Characterization of the Independent and Combined Vasoactive Abilities of H^+ and CO_2 in Skeletal Muscle Microvasculature

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ABSTRACT

CHARACTERIZATION OF THE INDEPENDENT AND COMBINED VASOACTIVE ABILITIES OF H^+ AND CO_2 IN SKELETAL MUSCLE MICROVASCULATURE

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Hydrogen ion (H^+) and carbon dioxide (CO_2) are products of skeletal muscle metabolism and have been implicated in the hyperaemic response to muscle contraction. The microvasculature is instrumental to the increase in blood flow during contraction and its distribution to active fibers. To investigate the vasodilatory ability of H^+ and CO_2 separately on the skeletal muscle microvasculature, Tris(hydroxymethyl)aminomethane (TRIS) buffer was substituted for bicarbonate buffer and increasing concentrations/pressures of H^+, CO_2 and H^+ with CO_2 were applied to the cremaster muscle. 100.0 nmol/L H^+, 60.7 mmHg CO_2, and 60.7 mmHg CO_2 with 158.5 nmol/L H^+ induced 9.5 ± 1.9, 10.4 ± 3.1 and 7.6 ± 2.5 μm vasodilations respectively. Micropipette application of H^+ and CO_2 independently and combined onto arterioles and capillaries did not induce upstream arteriolar vasodilation. Thus, H^+ and CO_2 independently and combined are weak vasodilators that do not stimulate capillaries or arterioles to conduct vasodilation.

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List of Abbreviations

1A: First order
2A: Second order
3A: Third order
4A: Fourth order
ACh: Acetylcholine
Ado: Adenosine
ADP: Adenosine diphosphate
ATP: Adenosine triphosphate
Ca^{2+}: Calcium ion
CO_2: Carbon dioxide
FITC: Fluorescein isothiocyanate-dextran
H^+: Hydrogen ion
H_2CO_3: Carbonic Acid
HCO_3^-: Bicarbonate
HCl: Hydrochloric acid
K^+: Potassium ion
K_{ATP}: ATP-dependent K^+ channel
KCl: Potassium chloride
K_{IR}: Inwardly rectifying potassium channel
n: Number of experiments
N_2: Nitrogen
NaOH: Sodium hydroxide
NO: Nitric oxide
O_2: Oxygen
pCO_2: Partial pressure CO_2
Phe: Phenylepherine
PSS: Physiological salt solution
SNP: Sodium nitroprusside
SNAP: S-Nitroso-N-acetyl-DL-penicillamine
TRIS: Tris(hydroxymethyl)aminomethane
Water: H_2O

Chapter 1. Introduction

1.1 Active Hyperaemia

Blood flow and metabolism are closely coupled in skeletal muscle. This has been observed through a variety of metabolic indicators like running speed, oxygen (O_2) consumption and contraction strength. As all three of these indices increase they produce an increase in blood flow (Laughlin & Armstrong 1982; Bockman 1983; Hamann *et al.* 2004). This relationship whereby blood flow increases with increasing metabolic need is known as active hyperaemia. It is a local phenomenon enacted by cells neighboring arterioles in skeletal muscle without input from the sympathetic nervous system (Bockman 1983). It is hypothesized that at the onset of contraction skeletal muscle cells release vasodilators, compounds which diffuse to arterioles, thereby decreasing resistance and increasing blood flow through the arteriole. Active hyperaemia is important in skeletal muscle as the metabolic demand varies greatly between rest and exercise.

1.1.1 Skeletal Muscle as the Source of Vasodilators

Active hyperaemia is hypothesized to be modulated by a large group of metabolites released from skeletal muscle cells during contraction. Vasodilatory metabolites include adenosine (Ado), hydrogen ion (H^+), decreased oxygen (O_2) partial pressure, and increased carbon dioxide (CO_2) (Hellsten *et al.* 1998; Rådegran & Calbet 2001; Toda *et al.* 1989; Fry *et al.* 1994; Peng *et al.* 1998; Daugherty *et al.* 1967; Duling 1973; Kontos *et al.* 1977; Gorczynski & Duling 1978). More recently, vasodilatory compounds have been identified that are not products of metabolism but rather

products of skeletal muscle activation. While they do not directly link active hyperaemia to metabolism, they still connect active hyperaemia with muscle activity. Two of these compounds are nitric oxide (NO) and potassium (K^+) (Kjellmer 1965; Hilton 1977; Hester *et al.* 1993; Gilligan *et al.* 1994; Armstrong *et al.* 2007; Ross *et al.* 2013). Thus, skeletal muscle contraction directly produces a variety of vasoactive compounds that may be able to increase blood flow during exercise.

1.1.2 Other Cell Types as the Source of Vasodilators

With continued research, vasodilatory compounds released from cell types other than skeletal muscle were identified. Endothelial cells lining arterioles release NO and prostaglandins in response to shear stress along the vessel wall that could contribute to vasodilation during exercise (Koller & Kaley 1990; Pohl *et al.* 2000). Red blood cells release adenosine triphosphate (ATP) during hypoxia or when they are compressed which may also contribute to vasodilation (Dietrich *et al.* 2000). While both endothelial cells and red blood cells are able to release vasoactive products, no connection has been made between muscle contraction (or metabolism) and the release of these vasodilators. Instead, endothelial cell and red blood cell vasodilators are hypothesized to be released after muscle contraction initiates a vasodilation, possibly allowing them to regulate the new increase in blood flow. While these hypotheses provide a framework for how active hyperaemia might work, there is still much to be understood about the variety of vasodilatory compounds released during contraction and the role of each vasodilator in active hyperaemia.

1.2. Vascular Architecture

To match blood flow distribution to metabolic demand, active hyperaemia requires coordination between skeletal muscle activity and blood vessels. To understand this coordination it is necessary to understand the architecture of the vascular tree, how it influences blood flow distribution and the relationship between the microvasculature, skeletal muscle fibers and motor unit recruitment. The vascular tree is categorized from proximal vessels to distal vessels (Figure 1). Arterioles paired with a vein are classified as first order arterioles (1A). Branches off of the 1A arterioles are classified as second order arterioles (2A). From 2A arterioles arise third order arterioles (3A). From 3A arterioles arise fourth order arterioles (4A). Each 4A arteriole branches into a group of capillaries called a capillary module. Capillary modules, are conserved in length and cross sectional vessel density and are perfused as a unit (Sweeney & Sarelius 1989; Berg & Sarelius 1995). Each skeletal muscle fiber is surrounded by 3-6 capillaries (Plyley & Groom 1975). The number of arteriolar levels in a vascular tree is variable across muscles and species, however in the cremaster muscle, a 1A arteriole is always a vessel paired with a vein and a 4A arteriole is always the terminal arteriole leading to a capillary module.

Arterioles paired with a vein (1A) deliver blood to the skeletal muscle tissue. Arterioles that are no longer paired with a vein (2A, 3A, 4A) distribute blood flow throughout the skeletal muscle tissue. Arteries and arterioles possess a smooth muscle layer and therefore are able to change diameter. This ability to alter resistance allows them to control blood flow supply. Alternatively, capillary walls are comprised only of endothelial cells, and therefore cannot change diameter to meet their own perfusion

requirements. Therefore, it is arterioles upstream of capillary modules that control capillary perfusion. 4A arterioles most directly regulate capillary perfusion as they are the last branches of arteriole before capillaries arise. If more flow is required, 3A and then 2A arterioles may be dilated to increase perfusion. Because capillaries exist as a

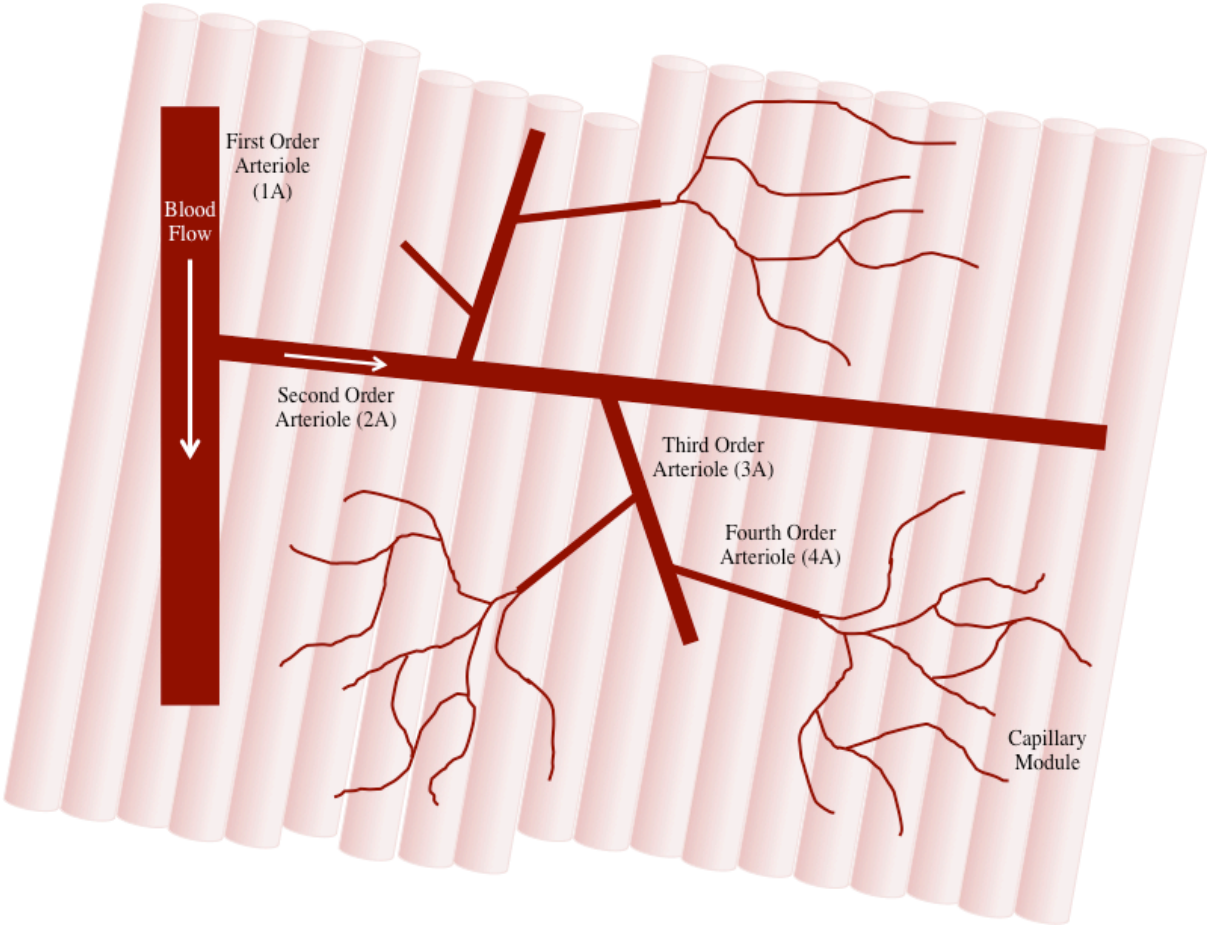


Figure 1. Schematic of part of the microvascular network in hamster cremaster muscle adapted from (Twynstra *et al.* 2012). Not drawn to scale. Not all capillary modules are drawn from each 4A arteriole for clarity. Background columns represent skeletal muscle fibers.

module, a single capillary may not be perfused but rather the entire capillary module will be perfused with 4A arteriolar vasodilation.

1.3. Maximal and Submaximal Contraction

1.3.1 Motor Unit Recruitment

Skeletal muscle fibers are activated through the recruitment of motor units. Motor units are defined as an alpha motor neuron and all of the skeletal muscle fibers that it innervates. They range broadly in innervation ratio from 1:5 to 1:2000 (for review see Enoka 1995). Muscle fibers of a single motor unit are not located beside each other, but rather are dispersed across a large region of the muscle amongst other motor units (Burke & Tsairis 1973; Bodine *et al.* 1987; Enoka & Fuglevand 2001). Motor units are recruited from smallest to largest, such that increasing demands for force are met with larger motor unit recruitment (for review see Enoka 1995). Thus, during submaximal contraction, the active fibers are interspersed among non-active fibers. Alternatively, during maximal contraction, all motor units are recruited and all fibers are active (Enoka & Fuglevand 2001). This creates two different physiological environments; maximal contraction where there is metabolic need at every muscle fiber, requiring capillary perfusion to increase to every fiber and submaximal contraction where there is metabolic need only at discrete muscle fibers, requiring capillary perfusion to increase only to specific fibers.

1.3.2 Maximal Contraction

During maximal contraction all muscle fibers are active and releasing vasoactive compounds. Therefore, the entire vascular network is in immediate proximity to vasoactive compounds. Vasoactive compounds can diffuse to nearby arterioles and dilate the entire vascular network. This increases capillary perfusion throughout the tissue and satisfies the universal metabolic need. However, the situation of maximal

contraction where all motor units have been recruited and all fibers are contracting only occurs when 50-85% of maximum voluntary contraction is reached (Enoka & Fuglevand 2001).

1.3.3 Submaximal Contraction

During submaximal contraction, not all muscle fibers are contracting. Only select fibers are contracting and these fibers are dispersed throughout the muscle. Therefore, the entire vascular network will not be in the immediate vicinity of vasodilators produced by contracting muscle. This presents an architectural problem. The arterioles supplying capillary modules of active muscle fibers may be too distant for vasodilatory compounds released by the contracting fibers to diffuse to (Murrant *et al.* 2016). Alternatively, even if the vasodilatory compounds are close enough to cause dilation at an arteriole, a single dilated area along an otherwise constricted vessel will not alter blood flow. Thus, a mechanism is required to spread the vasodilatory signal throughout the vasculature. This mechanism is called a conducted response.

1.4. Conducted Response

Conducted responses are most commonly thought to be a hyperpolarization transmitted through endothelial cells and smooth muscle cells along the arteriolar wall causing vasodilation (Welsh & Segal 1998; Emerson & Segal 2000; Murrant & Sarelius 2002). The hyperpolarization is thought to spread by gap junctions, relatively nonspecific ion channels between cells (Segal & Duling 1989). The hyperpolarization creates a pathway of decreased resistance along an arteriole (Segal & Duling 1986). This allows the vascular tree to spread a vasodilatory signal from the capillaries and

arterioles neighboring contracting fibers up through proximal arterioles to distribute blood flow to specific areas of the tissue. Thus, conducted responses are vasodilatory signals which travel along capillaries and arterioles, thereby increasing blood flow to a specific area.

1.4.1 Arteriolar Initiated Conducted Response

Historically, conducted responses have been investigated extensively on arterioles (Segal & Duling 1989; Segal *et al.* 1989; Doyle & Duling 1997; Budel *et al.* 2003). Arteriolar conducted responses have been shown to be initiated consistently by ACh, K^+, ATP, ATP-dependent K^+ channel (K_{ATP}) channel stimulation, and skeletal muscle contraction (Duling & Berne 1970; Murrant & Sarelius 2002; Twynstra *et al.* 2012; Dora 2016). They have been shown to travel bidirectionally along the arteriole (Duling & Berne 1970). They have been observed travelling upstream onto parent arterioles and downstream through daughter branches (Duling & Berne 1970; Segal *et al.* 1989; Twynstra *et al.* 2012). Thus arteriolar initiated conducted responses are important to study for two reasons: They can affect multiple vessel orders and significantly increase or decrease blood flow within a region; and they are the historical standard by which conducted responses are understood.

1.4.2 Capillary Initiated Conducted Response

More recently, capillaries have been shown to initiate conducted responses (Berg *et al.* 1997; Cohen *et al.* 2000; Twynstra *et al.* 2012). Capillaries initiate conducted responses in response to ACh, NO, Ado and K_{ATP} channel stimulation and muscle contraction (Berg *et al.* 1997; Cohen & Sarelius 2002). Capillary initiated conducted

responses follow a specific pathway of dilation up through proximal branch orders that increases blood flow directly to the stimulated capillary module (Twynstra *et al.* 2012). This suggests that while capillaries cannot dilate, they possess a mechanism to modulate their own perfusion (Dietrich 1989; Mitchell *et al.* 1997). This is necessary because capillary modules are the only vessels guaranteed to be in contact with skeletal muscle fibers (Murrant *et al.* 2016). Thus, capillaries initiate conducted responses in response to different vasodilators than arterioles and are the only vessels guaranteed architecturally to be in close proximity to a contracting fiber (for review see Murrant & Sarelius 2015). Capillary induced conducted responses are important to study because they allow skeletal muscle tissue to distribute flow directly to contracting muscle fibers without perfusing an entire region of non-active fibers during submaximal contraction.

