A Dissertation Entitled

The Role of Acidosis on Vascular Function during Dynamic Handgrip Exercise and Flow-mediated Dilation

By

John R. Thistlethwaite

Submitted as partial fulfillment of the requirements for
The Doctor of Philosophy degree in
Exercise Science

Advisor: Barry W. Scheuermann, Ph.D.

Thomas J. McLoughlin, Ph.D.

David L. Weldy, M.D., Ph.D.

Michael L. Troxell, Ph.D.

College of Health Science and Human Service

College of Graduate Studies

The University of Toledo
August 2008
This dissertation examined the effects of a chronic acidosis on vascular function during dynamic handgrip exercise and following post-occlusive reactive hyperemia. Seven males performed hand-grip exercise corresponding to 5% (moderate) and 10% (heavy) of maximal forearm strength during control (CON) and acidosis (acetazolamide 500 mg/d 3 d; AC) conditions. Flow-mediated dilation (FMD) was also measured on these subjects following a post-occlusive reactive hyperemia. Brachial artery diameters and velocities (MBV) were measured continuously during FMD and exercise using Doppler ultrasound. Muscle blood flow, shear rate, shear stress, mean arterial pressure, and vascular conductance were all computed. Muscle activity of the forearm flexors were measured using non-invasive electromyography. Arterialized venous blood was collected by catheter and analyzed for blood gases and ions.
Plasma [H+] were significantly higher during AC compared to CON for both moderate and heavy exercise as well as during FMD. Blood [HCO3\textsuperscript{-}] and blood gas P\textsubscript{CO\textsubscript{2}} were significantly decreased during AC compared to control for both moderate and heavy exercise as well as during FMD. Hematocrit was unchanged between rest and exercise for both conditions. As well, no differences in hematocrit were present between AC and CON conditions.

No difference in MBF or arterial diameters were detected between CON and AC conditions at rest or during moderate intensity exercise. As well, no significant difference was observed for MBF between CON and AC during heavy intensity exercise. However, heavy intensity exercise resulted in an increase in arterial diameter compared to rest for CON (p<0.05) but, no difference in arterial diameter was observed for AC. When expressed as a percent change from rest to heavy intensity exercise, the increase in diameter was greater (p<0.05) during CON compared to AC. FMD resulted in an increase in brachial artery diameter compared to rest (p<0.05). When expressed as %FMD, ACZ was significantly reduced compared to CON (p<0.05). In fact, the comparison resulted in a 48.5% difference in response between CON and ACZ conditions.

Shear rate (SR) and Shear stress (SS) values were well matched between conditions during both moderate and heavy exercise. Peak values for SR and SS were not significantly different between conditions during FMD. However, when FMD was normalized relative to shear rate (%FMD/SR), the ratio for ACZ was significantly lower (p<0.05) compared to CON.
Together these findings suggest that prior metabolic acidosis may alter vascular tone at rest and during heavy exercise but that MBF is unaltered between conditions during moderate and heavy exercise. As well, these results indicate that the endothelial-dependent FMD response is attenuated following chronic AC even after normalizing for changes in shear stress.
ACKNOWLEDGEMENTS

I would like to thank the entire Cardiopulmonary and Metabolism Research Laboratory: Joaquin Gonzales, Ben Thompson, Allison Harper, Mitch Stacy, and Dr. Barry Scheuermann for all their effort and support. Special thanks goes to Ben and Joaquin for their friendship and willingness to help in every possible way. I would not have been able to accomplish this feat without you. I would especially like to thank Dr. Barry Scheuermann for his wonderful guidance and insight into the world of physiology. You have not only peaked and persisted my interest in physiology, but you have taught me that it is an ever changing field and one in which I should contribute to through research. You have guided me through numerous presentations, lectures, acquaintances, and conferences. Not only have you been a remarkable teacher but a wonderful friend as well. A friendship that I hope lasts for many years. Thanks to you, I have learned more here than I thought possible. I would also like to thank the faculty of the Kinesiology Department for their friendship and encouragement. Further gratitude goes to my family for always reminding me that I am capable of achieving great things, no matter how difficult the venture. I would like to thank my fellow graduate assistants for all the friendship and support during this time.
# TABLE OF CONTENTS

Abstract iii

Acknowledgements vi

Table of Contents vii

List of Tables xi

List of Figures xii

I. Introduction 1

Purpose ................................................................................................................. 4

Hypothesis ............................................................................................................ 4

List of References ............................................................................................... 5

II. Literature Review 8

General Description of Muscle Blood Flow ....................................................... 10

Regulation of Blood Flow at Rest ................................................................. 11

Flow-mediated Dilation .................................................................................. 12

Regulation of Blood Flow at Exercise Onset ................................................. 13

Regulation of Blood Flow at Steady State Exercise ....................................... 17

Effect of Acidosis on Blood Flow ................................................................. 20

Effect of Carbonic Anhydrase on Vasodilation and Skeletal Muscle Blood Flow ................................................................................................................. 21

Reference List .................................................................................................. 24

III. Vascular Response to Acidosis during Dynamic Handgrip Exercise 35

Methods .......................................................................................................... 38
Subjects…………………………………………………………………………………38

General Experimental Protocol…………………………………………………38

Preliminary Testing……………………………………………………………..39

Constant-load Handgrip Exercise……………………………………………39

Measurement of Blood Velocity and Brachial Artery Diameter……………40

Measurement of muscle activation…………………………………………40

Analysis of Brachial Artery Diameter and Calculation of
Muscle Blood Flow, Shear Rate, Shear Stress,
Mean Arterial Pressure, and Vascular Conductance…………………41

Analysis of Blood Gases and Ions……………………………………………42

Statistical Analysis……………………………………………………………42

Results………………………………………………………………………………43

Subjects…………………………………………………………………………43

Effect of Acidosis on Blood Gases: H⁺, HCO₃⁻, and PCO₂……………….43

Effect of Acidosis on Muscle Activation…………………………………44

Effect of Acidosis on Brachial Artery Diameter and Mean
Blood Velocity…………………………………………………………………44

Effect of Acidosis on Muscle Blood Flow…………………………………45

Effect of Acidosis on Mean Arterial Pressure and Vascular
Conductance………………………………………………………………………45

Effect of Acidosis on Shear Rate and Shear Stress………………………46

Discussion……………………………………………………………………….53

Exercise and Acidosis: Effect on Brachial Artery Diameter,
Mean Arterial Pressure, and Mean Blood Velocity…………………...53

Exercise and Acidosis: Effect on Muscle Blood
Flow………………………………………………………………………………56
Exercise and Acidosis: Effect on Muscle Activation……………….…...58
Exercise and Acidosis: Effect on Shear Rate and Shear Stress……………………………………………………………………...……...59
Conclusion…………………………………………………………………………………………59
Reference List…………………………………………………………………………………………61

IV. The Effect of Acidosis on Endothelial Function 67

Methods………………………………………………………………………………………………69
Subjects……………………………………………………………………………………………69
General Experimental Protocol……………………………………………………………………69
Flow-Mediated Dilation…………………………………………………………………………………70
Measurement of Blood Velocity and Brachial Artery Diameter………71
Analysis of Brachial Artery Diameter and Calculation of % FMD, Shear Rate, and Shear Stress ……………………………………71
Analysis of Blood Gases and Ions……………………………………………………………………72
Statistical Analysis……………………………………………………………………………………73
Results…………………………………………………………………………………………………73
Subjects……………………………………………………………………………………………73
Effect of Acidosis on Blood Gases: H⁺, HCO₃⁻, and Pco₂……………….73
Effect of Acidosis on Brachial Artery Diameter……………………………………73
Effect of Acidosis on Mean Blood Velocity………………………………………74
Effect of Acidosis on Shear Rate and Shear Stress…………………………..74
Discussion……………………………………………………………………………………………..82
Acid-base Changes following Acz Administration…………………………82
Effect of Metabolic Acidosis on Brachial Artery Diameter……………………………………83
List of Tables

Table 3.1 – Subject characteristics..........................................................47

Table 3.2 – Cardiovascular and Blood Gas Data at Rest and during
Exercise for Acz and Control Conditions...........................................48

Table 4.1 – Subject Characteristics..........................................................76

Table 4.2 – Cardiovascular and Blood Gas Data at Rest and during
Flow-Mediated Dilation for Acz and Control Conditions......................77
List of Figures

Figure 3.1 – Diameter Response of a Single Individual for Moderate and Heavy Exercise during Acz and Control Conditions..........................49

Figure 3.2 – Group Diameter Response and Percent Change for Moderate and Heavy Exercise during Acz and Control Conditions..................................................50

Figure 3.3 – Group Mean Blood Velocities for Moderate and Heavy Exercise during Acz and Control Conditions...........................................51

Figure 3.4 – Group Muscle Blood Flow for Moderate and Heavy Exercise during Acz and Control Conditions..................................................52

Figure 4.1 – Flow-Mediated Dilation Response of a Single Individual during Acz and Control Conditions..................................................78

Figure 4.2 – Group Diameter Response and Percent Change following Post-Occlusive Reactive Hyperemia during Acz and Control Conditions..................................................79

Figure 4.3 – Group Mean Blood Velocity following Post-Occlusive Reactive Hyperemia during Acz and Control Conditions.........................80

Figure 4.4 – Group Shear Rate and Shear Stress Values following Post-Occlusive Reactive Hyperemia during Acz and Control Conditions..................................................81
Chapter I

Introduction

In today’s society, a sedentary lifestyle along with the increased consumption of foods high in fat content has lead to obesity reaching epidemic levels. Unfortunately, a significant relationship exists between the obese condition and the development of cardiovascular disease. While important medical advances have been made during the past two decades, cardiovascular disease is still prevalent and remains the leading cause of both morbidity and mortality in the United States today (Colleran et al., 2007).

Significant contributors to the prevention of cardiovascular disease are the cells lining the vasculature which are in direct contact with the blood – the vascular endothelium. Recently, changes in endothelial cell function have been gathering attention for their potential role in the development of vascular diseases such as atherosclerosis, vascular embolisms and aneurysms, and thrombosis (Bredt, 1999; Gimbrone, Jr. et al., 2000). In fact, endothelial dysfunction, or factors that negatively affect the health of endothelial cells, is considered by many to be the earliest marker of vascular disease (Mugge et al., 1991; Ohara et al., 1993). It is imperative that the regulation of endothelial cell function is well understood so that healthy endothelial function can be maintained or, endothelial dysfunction can be reversed and/or prevented.

Since blood behaves as a non-Newtonian fluid, meaning as flow increases the viscosity changes and therefore also affects shear rate, there is always some degree of
hemodynamic shear stress on the vascular endothelium, assuming some blood flow is present. Studies have shown that shear rate contributes significantly to maintaining vascular function including regulating vascular tone and diameter as well as preventing atherogenesis (Davies, 1995; Davies et al., 1995). Factors that cause changes in blood flow, and thus shear rate, are important because they can affect, as previously mentioned, the health of the vascular endothelium. Therefore, manipulation of those factors that alter blood flow to the muscle may provide investigators with an approach to further understand the mechanistic link between vascular function and prevention of diseases associated with the vasculature.

The results of previous studies have shown that an acidosis/alkalosis (or changes in pH) can affect vascular tone and therefore, blood flow to the limbs (Haddy & Scott, 1968; Hilton & Eichholtz, 1925; Ng et al., 1967; Daugherty, Jr. et al., 1967; Haddy & Scott, 1964). In fact, it has been recognized since the late 1800’s that an acidosis can alter muscle blood flow responses (Gaskell, 1877). Since this early report, other studies examining the effect of an acidosis on blood flow have demonstrated that a lower blood pH can cause vasodilation of the vascular smooth muscle thereby increasing blood flow (Haddy & Scott, 1964; Haddy & Scott, 1968). Therefore, an acidotic condition may potentially increase shear rate and thus, directly influence vascular endothelium function. While many studies have examined blood flow responses to an acidosis under resting conditions, there is a paucity of information regarding the effect of an induced acidosis on the regulation of blood flow to active muscles during exercise. One approach that has been used to induce a metabolic acidosis in humans is through the inhibition of the enzyme carbonic anhydrase (Scheuermann et al., 1998; Kowalchuk et al., 1994).
Carbonic anhydrase (CA) is the enzyme responsible for catalyzing the reversible hydration/dehydration reaction involving CO₂ and HCO₃⁻:

\[
\text{carbonic anhydrase} \quad H^+ + HCO_3^- \leftrightarrow H_2CO_3 \leftrightarrow H_2O + CO_2
\]

In addition to inducing a metabolic acidosis, studies inhibiting CA have shown impaired CO₂ elimination from the body, reduced exercise tolerance, an increase in minute ventilation as well as blunted lactate production (Kowalchuk et al., 1994; Scheuermann et al., 2000a).

Although several studies have been performed on the effects of CA inhibition on gas exchange kinetics (Scheuermann et al., 1998; Scheuermann et al., 1999; Jonk et al., 2007) and muscle metabolism (Scheuermann et al., 2000b; Scheuermann et al., 2000a) with exercise, very few studies have examined the skeletal muscle blood flow response to CA inhibition. This is quite surprising since muscle blood flow and gas exchange responses to exercise are closely coupled in a relatively linear manner (Paterson et al., 2005; Van Beekvelt et al., 2001). In addition, studies that have determined blood flow with CA inhibition have focused primarily on cerebral blood flow, (Wang et al., 1993), cerebral vascular reactivity, (Schwertfeger et al., 2006) or retinal circulation (Rassam et al., 1993). It has been demonstrated in isolated animal preparations that CA inhibition increases blood flow to major organs (Taki et al., 1998; Taki et al., 1999).

One study has confirmed that specific CA isoforms, CAI, CAII, and CAIII, exist in mammalian vascular smooth muscle (Berg et al., 2004) leading to the speculation that CA may play an important role in regulating vascular tone. It has also been demonstrated that CA inhibition can induce smooth muscle vasodilation through its action (whether directly or indirectly) on calcium activated potassium channels (Pickkers et al., 2001).
Despite the potential vasodilatory effects of CA, studies that have examined skeletal muscle blood flow adaptations to CA inhibition have found contrasting results (Jonk et al., 2007; Pickkers et al., 2001). Furthermore, it is also unclear as to whether CA inhibition can affect endothelial function in skeletal muscle since this has not yet been examined in the current literature.

**Purpose**

Since CA inhibition following chronic acetazolamide administration induces a metabolic acidosis which may influence resting and exercise vascular regulation, the objectives of this study are: i) to determine the effect of a metabolic acidosis (through CA inhibition) on muscle blood flow during dynamic handgrip exercise in both the moderate and heavy intensity domain and ii) to determine the effect of a metabolic acidosis (through CA inhibition) on endothelial function by examining the vascular response to flow mediated dilation following post-occlusive hyperemia.

