level of hypoxia reported to decrease HO activity is below 10 torr (Carraway *et al.*,

1998) (Carraway *et al.*, 2000), whereas the partial pressure of O_2 (PaO_2) measured in *in vivo* blood gases in rats under CH conditions is around 41 torr (Doyle & Walker, 1991). Furthermore, we have observed that the HO-dependent attenuation of vasoconstriction in intact animals is largely unaffected by acute restoration of normoxia (Doyle & Walker, 1991).

The present study also confirms the important regulatory role of cav-1 on EC BK function. Under control conditions, cav-1 potently inhibits HO-dependent activation of BK channels as evidenced by the lack of BK current under control conditions and the inability to activate channel activity with NS-1619 or CORM-2. However, CH exposure decreases association between cav-1 and BK channels (Riddle *et. al.,* 2011) enabling the channel activation by endogenous gaseotransmitters such as CO, and NO (Hughes JM *et al.*, 2010). Recent studies in vascular ECs demonstrate that HO isoforms 1 and 2 co-localize and are inhibited by association with cav-1 (Kim *et al.*, 2004). Thus a derangement in cav-1 function following CH exposure may enable not only accessibility of the channel to activators and permit channel opening, but could possibly increase endogenous HO activity. Although previous studies found enhanced vascular HO-1 expression following CH (Jernigan *et. al.,* 2001), both immunofluorescence on native cells and immunoprecipitation experiments on RAEC suggest that BK channels associate with HO-2 in endothelial cells and this enzyme isoform is likely responsible for channel activation. Interestingly, co-localization experiments demonstrated no decreases in association of either HO isoform with cav-1 in native cells following CH. These results suggest that the BK, cav-1, and HO-2 complex is intact in control conditions, but only becomes active following CH or cholesterol depletion due to decreased cav-1 inhibition

of the channel. Although HO-1 appears to also be associated with cav-1, it is not part of the BK signaling entity.

With sufficient levels of HO activity following CH to activate EC BK channels, the next question that arises is how HO activity is driving BK activity. In human BK channels, the breakdown of heme by heme oxygenase enables enhanced channel activity from production of CO, presumably through CO binding of a redox sensitive domain with the C-terminus (Christou *et al.*, 2000;Yi *et al.*, 2010). Our results confirm the ability of CO to activate EC BK channels and are consistent with earlier reports in human umbilical vein endothelial cells (Wang *et. al.,* 1997). Our results also indicate that channel activity is near maximum tonically in a HO-dependent fashion. This later result may be related to the greater calcium sensitivity that we have observed in EC BK channels compared to similar channels in VSM (Riddle *et. al.,* 2011) where localized calcium events (sparks) are required for full activation. In addition, the apparent enhanced calcium sensitivity of EC BK channels could be due to an effect of CO itself as previously suggested (Jaggar *et. al.,* 2002). We propose that HO-BK complexes are inhibited in control conditions due to channel association with cav-1 and its scaffolding domain. The rapid emergence of HO-dependent BK currents in cells from control animals treated with MBCD illustrates how cav-1 function either permits or inhibits this unique complex.

To conclude, CH exposure results in a loss of cav-1 inhibition of EC BK channels, that are dependent for activation by HO-derived CO. Activation of these normally dormant EC BK channels results in EC hyperpolarization (Hughes JM *et al.*, 2010) (Riddle *et. al*., 2011) and subsequent vascular wall hyperpolarization and

diminished vasoconstrictor reactivity as previously demonstrated (Caudill *et al.*, 1998;Earley & Walker, 2002;Hughes JM *et al.*, 2010;Naik & Walker, 2006).

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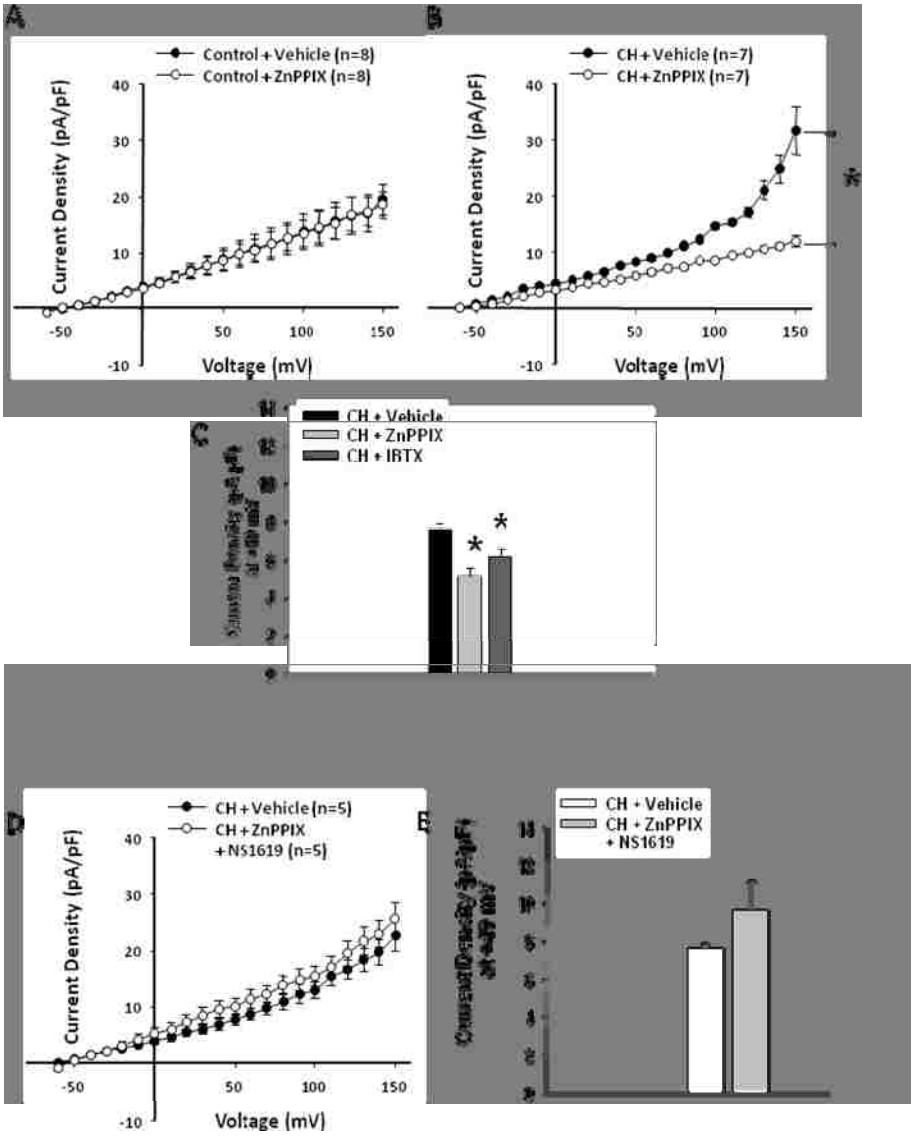

Figure 1. Heme oxygenase inhibition with ZnPPIX decreased outward currents only in ECs from CH rats (Panel B), while having no effect in controls (Panel A). The inhibitory effect of ZnPPIX in CH cells was not different from the effect of the BK specific inhibitor IBTX (Panel C). Panels D and E show the effect of the BK activator NS-1619 to restore outward current to CH vehicle levels following HO inhibition. NS-1619 is without effect in control cells (Hughes JM *et. al* 2010). * $p < 0.05$ vs. vehicle treated CH.