1.5. CO_2 and H^+

Extensive research is being carried out on the roles of O_2, K^+, NO, Ado, ATP and prostaglandins in active hyperaemia and local vasodilation in skeletal muscle (Buerk *et al.* 2011; Sprague & Ellsworth 2012; Pittman 2013; Murrant *et al.* 2014; Murrant & Sarelius 2015; Jackson 2016). However, two often overlooked metabolites are CO_2 and H^+.

H^+ is released every time ATP is hydrolyzed to adenosine diphosphate (ADP) (Boron & Boulpaep 2009). Therefore, with every completed actin-myosin cross bridge cycle, a molecule of H^+ is produced. Lactate has been shown to increase parabolically with increasing O_2 consumption during exercise (Bonen *et al.* 1998). Thus, H^+ is present

in great quantities any time a skeletal muscle fiber contracts, indicating that it could be an effective index of muscle contraction and metabolic need. CO_2 is present during skeletal muscle contraction as a byproduct of aerobic metabolism. CO_2 production and O_2 consumption increase linearly together during exercise (Jongbloed *et al.* 1957). Thus during almost all circumstances of submaximal and maximal exercise CO_2 is produced, also rendering it a potential indicator of metabolic need.

1.5.1 H^+ Induced Vasodilation

The majority of work done investigating H^+ has been in coronary, cerebral, mesentery and thoracic microvasculature, not skeletal muscle microvasculature (Aalkjaer & Peng 1997; Celotto *et al.* 2008). In cerebral arterioles endothelial cell derived NO and smooth muscle cell K_{ATP} channels have been implicated in the vasodilatory response to hydrochloric acid (HCl) (Horiuchi *et al.* 2002). In human internal mammary arteries K_{ATP} channels and voltage gated calcium (Ca^{2+}) channels were implicated as a mechanism for the observed HCl induced vasodilation (Rohra *et al.* 2005). In the rat aorta nitrite derived NO production and subsequent vasodilation was shown to increase with HCl and acetic acid (Modin *et al.* 2001). Supporting this evidence, NO was found to be easily produced and more stable in acidic conditions thereby increasing its bio-availability (Ignarro 1989). An outward, hyperpolarizing, K^+ current has been measured in response to HCl induced acidification of cerebral arterial smooth muscle cells (Bonnet *et al.* 1991). Therefore, NO and K_{ATP} channels, and a hyperpolarizing current have all been implicated as mechanisms by which HCl induces vasodilation in various vascular beds.

As stated previously, hyperpolarization through K_{ATP} channels or otherwise induces vasodilation and initiates conducted responses in skeletal muscle arterioles. NO induces vasodilation in skeletal muscle arterioles. Therefore, given that H^+ stimulates K_{ATP} channels, causes hyperpolarization, increases NO production and may increase the bioavailability of NO, it stands to reason that H^+ could cause vasodilation and a conducted response in skeletal muscle arterioles. Furthermore, hyperpolarization and NO can both initiate conducted responses from capillaries. Therefore, it stands to reason that H^+ could cause vasodilation and a conducted response in skeletal muscle capillaries.

1.5.2 CO_2 Induced Vasodilation

Hypercapnia has been shown to vasodilate cerebral and coronary arteries (Kontos *et al.* 1977; Ely *et al.* 1982; Peng *et al.* 1998). However, there lacks investigation of CO_2 in skeletal muscle. CO_2 has been implicated in rat cerebral arteries as a vasodilator by causing hyperpolarization and decreasing smooth muscle intracellular Ca^{2+} (Peng *et al.* 1998). In dog coronary arteries, hypercapnia vasodilated through an endothelial derived NO mechanism (Gurevicius *et al.* 1995). Lastly, in cat cerebral arteries CO_2 was shown to induce vasodilation through K_{ATP} channels (Kontos & Wei 1996). Therefore, there is evidence to indicate that hypercapnia, similar to H^+ also induces vasodilation through hyperpolarization, NO and K_{ATP} channel stimulation. As these mechanisms are involved in skeletal muscle arteriolar vasodilation and conducted responses at the arteriole and the capillary, it stands to reason that CO_2 should elicit vasodilation in skeletal muscle arterioles and a conducted response from skeletal muscle arterioles and capillaries.

1.5.3 Physiological Relationship Between CO_2 and H^+

Most of the studies investigating the vascular response to hypercapnia investigated hypercapnic acidosis. Thus, as CO_2 was elevated, H^+ was also elevated. In these studies H^+ was a confounding variable to the effects of CO_2 because H^+ is also a vasodilator. The observed vasodilatory effects of CO_2 on NO and K_{ATP} channels could have been in response to the presence of CO_2 or H^+. This overlap of experimental variables occurred because CO_2 and H^+ are linked through the bicarbonate (HCO_3^-) buffer (as shown in equation 1).

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+ \qquad (1)$$

Because of the bicarbonate buffer, H^+ and CO_2 each influence the other's presence. If CO_2 is increased, it will react with water (H_2O) to ultimately produce H^+ thereby directly changing H^+ concentration and vice versa. Therefore, it cannot be known what CO_2 and H^+ do independently in skeletal muscle microvasculature unless they are studied using a non-bicarbonate buffer system. A non HCO_3^- buffer would allow the isolation of CO_2 and H^+ so that the individual vasoactive characteristics of CO_2 and H^+ can be identified.

1.5.4 Vasodilator Interaction

Vasoactive metabolites interact with each other when inducing vasodilation. For example, K^+ has been shown to inhibit vasodilation produced by Ado and NO in skeletal muscle microvasculature (Lamb & Murrant 2015). Acidosis is well known to attenuate the effects of vasoconstrictors (for review, see Celotto *et al.* 2008). In human skeletal muscle feed arteries, which lie external to the skeletal muscle tissue, induced acidosis attenuates the response to known vasoconstrictors phenylephrine (Phe) and potassium

chloride (KCl) (Ives *et al.* 2013). In human internal mammary arteries, HCl induced vasodilation after preconstriction by Phe and KCl (Rohra *et al.* 2005). Thus H^+ has already exhibited interactive properties with known vasoconstrictors.

Due to the HCO_3^- buffer, CO_2 and H^+ are present during muscle contraction together. Based on the interaction of NO, Ado, and K^+ as well as the inevitable simultaneous presence of H^+ and CO_2 in skeletal muscle, it is reasonable that H^+ and CO_2 could interact when initiating vasodilation. Therefore, it is essential to investigate the combination of CO_2 and H^+.

1.6. Thesis Goal

The overall goal of this thesis was to characterize the independent and combined vasoactive abilities of H^+ and CO_2 in skeletal muscle microvasculature through their ability to induce vasodilation and initiate a conducted response at an arteriole and a capillary. This was accomplished through four aims.

Aim 1: To find a non HCO_3^- based buffer to separate CO_2 from H^+ so that each may be studied individually.

HCO_3^- buffered salt solution is used in superfusate to mimic extracellular fluid when studying *in situ* tissue in microvascular studies. Vessel responses using HCO_3^- buffered salt solutions represent the most physiologically accurate responses observable. Moving away from the HCO_3^- buffer marks a departure from almost all of the preceding skeletal muscle microvascular literature. Therefore, it is important to ascertain the effects, if any, that the replacement buffer has on the microvasculature.

Tris(hydroxymethyl)aminomethane (TRIS) buffer has been used successfully on the *in situ* hamster cheek pouch microvasculature preparation (Duling 1973). Therefore, TRIS was chosen as a replacement buffer. The effects of TRIS buffer on the microvasculature were evaluated A) To ensure TRIS buffer does not affect vascular tone. B) To determine how TRIS buffer affects vascular reactivity through smooth muscle cells and endothelial cells. C) To determine how TRIS buffer affects conducted responses initiated at an arteriole.

We hypothesized that TRIS buffer would not affect vascular tone, nor would it influence vascular reactivity or conducted responses from an arteriole.

Aim 2: To determine whether H^+ directly or indirectly stimulates arteriolar vasodilation and/or a conducted response from arterioles and/or capillaries.

Skeletal muscle interstitial pH during rest is 7.4 (Street *et al.* 2001). During resistance training skeletal muscle interstitial pH has been measured as low as 7.04, but during sprint exercise has been measured as low as 6.63 (Costill *et al.* 1983; Street *et al.* 2001). Therefore, to mimic exercise induced acidosis the vascular response to pH 7.0 and 6.6 was investigated A) To determine if physiological exercise levels of H^+ cause arteriolar vasodilation. B) To determine if physiological exercise levels of H^+ initiate a conducted response when applied to an arteriole. C) To determine if physiological exercise levels of H^+ initiate a conducted response when applied to a capillary.

We hypothesize that H^+ will induce vasodilation at an arteriole and a conducted response from an arteriole and a capillary.

Aim 3: To determine whether CO_2 stimulates arteriolar vasodilation and/or a conducted response from arterioles and/or capillaries.

Skeletal muscle venous CO_2 during rest is about 40 mmHg and can increase to almost 80 mmHg during exercise (Bangsbo *et al.* 1993). Therefore, to mimic exercise, we sought to test multiple CO_2 partial pressures between 40 mmHg and 80 mmHg. 5 and 10% CO_2 gas were used to induce increasing CO_2 partial pressures. pCO_2's of 48 mmHg and 61 mmHg CO_2 were investigated using three specific aims. A) To determine if CO_2 causes arteriolar vasodilation when applied directly to the arteriole. B) To determine if CO_2 initiates a conducted response when applied to an arteriole. C) To determine if CO_2 initiates a conducted response when applied to a capillary.

We hypothesize that CO_2 will cause vasodilation and conducted responses at the arteriole and the capillary.

Aim 4: To determine the vascular effect of CO_2 and H^+ in combination.

Physiologically, given the bodies' use of a HCO_3^- buffer system, CO_2 and H^+ will be directly related and will appear simultaneously. During exercise, as CO_2 is produced, H^+ is also produced. Therefore, low (48 mmHg CO_2) and medium (61 mmHg) levels of CO_2 were combined with low levels of H^+ (pH 6.8) and investigated using three specific aims. A) To determine whether CO_2 and H^+ together cause greater arteriolar vasodilation than either CO_2 or H^+ individually when applied directly to the arteriole. B) To determine whether CO_2 and H^+ together initiate a larger conducted response at an arteriole than either CO_2 or H^+ individually. C) To determine whether CO_2 and H^+

together initiate a larger conducted response from a capillary than either CO_2 or H^+ individually.

We hypothesize that CO_2 and H^+ together will cause a greater vasodilation and a more dilated conducted response at the arteriole and from the capillary than either H^+ or CO_2 individually.

Chapter 2. Materials and Methods

2.1 Ethical Approval

All experimental procedures were approved by the University of Guelph Animal Care and Use committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care as set out in the *Guide to the Care and Use of Experimental Animals.* After all experimental protocols, animals were euthanized with an overdose of sodium pentobarbital (0.26 mg ml^{-1} I. V. to effect).

2.2 Cremaster Muscle Preparation

Male golden Syrian hamsters, 8-14 weeks, were anesthetized using a 70 mg kg^{-1} intraperitoneal bolus of sodium pentobarbital. Hamsters were tracheotomized with polyethylene tubing (1.2 mm inner diameter, 1.7 mm outer diameter) to provide an unrestricted airway. The left femoral vein was catheterized with polyethylene tubing (0.6 mm inner diameter, 1.0 mm outer diameter) for supplemental anesthetic administration. Hamster esophageal temperature was maintained at 37˚C by a water-heated coil placed underneath the animal.

The right cremaster muscle was exteriorized for observation using light microscopy as described previously (Baez 1973). The muscle was detached from the skin and cut longitudinally. It was separated from the testis and epididymis, spread across a clear lucite surface and pinned flat at the edge. Once exposed, the muscle was superfused continuously with either bicarbonate buffered (PSS) physiological salt solution (in mMol: NaCl 131.9; KCl 4.7; $CaCl_2$ 2.0, $MgSO_4$1.2, $NaHCO_3$ 30.0, aerated with 5% CO_2, balance nitrogen (N_2) to a pH of 7.35-7.45) or

tris(hydroxymethyl)aminomethane (TRIS) buffered physiological salt solution (in mMol: 99.9 NaCl, 4.7 KCl, 2.0 $CaCl_2$, 1.17 $MgSO_4$, 25.0 Tris, adjusted to pH 7.35-7.45 using 2.4 M HCl) maintained between 33.5˚C and 34.5˚C. During experiments using muscle contraction, tubocurarine hydrochloride pentahydrate is added to the superfusate to ensure complete control of the contraction stimulus by the experimenter (Dua *et al.* 2009). While muscle contraction experiments were not performed for this thesis, 3.89 μmol L^{-1} tubocurarine hydrochloride pentahydrate was added to the superfusate to remain consistent with such previously published experimental conditions. After surgery the hamster was moved to the microscope stage and the anesthetic level was maintained using an anesthetic pump. 10 mg ml^{-1} sodium pentobarbital in saline was administered at 0.56 ml h^{-1}. After relocation to the microscope stage the cremaster tissue was allowed to equilibrate for 45 minutes before experimentation.

The cremaster microvasculature was observed using an Olympus BX51WI microscope and an Olympus UMPlanFI 40x/0.80 water immersion objective lens. The image was captured by a Sony DXC-390 color video camera and recorded digitally by EZGrabber or StreamCatcher software. The total magnification was approximately 1300x. 2A arterioles or 4A arterioles were the site of observation for all of the experimental protocols. Only vessels displaying consistent vascular tone, a steady resting diameter well between maximally dilated and maximally constricted, were examined. At the end of each experiment, the cremaster muscle was superfused with 10^{-4} M sodium nitroprusside (SNP), a nitric oxide donor, to obtain maximum vessel diameter (Murrant *et al.* 2014)

2.3 Microvasculature Stimulation Techniques

The microvasculature was stimulated either through the superfusate or through a micropipette. Stimulation of the microvasculature through the superfusate exposed the entire cremaster muscle including the entire microvascular tree and all other cell types present in the muscle tissue to the stimulus. Micropipettes were used to apply the stimulus to a small region (≈ 200 μm) of the microvasculature. Micropipettes were constructed from 4-inch thin wall glass tubes with a 1.5 mm outer diameter and 1.12 mm inner diameter. The micropipettes were pulled and beveled on one side to create an 8-10 μm diameter tip. Micropipettes were placed as close atop the vessel as possible without physically touching the cremaster muscle. Flow was ejected from the micropipette using a water manometer pressurized with 30 cm H_2O. 10^{-5} M Fluorescein isothiocyanate-dextran (FITC) was added to the micropipette contents to make them visible through epifluorescence and ensure flow from the micropipette. Care was taken to make sure micropipette flow did not travel across any upstream observation sites.

2.4 Aim 1 - To find a non HCO_3^- based buffer to separate CO_2 from H^+ so that each may be studied individually

Protocol 1.1 – 2A arteriole behavior between TRIS and PSS

To compare microvascular behavior during TRIS superfusion with that of PSS superfusion the cremaster was exposed to a range of doses of acetylcholine (ACh), an endothelial cell dependent vasodilator, S-Nitroso-N-acetyl-DL-penicillamine (SNAP), an endothelial cell independent vasodilator, and phenylephrine (Phe), a vasoconstrictor and a 2A arteriole was observed. The cremaster muscle was initially superfused with PSS. 10 fold increasing concentrations from 10^{-7} M to 10^{-4} M of either ACh (n = 9), Phe

(n = 7) or SNAP (n = 7) were added to the superfusate in consecutive 2 minute intervals. Following this, the tissue was superfused with TRIS for a 20-minute equilibration period. After equilibration with TRIS the dose response was repeated (Figure 2A).