**Hypothesis**

We hypothesize that a metabolic acidosis will lead to a higher hyperemic response during both moderate and heavy hand-grip exercises compared to control conditions consequent to induced vasodilation. Further, consistent with the first hypothesis that a metabolic acidosis will increase resting arterial diameter, endothelial function as assessed by flow-mediated dilation techniques will subsequently be reduced compared to control conditions.
Reference List


Chapter II

Literature Review

Cardiovascular disease (CVD) is the leading cause of mortality in the United States today (Colleran et al., 2007) and while there have been considerable medical advances over the last few years, the outlook for improvement does not appear particularly encouraging. As the average age of the population increases and the obesity epidemic in this country continues to increase at an alarming rate, the number of individuals diagnosed with some form of CVD is also expected to rise dramatically (Mokdad et al., 2000).

The cardiovascular system can be described as having three basic functions: first, to generate sufficient pressure to pump blood into the systemic, pulmonary, and coronary circulations; second, to deliver blood from the right and left ventricles to the capillaries of organs and tissues according to their requirements; third, to dampen the pulsations generated by ventricular contraction so that capillary blood flow is relatively continuous thus allowing efficient exchange of nutrients and by-products between the tissues and blood. Although the cardiovascular system may be subjected to considerable stresses including postural effects and the increased demands of exercise, the heart is able to generate sufficient pressure to maintain blood flow. The vasculature is an effective conduit which is able to redistribute blood flow to the regions that require it, and is very
efficient at dampening the pulsations in the capillary bed ensuring the efficient exchange of materials between tissue cells and blood.

The vascular endothelium, which is comprised of the single-cell layer of cells lining the walls of blood vessels, forms the interface between the blood and the smooth muscle of the vascular wall. In addition to its often ascribed protective anticoagulation and antithrombotic functions, the endothelium is now recognized as an important regulator of vascular tone. In addition, since the endothelium is located in an ideal position to respond to changes in hemodynamic forces as well as blood-borne compounds, the endothelium also plays a significant role in the regulation of tissue blood flow. Disruption of normal endothelial function (i.e. endothelial dysfunction) has been associated with increases in vascular tone or vasoconstriction, increased leukocyte adherence and platelet activation, and general vascular inflammation which leads to a prothrombotic condition. Thus, endothelial dysfunction may provide a mechanistic link between changes in hemodynamic conditions with the onset and progression of atherosclerosis (Gomez et al., 2007).

There is mounting evidence that regular physical activity is beneficial for maintaining or improving vascular health. Indeed, many of the risk factors associated with cardiovascular disease directly impact the vasculature resulting in altered structure of the endothelium suggesting that any cardioprotective effects achieved with physical activity are directly associated with endothelial function. It has been well established that an increase in physical activity increases blood flow to the active skeletal muscles and that the magnitude of this increase appears to be tightly coupled to the metabolic demands (Paterson et al., 2005). A very important mechanism responsible for the
increase in muscle blood flow is vasodilation occurring in both the local resistance 
vessels as well as in the conduit vessels. Since the endothelium plays a significant role in 
regulating vascular tone and contributes significantly to the vasodilatory response 
observed during increased levels of physical activity, gaining a further understanding of 
the role of the endothelium function (or dysfunction) is a necessary step in providing a 
framework from which exercise prescription (exercise modality) can be recommended 
that will promote the optimal stimulus for vascular adaptations.

General Description of Muscle Blood Flow

With the use of Doppler ultrasonography, changes in blood velocities can be 
obtained with almost instantaneous responses (Gill, 1985), and along with the 
measurement of vessel diameter, an accurate measurement of blood flow can be obtained 
with high resolution (Rådegran, 1997). Using this technique, it has been demonstrated 
that at the onset of rhythmic dynamic exercise in both the brachial and femoral arteries 
that there is a very rapid increase in muscle blood flow (Tschakovsky et al., 1995; 
Shoemaker et al., 1994; Walloe & Wesche, 1988; Rådegran & Saltin, 1998). The initial 
rapid increase in muscle blood flow occurs in approximately 10 s (phase I) and is 
followed by a second slower exponential increase towards a new steady state (phase II) 
value if the intensity of exercise is in the moderate domain (Shoemaker & Hughson, 
1999; Rådegran & Saltin, 1998) or may continue to increase if the exercise intensity is in 
the heavy domain (Rådegran & Saltin, 1998). Rådegran and Saltin (1998) demonstrated 
that at light workloads the time to reach half-peak blood flow value is less than 5 s and at 
heavier workloads the time increases but is still less than 10 s. This means that blood 
flow can increase from ~0.3 L/min at rest to upwards of ~10 L/min in less than 10 s.
The secondary increase in blood flow (i.e. phase II) that occurs around 10-20 s after exercise onset normally plateaus within 30-90 s depending on the exercise intensity and remains relatively constant even after prolonged periods of exercise (Savard et al., 1987). It is in general agreement that phase II is controlled by feedback regulation from metabolic factors but may also include feed-forward regulation from possible endothelial and neural factors (Hughson, 2003; Shoemaker & Hughson, 1999; Saltin et al., 1998a).

**Regulation of Blood Flow at Rest**

At rest, blood flow is believed to be regulated primarily by the vascular endothelium (Joannides et al., 1995; Bellien et al., 2005). The major vasoactive substances released from the endothelium are prostaglandins (PG; Koller & Kaley, 1990), endothelium-derived hyperpolarizing factor (EDHF; Coleman et al., 2004), and nitric oxide (NO; Rubanyi et al., 1986). Koller and Kaley (1990) showed that prostaglandins were released early following an increase in blood velocity. As well, Kilbom and Wennmalm (1976) and Carlsson and Wennmalm (1983) both showed that prostaglandins could have an effect on reactive hyperemia.

EDHF has been shown to aid in smooth muscle vasodilation and may be important in maintaining vascular tone (Coleman et al., 2004). It induces vasorelaxation by hyperpolarizing vascular smooth muscle through the opening of calcium-activated potassium (K$_{Ca}$) channels (Bellien et al., 2006). Studies examining EDHF have shown an increasing role in its ability to compensate for reduced NO production during flow mediated dilation (FMD; Bellien et al., 2006) and its role in exercise-induced skeletal muscle blood flow (Hillig et al., 2003).
Nitric oxide (NO) is released from endothelial cells through changes in shear stress (Joannides et al., 1995) and has been shown to increase blood flow at rest (Wilson & Kapoor, 1993) and during steady-state exercise (Dyke et al., 1995). It has also been demonstrated that NO synthesis blockade with N-monomethyl-L-arginine (L-NMMA) causes a reduction in blood flow at rest (Shoemaker et al., 1998). As well, NO plays an important role in flow-mediated dilation (FMD). It has been recently demonstrated that NO synthase inhibition with L-NMMA during sustained flow conditions (via hand heating) reduces FMD (Bellien et al., 2006).

Flow-mediated Dilation

Flow-mediated dilation (FMD) is a diagnostic tool used to non-invasively measure endothelial cell function and nitric oxide (NO) availability (Pohl et al., 1986). Through changes in shear stress, NO can be synthesized from L-arginine via NO synthase, thus allowing smooth muscle relaxation and increasing skeletal muscle blood flow. As well, FMD measured in the brachial artery has been shown to correlate with invasive measurements of endothelial function of the coronary artery (Anderson et al., 1995) and is now widely accepted as an accurate marker of endothelial function.

FMD is measured non-invasively by Doppler ultrasonography following a post-occlusive reactive hyperemia (PORH). A cuff is placed just distal to the elbow and is inflated to a suprasystolic pressure (~280 mmHg). Following a brief period (normally 5 minutes), the cuff pressure is released and a hyperemic response ensues, increasing blood flow to the arm. Through the changes in shear stress, NO is synthesized and vasodilation occurs to the artery of measurement.
There have been uncertainties to the reliability of FMD measurements, thus challenging its efficacy. In normal, healthy individuals, FMD is typically 7-10% above baseline values (Sorensen et al., 1995; Moens et al., 2005), while FMD measurements of individuals with CVD are typically blunted, reporting only 0-5% above baseline values (Clarkson et al., 1996). While values can be reported above or below the typical ranges, FMD is considered a reliable and reproducible measurement tool as determined through numerous studies (Shoemaker et al., 1996b; Corretti et al., 2002; Moens et al., 2005; Harris et al., 2007).

Regulation of Blood Flow at Exercise Onset

One confounding issue to the initial rapid increase in blood flow (phase I) is whether this is due to a feed-forward mechanism through inherent mechanical changes to the vasculature (i.e. the muscle pump) or by a feedback mechanism through vasoactive substances released at exercise onset to induce rapid vasodilation (for reviews see: Saltin et al., 1998a; Delp, 1999; Shoemaker & Hughson, 1999; Laughlin & Schrage, 1999; Hughson, 2003; Rowell, 2004; Tschakovsky & Sheriff, 2004).

There have been numerous attempts to determine if a feedback mechanism exists to induce rapid vasodilation in order to account for the rapid increase in blood flow during the initial seconds of muscular contraction (Shoemaker et al., 1997; Hughson, 2003; Tschakovsky & Sheriff, 2004; Delp, 1999). One important aspect in determining if a feedback mechanism exists at the onset of contraction is the time course required to induce vasodilation. It has been demonstrated that an increase in the hyperemic response can occur in less than 1 s (Delp, 1999) and elevated further within a few seconds (Sheriff & Hakeman, 2001) of muscular contraction. Therefore, vasodilation has to occur within
this time frame to account for the initial increases in blood flow after contraction. Studies supporting rapid dilation have used rat (Marshall & Tandon, 1984) and hamster (Mihok & Murrant, 2004; VanTeeffelen & Segal, 2006) models as well as stimulated (Hamann et al., 2004; Mohrman et al., 1973; Mohrman & Sparks, 1974; Naik et al., 1999) and voluntary (Hamann et al., 2003; Brock et al., 1998; Tschakovsky et al., 1996; Tschakovsky et al., 2004) contraction models. Direct evidence for rapid vasodilation indicates that upon twitch stimulation, dilation can occur within 1 s (Marshall & Tandon, 1984) but ranges have consistently been reported from 5 to 20 s (Cohen et al., 2000; Welsh & Segal, 1997; Wunsch et al., 2000). Using a whole muscle approach, however, points to a rapid vasodilation response. Tschakovsky et al. (2004) reported that with the arm positioned above heart level and performing exercise using isometric contractions (thereby minimizing the muscle pump effect), the hyperemic response (the first cardiac cycle after contraction) was proportional to contraction intensity. As well, Haman et al. (2004) using an isolated dog limb, infused potassium intra-arterially thereby eliminating smooth muscle membrane excitability and found an almost complete elimination of immediate hyperemia after brief muscular contraction.

A proposed feed-forward mechanism to explain the initial rapid increase in skeletal muscle blood flow deals with mechanical properties of vessels (veins) tethered to skeletal muscle. Upon muscle contraction, actin and myosin interact to shorten sarcomere length. As this occurs, compression is placed upon those vessels intertwined within the muscle. As the muscle relaxes, compression is relieved from the vessels and since the vessel walls are tethered to the muscle membranes, it is thought that the vessels are pulled open thereby decreasing transmural pressure. As a consequence, venous
pressure is reduced (Pollack & Wood, 1949) and a low pressure action occurs drawing in blood to the exercising muscle, an effect known as the muscle pump. This seems reasonable since it is unlikely that any metabolic vasodilator could evoke a response in such a short time frame.

Although the muscle pump seems reasonable, direct evidence for this action is lacking since to the required instrumentation needed to measure this effect would disrupt the internal structure of the vessels (Laughlin & Schrage, 1999). Studies disproving the muscle pump theory have ranged from altering work rate, and thus metabolic influence on rapid vasodilation (Shoemaker et al., 1998), to voluntary locomotion (Hamann et al., 2003). Shoemaker et al. (1998) used an experimental design comparing intensity vs. frequency effect on rapid hyperemia during dynamic hand-grip exercise. The results demonstrated that intensity determined the early increase in blood flow rather than frequency, thus giving evidence to local vasodilation as the primary mechanism for the early, rapid increase in blood flow. Haman et al. (2003) used voluntary locomotion along with adenosine administration and measured muscle blood flow. In this situation, adenosine maximally dilated the hindlimb, but an absence of a further increase in blood flow was observed at exercise onset. In discordance with the above evidence, many experiments have provided support to the muscle pump as the mechanistic reason for the increase in blood flow at exercise onset. For example, Almen and Nylander (1962) infused a contrast medium into superficial veins of the calf muscle during contractions and found that upon relaxation, the medium was “sucked” into the deep veins. They proposed that the rate of transfer was too fast to be accounted for by passive diffusion and that the muscle acted like a pump to transfer blood from the arteries to the veins. The
results of Sheriff and Van Bibber (1998) determined that muscular contraction and relaxation during rhythmic exercise had the ability to initiate muscle blood flow through mechanical influences on the vasculature. Other studies have used changes in contraction frequency, as the effect of the muscle pump is contingent upon frequency of contraction, and determined that changing contraction frequency can change blood flow (Folkow et al., 1970; Gotshall et al., 1996; Sheriff & Van Bibber, 1998; Sheriff & Hakeman, 2001).

A third theory for the initial increase in blood flow is through myogenic dilation, in such that compression of the arteries could cause dilation, much like the application of shear stress to the endothelium (Mohrman & Sparks, 1974). Subsequent studies have disproved this theory (Bacchus et al., 1981; Tschakovsky et al., 1996) in that mechanical compression of the vessels did not result in augmented dilation. However, more recent data have given new thought to this myogenic theory (Clifford et al., 2006; Kirby et al., 2007). The results of Clifford et al. (2006) revealed that mechanical compression could evoke rapid vasodilation in feed arteries of the soleus in a time course similar to the increase in hyperemia after muscular contraction. As well, the results of Kirby et al. (2007) demonstrated that a graded dilation response was observed with concomitant grades in cuff pressure. However it should be noted that mechanical compression can not fully account for changes in dilation caused by muscular contractions (Clifford & Jasperse, 2007).

Although there is much controversy surrounding the mechanistic cause of hyperemia at exercise onset, confounding direct evidence seems to point towards a rapid vasodilation albeit indirect evidence can support the muscle pump theory. Until such direct measures can be employed to support the muscle pump (which, as of now, may
negate the effect of the muscle pump itself), newer evidence is directed towards a rapid vasodilation, although the exact cause of the dilation remains speculative.

**Regulation of Blood Flow at Steady State Exercise**

The secondary phase of blood flow (i.e. 15 – 20 s post exercise onset) is believed to be regulated by feedback mechanisms, which include endothelial dependent factors as well as metabolic product accumulation from contracting muscles.

The vascular endothelium may play a role in regulating blood flow during steady state exercise through changes in blood velocities and shear rate (Rubanyi et al., 1986). It is known that increases in blood velocity can elicit production and release of PG (Koller & Kaley, 1990), and that PG can affect the reactive hyperemic response (Kilbom & Wennmalm, 1976). However, Shoemaker et al. (1996a) inhibited prostaglandin synthesis through ibuprofen administration and reported no changes in blood flow at any point during handgrip exercise, thus contradicting the contention that prostaglandins are significant in determining blood flow during exercise. Nitric oxide has been demonstrated to affect blood flow during steady state exercise (Dyke et al., 1995). While it has been demonstrated that NO synthesis blockade with N-monomethyl-L-arginine (L-NMMA) causes a reduction in blood flow at rest, a concomitant reduction in blood flow did not occur during voluntary or passive exercise (Saltin et al., 1998b). Also, by reducing blood flow through inhibition of muscarinic receptors by atropine, no further reduction was observed when L-NMMA was infused indicating a lack of contribution of NO on blood flow during exercise (Shoemaker et al., 1997). In relation, neurally released acetylcholine (ACh) has been shown to release NO through muscarinic receptors (Broten et al., 1992). As well, ACh can be released from endothelial cells and cause
release of NO, which has been shown to increase local vasodilation (Segal & Kurjiaka, 1995; Martin et al., 1996). However, Shoemaker et al. (1997) demonstrated that blockade of muscarinic receptors through atropine infusion had no effect on the magnitude of increase in blood flow suggesting that ACh was not significantly important in blood flow regulation during exercise.