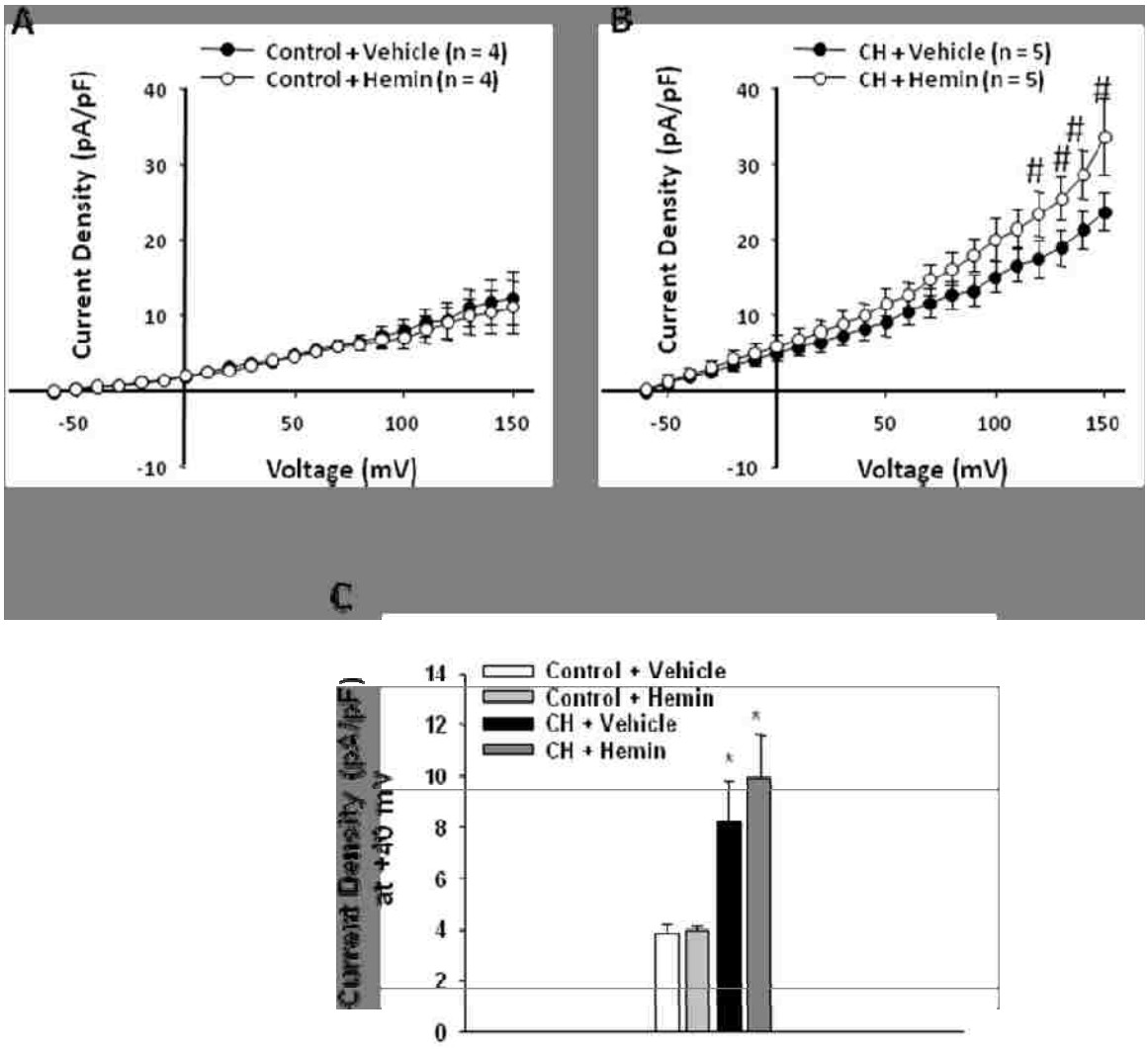

Figure 2. Addition of the HO substrate hemin increased outward currents in the CH group (Panel B) but was without effect in control cells (Panel A). Panel C shows the summary data at +40 mV. * $p < 0.05$ vs. vehicle treated Controls.

# $p < 0.05$ vs. vehicle treated CH.

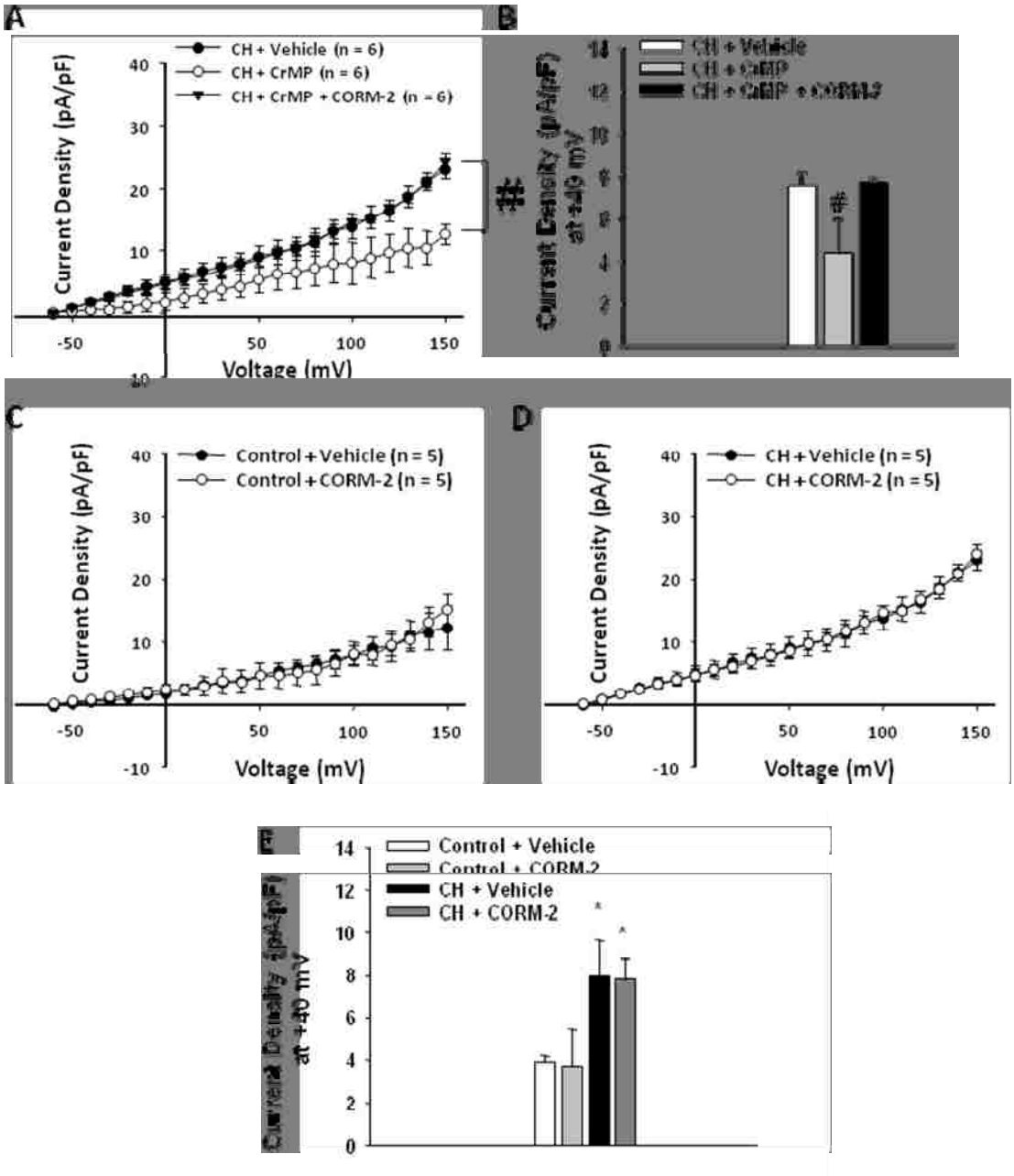

Figure 3. Similar to with ZnPPIX, outward currents were diminished in cells from CH rats by the HO inhibitor CrMP, however current was restored by the CO donor CORM-2 (Panel A). These data are summarized in Panel B. In contrast, CORM-2 was without effect on transmembrane currents in the absence of HO inhibition in either group (Panels C and D). * $p < 0.05$ vs. vehicle treated controls. # $p < 0.05$ vs. vehicle treated CH

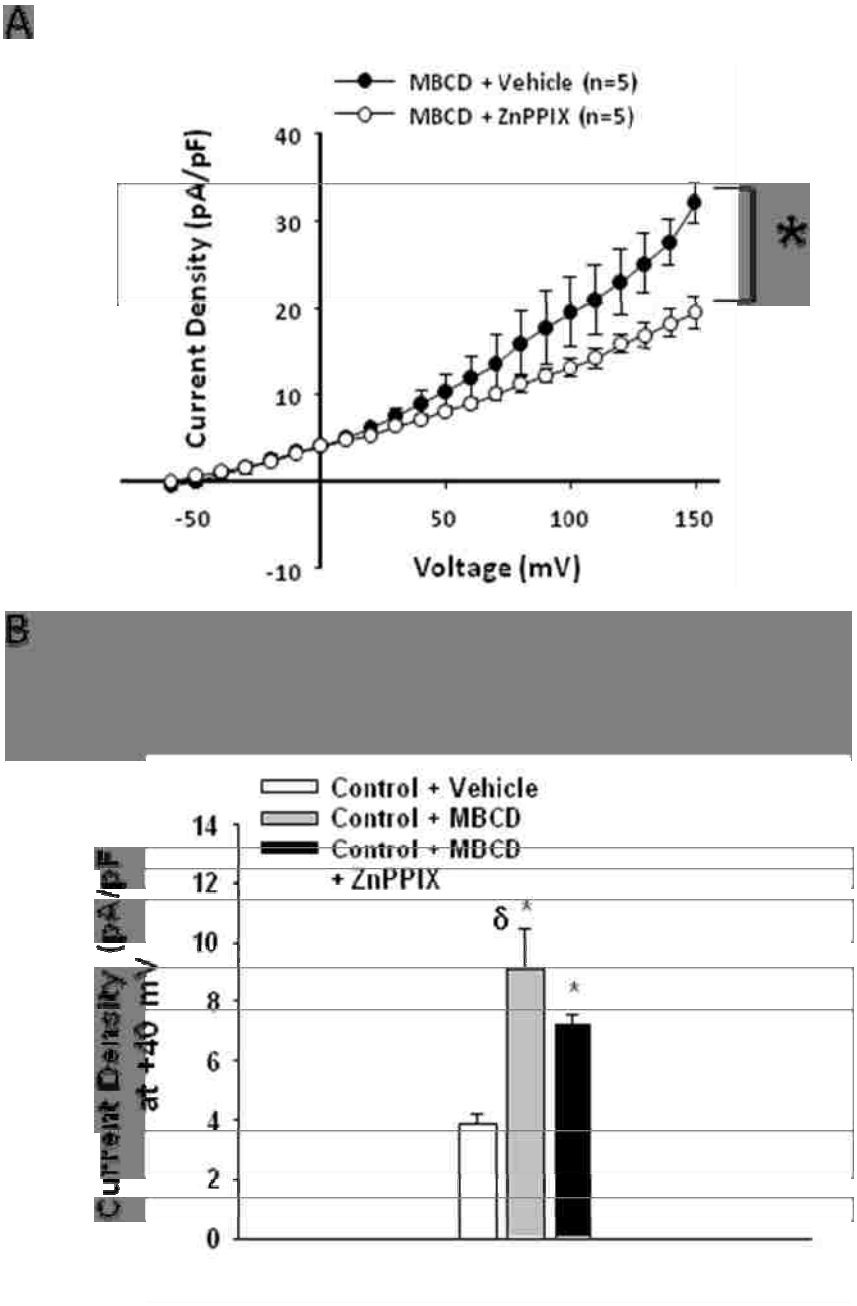

Figure 4. Cholesterol depletion in control ECs to disrupt cellular caveolin-1 function resulted in outward currents that were significantly larger than non cholesterol depleted control ECs (Panel B). The enhanced outward currents were additionally sensitive to HO blockade (Panels A/B). * $p < 0.05$ vs. MBCD treatment . δ $p < 0.05$ vs. vehicle treated controls