Protocol 1.2 – 2A arteriole responsiveness over time

Because ACh, Phe and SNAP applications in TRIS were always investigated after PSS in protocol 1.1 it was necessary to determine if time influenced microvascular reactivity. To determine whether the microvascular response to ACh in TRIS was consistent over time two dose responses of ACh were performed 60 minutes apart while a 2A arteriole was observed. The cremaster was superfused with TRIS. ACh was added to the superfusate in 10 fold increasing concentrations from 10^{-7} M to 10^{-4} M for consecutive 2-minute intervals. ACh was then washed out and the dose response was repeated 60 minutes later (n = 6) (Figure 2B).

Protocol 1.3 – 2A arteriole conducted response behavior under TRIS versus PSS

To investigate whether H^+ and CO_2 induced conducted responses, it had to first be determined whether conducted response behavior under TRIS was similar to conducted response behavior using PSS. The cremaster was superfused with PSS. A micropipette was used to expose a small region (≈ 200 μm) of the microvasculature to 10^{-4} M ACh, a known conducted response initiator. ACh was micropipetted for 2 minutes and microvasculature behavior at the application site (local site) was observed (Figure 2F). After washout, once the vessel regained consistent tone, the 10^{-4} M ACh micropipette application was repeated and a site 300 μm proximal (upstream) to the

local application site along the 2A arteriole was observed. This was repeated with observation sites 600, 900 and 1200 µm upstream (Figure 3). Then the superfusate was switched to TRIS and allowed to equilibrate for 20 minutes. After equilibration to TRIS, micropipette applications of 10^{-4} M ACh were repeated (n = 6).

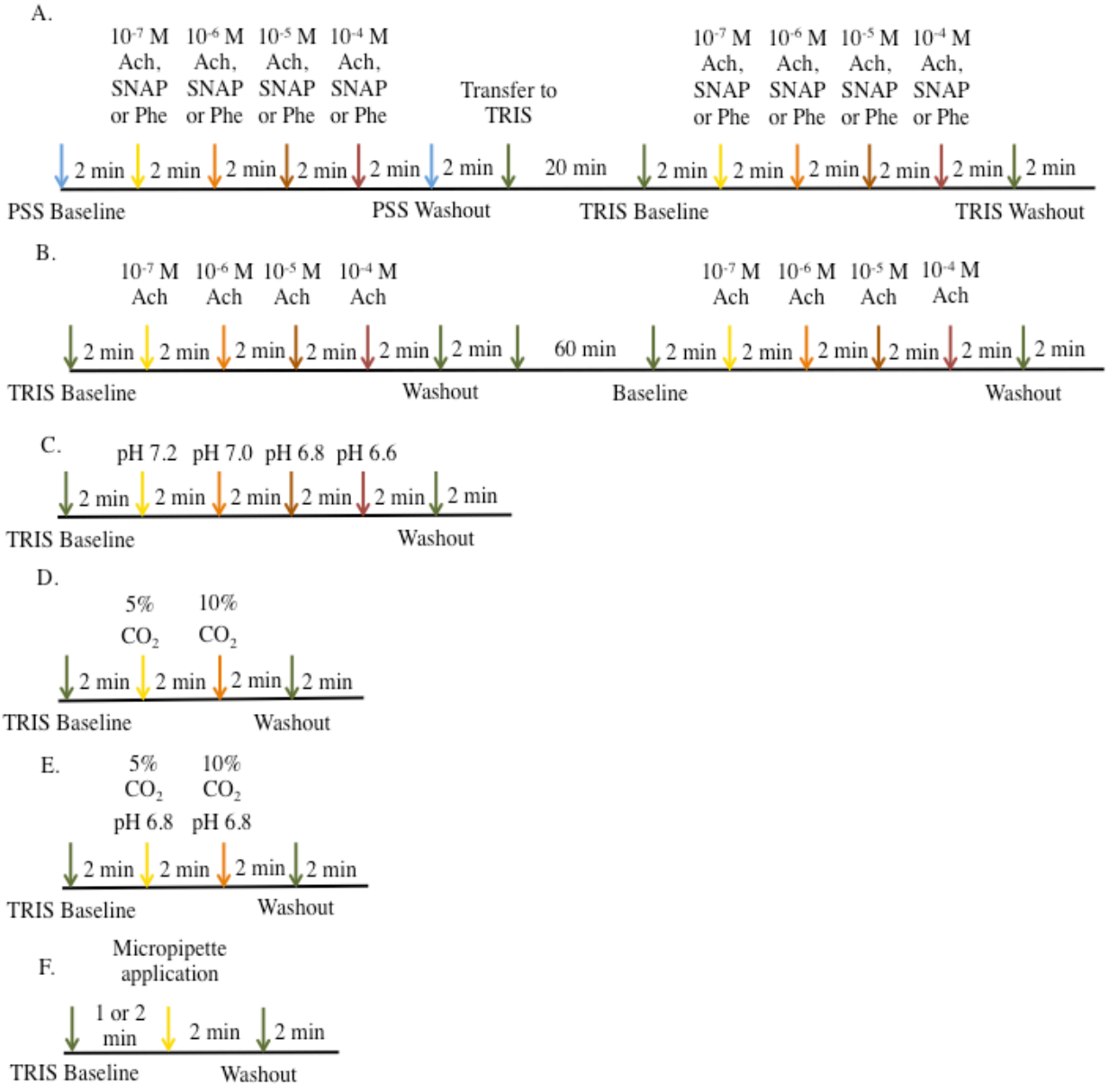


Figure 2. Timeline of protocols. A) Protocol 1.1 - Comparison of microvascular response to ACh, SNAP and Phe between PSS and TRIS superfusion. B) Protocol 1.2 – Comparison of microvascular response to ACh over time. C) Protocol 2.1 -

Investigation of microvascular response to H^+. D) Protocol 3.1 - Investigation of microvascular response to CO_2. E) Protocol 4.1 – Investigation of microvascular response to CO_2 combined with H^+. F) Micropipette application standard protocol.

2.5 Aim 2 - To determine whether H^+ directly or indirectly stimulates arteriolar vasodilation and/or a conducted response from arterioles and/or capillaries.

Protocol 2.1 – 2A arteriole response to H^+ applied in the superfusate

To determine the microvascular response to H^+ applied across the entire cremaster muscle a range of H^+ concentrations was added to the superfusate. The preparation was superfused with TRIS (pH 7.4) and then 63.1 nmol L^{-1} H^+ (pH 7.2), 100.0 nmol L^{-1} H^+ (pH 7.0), 158.5 nmol L^{-1} H^+ (pH 6.8) and 251.2 nmol L^{-1} H^+ (pH 6.6) were added in 2 minute consecutive intervals (n = 6) (Figure 2C). A 2A arteriole was observed. H^+ concentrations were achieved by adjusting TRIS with 2.4 M HCl. Osmolarity of each H^+ concentration of TRIS was measured (Advanced Instruments Micro Osmometer Model 3300) to ensure it was not a hyperosmotic solution.

Protocol 2.2 – H^+ induced vasodilation and conducted response on a 2A arteriole

To investigate whether H^+ can directly induce vasodilation or initiate a conducted response on a 2A arteriole, H^+ was micropipetted onto a 2A arteriole. Either TRIS adjusted to pH 7.0 with HCl (n = 9) or TRIS adjusted to pH 6.6 with HCl (n = 9) was micropipetted for 2 minutes and the local site was observed (Figure 2F). Then a washout period was allowed. After the vessel regained tone the same pH was reapplied while a site 1000μm upstream was observed (Figure 4). Every vessel examined using micropipette application of H^+, CO_2 or H^+ combined with CO_2 was also exposed to 10^{-4} M ACh at pH 7.4 micropipette applications to ascertain that local vasodilation and

conducted responses could be successfully initiated along each 2A arteriole. The 10^{-4} M ACh application verified that the vessel and experimental techniques were functional.

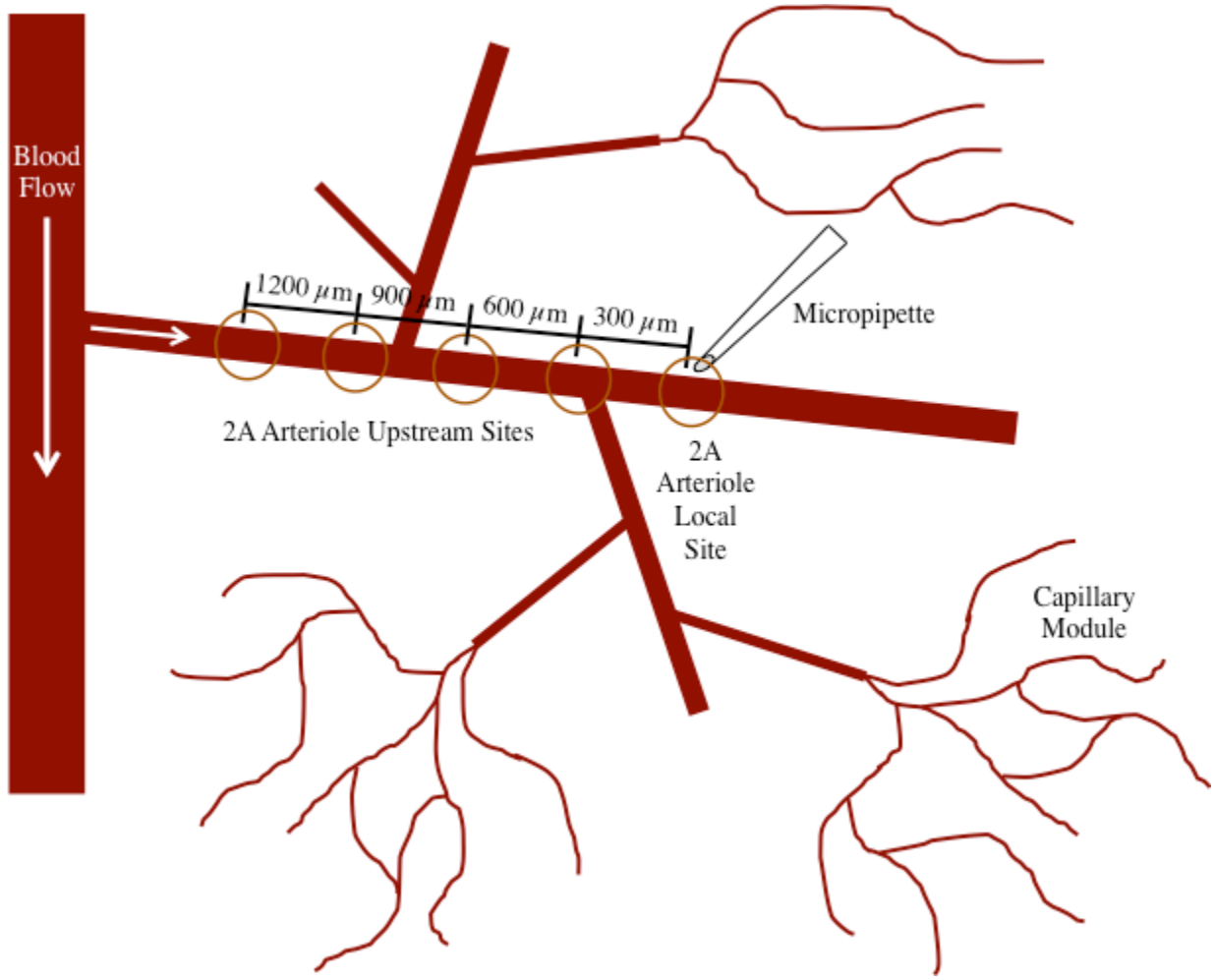


Figure 3. Schematic of micropipette placement and observation sites during protocol 1.3 adapted from (Twynstra *et al.* 2012). Micropipette indicates application site. Circles along the 2A arteriole indicate upstream observation sites.

Protocol 2.3 – H^+ induced conducted response from a capillary

To determine whether H^+ can stimulate a capillary to initiate a conducted response, H^+ was micropipetted onto a capillary and a site 1000μm upstream on the preceding 4A arteriole was observed (Figure 4). 2 minutes of either TRIS adjusted to pH 7.0 (n = 10) or pH 6.6 (n = 10) using HCl was applied while the upstream site was

observed. The protocol was repeated using 10^{-4} M ACh, pH 7.4 for each vessel observed.

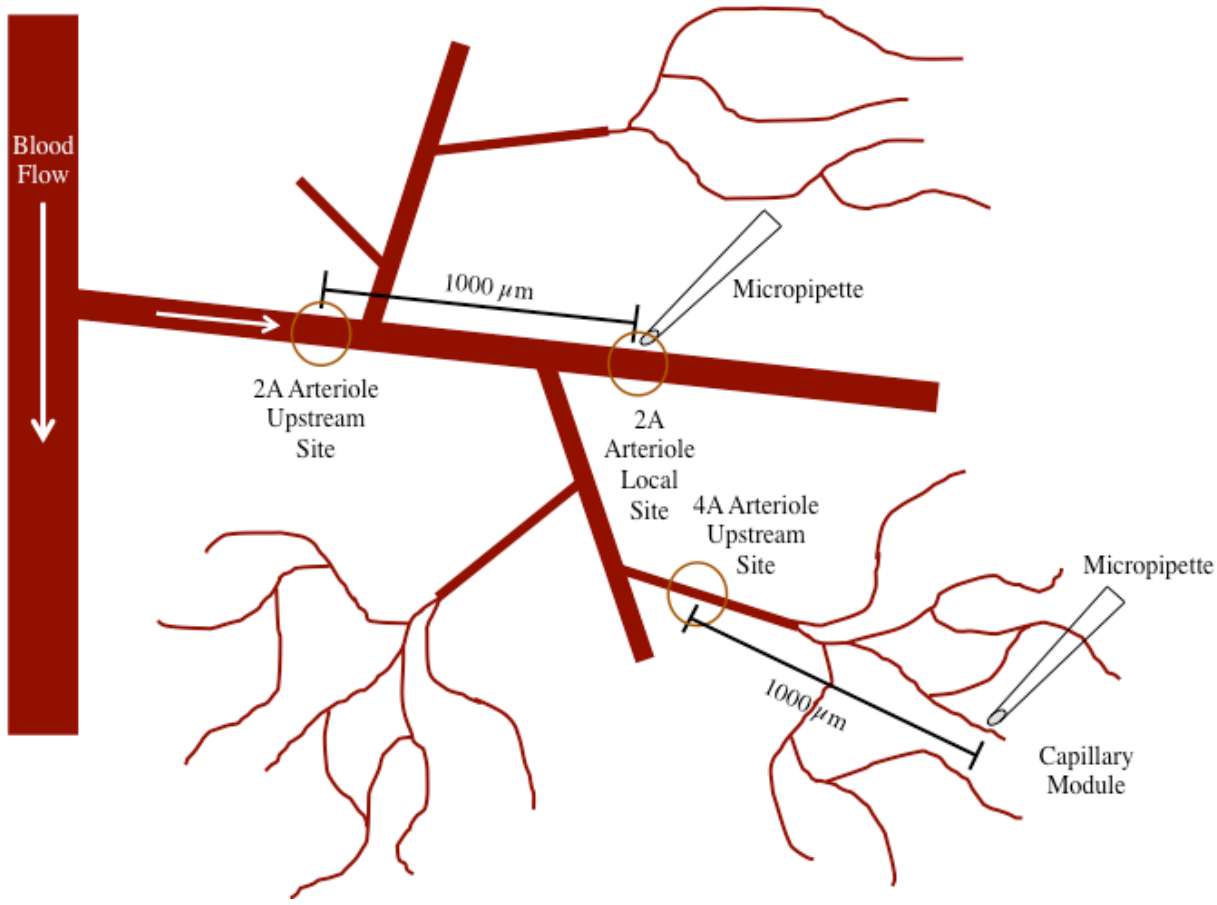


Figure 4. Schematic of micropipette placement and observation sites during protocols 2.2, 2.3, 3.2, 3.3, 4.2 and 4.3 adapted from (Twynstra *et al.* 2012). Micropipette indicates application site. Circles show local and upstream observation sites along the 2A arteriole and the upstream observation site along the 4A arteriole.

2.6 Aim 3 - To determine whether CO_2 directly or indirectly stimulates arteriolar vasodilation and/or a conducted response from arterioles and/or capillaries.