Metabolic by-products act as a feedback mechanism to control blood flow and match it to metabolic demand and, therefore, are believed to be the primary control mechanism during the secondary phase of blood flow (Skinner, Jr. & Powell, Jr., 1967; Skinner, Jr. & Costin, 1970; Haddy & Scott, 1968; Proctor & Duling, 1982; Skinner, Jr. & Powell, Jr., 1967). As well, these metabolites are believed to compete with sympathetic activity in what is termed functional sympatholysis (Remensnyder et al., 1962). Metabolic products that act as vasoactive substances override the signal from the SNS that wants to constrict local vessels by either desensitizing the vascular smooth muscle to catecholamines (Remensnyder et al., 1962) or by inhibiting norepinephrine (Vanhoutte et al., 1981). The vasodilatory effects of the by-products, then, allow for a balance of flow between working and non-working tissues as it has been demonstrated that increases in sympathetic activity via noradrenaline spillover does not result in a reduction in blood flow to exercising muscle (Savard et al., 1989). Of these metabolic by-products, those worth noting are: lactate (La\(^-\)), sodium (Na\(^+\)), hydrogen (H\(^+\)), carbon dioxide (CO\(_2\)), inorganic phosphate (P\(_i\)), ammonium (NH\(_4^+\)), potassium (K\(^+\)), and adenosine, as each is believed to have some impact on skeletal muscle blood flow (Mellander & Johansson, 1968). Lactate and sodium have been shown to increase osmolality during exercise as vasodilation ensues via a change in transmembrane
potential due to the dehydration of smooth muscle (Lundvall, 1972). Hydrogen ion concentration increases during exercise as glycolytic rates increase and muscle pH decreases (Gebert & Friedman, 1973). It has been shown that local administration of acid produces the same or similar effects as altering P_{CO_2} (Hilton & Eichholtz, 1925; Ng et al., 1967) and that alkalosis increases resistance to blood flow in the limb (Daugherty, Jr. et al., 1967; Haddy & Scott, 1964). Therefore, it seems as though H^{+} can affect blood flow through either vasodilation (increased [H^{+}]) or vasoconstriction (decreased [H^{+}]; (Haddy & Scott, 1968). Carbon dioxide, inorganic phosphate (Pi), and ammonia (NH_{3}) have been proposed to act together as potent vasodilators (Skinner, Jr. & Powell, Jr., 1967; Skinner, Jr. & Costin, 1970). Potassium is believed to be important in blood flow regulation due to the effect it has on muscle membrane excitability and its ability to induce vasodilation at low concentrations (Juel, 2007). Perhaps more importantly, potassium has several channels and transport systems within the sarcoplasm and T-tubules including: Na^{+}/K^{+}-pump, K_{ATP} channel, inward rectified K^{+} channel (Kir2.1 channel), Na^{+}-K^{+}-2Cl^{-} cotransporter (NKCC transporter), and the calcium activated K^{+} channel (KCa_{2+} channel). This could imply that K^{+} regulation may be integral in regulating blood flow due to the fact that the opening of these channels will cause hyperpolarization and lead to a reduction in intracellular Ca^{2+} and thereby induce vasodilation (Juel et al., 2007). Another important regulator of blood flow is adenosine. It has been demonstrated that arterial infusion of adenosine can increase blood flow concomitant with dose rates (Radegran & Calbet, 2001). As well, adenosine infusion can increase blood flow to values that match peak exercise flow (Radegran & Calbet, 2001). As previously mentioned, CO_{2} has the ability to induce vasodilation. It has been
demonstrated that increases in CO₂ tension can result in an increase in forearm blood flow as well as a decrease in vascular resistance (Kontos et al., 1967; Kontos et al., 1968a; Kontos et al., 1968b). These authors (Kontos et al., 1967) partly attribute this response to a possible decrease in the intracellular pH of smooth muscle cells, however these authors also noted that the changes in forearm vasodilation was primarily due to an increase in blood and tissue PCO₂ rather than a change in pH (Kontos et al., 1968a).

Effect of Acidosis on Blood Flow

Upon initiation of exercise, a series of reactions occur that allow for muscle to contract. As a result, many different anions and cations are released, many of which contribute to the pH environment within the muscle and vasculature. As mentioned above, many of these products are believed to play some role in their ability to vasodilate arterial smooth muscle. Using the physicochemical approach, the acid-base status of any biological solution is determined by three factors: weak acids, the partial pressure of CO₂ (PCO₂), and the strong ion difference (Stewart, 1981; Stewart, 1983). Weak acids are acids that do not completely ionize or dissociate in water and therefore, do not favor the formation of H⁺ ions. Strong ions are compounds that completely dissociate in aqueous solution and SID is the difference between the sum of strong cations and strong anions (SID = $\sum$ strong cations - $\sum$ strong anions). PCO₂ changes as exercise progresses, as CO₂ is produced through the formation of acetyl-CoA from pyruvate during the initial reaction of the tricarboxylic acid cycle (often referred to as metabolic CO₂ production) and through the buffering of H⁺ during the production of lactic acid in glycolysis (often referred to as non-metabolic CO₂ production).
PCO₂ is believed to play a role in vasodilation and has been shown to cause vasodilation when tissue levels become elevated (Kontos et al., 1967; Daugherty, Jr. et al., 1967). This is consistent with the view that increases in PCO₂ lead to increases in [H⁺], in agreement with the physicochemical approach to acid/base regulation (Kowalchuk & Scheuermann, 1994; Kowalchuk & Scheuermann, 1995). Of particular interest to the present study is how the manipulation of CO₂ production within the internal and external cellular environment would alter blood flow.

Carbonic anhydrase (CA) is the enzyme responsible for catalyzing the reversible hydration/dehydration reaction involving the production of CO₂ and buffering of H⁺ with bicarbonate (HCO₃⁻) according to the reaction: H⁺ + HCO₃⁻ ↔ H₂CO₃ ↔ H₂O + CO₂. A number of studies that have inhibited CA activity during exercise have shown impaired CO₂ elimination from the body as well as blunted lactate appearance in the blood (Kowalchuk et al., 1992; Scheuermann et al., 2000b). Perhaps more important, sixteen known isoforms of CA have been identified to date (Geers & Gros, 2000; Supuran & Scozzafava, 2007), three of which have been isolated in vascular smooth muscle (CA I, CAII, and CAIII; (Berg et al., 2004). Therefore, it may be speculated that CA may play an important role in regulating vascular tone and therefore muscle blood flow through a direct role acting on vascular smooth muscle or via alterations in CO₂ production which would be dependent on changes in the acid-base status in the vascular space.

Effect of Carbonic Anhydrase on Vasodilation and Skeletal Muscle Blood Flow

As previously mentioned, carbonic anhydrase is the enzyme responsible for driving the reversible reaction between HCO₃⁻ and CO₂. CA contains a hydrophobic pocket which is required for substrate association. As well, CA contains H⁺ bond
networks with Zn-bound hydroxides (Zn-OH⁻) required for chemical reactivity. Here, the Zn atom hydrolyzes H₂O to a reactive Zn-OH⁻ complex where a histidine residue shuttles H⁺ from the metal ion center and transfers it to any buffer molecule nearby. CO₂ then combines with Zn-OH⁻ and the HCO₃⁻ formed will rapidly dissociate from Zn. The turnover rate (k_{cat}) of CA is very high, with a single molecule of CA being able to convert 400,000 to 600,000 molecules of CO₂ per second. In fact, CA kinetics are determined by the ability of the surrounding buffers to provide/remove H⁺ from the enzyme.

Acetazolamide (Acz), molecular formula: C₄H₆N₄O₃S₂, is a member of the sulfonamide class of drugs. Acz is a non-specific inhibitor of CA, which will inhibit (~98%) at 5-20 mg · kg⁻¹. Also known as N-(5-(aminosulfonyl)-1,3,4-thiadiazol-2-yl) – acetamide, Acz has a specific half life of 3 – 9 hours and is over 90% excreted within 24 hours. Oral administration of Acz has a peak [plasma] within 1 – 3 hours. It is a diuretic (causes polyuria) which subsequently increases excretion of H₂O, K⁺, Na⁺, and HCO₃⁻.

While many studies have been performed on the effects of CA inhibition (through acetazolamide, Acz, administration) on gas exchange kinetics (Scheuermann et al., 1998; Scheuermann et al., 1999; Jonk et al., 2007) and muscle metabolism (Scheuermann et al., 2000b; Scheuermann et al., 2000a) with exercise, very few studies have examined skeletal muscle blood flow responses to CA inhibition. This is surprising since blood flow and gas exchange responses to exercise follow very similar time courses and metabolic rate is dependent upon adequate blood flow (Paterson et al., 2005; Van Beekvelt et al., 2001). Of the studies that have examined blood flow during CA inhibition, most have focused primarily on changes in cerebral blood flow (Wang et al.,
1993), cerebral vascular reactivity (Schwertfeger et al., 2006), retinal circulation (Rassam et al., 1993), or the splanchnic bed (Taki et al., 1998; Taki et al., 1999).

It has been confirmed that specific CA isoforms I, II, and III exist in mammalian vascular smooth muscle (Berg et al., 2004). It has also been demonstrated that CA inhibition has the ability to induce smooth muscle vasodilation through its action (whether directly or indirectly) on calcium activated potassium (KCa2+) channels (Pickkers et al., 2001). This is an important observation since it has been demonstrated that KCa2+ channels regulate conduit artery vascular tone when NO synthesis is inhibited (Bellien et al., 2005). Despite the potential vasodilatory action of CA inhibition, studies that have examined skeletal muscle blood flow adaptations using Acz treatment have found contrasting conclusions (Jonk et al., 2007; Pickkers et al., 2001). As well, it also remains unclear as to whether CA inhibition affects the flow mediated dilation response to post-occlusive hyperemia, as this has not yet been examined in the literature.
Reference List


Chapter III

Vascular Response to Acidosis during Dynamic Hand-grip Exercise

It has long been known that a metabolic acidosis can affect blood flow (Gaskell, 1877). More specifically, it has been demonstrated that an acidosis can elicit a vasodilatory response and therefore, increase blood flow to the limbs (Haddy & Scott, 1968; Haddy & Scott, 1964; Pickkers et al., 2001b). While many theories exist, the exact mechanism(s) responsible for vasorelaxation during acidosis remain speculative (Haddy & Scott, 1968; Juel, 2007; Aalkjaer & Poston, 1996). Plausible mechanisms include an increase in carbon dioxide (CO$_2$) tension (Kontos et al., 1967; Kontos et al., 1968) and changes in potassium (K$^+$) concentrations (Juel, 2007; Cho et al., 2007).

While many studies have examined blood flow changes in response to an acidosis at rest, there have been few studies investigating acidosis and regulation of blood flow to active muscle during exercise. Studies have speculated that prior heavy exercise resulting in an overall metabolic acidosis increases blood flow and perfusion to the working muscles (Gerbino et al., 1996; MacDonald et al., 1997) however, subsequent studies have provided evidence arguing against this idea (Burnley et al., 2002; Paterson et al., 2005). As well, it has been demonstrated that a metabolic acidosis can result in vasoconstriction during static exercise (Sinoway et al., 1989). Thus, it is unclear if an
induced acidosis will affect blood flow to the exercising muscles differently than the normal exercise condition (i.e. normal homeostasis).

One way to induce a metabolic acidosis is through the inhibition of the enzyme carbonic anhydrase (Scheuermann et al., 1998; Kowalchuk et al., 1994). Carbonic anhydrase (CA) is responsible for catalyzing the reversible hydration/dehydration reaction involving CO₂ and bicarbonate (HCO₃⁻):

\[ \text{HCO}_3^- + \text{H}^+ \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}_2\text{O} + \text{CO}_2. \]

An overall systemic acidosis results from increased renal excretion of HCO₃⁻, sodium (Na⁺), K⁺, and H₂O, while ammonia (NH₃⁺), hydrogen (H⁺), and chlorine (Cl⁻) are retained. Studies that have examined the effects of CA inhibition during exercise have consistently shown a reduction in arterial PCO₂ (PaCO₂), elevated arterial H⁺ concentration, and blunted lactate production (Kowalchuk et al., 1994; Scheuermann et al., 2000a).

It has been shown that CA inhibition can induce smooth muscle vasodilation through activation of calcium activated potassium channels (Pickkers et al., 2001b). Coincidently, specific CA isoforms (CAI, CAII, and CAIII) exist in mammalian vascular smooth muscle (Berg et al., 2004) implying that CA may function directly or indirectly to regulate vascular tone and thus, muscle blood flow. Evidence for a vasodilatory effect has been demonstrated through numerous studies that have measured increases in blood flow during CA inhibition, although most of these studies focused primarily on cerebral blood flow (Wang et al., 1993), cerebral vascular reactivity (Schwertfeger et al., 2006), retinal circulation (Rassam et al., 1993), and blood flow to major organs (Taki et al., 1998; Taki et al., 1999) with only a few measuring blood flow to the limbs (Pickkers et al., 2001b; Jonk et al., 2007).
To our knowledge, only one study to date has examined the effect of CA inhibition on blood flow during exercise in humans (Jonk et al., 2007). Jonk et al. (2007) measured femoral blood flow using the thermodilution technique during knee extension exercise at intensities ranging from 30% to ≥90% peak oxygen uptake (\(\dot{V}O_{2}\) max) following chronic acetazolamide (Acz) administration. The results of this study showed no differences in femoral blood flow following CA inhibition during normoxia or hypoxia at any exercise intensity in spite of the induced metabolic acidosis. While blood flow was not different between groups, femoral artery diameter and/or mean red blood cell velocity was not measured but may have been considerably different between conditions. Thus, a higher exercising diameter and lower exercising mean blood velocity would equate to similar blood flow values between groups but would provide insight into vascular control under acidotic conditions. Additionally, while the thermodilution approach directly measures blood flow, this method precludes the measurement of arterial diameter and blood velocity changes at rest or during exercise. The application of Doppler ultrasonography, where changes in blood velocities can be obtained with almost instantaneous responses (Gill, 1985), and along with the measurement of vessel diameter, provides an accurate measurement of blood flow with high temporal resolution (Rådegran, 1997).