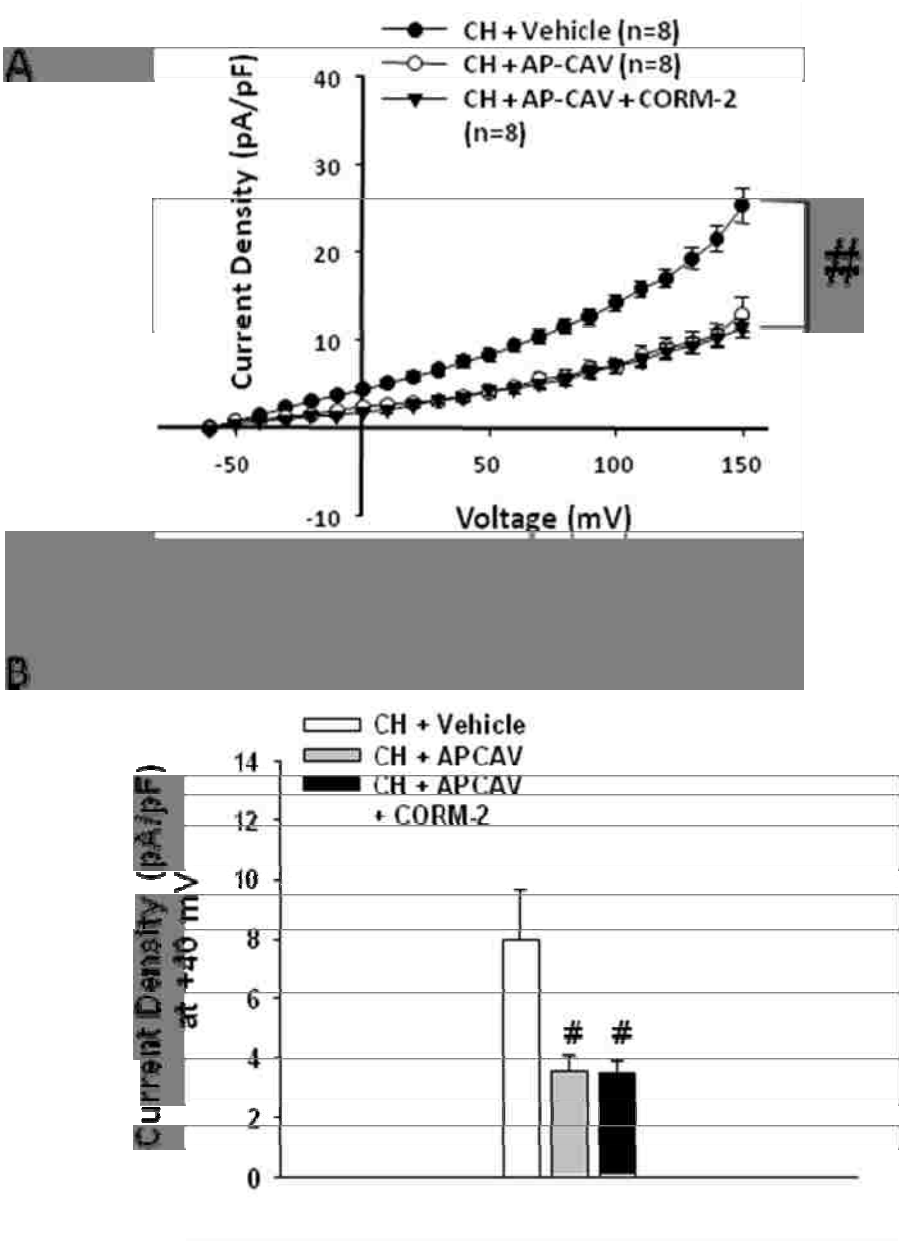

Figure 5. EC HO-dependent BK currents from CH rats were significantly reduced following treatment with a cell permeant peptide of the scaffolding domain of caveolin-1 (AP-CAV). Unlike following HO inhibition (Figure 3), current was not restored by exogenous CO (Panels A and B). # $p < 0.05$ vs. vehicle treated CH

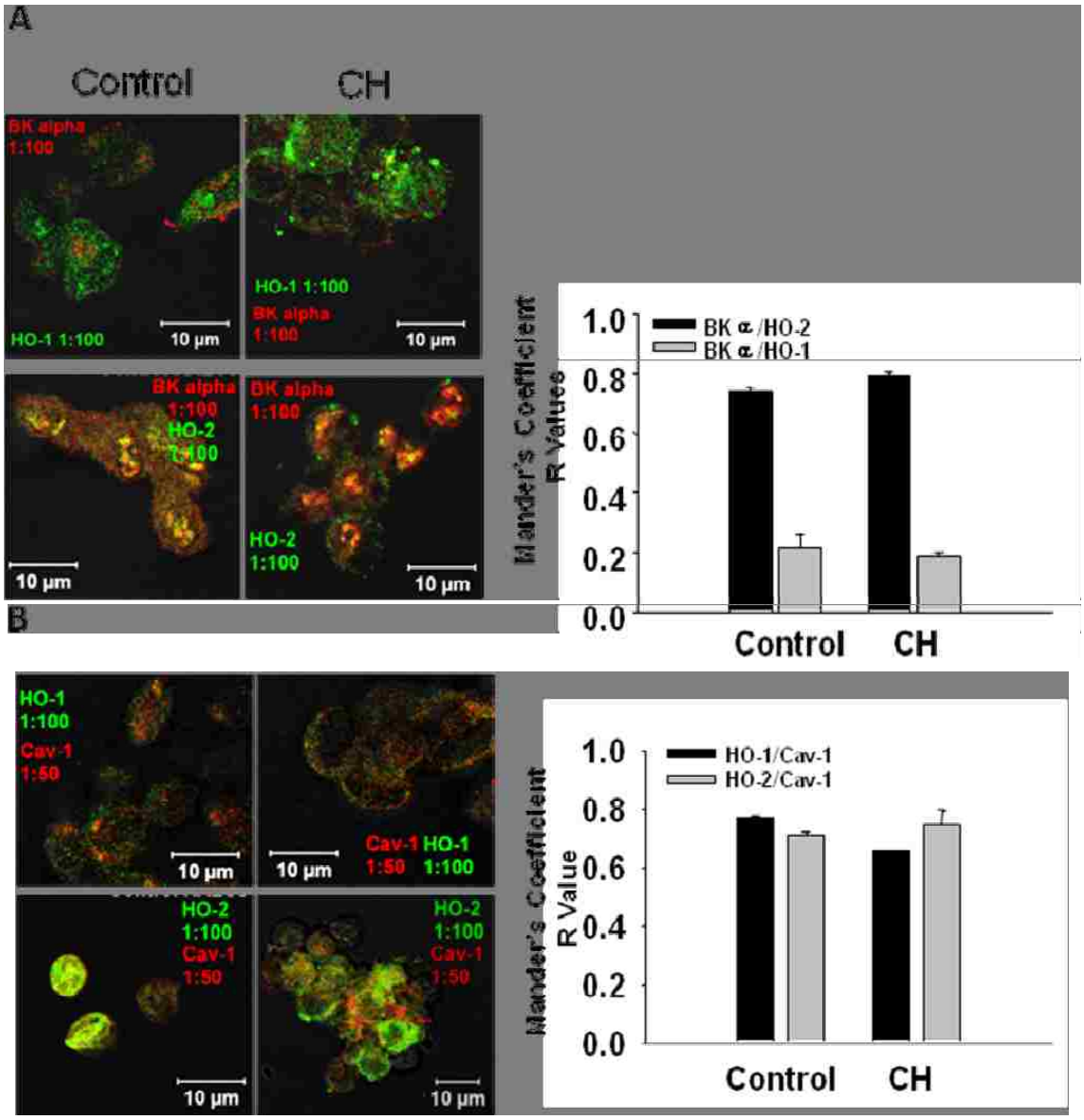

Figure 6. Freshly dispersed rat aortic ECs stained positive for BK α , HO-1, HO-2, and Cav-1. Co-localization between HO-2 and BK α did not differ between CH and control groups (Panel A). Cav-1 co-localized with both HO-1 and HO-2 in native tissue. No differences between HO-1/ Cav-1 or HO-2/Cav-1 association were detected in either group (Panel B).n = 4-5 animals, 5-8 images were obtained from each n.