Protocol 3.1 – 2A arteriole response to CO_2 applied in the superfusate

To understand the microvasculature response to carbon dioxide (CO_2) applied across the entire cremaster muscle, different partial pressures of CO_2 were added to the superfusate. The cremaster was superfused with TRIS and then 47.8 ± 0.7 mmHg CO_2

(5%) and 60.7 ± 1 mmHg CO_2 (10%) were added in consecutive 2-minute intervals (n = 9) (Figure 2D). A 2A arteriole was observed. 47.8 and 60.7 mmHg CO_2 partial pressures were obtained by aerating TRIS with 5 and 10% CO_2 balance N_2 gas, respectively. TRIS buffering capacity was found to be temperature dependent; therefore TRIS was maintained at 34.5˚C during and after aeration. After heating and aerating the solution with each CO_2 pressure, the pH was adjusted to physiological range (7.35-7.45) using either 2.4 M HCl or 2.4 M sodium hydroxide (NaOH). Osmolarity was measured to ensure each CO_2 partial pressure solution was not hyperosmotic. Partial pressure (pCO_2) of each CO_2 percentage used was measured using an ABL800 Flex blood gas analyzer.

Protocol 3.2 – CO_2 induced vasodilation and/or conducted response on a 2A arteriole

To explore whether CO_2 can directly induce vasodilation and/or initiate a conducted response along a 2A arteriole, TRIS aerated with 10% CO_2 (n = 7) was micropipetted onto a 2A arteriole for 2 minutes and the local application site was observed. After the vessel regained consistent tone, TRIS aerated with 10% CO_2 was reapplied and a site 1000μm upstream was observed (Figure 4). The protocol was repeated using 10^{-4} ACh, pH 7.4 for each vessel observed.

Protocol 3.3 – CO_2 induced conducted response from a capillary

To ascertain whether CO_2 can stimulate a capillary to initiate a conducted response, TRIS aerated with 10% CO_2 (n = 10) was micropipetted onto a capillary and a site 1000μm upstream along the preceding 4A arteriole was observed (Figure 4, Figure 2F). The protocol was repeated with 10^{-4} M ACh, pH 7.4 for each vessel observed.

2.7 Aim 4 - To determine the vascular effect of CO_2 and H^+ in combination.

Protocol 4.1 – 2A arteriole response to CO_2 combined with H^+ applied in the superfusate

To understand the microvasculature response to CO_2 combined with H^+ applied across the entire cremaster muscle, CO_2 and H^+ were added to the superfusate. The cremaster was superfused with TRIS and then TRIS aerated with 5% CO_2 (47.8 ± 0.7 mmHg) and 10% CO_2 (60.7 ± 1 mmHg) adjusted to pH 6.8 using HCl were added in consecutive 2 minute intervals (n = 8) (Figure 2E). A 2A arteriole was observed. Osmolarity was measured to ensure 5 and 10% CO_2 partial pressure at pH 6.8 in TRIS was not hyperosmotic.

Protocol 4.2 – CO_2 combined with H^+ induced vasodilation and conducted response on a 2A arteriole

To explore whether CO_2 combined with H^+ can directly induce vasodilation and/or initiate a conducted response along a 2A arteriole, CO_2 combined with H^+ was micropipetted onto a 2A arteriole. TRIS aerated with 10% CO_2 (n = 6) and adjusted to pH 6.8 using HCl was applied for 2 minutes and the local application site was observed. Then, after the vessel returned to baseline diameter, TRIS aerated with 10% CO_2 and adjusted to pH 6.8 using HCl was reapplied and a site 1000µm upstream was observed (Figure 4). The protocol was repeated using 10^{-4} ACh, pH 7.4 for each observed vessel.

Protocol 4.3 – CO_2 combined with H^+ induced conducted response from a capillary

To ascertain whether CO_2 combined with H^+ can stimulate a capillary to initiate a conducted response, CO_2 combined with H^+ was micropipetted onto a capillary and a

site 1000μm upstream along the preceding 4A arteriole was observed (Figure 4). TRIS aerated with 10% CO_2 (n = 7) and adjusted to pH 6.8 using HCl was applied for 2 minutes while the upstream site was observed (Figure 2F). The protocol was repeated with 10^{-4} M ACh, pH 7.4 for each vessel observed.

2.8 Data Collection and Analysis

During protocols 1.1, 1.2, 2.1, 3.1 and 4.1 the 2A arteriole was recorded for 2 minutes prior to drug or metabolite application, 2 minutes during each subsequent application and for 2 minutes following washout of the drug or metabolite (Figures 2A-D). During protocols 1.3, 2.2, 2.3, 3.2, 3.3, 4.2 and 4.3 the 2A arteriole or 4A arteriole site was recorded for 1 minute prior to micropipette application, 2 minutes during micropipette application and for 2 minutes after washout of the drug or metabolite (Figure 2F).

All measurements are reported as mean ± standard error. Baseline diameter is defined as the inner luminal vessel diameter 10 seconds prior to drug application. If the preparation retained vascular tone after a protocol was completed, a second protocol was performed using a different vessel. Thus, the number of experiments (n) is defined as the number of vessels studied, not the number of animals.

Video images were captured digitally using FrameShots Video Image Capture software. Vessel lumen diameter was measured every 10 seconds using ImageJ software. Measurements were organized in Microsoft Excel and graphs were created using Prism Graphpad. Graphs display change in vessel diameter, which was defined

as the difference between the baseline diameter and the measured diameter during drug or metabolite application.

Statistically, group means were compared with an ANOVA or a Student's *t*-test. Group means over time were compared with a repeated measures ANOVA. When the ANOVA identified significant differences a Protected LSD was used *post hoc* to determine which diameter changes were significantly different (Snedecor & Cochran 1989). Differences were considered statistically significant when $P < 0.05$.

Chapter 3. Results

3.1 Aim 1 - To find a non HCO_3^- based buffer to separate CO_2 from H^+ so that each may be studied individually

Protocol 1.1 – 2A arteriole behavior between TRIS and PSS

To characterize any differences in arteriolar response between TRIS and PSS superfusion we exposed the cremaster muscle to a dose response of ACh, Phe and SNAP in each buffer. Baseline and maximum diameters are reported in Table 1. There were no significant differences in the 2A arteriole baseline diameter between PSS and TRIS. There were no significant differences between the vasodilation induced by ACh in TRIS and PSS at 10^{-5} and 10^{-4} M (Figure 5A, 5B). The vasodilation induced by 10^{-7} and 10^{-6} M ACh was significantly attenuated in TRIS. All concentrations of SNAP and Phe elicited changes in arteriolar diameter that were not significantly different from each other in PSS and TRIS (Figure 5C, 5D, 5E, 5F). Thus, 2A arterioles display similar levels of tone between TRIS and PSS and 2A arterioles respond to pharmacological stimulation similarly between TRIS and PSS except to low concentrations of ACh in TRIS where vasodilation is attenuated.

Table 1. Average Baseline and Maximum Diameter for Experiments in Protocol 1.1

	Baseline Diameter (μm)			
Protocol	PSS	TRIS	Maximum Diameter (μm)	n
Dose Response of ACh from 10^{-7}-10^{-4} M	14.0 ± 1.8	16.2 ± 2.9	34.9 ± 3.4	9
Dose Response of SNAP from 10^{-7}-10^{-4} M	10.7 ± 1.8	9.6 ± 2.1	27.8 ± 3.2	7
Dose Response of Phe from 10^{-7}-10^{-4} M	14.8 ± 2.7	17.5 ± 1.5	30.7 ± 2.9	7

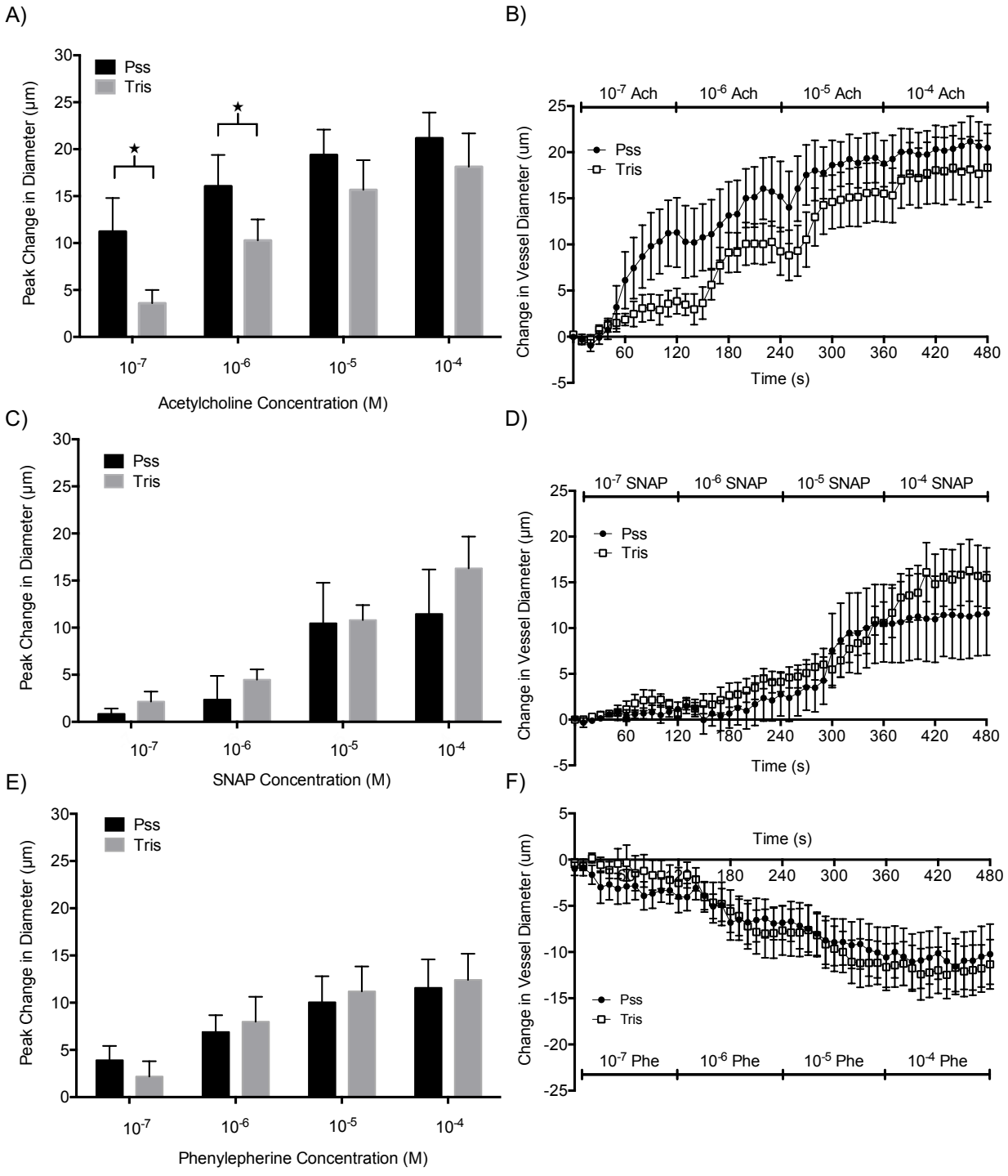


Figure 5. 2A arteriolar vasodilation in response to ACh was not significantly different in PSS and TRIS at 10^{-5} and 10^{-4} M. 2A arteriolar vasodilation in response to 10^{-7} and 10^{-6} M ACh was attenuated in TRIS. 2A arterioles did not differ significantly in response to SNAP and Phe in PSS and TRIS. **A)** Peak change in 2A arteriole diameter in response to dose response of ACh in PSS (■) and TRIS (■). **B)** Change in 2A arteriole diameter in response to dose response of ACh in PSS (•) and TRIS (□) over time. **C)** Peak

change in 2A arteriole diameter in response to dose response of SNAP in PSS (■) and TRIS (■). **D)** Change in 2A arteriole diameter in response to dose response of SNAP in PSS (•) and TRIS (■) over time. **E)** Peak change in 2A arteriole diameter in response to dose response of Phe in PSS (■) and TRIS (■). **F)** Change in 2A arteriole diameter in response to dose response of Phe in PSS (•) and TRIS (■) over time. ★ indicates where the change in 2A arteriolar diameter significantly differed between PSS and TRIS.

Protocol 1.2 – 2A arteriole responsiveness over time

To ensure that vascular reactivity in TRIS was consistent over time two dose responses of ACh were performed 60 minutes apart and a 2A arteriole was observed. There was no significant difference between the average 2A arteriole baseline diameter in TRIS immediately prior to the initial ACh application (16.5 ± 2.4 µm) and immediately prior to the second ACh application (16.3 ± 3.3 µm) (n = 6). The maximum vessel diameter was 34.2 ± 4.4 µm. 2A arterioles dilated similarly in response to cumulative applications of 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M ACh in the superfusate before and after 60 minutes (Figure 6A, 6B). There was a slight recovery of the dilation induced by 10^{-6} M ACh after 60 minutes, however this dilation did not differ significantly from the 10^{-6} M ACh dilation induced by the initial ACh application. Therefore 2A arterioles respond similarly to ACh stimulation before and after 60 minutes.

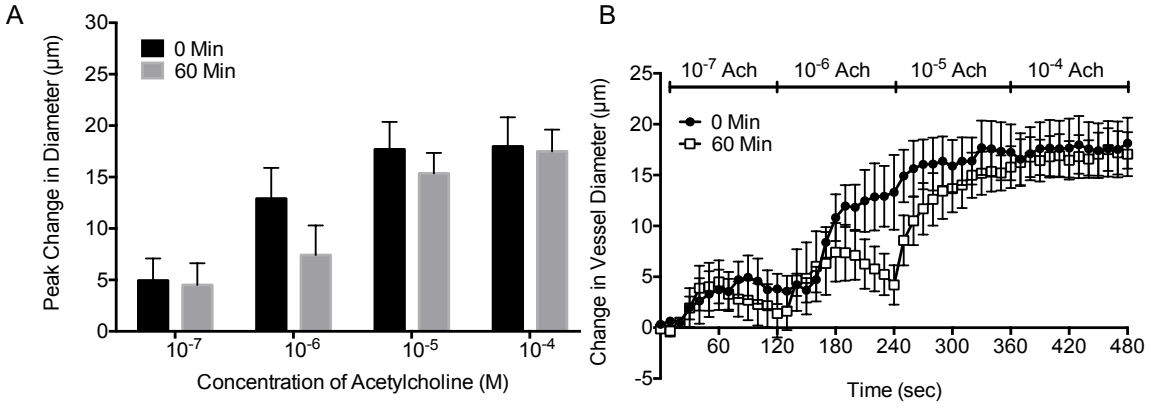


Figure 6. 2A arteriolar vasodilation responded similarly to ACh in TRIS before and after a 60 min wait period. **A)** Peak change in 2A arteriole diameter in response to dose response of ACh before (■) and after (■) 60 min wait period. **B)** Change in 2A arteriole diameter over time in response to dose response of ACh before (•) and after (■) 60 min wait period.

Protocol 1.3 – 2A arteriole conducted response behavior under TRIS versus PSS

To determine conducted response behavior when the preparation was superfused with TRIS, 10^{-4} M ACh was micropipetted onto a 2A arteriole in PSS and TRIS and the arteriole was observed locally, 300, 600, 900 and 1200 μm upstream. Average 2A arteriole baseline diameter was not significantly different between PSS and TRIS at each observed distance along the vessel prior to the ACh protocol (Table 2). The average vasodilation observed at each distance, 0, 300, 600, 900 and 1200 μm upstream of ACh application between PSS and TRIS was not significantly different (Figure 7). Thus, it was displayed again that 2A arterial tone does not differ between TRIS and PSS as well as that TRIS does not affect conducted response magnitude or propagation.