Therefore, it was the aim of this study to determine the effect of a metabolic acidosis (through CA inhibition) on muscle blood flow during dynamic handgrip exercise in both the moderate and heavy domains as measured by Doppler ultrasonography. Since CA inhibition following chronic Acz administration induces a metabolic acidosis which may influence blood flow to skeletal muscle, it was hypothesized that a metabolic
acidosis would induce a greater hyperemic response during both moderate and heavy hand-grip exercises compared to control conditions.

Methods

Subjects. Seven healthy male subjects participated in the research protocol in a repeated-measures design with an open-label method of treatment. All subjects were informed of potential risks, benefits, and exercise protocols prior to providing written consent. The experimental protocol was approved by the Institutional Review Board for Human Subjects Research and Review Committee at The University of Toledo and is in accordance with the guidelines set forth by the Declaration of Helsinki. Individuals with known cardiovascular disease, metabolic disease, limited physical arthritis, any abnormal cardiovascular response to exercise, on any medication at the time of screening, or smokers were excluded from the study. All testing for this study was performed in the Cardiopulmonary and Metabolism Research Laboratory located at The University of Toledo.

General Experimental Protocol. Each subject was asked to visit the laboratory on three separate occasions with no less than 4 d allowed between visits to the laboratory. During the first visit, the subject completed a medical questionnaire, provided written informed consent, underwent basic anthropometric measurements (height, weight, forearm volume, and forearm composition) and performed a preliminary handgrip exercise test for the determination of maximal handgrip force production. The subjects returned to the laboratory on two additional occasions: i) during control conditions (Con) and ii) following chronic acetazolamide administration (Acz; acetazolamide at 500 mg every 8 h po). The treatment order was randomized between and within subjects in order
to minimize any possible order effect. As well, since the side effects of Acz administration, although well-tolerated, are quite noticeable to the subject and therefore the administration of a placebo was not used since it would not sufficiently replicate the effects of Acz nor blind the subjects to the trial condition.

Preliminary Testing. A preliminary ramp exercise test (500 ml/min) using the forearm muscles was performed without pharmacologic intervention to determine peak exercise workload. Prior to the ramp test, each subject performed three maximal voluntary contractions (MVC) in which the subject contracted maximally for 5 s with no less than 2 min of rest between each maximal contraction using a standard hand-grip dynamometer to determine maximal force production of the forearm muscle group. The MVC reported was the average of the two highest values obtained.

Constant-load Handgrip Exercise. All exercise was performed in the supine position using a custom hand-grip ergometer involving a hand-grip device and a system of weights and pulleys. Intermittent dynamic handgrip exercise was performed at a contraction/relaxation ratio of 1:2 (i.e. 1s of contraction followed by 2s of relaxation). The workload determined for the constant-load exercise was set at 5% (moderate intensity) and 10% (heavy intensity) of the initial MVC. The step increase in work rate at 5% MVC began after one minute of rest and continued for 6 minutes followed by 10 minutes of recovery to allow the measured variables to return to baseline values (Hoelting et al., 2001). After the initial bout of exercise at 5% MVC and following 10 minutes of recovery, a subsequent bout of hand-grip exercise was performed at 10% MVC for 6 minutes.
Measurement of Blood Velocity and Brachial Artery Diameter. Measurements of blood velocities during exercise were measured using Doppler ultrasound velocimetry (500-V; Multigon Industries) of the brachial artery operating in pulsed mode. The Doppler transducer (frequency of 4 MHz and fixed-angle crystal of 45° relative to the skin) was placed flat on the medial aspect of the upper arm ~6-10 cm proximal to the antecubital fossa and parallel to the brachial artery. To ensure complete insonation, the gate was adjusted to the total width of the brachial artery. An audio demodulator was used to convert the frequency spectrum of the Doppler audio signal to an instantaneous mean blood velocity. The analog blood velocity signal was recorded at 100 Hz (PowerLab 16SP; ADInstruments, Grand Junction, CO) and stored on a computer for offline analysis. Mean brachial artery blood velocity was determined as the area under the curve for each cardiac cycle (i.e. each R-R wave interval) with blood velocity being expressed as minute values (i.e. cm/min) by multiplying the cardiac cycle-by-cycle values by the corresponding heart rate (Matlab Release 11.1, The Mathworks). Brachial artery diameters were measured at rest and after 4 minutes of steady-state exercise via echo-Doppler ultrasonography (GE Logiq 400CL, Milwaukee, WI) using a 7 MHz probe placed flat on the medial aspect of the upper arm ~6-10 cm proximal to the antecubital fossa and parallel to the brachial artery. Images were captured on computer using a frame-grabber interfaced directly to the ultrasound system at 10 Hz using commercially available software (Vascular Imager, Medical Imaging Applications, LCC, Coralville, IA).

Measurement of Muscle Activation. Muscle activation was measured non-invasively via electromyography (EMG). Single differential electrodes with a built-in biopotential
amplifier were placed over the main flexors of the forearm and the raw EMG signal was sampled at 2000 Hz, band-pass filtered at 20-400Hz, and notch filtered at 59-61 Hz using a commercially available data acquisition system (Bagnoli EMG system, Delsys, Inc.) and stored offline for later analysis. The raw EMG signal was analyzed in both the time and frequency domains to determine muscle activation and fiber type involvement during exercise (EMGworks, Delsys, Inc).

*Analysis of Brachial Artery Diameter and Calculation of Muscle Blood Flow, Shear Rate, Mean Arterial Pressure, and Vascular Conductance.* Brachial artery diameter was analyzed using an automated wall detection program using the stored images (Brachial Analyzer, Medical Imaging Applications, LCC, Coralville, IA) as previously described by Sonka et al. (2002). For detection of intimal wall far and near borders with each exercising file, a vascular region of interest (ROI) was determined by the operator. Quality tolerance parameters were used (i.e. gradient tolerance and shape tolerance) for determination of good quality vessel wall borders so that diameters could be detected frame-by-frame throughout the exercise duration. Calculation of blood flow was then determined using resting brachial artery velocities and diameter measurements as well as brachial artery velocities and diameter measurements during steady state exercise. Briefly, by multiplying the brachial artery diameter by the velocity of red blood cells moving through the brachial artery, muscle blood flow (MBF) can be calculated as follows: $\text{MBF} = (\text{Mean}_{\text{vel}})(\text{CSA})(60)$; where $\text{Mean}_{\text{vel}}$ is mean red blood cell velocities through the brachial artery (cm/s), CSA is the cross-sectional area of the brachial artery (as calculated by the equation $\text{CSA} = \pi r^2$, where $r$ is the radius of the brachial artery; Thompson *et al.*, 2007). Since an increase in MBF was expected as a function of
exercise intensity, the nitric oxide-dependent response to exercise was determined through the calculation of shear rate (SR): \[ SR = 4(\text{Mean}_{\text{vel}}/D); \] where \text{Mean}_{\text{vel}} is mean red blood cell velocities through the brachial artery (cm/s), and D is the brachial artery diameter (cm). Since shear stress (SS) is proportional to shear rate, SS was calculated as follows: \[ SS = (SR)(5.0 \times 10^{-2} \text{ dyn}\cdot\text{s}\cdot\text{cm}^{-2}); \] where SR is shear rate and 5.0 \times 10^{-2} dyn\cdot\text{s}\cdot\text{cm}^{-2} is the viscosity of blood through the brachial artery. Mean arterial pressure (MAP) was calculated: \[ \text{MAP} = \text{BP}_{\text{DIA}} + 1/3(\text{BP}_{\text{SYS}} - \text{BP}_{\text{DIA}}); \] where \text{BP}_{\text{DIA}} is diastolic blood pressure and \text{BP}_{\text{SYS}} is systolic blood pressure, as well as vascular conductance (VC): \[ \text{VC} = \text{MBF}/\text{MAP}; \] where MBF is muscle blood flow and MAP is mean arterial pressure.

**Analysis of Blood Gases and Ions.** Arterialized-venous blood was sampled from a superficial vein located on the back of the heated hand at specific times during rest, exercise, and recovery that was time aligned with blood flow measurements. Plasma was analyzed for concentrations of \( \text{HCO}_3^- \), \( \text{PCO}_2 \), and \( \text{pH} \) (\( \text{pH}^- = 10^{-\text{pH}} \)) using a commercially available blood-gas analyzer (pHox plus L, Stat Profile, Nova Biomedical; (Kowalchuk et al., 1988b; Kowalchuk et al., 1988a; Kowalchuk et al., 1992; Lindinger et al., 1990).

**Statistical Analysis.** A two-way analysis of variance with repeated measures was used to compare between conditions (Con versus Acz) and across time (rest versus exercise). A significant main effect or significant condition by time interaction were further analyzed using a Student-Neuman-Keuls post-hoc analysis. When time was not a factor, comparisons were made using a paired t-test to compare between conditions. Significance was set a priori at \( p \leq 0.05 \). All values are presented as the mean ± standard deviation unless stated otherwise.
Results

Subjects. Subject characteristics are displayed in Table 3.1. All subjects were healthy and recreationally active. Subjects weighed an average of 78.3 ± 17.3 kg and were 179.3 ± 8.1 cm tall. Mean forearm volume was 1237 ± 348.7 ml and average maximal forearm strength was 55 ± 6.4 kg. Based on this value, the average workload for moderate and heavy forearm exercises were 2.7 ± 0.3 kg and 5.5 ± 0.6 kg, respectively.

Effect of Acidosis on Blood Gases: H+, HCO3−, and PCO2. Chronic acetazolamide ingestion resulted in a significantly higher plasma [H+] for the Acz condition (Rest: 42 ± 3 nmol·L⁻¹; Exercise: 41 ± 4 nmol·L⁻¹) compared to Con (Rest: 35 ± 1 nmol·L⁻¹; Exercise: 31 ± 1 nmol·L⁻¹) prior to and during moderate exercise (p<0.05). Similarly, plasma [H+] was higher for the Acz (Rest: 40 ± 3 nmol·L⁻¹; Exercise: 39 ± 4 nmol·L⁻¹) compared to Con (34 ± 2 nmol·L⁻¹; Exercise: 35 ± 2 nmol·L⁻¹) at rest and during heavy exercise (p<0.05).

Plasma [HCO3−] was lower for Acz compared to Con at rest prior to both moderate (Acz:20.6 ± 1.11 mmol·L⁻¹; Con:29.0 ± 2.19 mmol·L⁻¹, p<0.05) and heavy intensity exercise (Acz:21.2 ± 1.68 mmol·L⁻¹; Con:30.6 ± 2.77 mmol·L⁻¹, p<0.05) as well as during moderate (Acz:22.7 ± 2.35 mmol·L⁻¹; Con:31.3 ± 0.90 mmol·L⁻¹, p<0.05) and heavy (Acz:22.06 ± 2.35 mmol·L⁻¹; Con:30.7 ± 1.40 mmol·L⁻¹, p<0.05) exercise.

Plasma PCO2 was also lower for the Acz condition compared to the Con condition at rest prior to moderate (Acz:34.0 ± 2.89 mmHg; Con:40.2 ± 2.95 mmHg, p<0.05) and heavy intensity exercise (Acz:33.1 ± 2.66 mmHg; Con:41.6 ± 5.08 mmHg, p<0.05) as
well as during moderate (Acz:36.2 ± 3.15 mmHg; Con:43.6 ± 2.24 mmHg, p<0.05) and heavy (Acz:33.8 ± 5.04 mmHg; Con:42.8 ± 3.80 mmHg, p<0.05) exercise.

**Effect of Acidosis on Muscle Activation.** Muscle activation (EMG activity) was not different during maximal voluntary contractions (MVC) between Con (2.1 x 10^{-4} ± 1.5 x 10^{-4} V) and Acz (2.5 x 10^{-4} ± 1.4 x 10^{-4} V) conditions. As well, muscle activation was similar during moderate (Con: 3.0 x 10^{-5} ± 1.6 x 10^{-5} V; Acz: 2.8 x 10^{-5} ± 1.4 x 10^{-5} V) and heavy (Con: 4.0 x 10^{-5} ± 2.5 x 10^{-5} V; Acz: 3.8 x 10^{-5} ± 1.8 x 10^{-5} V) exercise for both conditions.

**Effect of Acidosis on Brachial Artery Diameter and Mean Blood Velocity.** The change in arterial diameters at rest and during exercise in response to acetazolamide administration are presented in Table 3.2. Although not statistically significant, the resting arterial diameters prior to both moderate and heavy intensity exercise appeared to show a trend for larger arterial diameters for Acz (moderate: 4.93 ± 0.83 mm; heavy: 5.14 ± 0.75 mm) compared to Con conditions (moderate: 4.87 ± 0.58 mm; heavy: 4.87 ± 0.55) (Figure 3.1). No difference in brachial artery diameters were observed between Acz (5.05 ± 0.81 mm) and Con (4.95 ± 0.62 mm) conditions during moderate intensity exercise (Figure 3.2A).

While heavy intensity exercise did not result in a significant change in arterial diameter compared to resting values for Acz (rest: 5.14 ± 0.75 mm; exercise: 5.19 ± 0.54 mm), the increase was significantly higher (p<0.05) for Con conditions (rest: 4.87 ± 0.55 mm; exercise: 5.27 ± 0.60 mm) (Figure 3.2B). When expressed as a percent change from rest, the increase in arterial diameter during heavy exercise was significantly higher (p<0.05; Figure 3.2B) for Con (8.24 ± 4.06%) than Acz conditions (1.33 ± 4.19%).
Compared to resting values, both Acz and Con conditions resulted in higher mean blood velocities during both moderate (Acz: 5.06 ± 1.86 cm·s⁻¹ vs. 19.26 ± 3.54 cm·s⁻¹; Con: 5.34 ± 2.12 cm·s⁻¹ vs. 18.72 ± 4.70 cm·s⁻¹) and heavy (Acz: 5.78 ± 1.90 cm·s⁻¹ vs. 29.58 ± 3.92 cm·s⁻¹; Con: 5.54 ± 2.08 cm·s⁻¹ vs. 31.16 ± 8.36 cm·s⁻¹) exercise (p<0.05). However, when Acz and Con conditions were compared, no differences were detected between Acz and Con conditions at rest or during exercise for both moderate and heavy exercise intensities (Figures 3.3A and 3.3B). It is interesting to note that heavy intensity exercise resulted in a slightly lower mean blood velocity for the Acz condition (29.58 ± 3.92 cm·s⁻¹) compared to the Con condition (31.16 ± 8.36 cm·s⁻¹) even though arterial diameters were slightly lower (Acz: 5.19 ± 0.54 mm; Con: 5.27 ± 0.60 mm).

**Effect of Acidosis on Muscle Blood Flow.** All forearm muscle blood flow (MBF) values are reported in Table 3.2. When compared to resting values, Acz and Con conditions resulted in higher MBF during both moderate (Acz: 63.50 ± 41.69 ml·min⁻¹ vs. 243.34 ± 123.88 ml·min⁻¹; Con: 62.37 ± 33.42 ml·min⁻¹ vs. 222.52 ± 98.90 ml·min⁻¹) and heavy (Acz: 75.15 ± 37.11 ml·min⁻¹ vs. 374.58 ± 71.18 ml·min⁻¹; Con: 63.18 ± 28.80 ml·min⁻¹ vs. 420.84 ± 176.48 ml·min⁻¹) exercise (p<0.05). No differences were detected between Acz and Con conditions at rest or during exercise for both moderate and heavy exercise intensities (Figures 3.4A and 3.4B). While no significant differences were detected between conditions, it is important to note that MBF tended (p=0.14) to be lower during exercise following Acz (374.58 ± 71.18 ml·min⁻¹) compared to Con conditions (420.84 ± 176.48 ml·min⁻¹) during heavy exercise.