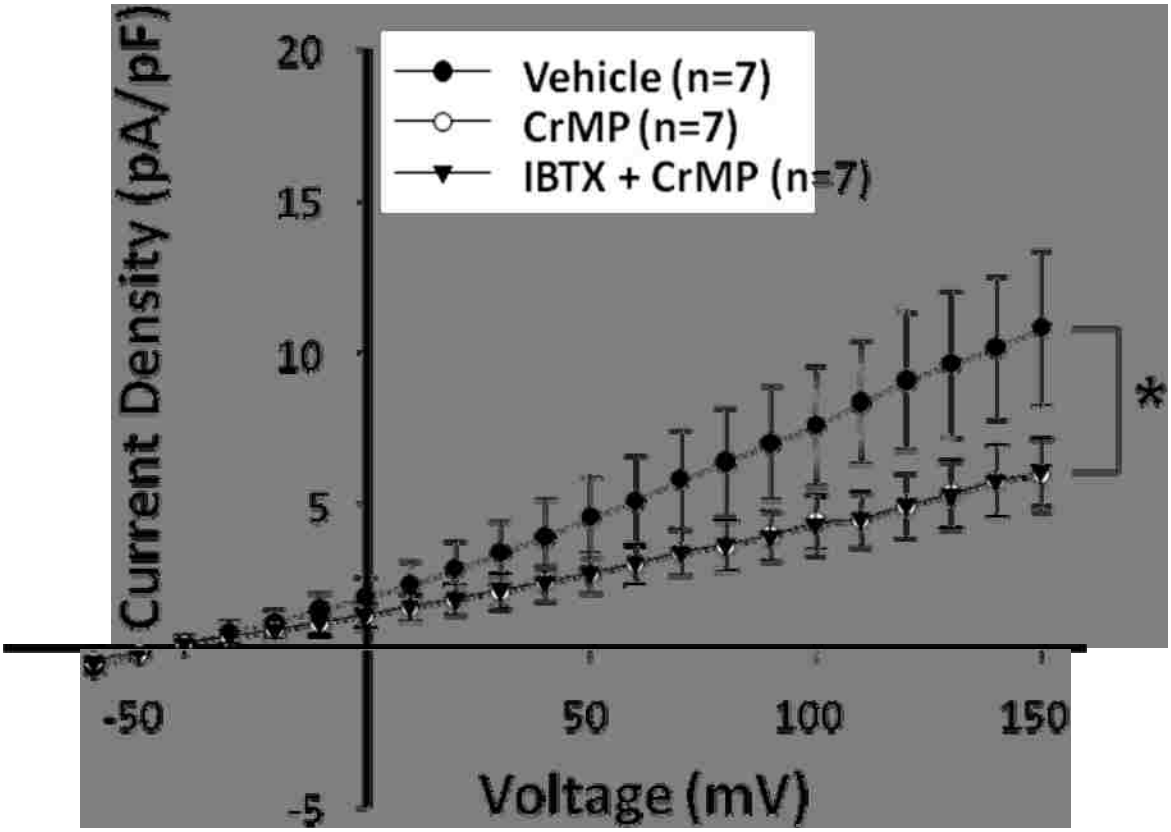

Figure 7. Cultured rat aortic endothelial cells (RAECs) demonstrated an outward current that was reduced by the HO inhibitor CrMP. Current was not further diminished by IBTX demonstrating that the HO-sensitive component was due to tonic BK activation.

* $p < 0.05$ vs. vehicle treated RAECs

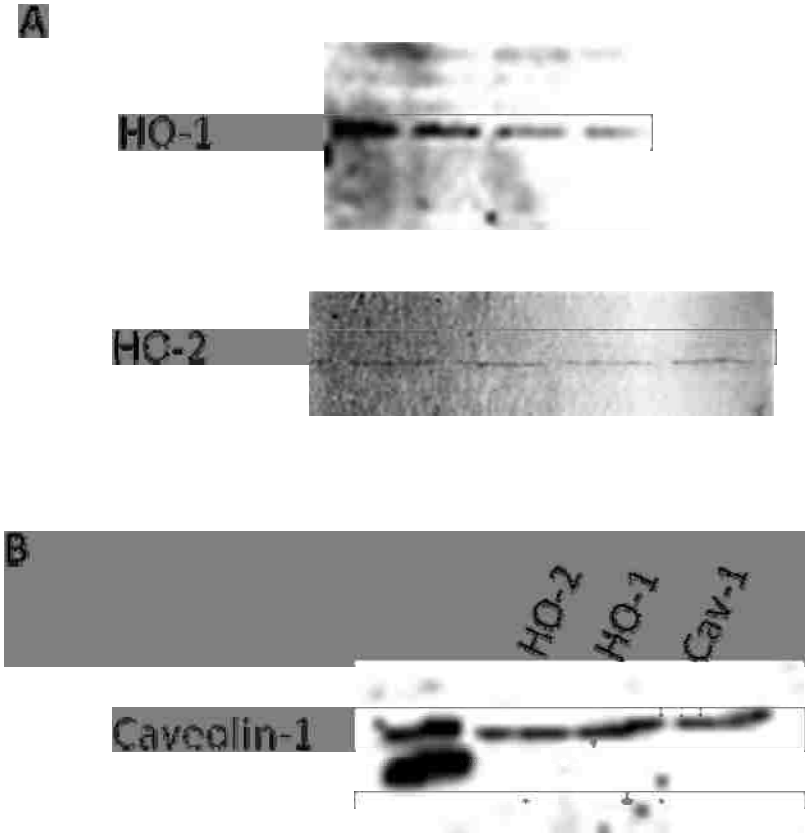

Figure 8. Western blots in RAECs (passage 6) detected HO-1 and HO-2 (Panel A). HO-1 and HO-2 co-immunoprecipitated with cav-1 (Panel B). Western blots and co-immunoprecipitation experiments were repeated three times for demonstration of reproducibility.

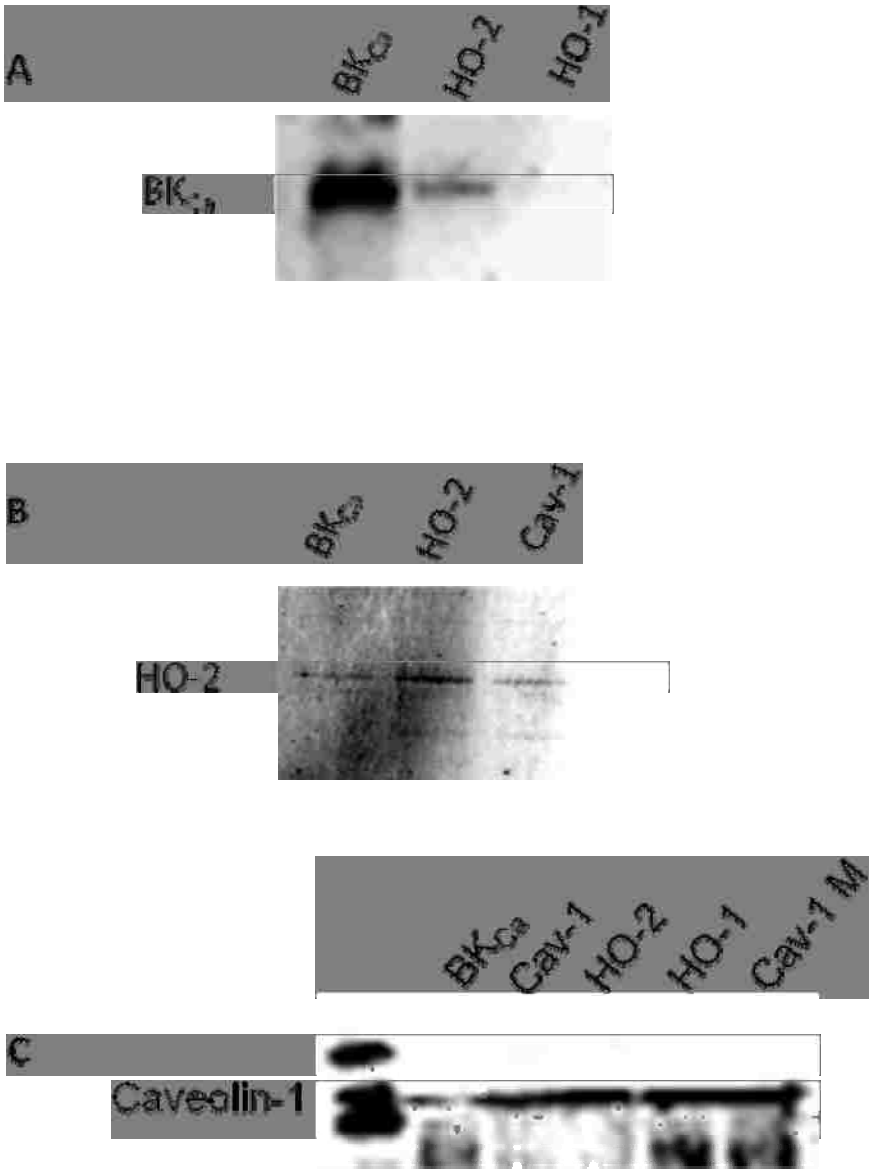

Figure 9. HO-2 but not HO-1 co-immunoprecipitated with BK α (Panel A) in RAECs.. In addition, cav-1 and BK α co-immunoprecipitated with HO-2 (Panel B). Caveolin-1 co-immunoprecipitated with HO-1, HO-2, and BK α (Panel C). co-immunoprecipitation experiments were repeated three times for demonstration of reproducibility.