Table 2. Average Baseline and Maximum Diameter for Experiments in Protocol 1.3

	Baseline (μm)			
Observation Distance (μm)	PSS	TRIS	Maximum Diameter (μm)	n
Local	12.2 ± 1.2	11.5 ± 2.4	36.5 ± 3.6	6
300	12.4 ± 1.4	14.0 ± 3.2	36.9 ± 2.6	6
600	13.4 ± 2.2	14.7 ± 3.1	37.7 ± 2.7	6
900	13.6 ± 2.0	13.5 ± 2.6	38.0 ± 2.0	6
1200	10.5 ± 1.1	12.2 ± 2.1	39.2 ± 2.8	6

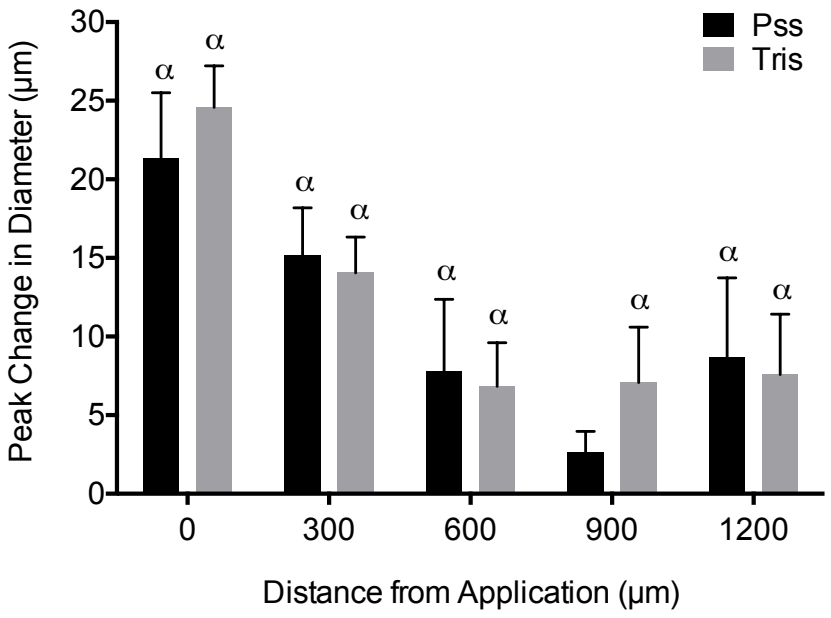


Figure 7. 2A arterioles responded similarly to 10^{-4} M ACh when the preparation was superfused with PSS (■) and TRIS (■) 0, 300, 600, 900 and 1200 μm upstream of the ACh application. α indicates significant vasodilation of 2A arterioles compared to baseline.

3.2 Aim 2 - To determine whether H^+ directly or indirectly stimulates arteriolar vasodilation and/or a conducted response from arterioles and/or capillaries.

Protocol 2.1 – 2A arteriole response to H^+ applied in the superfusate

To determine the vasodilatory capability of H^+ when applied to the entire cremaster muscle cumulative increasing concentrations of H^+ were applied through the superfusate. The average baseline diameter of 2A arterioles was 16.7 ± 1.7 μm and the average maximum diameter was 38.8 ± 3.3 μm (n = 6). TRIS adjusted to pH 7.2, 7.0, 6.8 and 6.6 using HCl all caused a significant increase in 2A arteriole diameter (Figure 8). TRIS adjusted to pH 7.2 (pCO_2 0 mmHg) caused a 5.9 ± 2.2 μm dilation. TRIS adjusted to pH 7.0 (pCO_2 0 mmHg) caused a 9.5 ± 1.9 μm vasodilation. TRIS adjusted to pH 6.8 (pCO_2 0 mmHg) caused a 9.4 ± 1.6 μm vasodilation and TRIS adjusted to pH 6.6 (pCO_2 0 mmHg) caused an 8.9 ± 1.8 μm vasodilation. Further addition of H^+ to lower

pH after 7.2 did not cause a significant change in 2A arteriolar diameter except for pH 6.8 which displayed a significantly greater peak vasodilation than pH 7.2. The addition of H^+ to TRIS to alter pH did not significantly change TRIS osmolarity (Table 3).

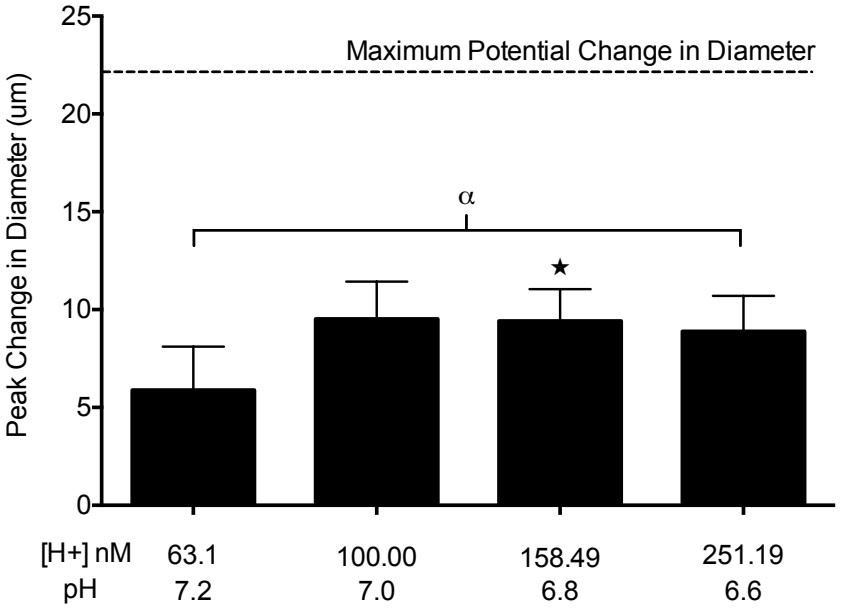


Figure 8. H^+ caused submaximal vasodilation in 2A arterioles. α indicates where H^+ induced significant vasodilation of 2A arterioles compared to baseline. ★indicates where additional H^+ induced a significant change in 2A arteriolar diameter from pH 7.2. -- indicates the maximum potential increase in diameter that may have occurred from the baseline diameter (average maximum diameter - average baseline diameter).

Table 3. Osmolarity of TRIS for Experiments in Protocols 1.1, 1.2, 1.3, 2.1, 2.2 and 2.3

pH	Osmolarity (mOsm)	n
7.4	294.4 ± 1.1	8
7.2	296.4 ± 1.6	5
7	297.6 ± 1.4	5
6.8	296.6 ± 2.0	5
6.6	299.4 ± 2.0	5

Protocol 2.2 – H^+ induced vasodilation and a conducted response on a 2A arteriole

To determine the ability of H^+ to induce vasodilation and/or initiate a conducted response directly on a 2A arteriole, TRIS adjusted to pH 7.0 and 6.6 using HCl was

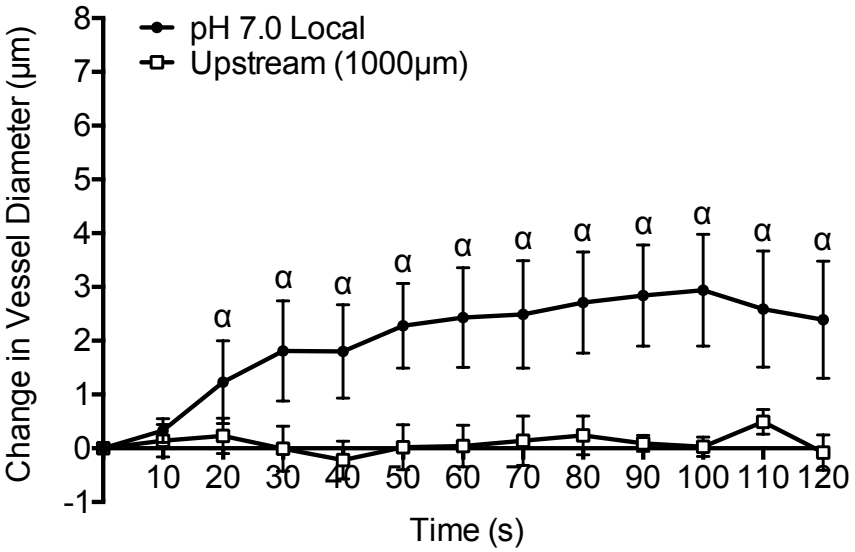
micropipetted onto the arteriole and the vessel was observed locally and 1000 μm upstream. 10^{-4} M Ach at pH 7.4 was also micropipetted onto each vessel that was studied to ensure that vasodilation and a conducted response could be elicited. ACh data was pooled between H^+, CO_2 and H^+ combined with CO_2 2A arteriole micropipette protocols.

Average local and upstream 2A arteriole baseline and maximum diameter was not significantly different between TRIS adjusted to pH 7.0, TRIS adjusted to pH 6.6 and 10^{-4} M ACh micropipette applications (Table 4). Micropipette application of TRIS adjusted to pH 7.0 elicited a significant increase in 2A arteriole diameter, 2.8 ± 1.0 μm, locally but did not induce any significant change in diameter 1000 μm upstream (Figure 9A). Micropipette application of TRIS adjusted to pH 6.6 elicited a significant increase in 2A arteriole diameter, 5.0 ± 2.0 μm, locally but did not induce any change in diameter 1000 μm upstream (Figure 9B). Micropipette application of 10^{-4} M ACh, pH 7.4 induced expected significant vasodilation locally and 1000 μm upstream (Figure 9C).

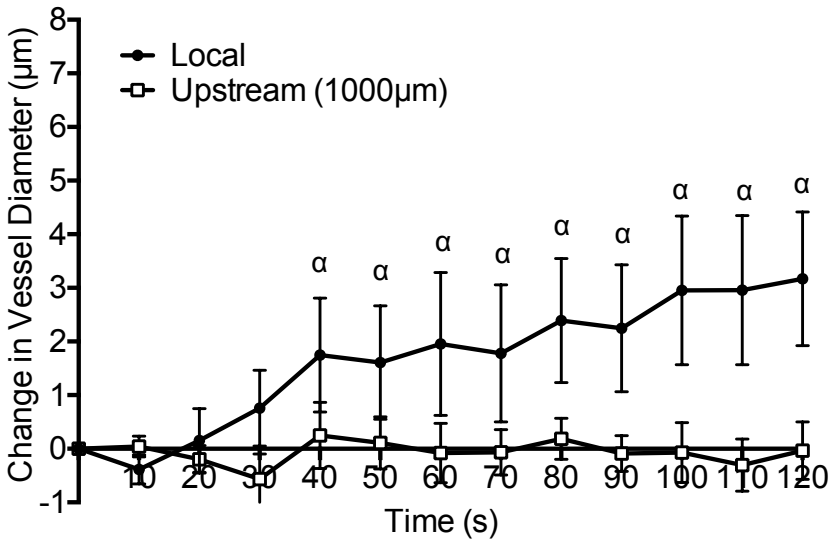
Table 4. Average Baseline and Maximum Diameter for Experiments in Protocol 2.2

	Baseline Diameter (μm)		Maximum Diameter (μm)		
Application	Local	Upstream	Local	Upstream	n
pH 7	12.8 ± 1.7	14.5 ± 1.2	32.4 ± 3.0	36.0 ± 2.9	10
pH 6.6	13.6 ± 1.9	13.1 ± 2.1	30.8 ± 2.9	33.8 ± 2.3	9
10^{-4} M ACh	13.5 ± 0.9	12.4 ± 1.0	32.0 ± 1.8	35.7 ± 1.5	28

A)


pH 7.0 Local
Upstream (1000µm)
Change in Vessel Diameter (µm)
Time (s)

B)


Local
Upstream (1000µm)
Change in Vessel Diameter (µm)
Time (s)

C)

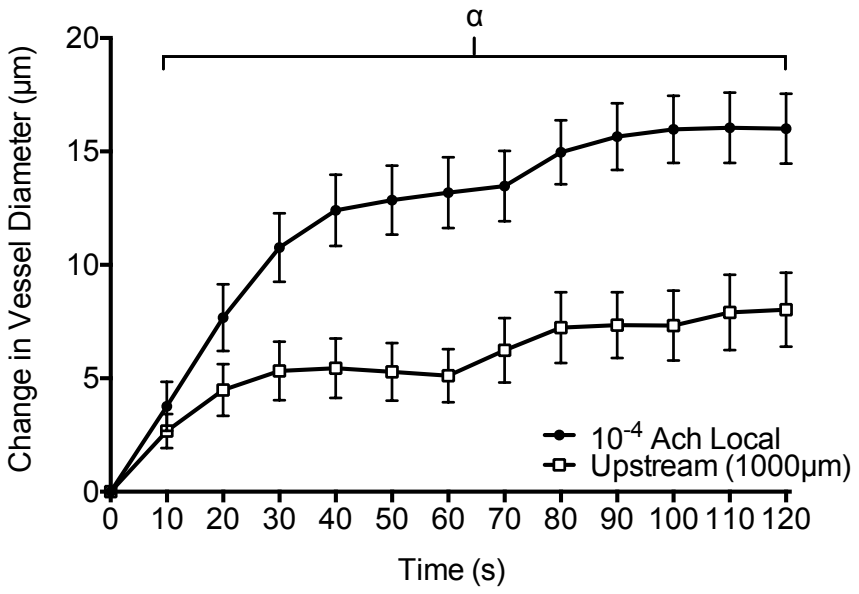

10-4 Ach Local
Upstream (1000µm)
Change in Vessel Diameter (µm)
Time (s)

Figure 9. 2A arteriolar diameter increased significantly in response to TRIS adjusted to pH 7.0 and 6.6 at local application site but not upstream. 10^{-4} M ACh elicited expected significant dilation at local site and 1000 µm upstream on 2A arterioles. **A)** Change in 2A arteriole diameter locally (•) and 1000 µm upstream (▣) in response to micropipette application of TRIS adjusted to pH 7.0. **B)** Change in 2A arteriole diameter locally (•) and 1000 µm upstream (▣) in response to micropipette application of TRIS adjusted to pH 6.6. **C)** Change in 2A arteriole diameter locally (•) and 1000 µm upstream (▣) in response to micropipette application of 10^{-4} M ACh. α indicates significant vasodilation of 2A arterioles compared to baseline.

Protocol 2.3 – H^+ induced conducted response from a capillary

To determine the ability of H^+ to initiate a conducted response from a capillary, TRIS adjusted to pH 7.0 and pH 6.6 using HCl was micropipetted onto a capillary and the 4A arteriole 1000 µm upstream was observed. 10^{-4} M ACh, pH 7.4, was also micropipetted onto to each vessel studied to ascertain that a conducted response could be elicited. ACh data was pooled between 4A arteriole micropipette protocols. Average 4A arteriole baseline and maximum diameter was not significantly different between TRIS adjusted to pH 7.0, pH 6.6 and 10^{-4} M ACh micropipette applications (Table 5). Micropipette application of TRIS adjusted to pH 7.0 or pH 6.6 onto a capillary did not elicit a significant increase in proximal 4A arteriole diameter (Figure 10A, B). Micropipette application of 10^{-4} M ACh, pH 7.4 onto a capillary induced a 4A arteriole vasodilation 1000 µm upstream (Figure 10C).