**Effect of Acidosis on Mean Arterial Pressure and Vascular Conductance.** Mean arterial pressure (MAP) was not different between conditions during moderate exercise.
(Acz 101 ± 14 mmHg vs. Con 99 ± 15 mmHg). However, during heavy exercise, Acz administration resulted in a significantly higher MAP compared to Con conditions (Acz 115 ± 14 mmHg vs. Con 108 ± 14 mmHg, p<0.05). Vascular conductance was not different between conditions during moderate or heavy exercise (Table 3.2), consistent with the findings for MBF.

**Effect of Acidosis on Shear Rate and Shear Stress.** Both shear rate and shear stress were higher during moderate exercise for Acz (SR:153.62 ± 26.64 s⁻¹; SS:6.14 ± 1.07 dyn·cm⁻²) and Con (SR:152.36 ± 39.06 s⁻¹; SS:6.09 ± 1.56 dyn·cm⁻²) conditions compared to rest values (Acz SR:40.67 ± 12.20 s⁻¹; Acz SS:1.63 ± 0.43 dyn·cm⁻²; Con SR:43.79 ± 16.25 s⁻¹; Con SS:1.75 ± 0.65 dyn·cm⁻²); no difference in shear stress or shear rate was observed between Acz and Con conditions. Similar to moderate intensity exercise, shear rate and shear stress were higher during heavy exercise for Acz (SR:230.92 ± 41.25 s⁻¹; SS:9.24 ± 1.65 dyn·cm⁻²) and control (SR:236.86 ± 57.94 s⁻¹; SS:9.47 ± 2.32 dyn·cm⁻²) conditions compared to rest (Acz SR:45.13 ± 14.13 s⁻¹; Acz SS:1.81 ± 0.57 dyn·cm⁻²; Con SR:45.69 ± 16.52 s⁻¹; Con SS:1.83 ± 0.66 dyn·cm⁻²; p<0.05), while no differences were observed between conditions. Indeed, both shear rate and shear stress values were well matched during each of exercise intensities examined between Acz and control conditions (Table 3.2).
<table>
<thead>
<tr>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Age (yrs)</th>
<th>Forearm volume (mL)</th>
<th>MVC (kg)</th>
<th>5% MVC (kg)</th>
<th>10% MVC (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>179.3</td>
<td>78.3</td>
<td>32</td>
<td>1237</td>
<td>55.0</td>
<td>2.7</td>
</tr>
<tr>
<td>SD</td>
<td>8.1</td>
<td>17.3</td>
<td>5</td>
<td>349</td>
<td>6.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>

MVC; maximal voluntary contraction
### Table 3.2 – Rest and exercising values for cardiovascular and blood-gas data during control and acetazolamide conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Acetazolamide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Moderate Rest</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBV (cm·s⁻¹)</td>
<td>5.3 ± 2.1</td>
<td>5.1 ± 1.9</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>4.9 ± 0.6</td>
<td>4.9 ± 0.8</td>
</tr>
<tr>
<td>MBF (ml·min⁻¹)</td>
<td>62.4 ± 33.4</td>
<td>63.5 ± 41.7</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>43.8 ± 16.3</td>
<td>40.7 ± 12.2</td>
</tr>
<tr>
<td>Shear stress (dyn·cm⁻²)</td>
<td>1.8 ± 0.7</td>
<td>1.6 ± 0.5</td>
</tr>
<tr>
<td>[H⁺] (nmol·L⁻¹)</td>
<td>35 ± 2</td>
<td>42 ± 3†</td>
</tr>
<tr>
<td>[HCO₃⁻] (mmol·L⁻¹)</td>
<td>29.0 ± 2.2</td>
<td>20.6 ± 1.1†</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>40.2 ± 3.0</td>
<td>34.0 ± 2.9†</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>44.0 ± 2.6</td>
<td>43.5 ± 2.4</td>
</tr>
<tr>
<td><strong>Moderate Exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBV (cm·s⁻¹)</td>
<td>18.7 ± 4.7*</td>
<td>19.3 ± 3.5*</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>4.9 ± 0.6</td>
<td>5.1 ± 0.8</td>
</tr>
<tr>
<td>%change from rest</td>
<td>1.7 ± 2.3</td>
<td>2.8 ± 6.1</td>
</tr>
<tr>
<td>MBF (ml·min⁻¹)</td>
<td>222.5 ± 98.9*</td>
<td>243.3 ± 123.9*</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>152.4 ± 39.1*</td>
<td>153.6 ± 26.6*</td>
</tr>
<tr>
<td>Shear stress (dyn·cm⁻²)</td>
<td>6.1 ± 1.6*</td>
<td>6.1 ± 1.1*</td>
</tr>
<tr>
<td>[H⁺] (nmol·L⁻¹)</td>
<td>35 ± 2</td>
<td>41 ± 4†</td>
</tr>
<tr>
<td>[HCO₃⁻] (mmol·L⁻¹)</td>
<td>31.3 ± 0.9</td>
<td>22.7 ± 2.4†</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>43.6 ± 2.2</td>
<td>36.2 ± 3.2†</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>43.8 ± 2.5</td>
<td>44.8 ± 1.6</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>99 ± 15</td>
<td>101 ± 14</td>
</tr>
<tr>
<td>VC (ml·min⁻¹·mmHg⁻¹)</td>
<td>2.3 ± 1.2</td>
<td>2.5 ± 1.6</td>
</tr>
<tr>
<td><strong>Heavy Rest</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBV (cm·s⁻¹)</td>
<td>5.5 ± 2.1</td>
<td>5.8 ± 1.9</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>4.9 ± 0.6</td>
<td>5.1 ± 0.8†</td>
</tr>
<tr>
<td>MBF (ml·min⁻¹)</td>
<td>63.2 ± 28.8</td>
<td>75.2 ± 37.1</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>45.7 ± 16.5</td>
<td>45.1 ± 14.1</td>
</tr>
<tr>
<td>Shear stress (dyn·cm⁻²)</td>
<td>1.8 ± 0.7</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>[H⁺] (nmol·L⁻¹)</td>
<td>34 ± 2</td>
<td>40 ± 3†</td>
</tr>
<tr>
<td>[HCO₃⁻] (mmol·L⁻¹)</td>
<td>30.6 ± 2.8</td>
<td>21.2 ± 1.7†</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>41.6 ± 5.1</td>
<td>33.1 ± 2.7†</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>42.6 ± 2.7</td>
<td>44.7 ± 2.0</td>
</tr>
<tr>
<td><strong>Heavy Exercise</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBV (cm·s⁻¹)</td>
<td>31.2 ± 8.4*</td>
<td>29.6 ± 3.9*</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>5.3 ± 0.6*</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>%change from rest</td>
<td>8.2 ± 4.1</td>
<td>1.0 ± 4.2†</td>
</tr>
<tr>
<td>MBF (ml·min⁻¹)</td>
<td>420.8 ± 176.5*</td>
<td>374.6 ± 71.2*</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>236.9 ± 57.9*</td>
<td>230.9 ± 41.3*</td>
</tr>
<tr>
<td>Shear stress (dyn·cm⁻²)</td>
<td>9.5 ± 2.3*</td>
<td>9.2 ± 1.7*</td>
</tr>
<tr>
<td>[H⁺] (nmol·L⁻¹)</td>
<td>35 ± 2</td>
<td>40 ± 4†</td>
</tr>
<tr>
<td>[HCO₃⁻] (mmol·L⁻¹)</td>
<td>30.7 ± 1.4</td>
<td>21.9 ± 2.4†</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>42.8 ± 3.8</td>
<td>33.8 ± 5.0†</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>43.4 ± 2.1</td>
<td>44.7 ± 1.9</td>
</tr>
<tr>
<td>MAP (mmHg)</td>
<td>108 ± 14</td>
<td>115 ± 14†</td>
</tr>
<tr>
<td>VC (ml·min⁻¹·mmHg⁻¹)</td>
<td>4.0 ± 1.8</td>
<td>3.3 ± 0.9</td>
</tr>
</tbody>
</table>

All values are reported as means ± SD. MBV, muscle blood velocity; MBF, muscle blood flow; [H⁺], hydrogen ion concentration; [HCO₃⁻], bicarbonate ion concentration; PCO₂, partial pressure of carbon dioxide. *Significantly different from rest (p<0.05). †Significantly different from control (p<0.05).
Figure 3.1 – Diameter response of a single individual for moderate and heavy rest and exercise bouts during Acz (red, dashed) and Con (blue, solid) conditions.
Figure 3.2 – Diameter comparisons for moderate (A) and heavy (B) rest and exercise bouts during Acz (open) and Con (closed) conditions. Bottom graphs represent percent change from rest between conditions for both moderate and heavy exercise. *Significantly different from rest (p<0.05). †Significantly different from Con (p<0.05).
Figure 3.3 – Mean blood velocity comparisons for moderate (A) and heavy (B) rest and exercise bouts during Acz (open) and Con (closed) conditions. *Significantly different from rest (p<0.05).
Figure 3.4 – Muscle blood flow comparisons for moderate (A) and heavy (B) rest and exercise bouts during Acz (open) and Con (closed) conditions. *Significantly different from rest (p<0.05).
Discussion

To our knowledge, this is the first study that has continuously measured brachial artery diameter and mean blood velocity during exercise in response to a metabolic acidosis following chronic acetazolamide administration. As well, this is the only study to measure hemodynamic shear stresses during exercise in response to a chronic metabolic acidosis. To date, only one other study has attempted to measure muscle blood flow during exercise following chronic acetazolamide administration (Jonk et al., 2007). This previous study examined femoral artery blood flow during knee extension exercise using the thermodilution technique and thus, the effects of the metabolic acidosis on changes in arterial diameter (i.e. the regulation of vascular tone) could not be determined using that approach. In contrast to our hypothesis, the main finding of the present study indicates that although muscle blood flow was not different between Acz and Con conditions, the ability of the brachial artery to vasodilate during exercise appeared to be attenuated following Acz administration. This may be due to the tendency for the resting arterial diameter to be greater in the Acz trial however, since the difference in arterial diameters were not different between conditions, other factors associated with either the metabolic acidosis and/or carbonic anhydrase inhibition may have contributed to the small increase in arterial diameter during heavy exercise in the Acz trial.

Exercise and Acidosis: Effect on Brachial Artery Diameter, Mean Arterial Pressure, and Mean Blood Velocity. Similar to previous studies, chronic acetazolamide ingestion resulted in a significantly higher plasma [H+] while [HCO3⁻] and PCO₂ were lower compared to control conditions prior to the onset of moderate and heavy intensity exercise (Kowalchuk et al., 1994; Scheuermann et al., 1999). Previous studies have
demonstrated that an increase in blood [H+] can induce vasodilation under resting conditions (Haddy & Scott, 1968; Juel et al., 2007). Furthermore, increases in tissue CO₂ tension can also induce vasodilation under resting conditions (Kontos et al., 1967). Although our results did not achieve statistical significance, resting arterial diameters were higher prior to the onset of both moderate and heavy exercise following Acz administration. Chronic Acz administration could lead to changes in smooth muscle tone during exercise through a systemic reduction in K⁺ (hypokalemia) thereby altering contractile function. Potassium is believed to be important in blood flow regulation due to the effect it has on muscle membrane excitability and its ability to induce vasodilation at low concentrations (Bellien et al., 2005; Juel et al., 2007). Additionally, it has also been demonstrated that Acz can induce vasodilation under resting conditions due to its effects on calcium activated potassium (KCa₂⁺) channels (Pickkers et al., 2001a).

The surprising finding in this study was the attenuated response in arterial diameter during heavy exercise with Acz administration. It is readily apparent that when the vasodilatory response was expressed as a percent change from resting values, the increase in arterial diameter from rest to heavy exercise was significantly attenuated during the metabolic acidosis. The extent that the higher initial arterial diameter at rest in the Acz trial may have contributed to the attenuated response cannot be excluded. Indeed, MBF was similar between the exercise conditions during both moderate and heavy intensity exercise and therefore, it appears as though muscle perfusion remained well matched to the requirements of the exercising muscle. However, since the difference in arterial diameters at rest was not significant, the possibility that other alternative mechanisms may have contributed the blunted response cannot be excluded.
A plausible explanation for the discrepancy between conditions could lie in nitric oxide (NO) production and regulation during exercise, more so as shear stimulus increases (Kingwell, 2000). It would seem reasonable for NO to contribute to vascular regulation during exercise although it has been demonstrated that NO inhibition does not significantly affect muscle blood flow at exercise onset but does contribute during recovery (Shoemaker et al., 1997; Rådegran & Saltin, 1999). The results of the previous studies can not preclude NO as a possible mechanism in regulating vascular function during exercise as other studies have implicated the importance of NO in regulating exercising hyperemia in the forearm (Dyke et al., 1995; Gilligan et al., 1994). Also, recent evidence points towards neuronal NO synthase (nNOS) as a regulator in basal and possibly exercising vascular tone (Seddon et al., 2008). Feed arteries are influenced to a greater extent by the sympathetic nervous system whereas resistance vessels are primarily influenced by local autoregulation. As sympathetic outflow increases (as during exercise), a greater contribution of nNOS on NO production would be expected. As well, nNOS plays a role in vascular regulation during mental stress (Seddon et al., 2008). In the present study, subjects anecdotally reported exercise to be considerably harder following Acz administration compared to Con conditions which may have lead to increased nNOS release. Since specific CA isoforms (CAI, CAII, and CAIII) exist in vascular smooth muscle (Berg et al., 2004), the existing metabolic acidosis brought about through carbonic anhydrase inhibition could have a direct action on the compliance of the vascular wall through NO production.

Interestingly, differences in mean arterial pressure (MAP) were observed during heavy intensity exercise as the Acz condition values were higher compared to Con trials.
Within the same vascular bed, differences in endothelial and smooth muscle function have been demonstrated (Hill et al., 2001). For example, it has been reported that changes in blood pressure can influence the sensitivity of these cells (Otsuka et al., 1988) thereby preventing maximal vasodilation (Gardiner et al., 1994). Our results did show the Acz condition as having a reduced arterial vasodilation during heavy exercise when compared to control, which would be in agreement with the aforementioned studies.

Another possible mechanism that could prevent further dilation of the brachial artery is K^+ and associated pumps (Juel, 2007; Juel et al., 2007). It has been demonstrated that Acz can directly affect calcium-activated potassium channels at rest (Pickkers et al., 2001a), thus evoking vasodilation. However, if K^+ is reduced (as one of the side-effects of Acz is hypokalemia) during exercise, this could possibly prevent hyperpolarization of smooth muscle to a further extent and thus a further dilation of the artery.