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CHAPTER 6: DISCUSSION

Aim 1: Investigation of the expression and activity of EC BK_{Ca} following CH

The general goal of this dissertation was to identify and characterize the physiological role of endothelial BK_{Ca} channels following chronic hypoxia. In the first aim we determined the localization and activity of EC BK_{Ca} channels following CH and tested the hypothesis that *the loss of vasoconstrictor reactivity following CH exposure results from increased EC BK_{Ca} channel activity and resultant membrane hyperpolarization* through whole cell patch clamp and single channel electrophysiology and immunofluorescence localization studies. The rationale behind these studies emerged from vascular reactivity and sharp electrode studies that determined that the endothelium and BK_{Ca} channels were responsible for decreased constrictor responses and hyperpolarization of the vascular wall. The controversy over the expression and physiological function of endothelial BK_{Ca} channels has been extensively debated the past two decades (Sandow & Grayson, 2009). It has been long accepted that endothelial BK_{Ca} were present in cultured cell lines such as human umbilical vein endothelial cells (HUVECs) (Sandow & Grayson, 2009;Jow & Numann, 1999;Wang *et al.*, 2005). In freshly isolated tissues however, the presence and/or function of the channel has been found to be absent in bovine coronary (Gauthier *et al.*, 2002), mouse aortic (Ledoux *et al.*, 2008), and rat aortic endothelial cells (Marchenko & Sage, 1996). The results from the aforementioned studies attempted to characterize EC BK_{Ca} through patch clamp. In agreement with the cell culture studies conducted in HUVECs, we identified EC BK_{Ca} currents in ECs from CH rats. In control animals, aortic and gracilis ECs did not

demonstrate outward currents that were sensitive to BK_{Ca} channel inhibitors or activators, supporting the previous findings in freshly isolated tissue. The inability to detect BK_{Ca} activity in controls may result from channel inhibition from Cav-1, as later discussed, or may be an artifact of experimental conditions. Endothelial BK_{Ca} channels are hypothesized to be localized to EC caveolae (Wang *et al.*, 2005) and are possibly sensitive to changes in vascular flow (Sun *et al.*, 2001), thus removal of endothelial cells from their native environment may result in decreased or absent activity of the channels. We have not conducted studies thus far in varied flow conditions in isolated or cultured ECs, nor have we investigated flow-induced dilation in arterioles from control and CH rats. Segmental differences in activity of endothelial BK_{Ca} channels may also occur as freshly isolated ECs from 120 μm mesenteric arterioles from normoxic control rats exhibit IBTX-sensitive currents (data not published). Thus, future studies addressing segmental differences and flow dependent dilation and the role of endothelial BK_{Ca} are warranted.

The emergence of active EC BK_{Ca} following CH may not be the only disease state where these channels become active, as the activity and expression of endothelial BK_{Ca} following disease has been documented in human mesenteric arteries from cancer patients (Kohler *et al.*, 2001). Changes in vascular flow over time from disease, such as in CH may permanently alter the membrane domain through changes to Cav-1 and/or cellular cholesterol and lead to chronic activation of endothelial BK_{Ca} channels. Investigations into other diseases that alter Cav-1 expression and intracellular cholesterol/lipids, such as intermittent hypoxia and obesity, may additionally provide

insight into how these channels function endogenously in disease and in normal conditions.

Characterization of endothelial K_{Ca} channels:

The present results demonstrate that EC BK_{Ca} have tonic activation following CH and contribute to resting membrane potential of ECs (**Figure 3: Chapter 2**). In addition to contributing to the membrane potential, ACh induced dilation following CH becomes dependent upon endothelial BK_{Ca} channels whereas the contribution of other calcium-activated K^+ channels is significantly decreased (**Figure 4: Chapter 2).** This switch of K_{Ca} channels in endothelium-derived hyperpolarizing factor (EDHF) dilation is a novel finding, suggesting BK_{Ca} channels following hypoxemic diseases may become responsible for hyperpolarizing the endothelium and leading to the release of vasodilators to regulate vascular tone. Several studies have recently demonstrated that endothelial BK_{Ca} channels function in mediating the release of NO upon channel stimulation and membrane hyperpolarization with NS-1619 (Calderone *et al.*, 2005;Calderone *et al.*, 2007). Thus it is possible that BK_{Ca} activity following CH may lead to the release of other important endothelial derived vasodilators, such as CO, NO, or EETs.

Electrophysiological characterization of EC K_{Ca} Channels:

Our initial observation of increased total EC transmembrane currents and the emergence of BK_{Ca} following CH exposure, was the first published documentation of enhanced BK_{Ca} currents in endothelial cells in a model of chronic hypoxemia. Interestingly, the aorta and gracilis displayed significantly larger transmembrane currents than controls (**Figures 6A-C: Chapter 2**), suggesting that CH treatment may enable EC BK_{Ca} activity throughout the systemic vasculature and not just in individual beds.

Experiments that characterized the role of all K_{Ca} channels in vascular EC outward currents displayed the importance of SK/IK_{Ca} currents in both control and CH groups (**Figures 1A-D: Chapter 2 Addendum**). Our results support previous experiments conducted in murine endothelial cells (Ledoux *et al.*, 2008) that demonstrated a vital role for SK/IK_{Ca} in endothelial membrane potential and outward currents in control non-diseased animals. Interestingly, following SK/IK_{Ca} blockade, CH ECs maintained significant residual currents. These currents were blocked by the BK_{Ca} specific blocker, IBTX. Application of the BK_{Ca}-specific activator, NS-1619 selectively enhanced currents in ECs from CH rats and was without effect in controls (**Figures 9A-C: Chapter 2**), providing more evidence for the presence of BK_{Ca} in ECs following CH.

Although ECs clearly express BK_{Ca} channels, a number of observations suggest that they are regulated differently from those in VSM. Previous experiments in our laboratory had demonstrated BK_{Ca} dependent responses to endogenous CO produced by HO that were independent of soluble guanylate cyclase (sGC) and activity of ryanodine receptors (Naik & Walker, 2003). The blockade of ryanodine receptors significantly decreases VSM BK_{Ca} activity which is dependent upon RyR derived calcium sparks acting on calcium sensitive β subunits for activation. Thus if EC BK_{Ca} are responsible for the previous observations, then calcium sensitivity in EC BK_{Ca} may be significantly different from VSM. To determine if BK_{Ca} activity in endothelial cells also utilized β subunits, which enhance channel opening by increasing calcium sensitivity, we studied outward currents in the presence of the β subunit activator tamoxifen, and found no apparent role for the accessory subunit in vascular endothelial cells (**Figure 3: Addendum Chapter 2**). These results are in agreement with previous studies conducted

in cultured ECs which have not found a physiological role for β1 subunits (McManus *et al.*, 2011;Eichhorn & Dobrev, 2007). Without a role for beta subunits, or RyR activity, activity of EC BK_{Ca} activity must be maintained through endogenous intracellular calcium and possess higher calcium sensitivity than VSMC BK_{Ca}.

EC BK_{Ca} channels have significantly greater calcium sensitivity than VSM BK_{Ca} :

To examine this possibility, we identified endothelial BK_{Ca} channels through single channel electrophysiology and determined their unitary conductance, voltage and calcium sensitivity, and inhibition by the channel specific inhibitor, iberiotoxin (IBTX) (**Figures 2A-C: Chapter 3**). EC BK_{Ca} unitary conductance was determined and contrasted to VSM BK_{Ca} unitary conductance to verify channel identity in whole cell-cell attached and inside-out patch clamp single channel recordings. No differences between cell types were found (**Figure 6: Addendum Chapter 2**). Calcium and voltage sensitivity of EC BK_{Ca} were performed in inside-out patches and contrasted to VSM BK_{Ca} calcium and voltage sensitivity and determined that EC BK_{Ca} exhibited enhanced calcium sensitivity (**Figure 2B: Chapter 3**). These results are in contrast to previous findings in cultured ECs which have shown lower calcium sensitivity of EC BK_{Ca} and activation at higher voltages (Nimigean & Magleby, 1999;Papassotiriou *et al.*, 2000;Eichhorn & Dobrev, 2007). The effects of culturing may significantly affect endothelial BK_{Ca} channel function compared to those in native tissue. With scant literature characterizing endothelial BK_{Ca} it will continue to be important to characterize channel sensitivity to voltage and calcium in each model presented to provide insight into the mechanisms responsible for driving EC BK_{Ca} activity, as it is apparent EC BK_{Ca} channel regulation differs from regulation in VSM.