Table 5. Average Baseline and Maximum Diameter for Protocol 2.3

Application	Baseline Diameter	Maximum Diameter	n
pH 7	5.5 ± 0.8	13.9 ± 1.4	10
pH 6.6	5.8 ± 0.8	13.9 ± 1.4	10
10^{-4} M ACh	5.0 ± 0.3	11.6 ± 0.6	28

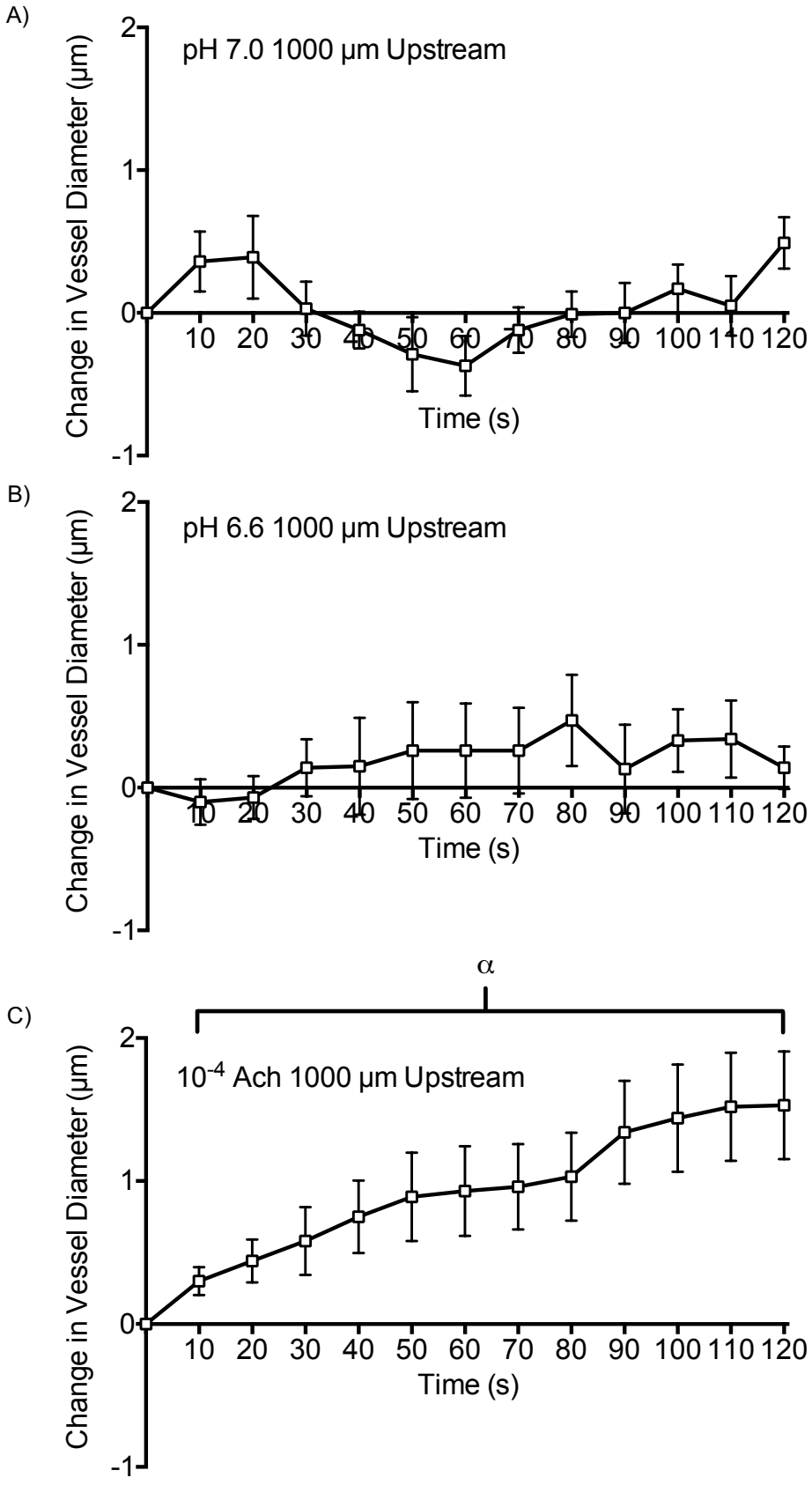

A)
pH 7.0 1000 µm Upstream
Change in Vessel Diameter (µm)
2
1
0
-1
10 20 30 40 50 60 70 80 90 100 110 120
Time (s)
B)
pH 6.6 1000 µm Upstream
Change in Vessel Diameter (µm)
2
1
0
-1
10 20 30 40 50 60 70 80 90 100 110 120
Time (s)
α
C)
10-4 Ach 1000 µm Upstream
Change in Vessel Diameter (µm)
2
1
0
-1
10 20 30 40 50 60 70 80 90 100 110 120
Time (s)

Figure 10. 4A arteriole diameter did not change significantly in response to TRIS adjusted to pH 7.0 and pH 6.6 micropipette application onto a capillary. 10^{-4} M ACh micropipette application onto a capillary elicited expected increases in 4A arteriole diameter. **A)** Change in 4A arteriole diameter in response to micropipette application of TRIS adjusted to pH 7.0 onto a capillary. **B)** Change in 4A arteriole diameter in response to micropipette application of TRIS adjusted to pH 6.6 onto a capillary. **C)** Change in 4A arteriole diameter in response to micropipette application of 10^{-4} M ACh onto a capillary. α indicates significant vasodilation of 4A arterioles compared to baseline.

3.3 Aim 3 - To determine whether CO_2 directly or indirectly stimulates arteriolar vasodilation and/or a conducted response from arterioles and/or capillaries.

Protocol 3.1 – 2A arteriole response to CO_2 applied in the superfusate

The microvasculature was exposed to cumulative increasing partial pressures of CO_2 in the superfusate to determine the vasodilatory capability of CO_2. First the partial pressure of CO_2 present in TRIS after aeration with 5 and 10% CO_2 gas balance N_2 was measured (Table 6).

Table 6. Average pCO_2 of TRIS for Experiments in Protocols 3.1, 3.2, 3.3, 4.1, 4.2 and 4.3

CO_2	Average Partial Pressure CO_2 (mmHg)	n
5%	47.8 ± .7	10
10%	60.7 ± 1.0	10

The average baseline diameter of 2A arterioles exposed to cumulative increasing partial pressures of CO_2 was 9.2 ± 1.1 μm and the average maximum diameter was 31.8 ± 2.5 μm (n = 9). CO_2 induced significant vasodilation of 2A arterioles (Figure 11). The maximum increases in average 2A arteriolar diameter in response to TRIS aerated with 5% CO_2 (pH 7.4, pCO_2 47.8 ± .7 mmHg) and 10% CO_2 (pH 7.4, pCO_2 60.7 ± 1.0 mmHg) were 7.3 ± 2.7 μm and 10.4 ± 3.1 μm respectively. The addition of 5% and 10% CO_2 to TRIS did not significantly change TRIS osmolarity (Table 7).

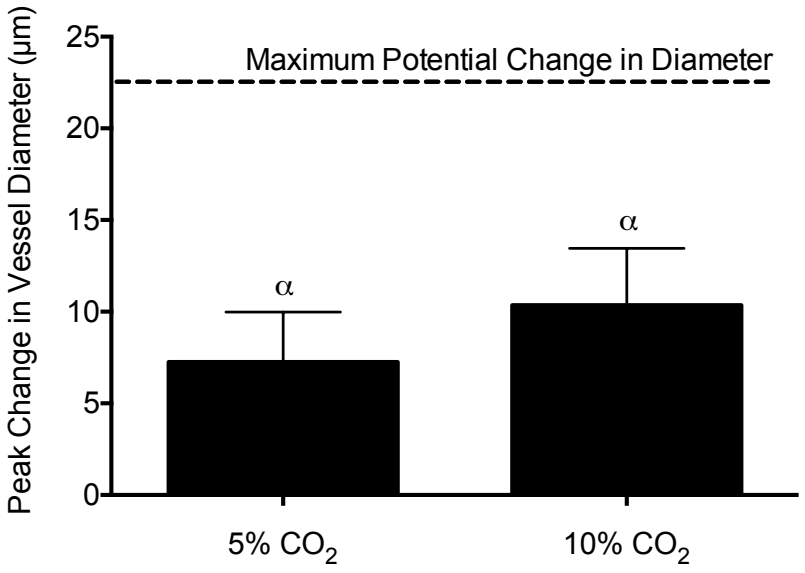


Figure 11. CO_2 caused submaximal vasodilation in 2A arterioles. α indicates where CO_2 induced significant vasodilation of 2A arterioles compared to baseline. -- indicates the maximum potential increase in diameter that may have occurred from the baseline diameter (average maximum diameter – average baseline diameter).

Table 7. Osmolarity of TRIS for Experiments in Protocols 3.1, 3.2 and 3.3		
CO2	Osmolarity (mOsm)	n
5%	297.6 ± 1.9	10
10%	295.8 ± 1.3	10

Protocol 3.2 – *CO_2 induced vasodilation and conducted response on a 2A arteriole*

To determine the ability of CO_2 to induce vasodilation and/or initiate a conducted response on a 2A arteriole TRIS equilibrated with 10% CO_2 (60.7 mmHg ± 1.0) was micropipetted onto a 2A arteriole and the vessel was observed locally and 1000 µm upstream. 10^{-4} M ACh with 0 mmHg CO_2 was also micropipetted onto each vessel studied to ascertain that vasodilation and a conducted response could be elicited. ACh data was pooled between 2A arteriole micropipette protocols. Average local and

upstream 2A arteriole baseline and maximum diameter was not significantly different between TRIS aerated with 10% CO_2 and 10^{-4} M ACh micropipette applications (Table 8). Micropipette application of TRIS equilibrated with 10% CO_2 did not elicit a significant vasodilation locally or 1000 μm upstream along a 2A arteriole (Figure 12A). Micropipette application of 10^{-4} M ACh induced expected significant vasodilation locally and 1000 μm upstream (Figure 12B).

Table 8. Average Baseline and Maximum Diameter for Protocol 3.2

	Baseline Diameter		Maximum Diameter		
Application	Local	Upstream	Local	Upstream	n
10% CO_2	15.5 ± 2.7	15.4 ± 1.9	37.1 ± 2.4	41.9 ± 2.4	7
10^{-4} M ACh	13.5 ± 0.9	12.3 ± 1.1	32.0 ± 2.1	36.6 ± 1.6	23

Protocol 3.3 – CO_2 induced conducted response from a capillary

To determine the ability of CO_2 to initiate a conducted response from a capillary TRIS aerated with 10% CO_2 was micropipetted onto a capillary and the 4A arteriole 1000 μm upstream was observed. 10^{-4} M ACh, 0 mmHg CO_2, was also micropipetted onto each vessel studied to ascertain that a conducted response could be elicited. ACh data was pooled between 4A arteriole micropipette protocols. Average 4A arteriole baseline and maximum diameter was not significantly different between TRIS aerated with 10% CO_2 and 10^{-4} M ACh micropipette applications (Table 9). Micropipette application of TRIS aerated with 10% CO_2 onto a capillary did not elicit a significant increase 1000 μm upstream in 4A arteriole diameter (Figure 13A). Micropipette application of 10^{-4} M ACh onto a capillary induced expected 4A arteriole vasodilation 1000 μm upstream (Figure 13B).

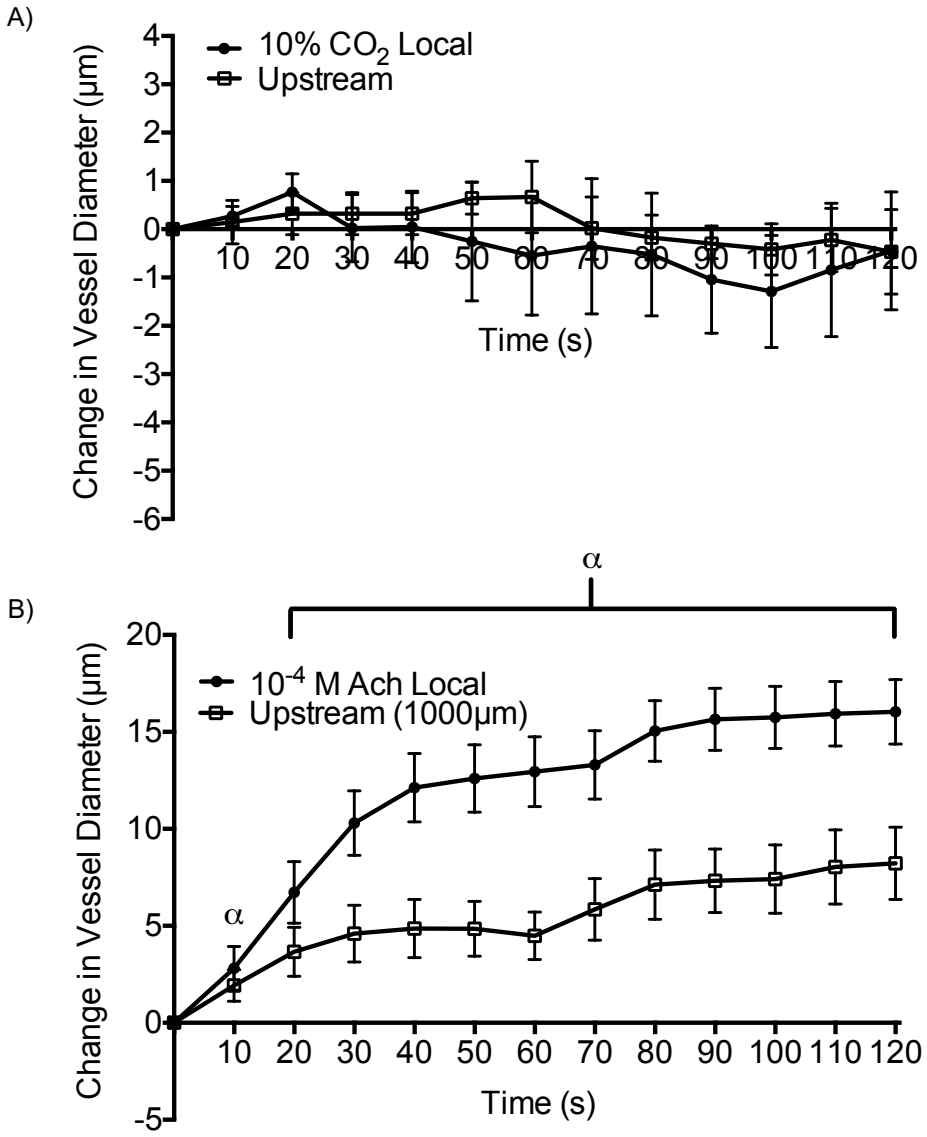


Figure 12. 2A arteriolar diameter did not increase significantly in response to micropipette application of TRIS aerated with 10% CO_2 at the local application site or 1000 µm upstream. 10^{-4} M ACh elicited expected significant dilation at local site and 1000 µm upstream on 2A arterioles. **A)** Change in 2A arteriole diameter locally (•) and 1000 µm upstream (▣) in response to micropipette application of TRIS aerated with 10% CO_2. **B)** Change in 2A arteriole diameter locally (•) and 1000 µm upstream (▣) in response to micropipette application of 10^{-4} M ACh. α indicates significant vasodilation of 2A arterioles compared to baseline.

Table 9. Average Baseline and Maximum Diameter for Protocol 3.3			
Application	Baseline Diameter	Maximum Diameter	n
10% CO_2	4.4 ± 0.3	11.0 ± 0.7	10
10^{-4} M ACh	4.7 ± 0.2	11.0 ± 0.7	24

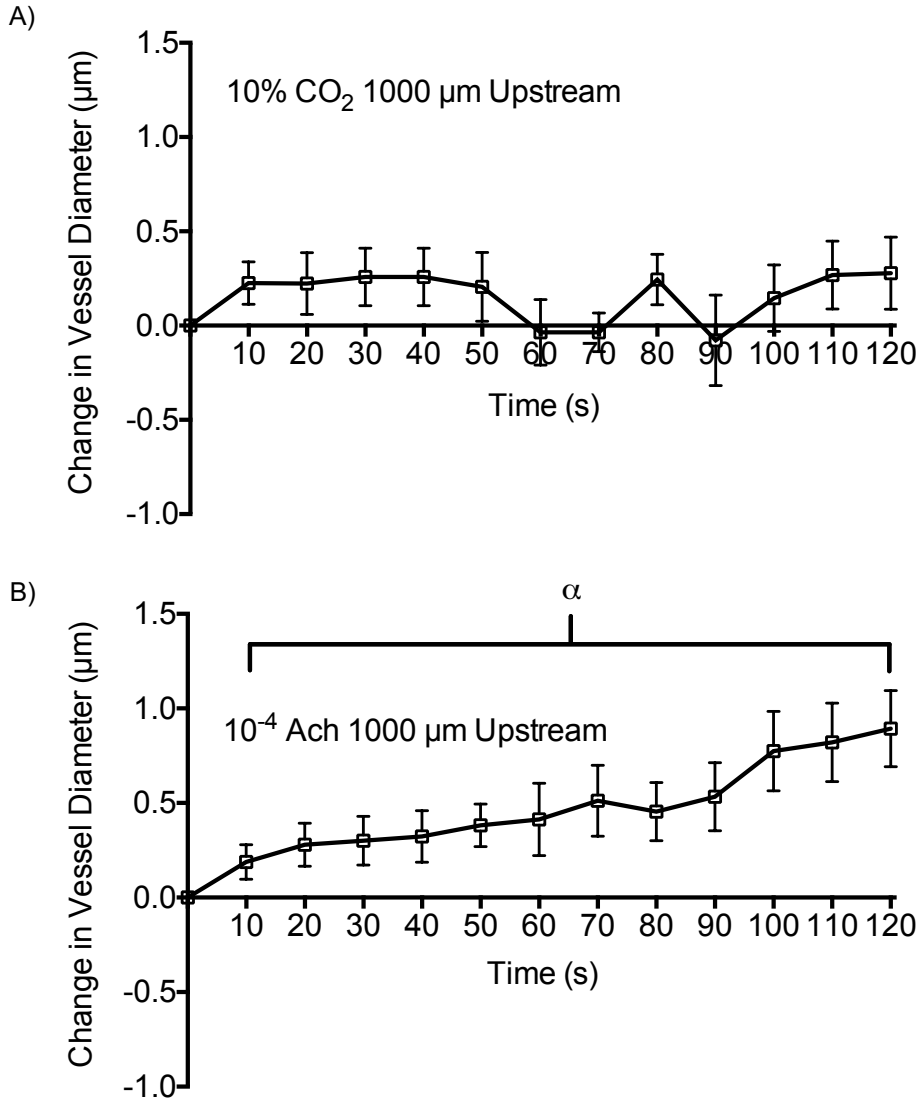


Figure 13. 4A arteriole diameter did not change significantly in response to TRIS aerated with 10% CO_2 micropipette application onto a capillary. 10^{-4} M ACh micropipette application onto a capillary elicited expected increases in 4A arteriole diameter. **A)** Change in 4A arteriole diameter in response to micropipette application of TRIS aerated with 10% CO_2 onto a capillary. **B)** Change in 4A arteriole diameter in response to micropipette application of 10^{-4} M ACh onto a capillary. α indicates significant vasodilation of 4A arterioles compared to baseline.