No significant differences were present between conditions in mean blood velocities at rest or during moderate or heavy exercise. However, heavy intensity exercise resulted in a slightly lower mean blood velocity for the Acz condition compared to control (see Table 3.2) even though arterial diameters were slightly lower, which would contribute to the lower trend in blood flow observed during heavy exercise between the two conditions, as will be discussed.

Exercise and Acidosis: Effect on Muscle Blood Flow. While resting muscle blood flow values for the Acz condition showed a higher trend compared to control (Table 2, Figure 4), there was no significant difference between the two conditions. The results from Pickkers et al. (2001a) showed an increase in resting forearm blood flow following
Acz administration compared to control. While our results showed no differences between conditions, the discrepancy between results might be due to the differences in Acz administration. While Pickkers et al. (2001a) directly infused Acz into the brachial artery, we administered Acz orally over several days. Acz might have a more significant effect if localized to a specific area rather than systemically, especially due to its non-specific inhibition of carbonic anhydrase isoforms (Maren et al., 1954; Supuran et al., 2003).

Forearm blood flow was not different between conditions during moderate exercise, in agreement with the results of Jonk et al. (2007). During heavy exercise, a 46 ml·min⁻¹ difference in MBF between Acz and control conditions were observed (p=0.14). Although this was not statistically significant, the difference in magnitude of blood flow between conditions was close to resting values of the forearm (Table 3.2). The results of Jonk et al. (2007) reported no differences in muscle blood flow during heavy exercise. The difference between our study and that of Jonk et al. (2007) are several fold. Our study measured forearm blood flow while Jonk et al. (2007) measured femoral blood flow. There are vast differences between the conduit arteries involved in each study (Newcomer et al., 2004). As Newcomer et al. (2004) have shown, differences in limb responses to endothelium dependent and independent vasodilators exist, as the leg response was blunted compared to the arm. Since the femoral artery is such a large feed artery, a large change in blood flow would be necessary to show differences between conditions, possibly much larger than physiological conditions can present. By using a smaller artery, a smaller change in blood flow could possibly be detected as different between conditions. Also, the quadriceps muscle group is a large muscle group.
Therefore, if any differences in muscle metabolism existed as previously reported (Scheuermann et al., 2000b), a large difference could presumably be needed in order to affect blood flow to the exercising muscles, possibly much larger than a smaller muscle mass.

It is well understood that many factors can influence blood flow during exercise (Saltin et al., 1998). One reason for the lower trend in blood flow during heavy exercise in this study could possibly be due to lactate within the red blood cell. If lactate efflux is affected during Acz administration (Scheuermann et al., 2000b), a reduction in adenosine (via ATP) release from red blood cells could occur as it has been reported that increased levels of intracellular lactate reduces ATP release (Rozier et al., 2007). The ensuing response would be a reduction in blood flow. Another possible metabolic factor that could influence blood flow during heavy exercise is K⁺ (Juel et al., 2007). Due to the inherent number of K⁺ pumps within the body, the implications for K⁺ to play a significant role during exercise has been discussed. Potassium has several channels and transport systems within the sarcoplasm and T-tubules including: Na⁺/K⁺-pump, Kₐ₅, channel, inward rectified K⁺ channel (Kir2.1 channel), Na⁺-K⁺-2Cl⁻ cotransporter (NKCC transporter), and the calcium activated K⁺ channel (KCa²⁺ channel). This could imply that K⁺ regulation may be integral in regulating blood flow due to the fact that the opening of these channels will cause hyperpolarization and lead to a reduction in intracellular Ca²⁺ and thereby induce vasodilation (Juel, 2007).

**Exercise and Acidosis: Effect on Muscle Activation.** Forearm flexor muscle activation was similar between Con and Acz conditions during both moderate and heavy exercise. Thus the ability to maintain force production was similar between conditions,
similar to previous studies (Scheid & Siffert, 1985; Kowalchuk et al., 1994; Kowalchuk et al., 2000). Also, EMG activity was similar during MVC between conditions. Differences in EMG activity during MVC could cause differences in relative workload between conditions during exercise which could explain the differences in MAP between conditions, however this was not the case in the present study. It is important to note that individuals reported heavy exercise as being “harder” following chronic Acz administration compared to Con although perceived exertion was not measured in the present study. This could have implications on increased sympathetic activity and, as such, increased MAP observed during heavy exercise for the Acz compared to the Con condition. As well, increased sympathetic activity would have an impact on neuronal NOS activation and subsequent NO production and release.

*Exercise and Acidosis: Effect on Shear Rate and Shear Stress.* The present study found no differences in either shear rate or shear stress between experimental conditions during moderate or heavy intensity exercise. In fact, both shear rate and shear stress values were well matched during associated exercise intensities between Acz and control conditions (Table 3.2). Assuming plasma viscosity was maintained during exercise (as indicated by similar hematocrit values between conditions), the associated viscosity of 5.0 mPa·s (Reneman et al., 2006) was used to calculate shear stress, thus accounting for possible differences in plasma volume between conditions with Acz treatment.

*Conclusion.* In conclusion, the results of the present study indicate a blunted response in vasodilation of the Acz condition during heavy exercise compared to control. As well, the Acz condition resulted in a significantly higher MAP compared to control, which could prevent a maximal dilatory response during exercise. While no differences
existed between conditions, resting arterial diameters tended to be higher for the Acz condition compared to control which could also explain the lack of a significant effect between rest and heavy intensity exercise. Further studies are warranted to elucidate the specific mechanisms and to determine the extent that a prior condition of metabolic acidosis affects vascular regulation during exercise versus the role that chronic inhibition of carbonic anhydrase may play in regulating vascular tone.
Reference List


Chapter IV

The Effect of a Metabolic Acidosis on Endothelial Function

As normalcy in the United States progresses towards obesity, vascular-related
diseases with an etiology related to blood flow are becoming more prevalent despite
numerous advances in medical technology (Bredt, 1999; Gimbrone, Jr. et al., 2000). This
growing epidemic is quite alarming since cardiovascular disease is the leading cause of
morbidity and mortality in the United States today (Colleran et al., 2007). In addition,
the number of individuals with a vascular-related disease has been increasing
exponentially and this trend is expected to continue in the years to come (Mokdad et al.,
2000). Many investigators concerned with the rising incidence of vascular related
diseases consider one of the earliest markers of these diseases to be the dysfunction of the
endothelial cells that line the vasculature which are in direct contact with blood (Ohara et
al., 1993; Mugge et al., 1991).

The vascular endothelium is extremely important in the regulation of blood flow
(Koller & Kaley, 1991; Kingwell, 2000) as well as preventing thrombus formation by
inhibiting monocyte adhesion and platelet aggregation to the vessel surface (Bredt, 1999).
It is known that the hemodynamic force placed upon the vascular endothelium (i.e. shear
stress) plays a large role in maintaining vascular function including regulating vascular
tone and diameter as well as preventing atherogenesis (Davies, 1995; Davies et al., 1995;
Corson et al., 1996). Due to the pulsatility of blood flow and its non-Newtonian
properties, some degree of shear stress is always applied to the endothelium, unless blood flow is not present. Conditions that induce changes in blood flow are an important consideration since they can affect the “health” of the vascular endothelium through changes in shear stress. Therefore, experimental manipulations that can affect blood flow may provide further mechanistic links between vascular function and prevention of vascular diseases.

One way to induce a change in blood flow is through changes in acid-base status of the blood. It has long been known that an acidosis condition (or drop in pH) can alter blood flow appreciably (Gaskell, 1877). More specifically, it has been demonstrated that an acidosis can elicit a vasodilatory response and thus, increase blood flow (Haddy & Scott, 1968; Haddy & Scott, 1964). Therefore, a metabolic acidosis could create a cascade effect through its ability to change blood flow thereby affecting shear rate and thus, possibly the function of the vascular endothelium. In past research, a metabolic acidosis has been induced through the inhibition of the enzyme carbonic anhydrase (CA; Scheuermann et al., 1998; Kowalchuk et al., 1994), which is responsible for catalyzing the reversible hydration/dehydration reaction involving carbon dioxide (CO₂) and bicarbonate (HCO₃⁻): $\text{HCO}_3^- + \text{H}^+ \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}_2\text{O} + \text{CO}_2$.

Studies that have measured blood flow during CA inhibition have focused primarily on cerebral blood flow (Wang et al., 1993), cerebral vascular reactivity (Schwertfeger et al., 2006), and retinal circulation (Rassam et al., 1993). To date, no studies have looked at the effects of an acidosis (through CA inhibition) on endothelial function in the peripheral vasculature. Interestingly, specific CA isoforms (CAI, CAII,
and CAIII) exist in mammalian vascular smooth muscle (Berg et al., 2004) and therefore, could potentially have a direct effect on regulating vascular tone.

Since CA inhibition, through chronic acetazolamide (Acz) administration, induces a metabolic acidosis which may influence muscle blood flow, the objective of this study is to examine the effect of a metabolic acidosis (through CA inhibition) on endothelial function using measurements of flow-mediated dilation following a post-occlusive reactive hyperemia. We hypothesize that a prior condition of metabolic acidosis will induce a greater vasodilatory response at rest thereby reducing the magnitude of change in arterial diameter from rest to maximal dilation compared to a control condition.

Methods

Subjects. Seven healthy male subjects participated in the research protocol in a repeated-measures design with an open-label method of treatment. All subjects were informed of potential risks, benefits, and exercise protocols prior to providing written consent. The experimental protocol was approved by the Institutional Review Board for Human Subjects Research and Review Committee at The University of Toledo and is in accordance with the guidelines set forth by the Declaration of Helsinki. Individuals with known cardiovascular disease, metabolic disease, limited physical arthritis, any abnormal cardiovascular response to exercise, on any medication at the time of screening, or smokers were excluded from the study. All testing for this study was performed in the Cardiopulmonary and Metabolism Research Laboratory (CMRL) located on the main campus of The University of Toledo.

General Experimental Protocol. Each subject was asked to visit the laboratory on three separate occasions with no less than 4 d allowed between visits to the laboratory.
During the first visit, the subject completed a medical questionnaire, provided written informed consent and underwent basic anthropometric measurements (i.e. height, weight, forearm volume, and forearm composition). The subjects were studied on two additional occasions: i) during control conditions (Con) and ii) following chronic acetazolamide administration (Acz; acetazolamide at 500 mg every 8 h po). The treatment order was randomized between and within subjects in order to minimize any order effect. Although the side effects of Acz administration, although well-tolerated by most subjects, are quite noticeable and therefore, the administration of a placebo was not used since it would not sufficiently replicate the effects of Acz nor blind the subjects to the trial condition.

*Flow-mediated Dilation.* Prior to each condition, endothelial cell function was assessed using the flow mediated dilation (FMD) technique following post-occlusive reactive hyperemia (Corretti et al., 2002). FMD measurements were performed using echo-Doppler ultrasonography (GE Logiq 400CL, Milwaukee, WI) using a 7 MHz linear array probe. The subject was asked to rest comfortably for approximately 20 min in a supine position while being instrumented. A turnicuff was placed distal to the elbow and inflated to a suprasystolic pressure of ~270 mmHg for 5 min. Scans of the brachial artery were obtained in longitudinal sections 5 to 10 cm proximal to the elbow at rest for 30 s prior to the application of the occluding pressure and for 90 s following release of the turnicuff. Images of the brachial artery were captured on an off-line PC directly from the ultrasound system at 10 Hz using commercially available software (Vascular Imager, Medical Imaging Applications, LLC, Coralville, IA). Maximum, minimum, and mean blood velocities (Vmax, Vmin and Vmean) were measured simultaneously with scans of
the brachial artery thereby enabling continuous measurements of blood flow during the FMD procedure.

**Measurement of Blood Velocity and Brachial Artery Diameter.** Measurements of blood velocities (MBV) during rest and following post-occlusive reactive hyperemia were measured using Doppler ultrasound velocimetry (500-V; Multigon Industries) of the brachial artery operating in pulsed mode. The Doppler transducer (frequency of 4 MHz and fixed-angle crystal of 45° relative to the skin) was placed flat on the medial aspect of the upper arm ~6-10 cm proximal to the antecubital fossa and parallel to the brachial artery. To ensure complete insonation of the brachial artery, the gate was adjusted to the total width of the artery. An audio demodulator was used to convert the frequency spectrum of the Doppler audio signal to an instantaneous mean blood velocity. The analog blood velocity signal was recorded at 100 Hz (PowerLab 16SP; ADInstruments, Grand Junction, CO) and stored on a computer for offline analysis. Mean brachial artery blood velocity was determined as the area under the curve for each cardiac cycle (i.e. each R-R wave interval) with blood velocities being expressed per minute (i.e. cm/min) by multiplying the cardiac cycle-by-cycle values by the corresponding heart rate (Matlab Release 11.1, The Mathworks). MBV were subsequently used to estimate the amount of stress placed upon the endothelial cells through the calculation of shear rate (see below).

**Analysis of Brachial Artery Diameter and Calculation of %FMD, Shear Rate, and Shear Stress.** Brachial artery diameter was analyzed using an automated wall detection program (Brachial Analyzer, Medical Imaging Applications, LCC, Coralville, IA) as previously described by Sonka et al. (2002). For detection of intimal wall far and near borders, a vascular region of interest (ROI) was determined by the operator. Quality
tolerance parameters were used (i.e. gradient tolerance and shape tolerance) for
determination of acceptable quality vessel wall borders so that diameters could be
detected frame-by-frame throughout the entire data collection duration. Calculation of
%FMD was then determined using resting brachial artery velocities and diameter
measurements as well as brachial artery velocities and diameter measurements following
post-occlusive reactive hyperemia: \( \%FMD = \frac{(\text{Dia}_{\text{Peak}} - \text{Dia}_{\text{Rest}})}{\text{Dia}_{\text{Rest}}} \times 100 \), where
\( \text{Dia}_{\text{Peak}} \) is peak arterial diameter and \( \text{Dia}_{\text{Rest}} \) is resting arterial diameter. Peak diameters
were determined as the highest diameter measurements averaged over a 5 second period.

Changes in MBV were expected following cuff release, and since the ability of
the brachial artery to dilate is determined through the endothelium-dependent response
(through nitric oxide production), nitric oxide bioavailability was assumed from the
calculation of shear rate (SR): \( \text{SR} = 4(\text{Mean}_{\text{vel}}/D) \); where \( \text{Mean}_{\text{vel}} \) is mean red blood cell
velocities through the brachial artery (cm/s), and \( D \) is the brachial artery diameter (cm).
Since shear stress (SS) is proportional to SR and the viscosity of blood plasma can
determine the amount of pull or force placed on the endothelium, SS was calculated as
follows: \( \text{SS} = (\text{SR}) (5.0 \times 10^{-2} \text{ dyn·s·cm}^{-2}) \); where \( \text{SR} \) is shear rate and \( 5.0 \times 10^{-2} \text{ dyn·s·cm}^{-2} \)
is the viscosity of blood through the brachial artery as previously determined (Reneman
\textit{et al.}, 2006).