CH ECs demonstrated EC BK_{Ca} activity at the single channel level in whole-cell cell attached or inside-out patches. These results provide evidence that the mechanism responsible for enabling BK_{Ca} activity following CH permits channel activity in a membrane-delimited fashion. However, studies using a variety of electrophysiological methods including perforated patch, whole cell patch clamp, and single channel studies never isolated BK_{Ca} activity in control ECs from rat gracilis and aortae. These studies support previous observations of no electrophysiological evidence for these channels within ECs from control animals (Ledoux *et al.*, 2008). Though a powerful technique for identifying and characterizing the role of ion channels in cells, electrophysiological experiments are not able to discriminate between a lack of expression, or the presence of functional endogenous channel inhibitors. Indeed experiments within control animals suggested no functional role for EC BK_{Ca}, thus additional methods to characterize and identify EC BK_{Ca} were employed to either prove or disprove the lack of physiological function of BK_{Ca} in control ECs.

Control and CH animals have EC BK_{Ca} channels:

Endothelial expression of BK_{Ca} α and β1 subunits was confirmed in sectioned resistance arteries and in freshly isolated aortic endothelial cells using confocal immunoflourescence microscopy (**Figures 10A-H & Supplemental Figure 2: Chapter 2**). These results were in contrast to our aforementioned data that only demonstrated the functional presence of BK_{Ca} in CH ECs through electrophysiological characterization. The presence of β subunits in the localization experiments is interesting as they do not appear to be functionally coupled to the channel (**Figures 10C-D: Chapter 2**). The localization of β subunits in isolated cells does not match localization of the α subunit and

appears to be restricted to cytosolic domains. However, without the use of membrane specific markers, we cannot discount plasma membrane β subunit localization. Nevertheless, their lack of functional coupling to the α subunit and irregular staining may suggest endosomal compartmentalization. Currently their function in the vascular endothelium is unknown and warrants further investigation.

RAECs express BK_{Ca}:

Immunofluorescence localization experiments provided evidence for channel expression in control ECs, but did not discriminate changes in channel expression. These results led us to investigate how EC BK_{Ca} channels were regulated and if BK_{Ca} expression could be maintained in culture. Due to the limitations of sample purity and quantity from freshly dispersed ECs, we utilized a cultured EC line from rat aorta (RAECs) to characterizethe presence of BK_{Ca} in RAECs through immunofluorescence and western blot. RAEC expression of BK_{Ca} α was confirmed using confocal immunoflourescence microscopy (**Figure 1: Addendum Chapter 3**). Western blot detection also confirmed BK_{Ca} α expression in RAECs (**Supplemental Figure 1: Chapter 3**). The use of cultured cells does not address changes in expression that may exist following CH in native cells. The use of cultured cells, however does enable the use of molecular methods that are not feasible in native tissue. Results from these studies support previous studies also identifying BK_{Ca} in cultured ECs (Wang *et al.*, 2005;Dong *et al.*, 2007;Sandow & Grayson, 2009) and warrant their use as a tool for investigations of cellular mechanism responsible for BK_{Ca} regulation in native ECs.

Aim2 : Assess the role of the Cav-1 scaffolding domain in regulating EC BK_{Ca} channels following CH.

The second aim of this dissertation project assessed the role of the Cav-1 scaffolding domain in regulating EC BK_{Ca} channels following CH by testing the hypothesis that *CH exposure results in a loss of Caveolin-1 inhibition of EC BK_{Ca} enabling increased channel activity and a loss in vasoconstrictor reactivity*. Following immunofluorescent localization of BK_{Ca} channels within ECs from control rats, we sought to find an inhibitory mechanism which may be responsible for the lack of activity found in controls. BK_{Ca} in cultured ECs are hypothesized to be localized within caveolae and inhibited by the scaffolding domain of caveolin-1 (Wang *et al.*, 2005). Loss of caveolar structure, caveolin presence, or the ability of Cav-1 to directly regulate the channel through its scaffolding domain following CH would result in increased channel activity and subsequent diminished vasoconstrictor reactivity. Experiments in this aim addressed alterations to caveolae, Cav-1, and channel inhibition by Cav-1 following CH exposure. Patch clamp electrophysiology, membrane potential recordings, and isolated arterioles were utilized for functional experiments determining the role of Cav-1 and its scaffolding domain on BK_{Ca} activity; and immunofluorescent localization and co-immunoprecipitation experiments were conducted to investigate association between Cav-1 and BK_{Ca}. EC BK_{Ca} channels (in HUVECs) are inhibited by association with caveolin-1 and its scaffolding domain (Wang *et al.*, 2005) and are tightly regulated by lipid content of cellular membranes (Thompson & Begenisich, 2006;Weaver *et al.*, 2007). Two consensus caveolin-1 binding sites exist on BK_{Ca}, with previous studies

demonstrating that caveolin-1 siRNA acts similarly to cholesterol depletion (Lu *et al.*, 2006).

Caveolin-1 and cholesterol regulation of EC BK_{Ca} :

To determine the role of caveolin-1 in regulation of EC BK_{Ca} channels,, we examined the effect of cholesterol depletion, cholesterol repletion, and the administration of a cell permeant peptide of the scaffolding domain of Cav-1 on BK_{Ca} currents in freshly dispersed cells from control and CH rats. For cholesterol depletion experiments, the agent methyl-β-cylcodextrin (MBCD) was administered. MBCD treatment resulted in significantly larger currents in control ECs in (**Figures 11A-D: Chapter 2** / **Figure 1D: Chapter 3**) that were inhibited by IBTX (**Figures 12A-C: Chapter 2** / **Figures 3C/D: Chapter 3**) and augmented by NS-1619 (**Figures 4C/D: Chapter 3).** These results are in agreement with previous studies that determined that depletion of cellular cholesterol with MBCD enhanced BK_{Ca} currents in HUVECs (Wang *et al.*, 2005f) and in other volume sensitive cell types, such as acinar cells (Tajima *et al.*, 2011;Thompson & Begenisich, 2006b). Interestingly in myometrial myocytes cholesterol depletion may cause significant decreases or increases in BK_{Ca} currents. In freshly isolated myocytes from non-pregnant and pregnant female human donors, Brainard demonstrated increases in BK_{Ca} activity (Brainard *et al.*, 2005), however these results are in sharp contrast to observations in freshly isolated rat myometrial myocytes (Shmygol *et al.*, 2007) which demonstrated decreased BK_{Ca} function. The relationship between BK_{Ca} and Cav-1 and cholesterol rich domains was further explored in studies with cholesterol repletion, in which we found the addition of cholesterol significantly decreased currents in CH ECs to the level of controls (**Figures 5A-D : Chapter 3**). Co-immunoprecipitation experiments

conducted by our group and others (Brainard *et al.*, 2005;Shmygol *et al.*, 2007;Wang *et al.*, 2005) have provided evidence for possible direct association between Cav-1 and BK_{Ca}, however it is unclear if cholesterol limits Cav-1 association with the channel, as free cholesterol is necessary for its plasma membrane localization (Pol *et al.*, 2005) or if cholesterol itself may bind and inhibit the channel (Bukiya *et al.*, 2011).

It has been demonstrated that either large scale depletion or repletion of cellular cholesterol content, or changes in the contents of the lipid bilayer result in decreased BK_{Ca} channel activity (Romanenko *et al.*, 2009;Yuan *et al.*, 2007). Cholesterol "manipulation" affects membrane fluidity and may possibly result in the activation of multiple signaling cascades, not just those localized and dependent on caveolae. It is possible that MBCD treatment increases the production of endogenous channel activators, such as NO derived from eNOS, or CO derived from heme oxygenase. It is also possible that the removal of cholesterol, may directly alter in channel activity. BK_{Ca} channels are sensitive to changes in lipid environments and are hypothesized to have direct sterol-channel interactions (Bukiya *et al.*, 2011), thus manipulation of cholesterol content with cyclodextrins likely affects channel activity through Cav-1 dependent and independent mechanisms.

The use of the scaffolding domain is thought to be a more direct approach tostudy the effect of Cav-1 function. Several studies have demonstrated the scaffolding domain of Cav-1 acts similarly to overexpression or rescue of Cav-1 (Bucci *et al.*, 2000;Couet *et al.*, 1997;Oka *et al.*, 1997) suggesting that AP-CAV affects Cav-1 function more directly than cyclodextrins. Other studies have supported the specificity of the scaffolding domain peptide on Cav-1 function by utilizing the peptide as an inhibitor of caveolin-1

function. In these studies, the administration of AP-CAV acted similarly to cholesterol depletion with both treatments resulting in hindering of Cav-1 function but through very different mechanisms (Sathish *et al.,* 2011; Adebiyi *et al.,* 2010). The scaffolding domain peptide in these studies overloaded binding of endogenous Cav-1 to target proteins, resulting in a decrease in Cav-1 dependent signaling. In the present studies, AP-CAV administration significantly inhibited BK_{Ca} currents. This is in agreement with previous studies in which the scaffolding domain of Cav-1 inhibited BK_{Ca} channels (Lu *et al.*, 2006), however the nature of this inhibition is unclear. Currently, we believe that Cav-1 may inhibit BK_{Ca} channels in endothelial cells through its scaffolding domain. Future studies addressing whether the scaffolding domain of Cav-1 alters Cav-1 aggregation and targeting of BK_{Ca} channels or cellular cholesterol levels are warranted and would provide clarification between the observed effects of cholesterol manipulation and AP-CAV on BK_{Ca} channel function.