3.4 Aim 4 - To determine the vascular effect of CO_2 and H^+ in combination.

Protocol 4.1 – 2A arteriole response to CO_2 combined with H^+ applied in the superfusate

To determine the vasodilatory capability of CO_2 and H^+ together cumulative increasing partial pressures of CO_2 combined with H^+ were added to the superfusate. The average baseline diameter of 2A arterioles exposed to cumulative increasing partial pressures of CO_2 combined with H^+ was 12.6 ± 1.6 µm and the average maximum diameter was 26.1 ± 3.3 µm (n = 8). The peak increases in average 2A arteriolar diameter in response to TRIS equilibrated with 5% and 10% CO_2 adjusted to pH 6.8 using HCl were 7.0 ± 1.9 µm and 7.6 ± 2.5 µm respectively (Figure 14). These increases in diameter when compared as percentage of vasodilatory potential were not significantly different from the increases observed in response to CO_2 and H^+ applications in protocols 2.1 and 3.1 (Figure 8, 11). The osmolarities of TRIS equilibrated with 5 and 10% CO_2 adjusted to pH 6.8 using HCl were not significantly different from each other (Table 10).

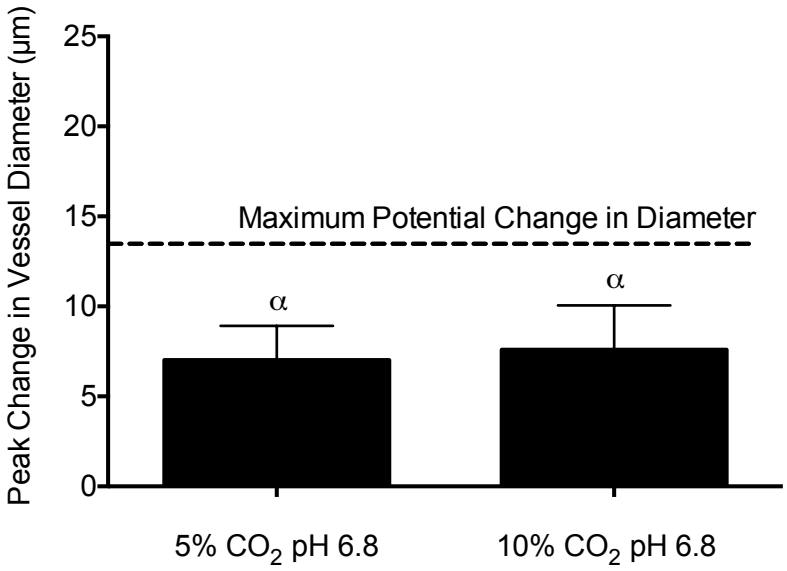


Figure 14. CO_2 and H^+ combined caused submaximal vasodilation in 2A arterioles. α indicates where CO_2 and H^+ combined induced significant vasodilation of 2A arterioles compared to baseline. -- indicates the greatest potential increase in diameter that may have occurred from the baseline diameter (average maximum diameter – average baseline diameter).

Table 10. Osmolarity of TRIS for Experiments in Protocols 4.1, 4.2 and 4.3

CO_2, pH	Osmolarity (mOsm)	n
5%, 6.8	311.7 ± 5.5	3
10%, 6.8	309.3 ± 1.8	3

Protocol 4.2 – CO_2 combined with H^+ induced vasodilation and conducted response on a 2A arteriole

To determine the ability of CO_2 combined with H^+ to induce vasodilation and/or initiate a conducted response on a 2A arteriole TRIS equilibrated with 10% CO_2 and adjusted to pH 6.8 with HCl, was micropipetted onto a 2A arteriole and the vessel was observed locally and 1000 µm upstream. 10^{-4} M ACh, pH 7.4, 0 mmHg CO_2, was also micropipetted onto each vessel studied to ascertain that vasodilation and a conducted response could be elicited. ACh data was pooled between 2A arteriole micropipette protocols. Average local and upstream 2A arteriole baseline and maximum diameters were not significantly different between TRIS aerated with 10% CO_2 adjusted to pH 6.8 and 10^{-4} M ACh micropipette applications (Table 11). Micropipette application of TRIS aerated with 10% CO_2 adjusted to pH 6.8 did not elicit a significant vasodilation locally or 1000 µm upstream along a 2A arteriole (Figure 15A). Micropipette application of 10^{-4} M ACh induced expected significant vasodilation locally and 1000 µm upstream (Figure 15B).

Table 11. Average Baseline and Maximum Diameter for Protocol 4.2					
	Baseline Diameter		Maximum Diameter		
Application	Local	Upstream	Local	Upstream	n
10% CO_2 pH 6.8	12.8 ± 1.6	15.4 ± 1.5	32.9 ± 3.4	38.2 ± 2.5	6
10^{-4} M ACh	13.5 ± 0.9	12.3 ± 1.1	32.0 ± 2.1	36.6 ± 1.6	23

Protocol 4.3 – CO_2 combined with H^+ induced conducted response from a capillary

To determine the ability of CO_2 combined with H^+ to initiate a conducted response from a capillary TRIS aerated with 10% CO_2 and adjusted to pH 6.8 using HCl was micropipetted onto a capillary and the 4A arteriole 1000 µm upstream was observed. 10^{-4} M ACh, pH 7.4, 0 mmHg was also micropipetted onto each vessel studied to ascertain that a conducted response could be elicited. ACh data was pooled between 4A arteriole micropipette protocols. Average 4A arteriole baseline and maximum diameter was not significantly different between TRIS aerated with 10% CO_2 adjusted to pH 6.8 and 10^{-4} M ACh micropipette applications (Table 12). Micropipette application of TRIS aerated with 10% CO_2 adjusted to pH 6.8 onto a capillary did not elicit a significant increase 1000 µm upstream in 4A arteriole diameter (Figure 16A). Micropipette application of 10^{-4} M ACh onto a capillary induced expected 4A arteriole vasodilation 1000 µm upstream (Figure 16B).

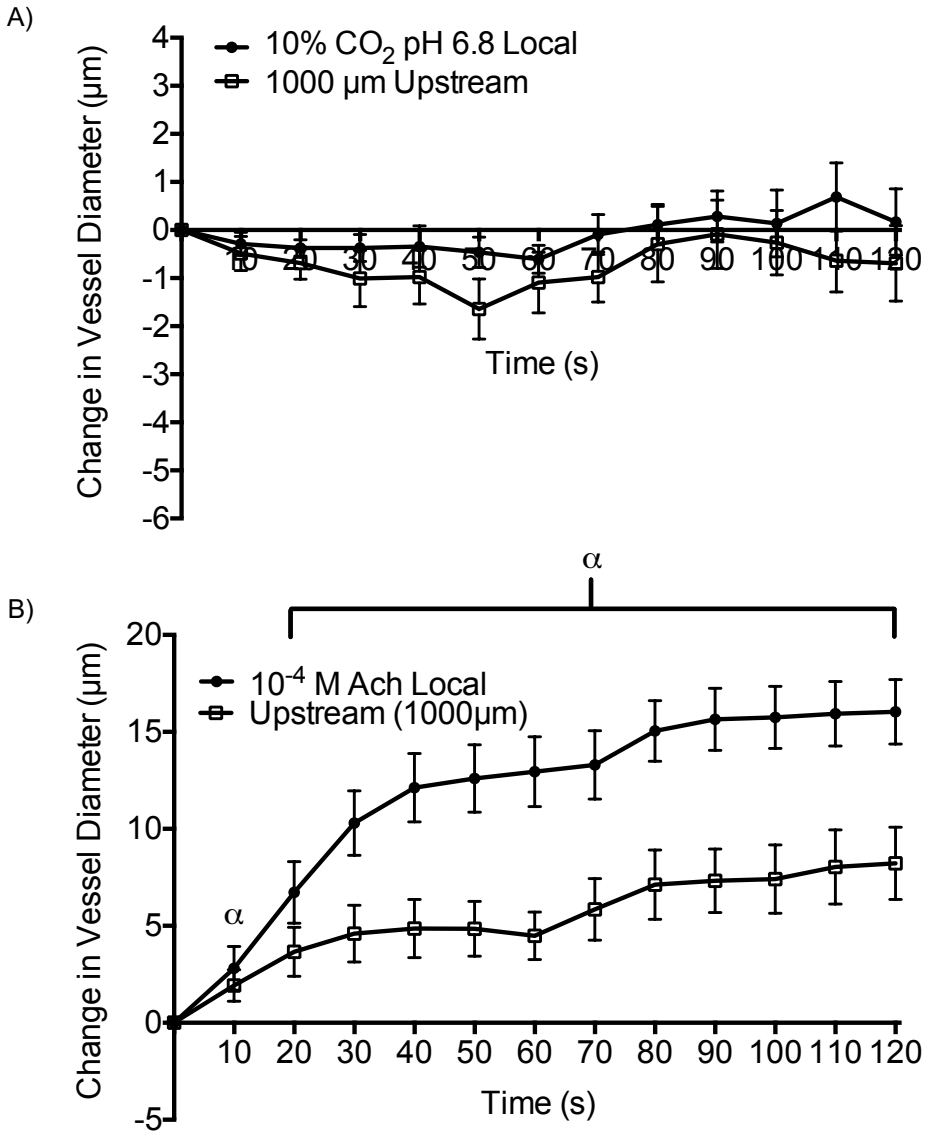


Figure 15. 2A arteriolar diameter did not increase significantly in response to micropipette application of TRIS aerated with 10% CO_2 adjusted to pH 6.8 using HCl locally or 1000 µm upstream. 10^{-4} M ACh elicited expected significant dilation at local site and 1000 µm upstream on 2A arterioles. **A)** Change in 2A arteriole diameter locally (•) and 1000 µm upstream (▣) in response to micropipette application of TRIS aerated with 10% CO_2 adjusted to pH 6.8 with HCl. **B)** Change in 2A arteriole diameter locally (•) and 1000 µm upstream (▣) in response to micropipette application of 10^{-4} M ACh. α indicates significant vasodilation of 2A arterioles compared to baseline.

Table 12. Average Baseline and Maximum Diameter for Protocol 4.3			
Application	Baseline Diameter	Maximum Diameter	n
10% CO_2 pH 6.8	4.6 ± 0.3	10.4 ± 0.7	7
10^{-4} M ACh	4.7 ± 0.2	11.0 ± 0.7	24

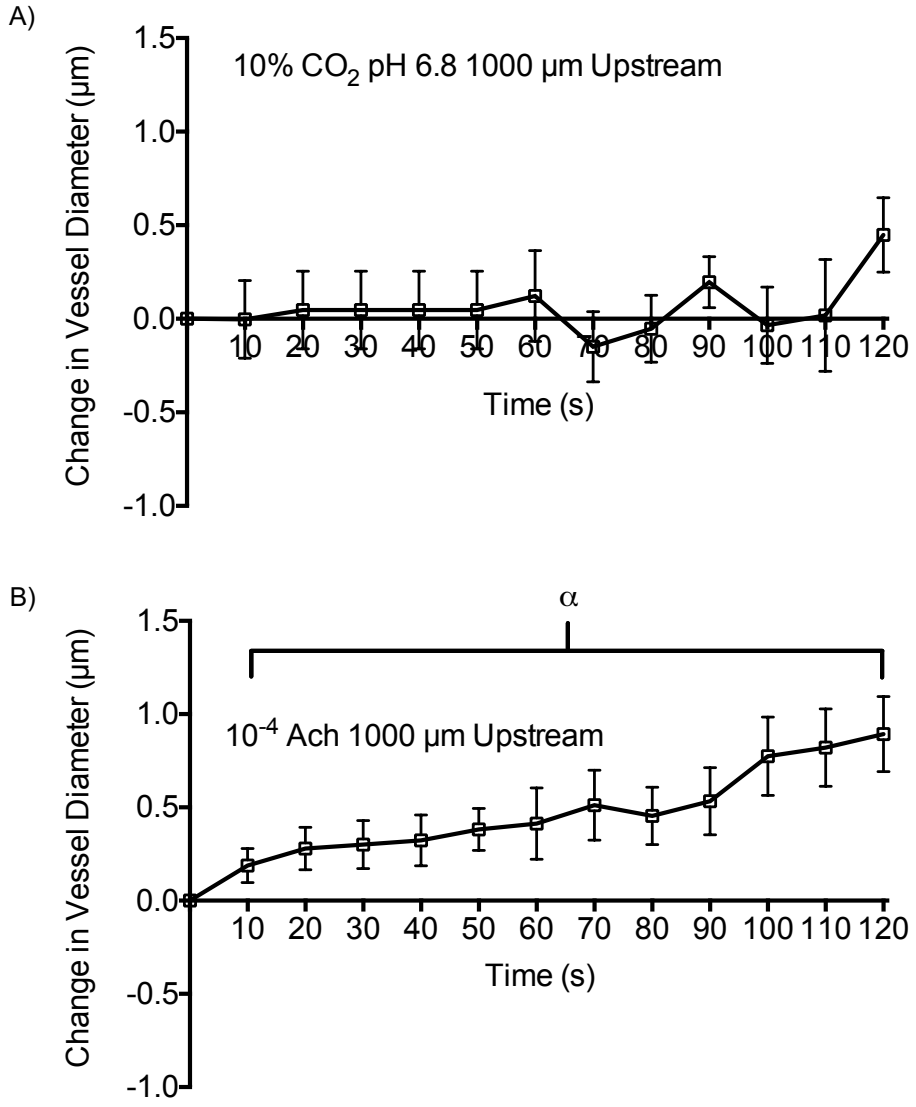


Figure 16. 4A arteriole diameter did not change significantly in response to micropipette application of TRIS aerated with 10% CO_2 adjusted to pH 6.8 onto a capillary. 10^{-4} M ACh micropipette application onto a capillary elicited expected increases in 4A arteriole diameter. **A)** Change in 4A arteriole diameter in response to micropipette application of TRIS aerated with 10% CO_2 adjusted to pH 6.8 using HCl onto a capillary. **B)** Change in 4A arteriole diameter in response to micropipette application of 10^{-4} M ACh onto a capillary. α indicates significant vasodilation of 4A arterioles compared to baseline.

Chapter 4. Discussion

These data show that H^+ caused submaximal arteriolar vasodilation directly on skeletal muscle arterioles. CO_2, and H^+ combined with CO_2 did not directly cause skeletal muscle arteriolar vasodilation, however when added to the superfusate and exposed to extravascular cell types were able to induce submaximal vasodilation. H^+, CO_2 and CO_2 combined with H^+ do not initiate conducted responses anywhere in the terminal microvasculature. Therefore, our data show that H^+ is a weak direct vasodilator in skeletal muscle that does not initiate conducted responses. Alternatively, CO_2 and H^+ combined with CO_2 are weak indirect vasodilators in skeletal muscle that also do not initiate conducted responses.