\textit{Analysis of Blood Gases and Ions.} Arterialized-venous blood was sampled from a
superficial vein located on the dorsal aspect of a heated hand at rest and following flow-
mediated dilation. Plasma was analyzed for concentration of \( \text{HCO}_3^- \), pH and the partial
pressure of \( \text{CO}_2 \) (\( \text{PCO}_2 \)) using a commercially available blood-gas analyzer (pHox plus L,
Stat Profile, Nova Biomedical; Kowalchuk et al., 1988b; Kowalchuk et al., 1988a; Kowalchuk et al., 1992; Lindinger et al., 1990).

**Statistical Analysis.** A two-way analysis of variance with repeated measures was used to compare between conditions (Con versus Acz) and across time (rest versus post-occlusion). A significant main effect or significant condition x time interaction was further tested using Student-Neuman-Keuls post-hoc analysis. A paired t-test was used to compare between conditions (Con and Acz) when time was not a factor (i.e. %FMD and shear rate). Significance was set a priori at p≤0.05. All values are presented as the mean ± standard deviation unless stated otherwise.

**Results**

**Subjects.** Subject characteristics are displayed in Table 4.1. All subjects were healthy and were considered recreationally active. Subjects weighed an average of 78.3 ± 17.3 kg and were 179.3 ± 8.1 cm tall. Mean forearm volume was 1237 ± 349 ml and average maximal forearm strength was 55 ± 6 kg.

**Effect of Acidosis on Blood Gases: H⁺, HCO₃⁻, and PCO₂.** As shown in Table 4.2, chronic Acz ingestion resulted in a significantly higher plasma [H⁺] for the Acz condition (42 ± 3 nmol·L⁻¹) compared to the Con condition (35 ± 1 nmol·L⁻¹; p<0.05). Plasma [HCO₃⁻] was significantly lower following Acz compared to Con (Acz: 20.6 ± 1.11 mmol·L⁻¹; Con: 29.0 ± 2.19 mmol·L⁻¹; p<0.05). Similar, plasma PCO₂ was also lower following Acz administration compared to Con (Acz: 34.0 ± 2.89 mmHg; Con: 40.2 ± 2.95 mmHg; p<0.05) under resting conditions.

**Effect of Acidosis on Brachial Artery Diameter.** Table 4.2 shows diameter differences between control and Acz conditions. For both Acz and control conditions,
flow-mediated dilation resulted in higher brachial artery diameters (Acz:4.79 ± 0.56 mm; Con:4.85 ± 0.59 mm) compared to rest (Acz:4.60 ± 0.49 mm; Con:4.49 ± 0.51 mm). Although not statistically significant, there was a trend for a higher resting arterial diameter for the Acz condition (4.60 ± 0.49 mm) compared to Con (4.49 ± 0.51 mm). When expressed as %FMD, Acz administration resulted in a significantly lower response compared to Con (Acz:4.11 ± 3.55% vs. Con:7.98 ± 2.96%; p<0.05). The difference in %FMD responses between the Acz and Con conditions equates to a 48.5% difference which is shown in Figure 4.1 which also shows the diameter change for a single individual in response to FMD for both Acz and Con conditions.

*Effect of Acidosis on Mean Blood Velocity.* Compared to resting values, both Acz and Con trials resulted in higher mean blood velocities (MBV) in response to FMD (Acz:5.53 ± 2.78 cm·s⁻¹ vs. 42.91 ± 14.37 cm·s⁻¹; Con:4.29 ± 1.55 cm·s⁻¹ vs. 36.60 ± 12.59 cm·s⁻¹; p<0.05), as shown in Table 4.2. When conditions were compared, the Acz condition (42.91 ± 14.37 cm·s⁻¹) and Con conditions (36.60 ± 12.59 cm·s⁻¹), the difference in MBV responses did not achieve statistical significance (p=0.089).

*Effect of Acidosis on Shear Rate and Shear Stress.* As shown in Table 4.2, both shear rate and shear stress were higher in response to FMD for Acz (SR:354.98 ± 98.21 s⁻¹; SS:14.20 ± 3.93 dyn·cm⁻²) and control (SR:295.20 ± 72.15 s⁻¹; SS:11.81 ± 2.89 dyn·cm⁻²) conditions compared to resting values (Acz SR:48.18 ± 23.51 s⁻¹; Acz SS:1.93 ± 0.94 dyn·cm⁻², Con SR:37.62 ± 11.18 s⁻¹; Con SS:1.50 ± 0.45 dyn·cm⁻²; p<0.05). While no significant difference was observed between conditions, both shear rate and shear stress tended to be higher for Acz conditions (SR:354.98 ± 98.21 s⁻¹; SS:14.20 ± 3.93 dyn·cm⁻²) compared to Con conditions (SR:295.20 ± 72.15 s⁻¹; SS:11.81 ± 2.89 dyn·cm⁻²; p=0.121).
When the %FMD response was normalized for differences in shear rate (i.e., %FMD/shear rate), the Acz condition resulted in a significantly lower response compared to Con (Acz:0.0110 ± 0.010; Con:0.0287 ± 0.005; p<0.05) indicating that the endothelial response was blunted considerably following Acz administration.
Table 4.1 – Subjects’ physical characteristics and maximal forearm strength

<table>
<thead>
<tr>
<th></th>
<th>Height (cm)</th>
<th>Weight (kg)</th>
<th>Age (yrs)</th>
<th>Forearm volume (mL)</th>
<th>MVC (kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>179.3</td>
<td>78.3</td>
<td>32</td>
<td>1237</td>
<td>55</td>
</tr>
<tr>
<td>SD</td>
<td>8.1</td>
<td>17.3</td>
<td>5</td>
<td>348</td>
<td>6</td>
</tr>
</tbody>
</table>

MVC; maximal voluntary contraction
### Table 4.2 – Cardiovascular and blood-gas values during control and acetazolamide conditions.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Acetazolamide</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Flow-mediated Dilation Rest</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBV (cm·s⁻¹)</td>
<td>4.3 ± 1.6</td>
<td>5.5 ± 2.8</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>4.5 ± 0.5</td>
<td>4.6 ± 0.5</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>37.6 ± 11.2</td>
<td>48.2 ± 23.5</td>
</tr>
<tr>
<td>Shear stress (dyn·cm⁻²)</td>
<td>1.5 ± 0.5</td>
<td>1.9 ± 0.9</td>
</tr>
<tr>
<td>[H⁺] (nmol·L⁻¹)</td>
<td>35 ± 2</td>
<td>42 ± 3†</td>
</tr>
<tr>
<td>[HCO₃⁻] (mmol·L⁻¹)</td>
<td>29.0 ± 2.2</td>
<td>20.6 ± 1.1†</td>
</tr>
<tr>
<td>PCO₂ (mmHg)</td>
<td>40.2 ± 3.0</td>
<td>34.0 ± 2.9†</td>
</tr>
<tr>
<td><strong>Flow-mediated Dilation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MBV (cm·s⁻¹)</td>
<td>36.6 ± 12.6*</td>
<td>42.9 ± 14.4*#</td>
</tr>
<tr>
<td>Diameter (mm)</td>
<td>4.9 ± 0.6*</td>
<td>4.8 ± 0.6*</td>
</tr>
<tr>
<td>%FMD</td>
<td>8.0 ± 3.0</td>
<td>4.1 ± 3.6†</td>
</tr>
<tr>
<td>FMD/Shear Rate</td>
<td>0.029 ± 0.005</td>
<td>0.011 ± 0.010†</td>
</tr>
<tr>
<td>Shear rate (s⁻¹)</td>
<td>295.2 ± 72.2*</td>
<td>355.0 ± 98.2*</td>
</tr>
<tr>
<td>Shear stress (dyn·cm⁻²)</td>
<td>11.8 ± 2.9*</td>
<td>14.2 ± 3.9*</td>
</tr>
</tbody>
</table>

All values are reported as means ± SD. MBV, muscle blood velocity; MBF, muscle blood flow; [H⁺], hydrogen ion concentration; [HCO₃⁻], bicarbonate ion concentration; PCO₂, partial pressure of carbon dioxide; FMD, flow-mediated dilation. *Significantly different from rest (p<0.05). †Significantly different from control (p<0.05). #Comparison between Con and Acz (p=0.089).
Figure 4.1 – Diameter response of a single individual following post-occlusive reactive hyperemia during Acz (blue) and Con (red) conditions.
Figure 4.2 – Diameter comparisons during Acz (open) and Con (closed) conditions. Bottom graph represents percent change from rest between conditions. *Significantly different from rest (p<0.05). †Significantly different from Con (p<0.05).
Figure 4.3 – Mean blood velocity comparisons during Acz (open) and Con (closed) conditions. *Significantly different from rest (p<0.05).
Figure 4.4 – Shear rate (top) and shear stress (bottom) comparisons during Acz (open) and Con (closed) conditions. *Significantly different from rest (p<0.05).
Discussion

To our knowledge, this is the first study to examine endothelial function using the flow-mediated dilation (FMD) technique in response to an induced metabolic acidosis following chronic Acz administration. Additionally, this is the first study to assess changes in hemodynamic shear stresses following a post-occlusive reactive hyperemia in response to a chronic metabolic acidosis. In agreement with our original hypothesis, the primary finding of this study indicates that the FMD response is appreciably attenuated such that the overall ability of the brachial artery to vasodilate relative to control conditions is impaired. Furthermore, this attenuation of the FMD response following Acz administration occurred in spite of the hemodynamic shear stimulus being similar between the experimental conditions indicating that endothelial cell function was diminished in the Acz condition (Pyke & Tschakovsky, 2005). Although we are not able to discern a specific mechanism to explain the attenuated FMD in response to the metabolic acidosis from the results of the present study, it may be that the tendency for the resting arterial diameter to be larger during Acz resulted in a blunted vasodilatory response.

Acid-base Changes Following Acz Administration. Similar to the results of previous studies, chronic acetazolamide ingestion resulted in a significantly higher plasma [H+] while plasma [HCO3−] and PCO2 were significantly lower compared to control conditions (Kowalchuk et al., 1994; Scheuermann et al., 1999). It has been demonstrated that increases in [H+] can induce vasodilation at rest (Haddy & Scott, 1968; Juel et al., 2007) and that an increase in PCO2 within tissue, especially within the cerebral circulation, can induce vasodilation as well (Kontos et al., 1967). The results of the
present study are consistent with these previous findings since resting arterial diameter values tended to be higher following Acz administration compared to Con trials. Although speculation only, chronic Acz administration has been shown to change smooth muscle function through a systemic reduction in K\(^+\) (i.e. induced hypokalemia) thereby affecting the ability of smooth muscle cell membranes to maintain excitability characteristics (Bellien et al., 2005; Juel et al., 2007). This leads to the smooth muscle cells to relax and as a consequent to their proximity to the endothelial cells, vascular tone is reduced (i.e. vasodilation occurs). In additions, it has been demonstrated that Acz can induce vasodilation under resting conditions by acting at the calcium activated potassium (K\(\text{Ca}^{2+}\)) channels (Pickkers et al., 2001).

**Effect of Metabolic Acidosis on Brachial Artery Diameter.** The major finding in this study was the attenuated FMD response with the Acz condition. When expressed as a percent change from rest, the control condition had a considerably higher increase in peak diameter than the Acz condition, about a 50% difference. There are a number of mechanism(s) that may account for the significant attenuation of the FMD response following Acz administration compared to Con conditions.

The first could be due to nitric oxide (NO) production and regulation, more so as shear stimulus increases (Kingwell, 2000). It has been well documented that as red blood cells exert considerable hemodynamic stress on the endothelium lining vascular smooth muscle (i.e. shear stress). This increase in shear stress results in the stimulation of endothelial nitric oxide sythase (eNOS) which drives the reaction of L-Arginine to L-Citrulline and the production of NO (Pohl et al., 1986; Segal, 1994). The subsequent production of NO is paramount in controlling basal vascular tone (Rådegran & Saltin,
1999; Shoemaker & Hughson, 1999; Bellien et al., 2006). Also, recent evidence points towards neuronal NO synthase (nNOS) as a regulator in basal vascular tone (Seddon et al., 2008), as a basal level of sympathetic outflow does exist at rest. Since specific CA isoforms (CAI, CAII, and CAIII) exist in vascular smooth muscle (Berg et al., 2004), the existing acidosis brought about through CA inhibition could have a direct action on the compliance of the vascular wall through NO production. More so, a direct action on the function of NO could occur, once in the smooth muscle. Here, it would seem plausible that perhaps one role of carbonic anhydrase is to aid NO in maintaining vascular tone. Due to the inherent limitations of this study we can not deduce exactly how this might occur as further studies examining the interaction between carbonic anhydrase inhibition and NO bioavailability are warranted.

Another mechanism that could cause a reduction in FMD of the brachial artery involves changes in K$^+$ and associated pumps (Juel, 2007; Juel et al., 2007). It has been demonstrated that Acz can directly affect calcium-activated potassium channels at rest (Pickkers et al., 2001) thus evoking vasodilation through hyperpolarization of smooth muscle causing a reduction in intracellular Ca$^{2+}$ (Juel, 2007). As previously discussed, specific isoforms of carbonic anhydrase have been identified in the vascular smooth muscle. A common mechanism for vasodilation of smooth muscle is through the production of cGMP thus stimulating associated K$^+$ channels. If carbonic anhydrase inhibition somehow inhibits this process (at the level of the channels), inhibition would lead to a further relaxation of smooth muscle. The results of the present study did indicate a tendency for brachial artery diameter to increase under resting conditions and an attenuated FMD response did follow consistent with our hypothesis based on previous
resting data from other studies (Taki et al., 1998; Pickkers et al., 1999; Pickkers et al., 2001).

Perhaps the most plausible explanation for the attenuated FMD response to the metabolic acidosis is through sympathetic vasoconstriction. It has been reported that NO can inhibit sympathetic constriction by preventing norepinephrine from binding to \( \alpha \)-adrenergic receptors (Thomas & Victor, 1998). As discussed already, during the FMD response, an increase in shear stimulus results in the production of NO through stimulation of eNOS. Hence, NO production may exert its effect through activation of guanaryl cyclase and subsequent relaxation of vascular smooth muscle. As well, NO release may prevent vasoconstriction through inhibition of basal sympathetic outflow. Perhaps one function of the smooth muscle carbonic anhydrase isoform(s) is to modulate NO release and prevent sympathetic vasoconstriction from occurring. If carbonic anhydrase is inhibited, this will prevent NO from allowing norepinephrine to bind to \( \alpha \)-receptors thereby preventing inhibition of sympathetic-mediated vasoconstriction. This would seem possible since vasodilation did occur following the post-occlusive reactive hyperemia but the magnitude was considerably less compared to Con conditions.

**Effect of a Metabolic Acidosis on Mean Blood Velocity, Shear Rate and Shear Stress.** While no significant differences were present between conditions in mean blood velocities (MBV) at rest, an increase in MBV was observed following a post-occlusive reactive hyperemia for the Acz group and Con conditions. Although the difference was not significantly different between conditions \((p=0.089)\), a higher MBV might be expected given the relatively smaller change in arterial diameter observed in the Acz condition.
The results of the present study found that shear rate (SR) was higher for the Acz group compared to Con conditions following post-occlusive reactive hyperemia by about 60 s⁻¹ (higher than resting values) however, due to the large variability, statistical significance could be detected (p=0.122). Due to the proportionality between SR and shear stress (SS), SS values also appeared to be higher, although not significantly. However, when the FMD response was expressed relative to the hemodynamic shear stresses as performed in a previous study (Pyke & Tschakovsky, 2005), the FMD-shear rate response during the Con trial was over 2-fold higher than the Acz condition. In other words, the FMD response during metabolic acidosis was considerably lower following Acz administration indicating that even in the presence of similar hemodynamic shear stress, the ability of the endothelial cells to vasodilate was significantly impaired following Acz administration.