Endothelial hyperpolarization following CH is restored through inhibition of EC BK_{Ca}:

Following characterization of endothelial BK_{Ca} inhibition by Cav-1, we next investigated if manipulation of Cav-1 activity would translate to alterations in vascular function. We found that endothelial cell E_m in gracilis arteries from MBCD treated controls was hyperpolarized compared to controls, similar to findings in the CH model. The MBCD induced hyperpolarization was inhibited by IBTX (**Figure 3: Chapter 2** / **Figure 1B: Chapter 3**) and by AP-CAV (**Figure 1C: Chapter 3**). Thus, caveolar disruption with MBCD also resulted in hyperpolarization of the endothelium. These results support a role for EC BK_{Ca} in mediating the vascular wall hyperpolarization

following CH due to a loss in Cav-1 inhibition of the channel through its scaffolding domain.

Diminished vasoconstrictor reactivity following CH is restored through the Cav-1 scaffolding domain :

Previous studies demonstrated that vasoconstrictor responsiveness of the systemic circulation is attenuated following prolonged exposure to hypoxia (Auer & Ward, 1998a;Toporsian & Ward, 1997;Hu *et al.*, 1996;Doyle & Walker, 1991). Diminished vasoconstrictor reactivity following CH is observed both systemically and in several individual vascular beds (O'Donaughy & Walker, 2000;Jernigan *et al.*, 2001;Auer & Ward, 1998;Caudill *et al.*, 1998) as reduced vascular resistance response to vasoconstrictor agonists (Doyle & Walker, 1991). The role of Cav-1 in vascular reactivity has not been defined in this setting. Due to expression of Cav-1 in both VSM and the endothelium, we designed our studies to focus on the effects of endothelial Cav-1 in vascular reactivity by employing intraluminal administration of agents affecting caveolar function. Myogenic and PE responses of isolated gracilis arteries displayed decreased vasoconstrictor reactivity in CH and MBCD treated controls. Luminal administration of the scaffolding domain peptide AP-CAV restored myogenic tone and vasoconstriction to PE to levels of controls. These results support a role for Cav-1 and its scaffolding domain in maintaining vascular reactivity. Whether Cav-1, the scaffolding domain peptide, or both are acting directly upon EC BK_{Ca} channels or through indirect mechanisms is unclear from these results. Interestingly, these results are in sharp contrast to much of the

literature describing the role of Cav-1 and MBCD treatment on VSM and vasoconstrictor reactivity (Potocnik *et al.*, 2011).

Caveolar structure and relative number does not change with CH:

The concentration of MBCD utilized by Potocnik is 100 fold greater than the concentration of MBCD that we used in our studies to mimic the effects of CH. The concentration of 10 mM of MBCD has been previously demonstrated to ablate caveolae, and following structural studies conducted in rat aorta we additionally found the concentration was sufficient to eliminate cellular caveolae. The 100 μM concentration that we utilized did not alter the structure or number of cellular caveolae. Neither did CH treatment (**Supplemental Figure 4: Chapter 2**). These results suggest that CH exposure is not resulting in a dramatic change in plasma membrane caveolae, but rather BK_{Ca} activity is enabled by more subtle alterations in caveolar function.

Decreased Cav-1 and BK_{Ca} association following CH:

Drastic changes in localization or relative fluorescence between Cav-1 or BK_{Ca} α were not documented in immunofluorescence studies following CH. However, determination of BK_{Ca}/Cav-1 co-localization in arteriole cross sections did demonstrate a significant decrease in association (**Figure 7 bottom panel: Chapter 3**). These results suggest that CH exposure results in decreased association between the channel and Cav-1 and not through changes in expression *per-se*. This could be through decreased plasma membrane targeting of EC BK_{Ca} associated with diminished cellular cholesterol, which is required for Cav-1 localization at the plasma membrane (Pol *et al.*, 2005). Increased Cav-1 phosphorylation or decreased post-translation modifications such as palmitoylation, have also been demonstrated to be important for Cav-1 inhibition of its various signaling

partners (Cohen *et al.*, 2004;Parat *et al.*, 2002), and could also explain the decrease in association between the channel and Cav-1. However, although immunofluorescent localization provided basic assessment of Cav-1/BK_{Ca} association in ECs, it has limitations by not discriminating possible small but significant changes in localization and targeting at the plasma membrane. Further exploration into the effect of hypoxia on Cav-1 post-translational modifications, phosphorylation, and targeting of BK_{Ca} through direct association with the channel in cultured cell systems may provide further insight into the direct mechanism responsible for the decreased association amongst BK_{Ca} and Cav-1.

Caveolin-1 and BK_{Ca} co-immunoprecipitate in RAECs:

We further determined association of BK_{Ca} and Cav-1 through co-immunoprecipitations of cultured RAECs, and found that BK_{Ca} and Cav-1 immunoprecipitate (**Supplemental Figure 2: Chapter 3**). These results suggest that BK_{Ca} and Cav-1 are structurally associated in ECs. The use of cultured cell systems has limitations as characteristics of cultured cell lines may be drastically different from native cells. Characterization of Cav-1/BK_{Ca} association in native endothelium would be ideal, but due to sample size and purity limitations we chose to use RAECs to conduct these studies. We chose to only conduct experiments in normoxic conditions as we have not characterized the effects of hypoxia in these cells. Current investigations using RAECs demonstrated expression of BK_{Ca}. However, unlike results obtained in native tissues, cholesterol depletion was not required to unmask BK_{Ca} activity (**Figure 7: Chapter 4**). Thus, although BK_{Ca} channels are associated with caveolin-1 in these cells, channel

function is not inhibited. However, it must be pointed out that even in native cells from CH rats, there is a level of maintained association between these proteins. Therefore, the disinhibition of BK_{Ca} channels in CH or in cultured cells does not require complete dissociation from Cav-1.

<u>Aim 3:</u> Investigate caveolin-1 regulation of heme oxygenase and HO-dependent BK_{Ca} activity following CH.

In the third aim of the project we directed our attention on the relationship between BK_{Ca} and HO, and their regulation by Cav-1. Previous data have linked HO activity and BK_{Ca} activity resulting in hyperpolarization of the vascular wall and decreased vasoconstrictor reactivity (Jernigan *et al.*, 2001;Naik & Walker, 2003;Naik & Walker, 2006;Wang *et al.*, 2005). We tested the hypothesis *that CH treatment results in a loss of Cav-1 inhibition of HO dependent BK_{Ca} activity resulting in EC hyperpolarization and diminished vasoconstriction* through electrophysiology, immunofluorescent colocalization, and co-immunoprecipitation experiments.

HO dependent activation of EC BK_{Ca}:

The HO product, CO can activate VSM BK_{Ca} channels (Jaggar *et al.*, 2002) and HO-dependent activation of BK_{Ca} channels has been additionally found in carotid body glomus cells where it acts as an oxygen sensor (Williams *et al.*, 2004). Patch clamp experiments in freshly isolated ECs found HO-dependent activation of EC BK_{Ca} channels (**Figure 1: Chapter 4**). It was further determined that the HO product responsible for

activating EC BK_{Ca} was likely CO, as the effects of enzyme blockade could be restored by exogenous CO treatment (**Figure 3: Chapter 4**). These results are in agreement with previous studies demonstrating HO derived CO directly activates BK_{Ca} channels in functional oxygen sensing units (Williams *et al.*, 2004;Kooli *et al.*, 2008). Following cholesterol depletion to unmask BK_{Ca} channel activity, currents from control ECs were similarly sensitive to HO blockade (**Figure 4: Chapter 4**). Thus, a loss in Cav-1 regulation unmasks HO-dependent BK_{Ca} currents in ECs. Subsequent experiments determined that the scaffolding domain of Cav-1 inhibits HO-dependent BK_{Ca} currents that were not restored by exogenous CO (**Figures 5A/B: Chapter 4**). Furthermore the use of a channel activator could stimulate channel activity following blockade of HO, providing evidence that blockade of the enzyme does not affect the ability of the channel to be activated by channel openers or CO. However, the scaffolding peptide AP-CAV does not enable subsequent channel activity. To corroborate findings in freshly dispersed cells, we additionally performed experiments examining HO-dependent BK_{Ca} activity in cultured RAECs and also observed HO-dependent BK_{Ca} currents (**Figure 7: Chapter 4**). The HO-dependent BK_{Ca} currents were present without the need for cholesterol depletion, suggesting culturing of these cells alters Cav-1 and membrane properties as discussed above. As reviewed by Sandow and Grayson, the effects of culturing may significantly alter phenotypic properties of endothelial cells enabling the activity of BK_{Ca} in cells that have quiescent channels *in vivo* or in *ex vivo* (freshly dissociated) conditions (Sandow & Grayson, 2009). Thus, RAECs cultured in normoxic conditions do not display identical properties to freshly isolated ECs from normoxic rat aorta; however both models do contain functional HO-BK_{Ca} units.