4.1 Experimental Considerations

4.1.1 Baseline and Maximum Diameter

Baseline diameter provides an important indication of the potential for vascular smooth muscle to change diameter during experimentation. If a vessel is either fully constricted or fully dilated it is no longer an appropriate assay to measure vascular constriction or dilation, respectively. For example, a vessel with a baseline of 0 μm will not show a change in diameter when exposed to vasoconstrictors. Thus, potent vasoconstrictors could be falsely interpreted as having no effect on vascular diameter. Maximum diameter provides context to interpret the baseline diameter. It displays the vasodilatory potential, the greatest possible amount that a given vessel can increase in diameter (maximum diameter – baseline diameter). Therefore, observing a vessel with a

baseline diameter that lies well between minimal and maximum diameter ensures the vascular assay is sensitive to vasodilation or vasoconstriction.

The lack of significant differences within any protocol between baseline diameters and maximum diameters establishes that the experimental vessels maintained a consistent ability to constrict or dilate prior to each experimental stimulus. Thus, the observed increases and decreases in diameter were not confounded by the resting diameter of the vessel.

4.1.2 Vascular Responses in TRIS Buffer

The vascular responses in TRIS buffer were similar to the more common bicarbonate buffer in PSS. The consistency of baseline diameter between TRIS and PSS superfusion during the ACh, Phe and SNAP dose responses indicates that TRIS does not alter vascular tone compared to PSS (Table 1). TRIS displayed no effect on the vascular response to high concentrations of ACh, and all concentrations of Phe and SNAP. TRIS attenuated the vasodilatory response to low concentrations of ACh in protocol 1.1 and during both applications in protocol 1.2 (Figures 5A, 6A). There is no clearly documented effect of TRIS on ACh or endothelial cell induced vasodilation and thus it is not clear why the vasodilatory response was attenuated. However, the data indicate that TRIS does not affect endothelial independent vasodilation or vasoconstriction but it may blunt the response to low doses of endothelial dependent vasodilators.

There was no significant difference between the ACh response in TRIS at two different time points 60 minutes apart, therefore microvasculature retained the same

ability to vasodilate over time (Figure 6A). However, in response to 10^{-6} M ACh applied after 60 minutes, the vasodilation was transient and the vessel began to recover (Figure 6B). Transient vasodilation over 2 minutes in response to 10^{-7} and 10^{-6} M ACh application was observed during protocol 1 in response to ACh applied in PSS and TRIS alike (Figure 5B). It was also observed before and after 60 minutes in response to 10^{-7} M in protocol 2. It has also been observed in hamster cheek pouch vessels (Doyle & Duling 1997). Therefore, because a similarly robust vasodilation was observed in response to each ACh application before and after 60 minutes and transient vasodilations in response to low concentrations of ACh are often observed in a variety of experimental conditions, time was not considered to affect vasodilatory ability.

The conducted response behavior in TRIS was not significantly different from that of PSS. Consistent vasodilations were observed 300, 600, 900 and 1200 μm upstream from the local application site, indicating that TRIS does not affect conducted response magnitude or propagation (Figure 7). Therefore, overall the reactivity of the vasculature to vasodilate, vasoconstrict, transmit conducted responses, and over time in TRIS, for the agonists studied, was similar to the results in PSS.

4.1.3 Osmolarity

Hypertonicity has been identified as a cause of vasodilation in skeletal muscle microcirculation (Gray 1971; Duling & Staples 1976). Each protocol required that TRIS be adjusted to a variety of pH values by adding HCl or a combination of HCl or NaOH with CO_2. It was necessary to ensure that these additions did not increase osmolarity enough to cause vasodilation and confound the experimental response; so TRIS

osmolarity was monitored throughout each protocol (Tables 3, 7 and 10). According to Duling and Staples (1976), in the hamster cremaster muscle, osmolarity increases from 292 mOsm/l to 302, 312, and 332 mOsm/l for 2 minutes did not cause significant changes in diameter. In the current experiments the highest osmolarity of TRIS measured was 311.7 mOsm. Thus it can be concluded that the osmolarity of TRIS did not independently produce vasodilation during this protocol.

4.2 Vasodilatory Capability of H^+

H^+ causes arteriolar vasodilation in skeletal muscle microvasculature. When the pH of the superfusate was decreased from 7.4 (40 nmol/L) to 7.2 using HCl (63 nmol/L), a significant vasodilation occurred, indicating that small increases in H^+ are vasoactive (Figure 8). This degree of sensitivity was also observed in porcine coronary arteries which increased in diameter incrementally in response to cumulative exposures to pH 7.3, 7.2, 7.1 and 7.0 (Ishizaka & Kuo 1996). However, in the hamster cremaster muscle, 2A arteriolar diameter did not continue to increase with decreasing pH. Instead, the vasodilatory effect appeared to plateau at pH 7.0.

Arteriolar vasodilation in skeletal muscle in response to pH lower than 7.0 has not been well investigated. However, the observed plateau in response to H^+ contradicts data collected on rat and cat cerebral vasculature which shows increasing vasodilation at pH 6.8 and 6.5 (Dacey Jr. & Duling 1982; Kontos *et al.* 1977; Horiuchi *et al.* 2002). These differing results indicate that cerebral microvasculature is more sensitive to decreases in pH than skeletal muscle microvasculature. This could mean that H^+ plays a greater role in cerebral blood flow regulation than in skeletal muscle.

H^+ applied to discrete areas of 2A arterioles through a micropipette also induced significant vasodilation, albeit a much smaller dilation. This indicates that H^+ was able to cause arteriolar vasodilation directly by stimulating either vascular smooth muscle cells or endothelial cells. Unlike when applied through the superfusate, micropipette applied TRIS adjusted to pH 6.6 induced a larger average vasodilation than TRIS adjusted to pH 7.0 (Figure 9). This could be due to the inherent dilution factor that micropipette contents experience upon exiting the micropipette and mixing with the superfusate. It is likely that micropipette application of TRIS adjusted to pH 7.0 exposed the vessel to a pH that is greater than 7.0 and micropipette application of TRIS adjusted to 6.6 exposed the vessel to a pH that is greater than 6.6.

While H^+ applied to discrete locations on arterioles caused vasodilation, it did not cause a conducted response. This indicates that H^+ may have caused vasodilation through a vasodilatory mechanism that is not also a putative conducted response initiator such as endothelial cell production of NO (Chen & Rivers 2001). H^+ applied to capillaries also did not cause a conducted response (Figure 10). Therefore, any stimulation of the vasculature by H^+ during muscle contraction will remain local to the stimulation site and not spread to other levels of the microvasculature. This indicates that H^+ is not directly involved in active hyperaemia during submaximal contraction where blood flow is more dependent on conducted responses.

The local vasodilation observed in response to micropipette application of H^+ and superfusate application of H^+ supports a role for H^+ in active hyperaemia during maximal muscle contraction where blood flow distribution is not dependent on

conducted responses. When all fibers are producing H^+ and the entire vascular tree is within diffusion distance of increased H^+ concentrations, H^+ can contribute an approximate 43 ± 9% increase in vessel diameter to increase blood flow throughout the muscle.

4.3 Vasodilatory Capability of CO_2

CO_2 also caused vasodilation in skeletal muscle arterioles when applied through the superfusate. When the CO_2 application was increased from 0% to 5% a significant vasodilation occurred indicating that CO_2 is vasoactive (Figure 11). This is consistent with a previous study of hypercapnia in the hamster cheek pouch in which pCO_2 was increased from 0 mmHg to 32 mmHg using 5% CO_2 and caused an 18 ± 7% increase in microvascular diameter (Duling 1973). Similar to H^+, increasing the CO_2 applied through the superfusate from 5% to 10% did not induce a significantly different vasodilation. This plateau in vasodilation is inconsistent with data collected from canine coronary arterioles in which an increase in pCO_2 from 30 mmHg to 60 mmHg caused coronary arterial pressure to drop linearly from approximately 180 mmHg to 60 mmHg (Case *et al.* 1978). The plateau in vasodilation is also inconsistent with the observed effects of 5% and 10% CO_2 on rat mesentery arteries which increased active tension in response to 5% CO_2 and decreased active tension in response to 10% CO_2 (Carr *et al.* 1993).

The difference in response to hypercapnia could be due to vessel order, type and location. The vascular tree has been shown to be heterogeneous in its response to vasodilatory stimuli (Jacobs & Segal 2000). Vessels that reside outside of the tissue are not exposed to the same microenvironments as vessels that reside in the tissue.

Therefore, their sensitivity to vasoactive stimuli may vary. The mesentery arteries examined likely did not penetrate the intestinal wall, though their location was not explicitly mentioned (Carr *et al.* 1993). Therefore, it is possible that this study displayed increased sensitivity to CO_2 because it incorporated vessels that lay outside of the tissue which may have different a CO_2 sensitivity than vessels that lie within the tissue. The coronary vasculature investigated included all of the vasculature between the left main coronary artery and the coronary sinus, which represents a combination of arteriolar vessel orders as well as capillaries and veins within the heart (Case *et al.* 1978). Therefore it is possible that this study displayed increased sensitivity to CO_2 because it involved other arteriolar vascular orders and vessel types that may have different CO_2 sensitivities than 2A arterioles specifically.

During micropipette application, the vasodilatory capability of CO_2 was eliminated (Figure 12). It was initially thought that the TRIS aerated with 10% CO_2 micropipette contents were being diluted and the vessel was not receiving a great enough stimulus to dilate. However, logically, when applying 10% CO_2 though a micropipette, even if there was a 50% dilution factor, the vessel would be exposed to 5% CO_2 which should still elicit vasodilation (Figure 11). Thus, the lack of vasodilation observed indicates a true lack of vascular response to localized micropipette application of 10% CO_2.

When a stimulus is applied to the vasculature through the superfusate it interacts with all of the different cell types in the muscle; nerves, skeletal muscle cells, red blood cells as well as vascular smooth muscle cells and endothelial cells. Alternatively, when the stimulus is applied through a micropipette, it is only guaranteed to interact with

vascular smooth muscle cells, endothelial cells and a small portion of skeletal muscle cells. Red blood cells will experience the stimulus transiently as they pass through the vessel and nerves are not guaranteed to lie near the stimulation site. Therefore, stimuli applied through the superfusate may induce vasodilation through interactions with other cell types that are not stimulated using a micropipette.

Human erythrocytes have been shown to release ATP, a known vasodilator, during situations of simultaneous hypoxia and hypercapnia (Bergfeld & Forrester 1992). Hypercapnia was found to increase Ado release from skeletal muscle during twitch contraction (Mo & Ballard 1997). Thus, it is possible that the vasodilation observed in response to CO_2 in the superfusate was caused by an increase in ATP release from red blood cells or Ado from skeletal muscle cells, while the micropipette application of CO_2 not affect these cells.

CO_2 did not cause a conducted response on an arteriole or from a capillary when applied through a micropipette (Figure 12, 13). The lack of a conducted response indicates that CO_2 is not likely involved in active hyperaemia during submaximal contraction when blood flow distribution is dependent on conducted responses. The response curve data for CO_2 support a role for CO_2 in active hyperaemia during maximal contraction when all vessels are within diffusion distance of contracting fibers. When all muscle fibers are contracting and high partial pressures of CO_2 are present throughout the vascular tree, CO_2 can contribute approximately a 46 ± 14% increase in vessel diameter to increase blood flow, not by acting on blood vessels directly, but likely through secondary release of a product from another tissue.

4.4 Vasodilatory Capability of CO_2 and H^+ Combined

Vasodilators have only recently been hypothesized to interact with each other (Lamb & Murrant 2015). Due to the inevitable simultaneous presence of H^+ and CO_2 together, physiologically or in a bicarbonate buffer, it was hypothesized that when H^+ and CO_2 were applied together they might interact to affect each other's vasoactivity. The vasodilations induced by application of H^+ through the superfusate, application of CO_2 through the superfusate or application of H^+ and CO_2 combined through the superfusate were not statistically different from each other (Figures 8, 11, 14). This indicates that H^+ and CO_2 did not display additive vasodilatory properties.

Micropipette application of TRIS adjusted to pH 7.0 and pH 6.6 consistently induced vasodilation while micropipette application of TRIS aerated with 10% CO_2 alone and 10% CO_2 adjusted to pH 6.8 did not produce a vasoactive response (Figures 9, 12, 15). Thus, adding CO_2 to H^+ abolished the vasodilation seen from H^+ alone, demonstrating an inhibitory interaction of CO_2 on H^+. This may indicate that during the CO_2 and H^+ combined response curve, the dilatory response was not additive because CO_2 may have been inhibiting H^+. Thus the only vasodilation observed would have been that resulting from CO_2.

K^+ has been demonstrated to inhibit NO and Ado induced vasodilation through a inward rectifying potassium channel (K_{IR}) dependent mechanism (Lamb & Murrant 2015). The role of K_{IR} channels has not been investigated in CO_2 induced vasodilation. However, K_{IR} channels have been shown to be sensitive to CO_2 in cell culture and K_{ATP} channels, which involve K_{IR} subunits, have been implicated in the rat mesenteric

vasodilatory response to hypercapnic acidosis (Wang *et al.* 2003; Huckstepp & Dale 2011). Thus it can be speculated that CO_2 may inhibit the H^+ induced vasodilation through activating K_{IR} channels.

Regardless of mechanism, it can be concluded that H^+ and CO_2 in combination do not cause conducted responses on arterioles or from capillaries (Figure 15, 16). Thus H^+ and CO_2 combined do not directly contribute to active hyperaemia during submaximal contraction, when blood flow distribution is dependent on conducted responses. They remain relevant to active hyperaemia during maximal contraction during which they can contribute up to approximately 56 ± 19% of the observed vasodilation.

4.5 Conclusion

H^+ can induce vasodilation directly on an arteriole and may be relevant to active hyperaemia during maximal contraction when all muscle fibers are contracting. CO_2 and CO_2 combined with H^+ can only induce vasodilation indirectly on an arteriole when exposed to extravascular cell types and may be relevant to active hyperaemia during maximal contraction when all muscle fibers are contracting. H^+ and CO_2 independently or combined do not stimulate an arteriole or a capillary to induce a conducted response and therefore do not play a role in initiating increases in blood flow during submaximal contraction. Considering maximal contraction is a rare occurrence and most actions occur at submaximal contraction, CO_2 and H^+ are very weak vasodilators.

4.6 Future Directions

To further investigate the physiological role of CO_2 and H^+ in active hyperaemia, the role of H^+, CO_2 should be investigated during muscle contraction. Muscle contraction releases a variety of vasodilators that mediate active hyperaemia (Dua *et al.* 2009). CO_2 and H^+ scavengers can be applied to the cremaster muscle during contraction to determine the strength and importance of CO_2 and H^+ in active hyperaemia.

To further investigate a role for H^+ and CO_2 in active hyperaemia, their ability to interact with other vasodilators should be examined. H^+ and CO_2 can be added in the presence of other vasodilators like K^+, NO and Ado to determine their potential to interact with other vasodilators during active hyperaemia.

The ability of H^+ and CO_2 to influence arterioles directly as opposed to extravascular cell types should be investigated. Therefore isolated vessels should be exposed to H^+ and CO_2 to determine their direct effects on smooth muscle cells and endothelial cells. Additionally, the ability of H^+ and CO_2 to change smooth muscle cell membrane potential in skeletal muscle arterioles should be examined.

The cellular mechanism by which H^+ and CO_2 induce vasodilation should be investigated to understand how CO_2 may inhibit H^+ or any other vasodilators. Thus H^+ and CO_2 should be investigated in the presence of nitric oxide synthase inhibitors and K^+ channel antagonists to try to elucidate their respective and combined vasodilatory mechanisms.

Lastly, the effect of H^+ and CO_2 should be investigated on different vascular orders and on vessels that lie inside and outside of the tissue. Different order vessels may react differently to vasoactive stimuli and vessels outside of the muscle are exposed to a different environment (Hebert & Marshall 1985). Therefore, to understand the full vasoactive abilities of H^+ and CO_2 independently and combined, it is important to investigate their effects on the rest of the vascular tree.

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