Co-immunoprecipitation experiments demonstrate association amongst HO-2 and BK_{Ca} in RAECs:

Without the capacity to perform co-immunoprecipitations and western blots on freshly isolated ECs without contamination from VSMCs, we utilized RAECs and found HO-2 and BK_{Ca}, and Cav-1 and both HO isoforms co-immunoprecipitated (**Figures 8 and 9: Chapter 4**). These results are in agreement with previous studies which demonstrated HO and Cav-1 association in pulmonary vascular endothelial cells (Kim *et al.*, 2004b) and HO-2 and BK_{Ca} in cerebral microvascular ECs and carotid body glomus cells (Kooli *et al.*, 2008;Roth *et al.*, 2009;Williams *et al.*, 2004). Supporting these findings, imunnofluorescence co-localization experiments in freshly dispersed ECs for CH found association between HO-1 and Cav-1, HO-2 and Cav-1, and BK_{Ca} and HO-2. Co-localization was quantitated and no differences were observed in the degree of association following CH (**Figure 6 : Chapter 4**) in contrast to previous results in which BK_{Ca} and Cav-1 association decreased with CH..

These studies are the first investigation of the role of HO on BK_{Ca} activity in native endothelial cells, although others have provided evidence for this pathway in cultured HUVECs (Wang. *et al.,* 2005). However, the role of Cav-1 in this signaling pathway was not been examined. The limitations of the study include: the inability to determine changes in BK_{Ca}/ HO/ and Cav-1 association through co-immunoprecipitation in native tissues; and a lack of discrimination of direct / indirect actions of Cav-1 on BK_{Ca} function. The strengths of this study include: establishment of EC BK_{Ca} regulation by Cav-1 and HO in native tissues and cultured RAECs enabling a variety of models to study detailed mechanisms of this novel regulatory unit in ECs. These observations were

also translated into *in vivo* experiments where we observed that long term and short term administration of the scaffolding domain peptide restored vascular reactivity following CH (these data were not in the scope of the dissertation and are not included). Coupled with the observation that acute luminal cholesterol depletion of arterioles mimicked the effects previously described in CH, our data suggest an important role for Cav-1 and cellular cholesterol in mediating HO-dependent BK_{Ca} activity in the vasculature.

The question of whether Cav-1 function is decreased initially and cholesterol decrease, or if cholesterol levels decrease leading to derangements in Cav-1 activity may be a difficult task to delineate. Unpublished observations from our laboratory demonstrate that in a 4 wk model of CH, pulmonary ECs have significantly less cholesterol than controls. Previous studies have demonstrated that enzymes responsible for cholesterol synthesis are hypoxia sensitive and alter cholesterol synthesis and metabolism in vascular tissues (Arai *et al.*, 1996;Nguyen *et al.*, 2007). In addition, hypoxia has been demonstrated to increase HO expression and activity (Jernigan *et al.*, 2001;Lee *et al.*, 1997;Motterlini *et al.*, 2000). Interestingly, recent studies have also provided evidence for HO expression and activity driving cholesterol synthesis and efflux in cultured astroglia (Hascalovici *et al.*, 2009). In addition, Cav-1 negatively regulates HO activity (Kim *et al.*, 2004).

Adding to the complexity of the scenario, oxidative stress which is elevated with CH, can increase HO expression and activity (Abraham & Kappas, 2008) and have affect Cav-1 function. Oxidative stress has been shown to increase Cav-1 expression in fibroblasts (Bartholomew & Ganetzky, 2010), while in vascular smooth muscle cells Cav-1 expression in unchanged. However there is an increase Cav-1 phosphorylation

which regulates Cav-1 trafficking and localization (Basset *et al.*, 2009). Interestingly, different types of ROS may also contribute to either increases or decreases in Cav-1 expression. In human lung carcinoma cells, superoxide anions and hydrogen peroxide down-regulated Cav-1 expression, while hydroxyl radicals upregulated Cav-1 expression (Luanpitpong *et al.*, 2010). Thus depending upon which type of ROS is present and which particular cell is exposed, differences in Cav-1 expression and/ or activity may occur. The effect of hypoxemia and ROS on Cav-1 in systemic endothelial cells has not been characterized and warrants further investigation. Recent data from our laboratory have demonstrated roles for both HO and ROS contributing to impaired vasoconstriction following CH (Sweazea & Walker, 2009). The blockade of both ROS and HO increased CH vascular reactivity similarly to our present findings with AP-CAV treatment. However, we did not examine the role of ROS in the

current studies and it is a potential future direction of this research.

LIMITATIONS OF STUDIES

Non-specific actions of AP-CAV:

The experiments and studies conducted within the scope of this project are not without limitations. As previously discussed, the actions of AP-CAV on BK_{Ca} channel activity do not permit channel activation by exogenous CO or channel openers, however these experiments have not demonstrated physical association between the peptide and BK_{Ca}. Additionally, AP-CAV may target other Cav-1 regulated kinases and phosphatases. It is possible that kinase or phosphatase activity may result in post-translational modification of BK_{Ca} and cause the decreased channel activity. Thus, from

our results it is unclear if AP-CAV is decreasing BK_{Ca} activity through indirect or direct mechanisms. Investigations into possible post-translational modifications of EC BK_{Ca} channels may provide greater insight into channel regulation following treatments with the scaffolding peptide.

Limitations on the resolution of BK_{Ca} and Cav-1 association:

We have hypothesized that EC BK_{Ca} are localized to caveolae and are directly associated with Cav-1. Immunofluorescence data support this claim by demonstrating a reduced BK_{Ca} / Cav-1 overlap following CH. Immunofluorescence studies are limited by the size and bulk of fluorescent proteins which determine "association" between labeled proteins. The overlap of large fluorescent proteins attached to secondary antibodies determines co-localization, which may not be indicative of physical interactions between two proteins *in vivo*. Additionally, confocal microscopy does not have the resolution to view cellular structures such as caveolae. Thus, without determining association between Cav-1 and BK_{Ca} in caveolae or the plasma membrane we cannot definitively claim BK_{Ca} and Cav-1 directly interact in EC caveolae.

Limitations on the study of isolated arterioles in no flow conditions:

Current studies investigated the role of Cav-1 and BK_{Ca} on vasoconstrictor reactivity following CH in isolated arterioles. By removing the arteriole from native conditions in the animal we are removing blood flow and studying the arteries under static conditions. We hypothesize that EC BK_{Ca} channels are localized to cellular caveolae and regulated by Cav-1 in the plasma membrane. Therefore, changes in flow

may result in significant changes in membrane fluidity, Cav-1 association with the channel, and ultimately BK_{Ca} activity.

Limitations of the model used for these studies:

We chose to study the model in the Sprague Dawley rat, which has been previously used to determine HO-dependent hyperpolarization and losses in vasoconstrictor reactivity following CH. The use of knockout animals for HO, Cav-1, or BK_{Ca} would have strengthened these studies by allowing investigation of each protein in the regulation of vascular tone in conscious animal, isolated arteriole, and isolated cell experiments. Whether HO-BK_{Ca} dependent regulation of vascular tone occurs in humans is unclear. Our studies assessed physiological adaptations in rats following CH exposure, which may not reflect physiological adaptations to hypoxia or COPD in humans. Additionally, by studying how Cav-1 and HO-BK_{Ca} complexes are regulating vascular tone within isolated cell systems our interpretation on whether these complexes are important in an intact animal or human is limited.

SUMMARY

To summarize, vascular endothelial cells contain BK_{Ca} channels which are inhibited by Cav-1 and cellular cholesterol and are activated endogenously by CO derived from HO. These functional oxygen sensing units become active following CH with the removal of Cav-1 inhibition and causing endothelial and vascular wall hyperpolarization leading to diminished vasoconstrictor reactivity. The role of BK_{Ca} channels in regulating vascular tone following hypoxia and a possible contribution to vascular tone in normoxia appears to depend upon the vascular bed and size of the

arteriole, and on whether the arteries are studied under constant flow or constant pressure conditions. Activation of these functional units may additionally contribute to increased release of EDHFs as ACh-induced dilations following CH become dependent upon the activity of EC BK_{Ca}. Thus HO-dependent activation of EC BK_{Ca} channels may function in flow-induced vasodilation and regulation of vascular tone through modulation of endothelial membrane potential and the release of EDHFs.

The removal of Cav-1 inhibition may be due to a loss in cellular cholesterol synthesis and Cav-1 targeting of the ion channel to the plasma membrane. Enhanced HO activity, as previously characterized (Jernigan *et al.*, 2001), may additionally contribute to Cav-1 dysfunction through reductions in cholesterol synthesis; as could increases in ROS as both have been recently implicated in dampening vasoconstrictor reactivity following CH.

Further elucidation into how HO-BK_{Ca} is unmasked in vascular ECs will be of importance in models of increased vasoconstriction such as systemic hypertension and obesity. For example, in a model of diet-induced obesity, increased ROS and decreased HO expression contributed to endothelial dysfunction and impaired vasodilation (Roberts *et al.*, 2006). The ability to activate and target EC HO-dependent BK_{Ca} to oppose vasoconstriction may restore vasodilation and endothelial function in this model. In contrast, in settings with enhanced vasodilation in the systemic circulation as in animal models of cirrhosis, where enhanced production of CO and increased BK_{Ca} activity have been documented (Bolognesi *et al.*, 2007) , inhibition of HO-dependent BK_{Ca} activity within the endothelium could prove beneficial.

To conclude, the delicate balance of vasoconstriction and vasodilation has long been connected with BK_{Ca} function in the vascular smooth muscle. However, our current studies clearly establish a prominent role for the regulation of vascular tone by endothelial BK_{Ca}. This represents a truly novel mode of vascular regulation of potential significance in a variety of physiological and pathophysiological conditions.

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