As previously mentioned, NO is important in regulating basal vascular tone and that shear stress is an important stimulus in the production of NO (Pohl et al., 1986; Bellien et al., 2006). The Acz condition had a tendency towards a higher shear stimulus compared to control, which should have presented a response for further production of NO and a subsequent increase in smooth muscle dilation. As we have shown, the Acz condition failed to present a further increase in vasodilation compared to the control condition. The resulting differences in shear values between conditions following occlusion were greater than the resting SR and SS values. Due to the smaller size of the brachial artery, a smaller change in shear stimulus should be sufficient to evoke a change in diameter, although due to the heterogeneity of endothelial and smooth muscle cells (Hill et al., 2001; Newcomer et al., 2004) functional differences may exist between
conditions such that an acidosis (or carbonic anhydrase itself) could impede receptor or protein function at the level of the endothelium or smooth muscle. Regardless, the limitations of the present study preclude specific insight to the mechanism for the trend in shear differences between conditions as further studies are warranted.

Clinical Significance. Our discovery that a blunted FMD response occurred during Acz administration is an interesting find and could have clinical implications. If carbonic anhydrase is directly involved in smooth muscle function and its inhibition detrimentally affects the ability of smooth muscle cells to vasodilate, then a correlation between protein levels of carbonic anhydrase and FMD could exist. Such that individuals with some form of vascular disease would have decreased protein levels of associated smooth muscle carbonic anhydrase isoforms.

Conclusion. In conclusion, the results of the current study suggest that a prior condition of metabolic acidosis through Acz administration significantly attenuates the FMD response in the brachial artery in spite of a similar shear stimulus (FMD/SR). While no differences were detected, a slightly higher resting arterial diameter existed for the Acz group compared to control, which could possibly explain the reduction in the FMD response. As noted, further studies are warranted to dissociate the mechanism(s) involved in the attenuated FMD response following Acz administration and in particular, the effects a metabolic acidosis versus the effect of carbonic anhydrase inhibition need to be identified.


The specific aims of this dissertation were to i) determine if a metabolic acidosis (through chronic acetazolamide administration, Acz) would affect blood flow differently than control (Con) conditions during dynamic hand-grip exercise at moderate and heavy intensities, and ii) determine if a metabolic acidosis would affect endothelial function as measured by flow-mediated dilation (FMD). In contrast to our first hypothesis, our main finding indicated that there was a blunted response during heavy exercise in the overall ability of the brachial artery to dilate compared to Con conditions. When resting diameters were compared between conditions prior to heavy exercise, the Acz condition had a significantly higher resting arterial diameter compared to Con. When expressed as a percent change from rest, the Con condition had a significantly greater increase in diameter than the Acz condition. As well, only the Con condition had a significant increase in diameter from rest to heavy exercise. In addition, muscle blood flow was lower by ~46 ml·min^{-1} during heavy exercise following chronic Acz treatment compared to control, although this did not reach significance either. Interestingly, mean arterial pressure (MAP) was significantly higher during the Acz condition compared to control. Since endothelial cells as well as smooth muscle cells are heterogeneous in nature and can be influenced by changes in MAP, this could be attributed to the differences in not only diameter but also to muscle blood flow. Additionally, the difference in heavy
exercising blood flow between conditions could have contributed to the lower exercising arterial diameter for the Acz condition or by functional sympatholysis, as a function of smooth muscle carbonic anhydrase (CA) could be to facilitate nitric oxide (NO) in preventing norepinephrine from binding to α-adrenergic receptors.

Consistent with our second hypothesis, our main finding indicated that the FMD response was blunted during the Acz condition following a post-occlusive reactive hyperemia compared to the Con condition. As with the exercising data, this could be contributed to a higher resting arterial diameter for the Acz condition, however no significant differences existed between conditions at rest. Also, despite a higher shear stimulus during Acz, the FMD response was still blunted indicating that the vasodilatory capacity of the endothelial cells were affected by carbonic anhydrase inhibition. This is an interesting finding since the stimulus for the production of nitric oxide (NO) appeared to be similar between Acz and Con conditions. The exact mechanism involved could not be deduced since the study methodology did not directly test for that question, however possible mechanisms do exist. These mechanisms range from an increased intracellular $\text{PCO}_2$ causing a higher resting arterial diameter and thus preventing a large percent change from rest to peak diameter, to possible direct effects of carbonic anhydrase on smooth muscle function.

**Future Direction**

Our discovery that a blunted FMD response occurred during Acz administration is an interesting finding and could have significant clinical implications. If carbonic anhydrase is directly involved in smooth muscle function and its inhibition detrimentally affects the ability of smooth muscle cells to dilate, then a correlation between either the
amount or the isoform of carbonic anhydrase present in the endothelial cells may be a significant regulator of endothelial function; such that individuals with some form of vascular disease would have decreased protein levels of carbonic anhydrase associated with the vascular smooth muscle. If carbonic anhydrase is not directly involved in the function of smooth muscle and the blunted FMD response is simply a consequence of a higher resting arterial diameter due to the induced acidosis, then further investigations into the effects of acid-base status on endothelial function are warranted. As well, if the function of NO is compromised during Acz administration, then inhibiting NO would be of interest. This can be accomplished through the administration of L-NMME or L-NAME, both of which have the ability to completely inhibit NO.

An interesting finding from our exercising study was the blunted response in vasodilation of the brachial artery during heavy exercise for the Acz condition. The same problems reside as mentioned above, however confounding factors are also involved. Since muscle contraction was involved in this study, the effects of potassium (K+) might have a significant effect on contractile muscle as well as smooth muscle function. Since Acz induces a systemic hypokalemic effect through polyuria, membrane excitability might be depressed. Also, it has been demonstrated that Acz directly affects chloride channels in skeletal muscle in vitro, which control resting membrane conductance and excitability. However, this might have an opposite effect during exercise. By specifically inhibiting the skeletal muscle chloride channel or by infusing an H+ buffer, the effects of Acz can be attenuated. Another possible influence could be the heterogeneity of smooth muscle cells. It has been shown that phenotypic changes can occur in smooth muscle cells as a result of pressure changes (i.e. changes in MAP). Our
results indicated there was a significant difference in MAP during heavy exercise between conditions. This could be corrected by manipulating the arm angle during Con conditions to increase MAP of the arm (increasing the hydrostatic column) to Acz levels during heavy exercise. Along similar lines, sympathetic vasoconstriction exists during exercise and is increased as intensity levels increase. Sympathetic outflow can be inhibited by NO. If the anti-constrictive effect of NO is inhibited during Acz treatment, norepinephrine would be able to induce vasoconstriction and thus prevent a further increase in dilation during exercise. As mentioned previously, inhibition of NO through L-NMME or L-NAME would resolve an existing effect of NO on sympathetic-mediated constriction. Also, by eliminating the sympathetic response through the cold-pressor test, a better understanding of NO regulation on sympathetic-mediated vasoconstriction could be determined during Acz treatment.
Appendix A

Research Consent Form………………………………………………………………………………….98
ADULT RESEARCH SUBJECT INFORMATION AND CONSENT FORM

THE ROLE of ACID-BASE STATUS ON MUSCLE BLOOD FLOW DURING INTERMITTENT ISOMETRIC HANDGRIP EXERCISE AND FLOW-MEDIATED DILATION

Principal Investigator: Dr. Barry Scheuermann, Ph.D.
Other Staff (identified by role): John Thistlethwaite, M.S.
                                Benjamin Thompson, M.A.
                                Joaquin Gonzales, M.S.

Contact Phone number(s): (419) 530-2058

What you should know about this research study:

• We give you this consent/authorization form so that you may read about the purpose, risks, and benefits of this research study. All information in this form will be communicated to you verbally by the research staff as well.

• Routine clinical care is based upon the best-known treatment and is provided with the main goal of helping the individual patient. The main goal of research studies is to gain knowledge that may help future patients.

• We cannot promise that this research will benefit you. Just like routine care, this research can have side effects that can be serious or minor.

• You have the right to refuse to take part in this research, or agree to take part now and change your mind later.

• If you decide to take part in this research or not, or if you decide to take part now but change your mind later, your decision will not affect your routine care.

• Please review this form carefully. Ask any questions before you make a decision about whether or not you want to take part in this research. If you decide to take part in this research, you may ask any additional questions at any time.

• Your participation in this research is voluntary.
PURPOSE (WHY THIS RESEARCH IS BEING DONE)
You are being asked to take part in a research study on the effect of Acetazolamide administration on skeletal muscle blood flow. The purpose of the study is to determine whether carbonic anhydrase (an enzyme that is responsible for the formation of CO$_2$) inhibition plays a factor in determining blood flow to exercising muscle.

You were selected as someone who may want to take part in this study because you meet the age, health, and gender criteria for subjects to be involved in this research. There will be approximately 10 healthy male subjects involved in this research project.

DESCRIPTION OF THE RESEARCH PROCEDURES AND DURATION OF YOUR INVOLVEMENT
If you decide to take part in this study, you will be asked to make 4 separate visits to the Cardiopulmonary and Metabolism Research Laboratory (CMRL) with at least 1 week between visits. You are being asked to participate in a study examining the relationship between the regulation of acid-base balance and muscle blood flow during handgrip exercise. The purpose of this research study is to determine if the inhibition of carbonic anhydrase (CA), a specific enzyme that is important for the normal regulation of acid-base balance in the blood alters muscle blood flow during intermittent isometric handgrip exercise. In addition, the endothelial response to CA inhibition will be determined by examining the effect of acetazolamide administration on flow-mediated dilation (i.e. the vasodilatory response to 5 min of forearm cuff occlusion). In order to examine the role of CA, a drug called acetazolamide (trade name Diamox) will be administered orally in order to temporarily inhibit the normal function of the enzyme.

The 3 treatments include: i) a “control condition where CA is normal and there is no acidosis, ii) an acetazolamide trial where CA is inhibited and a metabolic acidosis is present and iii) an ammonium chloride trial where CA is normal but a metabolic acidosis is present. This study design will allow us to differentiate between the effects of the metabolic acidosis condition that typically accompanies acetazolamide administration and the direct influence of CA. The treatment order will be randomized.

Experimental Procedures;
Flow Mediated Dilation (FMD): This test will last approximately 10 minutes. You will be asked to lie down quietly for 15 minutes on a standard treatment table. During this time, your blood pressure will be monitored by placing a small device around your wrist. Heart rate will be monitored by placing three electrodes on your skin.
• A blood pressure cuff will be placed around your forearm.
• A plastic probe (Doppler ultrasound) with gel will be placed on your upper arm to acquire an image of your artery.
• The blood pressure cuff on your upper arm will be inflated to a high pressure for 5 minutes. Following the 5 minutes of occlusion, the cuff around your upper arm will be rapidly deflated and an image of your artery will be measured continuously for another 2 minutes.
Blood Flow Measurements:
Continuous measurements of blood velocity in the large artery of your upper arm will be obtained using a Doppler ultrasound velocimetry system operating in pulsed mode.
- A small plastic transducer with gel will be placed flat on your skin over a large artery in the upper arm.
- The transducer will be held in place by a technician at rest and during exercise and recovery.
- This procedure is non-invasive and will not cause you any discomfort.

Experimental visits;
First visit;
During your first visit, all of the experimental procedures will be explained to you and you will be asked to complete an informed consent form and medical history questionnaire. You will be asked to arrive in a fasted state (no food or drink) for 8 hours prior to your arrival and not to have performed any strenuous physical activity for 24 hours prior to arriving to the laboratory. Standard measurements of height, weight, forearm volume, forearm muscle strength will also be made during the first visit. Forearm volume will be measured by placing your hand and forearm in a container of water and maximal forearm muscle strength will be determined using a standard handgrip dynamometer.

You will then be asked to perform a progressive ramp exercise test using your handgrip muscles. The resistance is incremented 0.5 kg each minute until you can no longer lift the weight or maintain the contraction rate of 1 s contraction to 2 s relaxation. The peak workload achieved during this initial ramp test will be used to set the exercise intensity to be used during your subsequent visits to the laboratory.

Second visit (control condition);
You will be asked to arrive in a fasted state (no food or drink) for 8 hours prior to your arrival and not to have performed any strenuous physical activity for 24 hours prior to arriving to the laboratory. After a brief rest period, you will have adhesive electrodes placed on your chest for monitoring heart rate and over the muscles of your forearm to monitor muscle activation patterns. In addition, a small Teflon catheter will be placed in a dorsal hand vein to allow a small sample of blood to be withdrawn and analyzed for acid-base changes. Each sample will amount to approximately 1 teaspoon; samples will be obtained at rest and every 3rd minute of exercise.

Once the electrodes are in place and the catheter secured, you will be asked to rest comfortably for 20 min followed by a test that measures endothelial function in the brachial artery in your upper arm. The flow-mediated dilation (FMD) technique is used to assess how well your blood vessels respond to a brief increased in elevated flow. Following the FMD test, you will be asked to perform intermittent isometric handgrip exercise (1 s contraction; 2 s relaxation) at an intensity corresponding to 20% of their peak workload achieved during the initial ramp test. You will perform 2 bouts of this constant load exercise, each bout lasting for 6 min in duration with 10 min of rest.
allowed between each bout. Blood flow will be measured continuously using noninvasive Doppler ultrasonography throughout exercise and recovery.

*Third visit (acetazolamide administration; carbonic anhydrase inhibition and metabolic acidosis)*;

The protocol and measurements made during third visit are the same as those described for the second visit with the exception that you will be provided with 6 tablets containing 250 mg of acetazolamide (Diamox). You will be asked to ingest 1 tablet in the morning and 1 again in the evening for a period of 3 days prior to your next visit to the laboratory. Upon your arrival to the CMRL, the preparation and protocol is the same as the second visit.

*Fourth visit (ammonium chloride administration; metabolic acidosis only)*;

The protocol and measurements made during fourth visit are the same as those described for the second visit with the exception that you will asked to ingest tablets containing the equivalent of 300 mg/kg body weight of ammonium chloride 3 hours prior to start of the FMD test in order to induce a metabolic acidosis.

**RISKS AND DISCOMFORTS YOU MAY EXPERIENCE IF YOU TAKE PART IN THIS RESEARCH**

As with any exercise regimen, there is a very small risk of heart attack. Heart rate and EKG will be monitored closely throughout each test. As well, you may stop the test at any time if you feel inclined to do so. There is also the possibility of soreness 24-48 hours post-exercise. By performing the initial ramp exercise protocol, you may experience soreness 24-48 hours post-testing. However, the subsequent exercise protocols are of moderate intensity and therefore, the potential for soreness is minimal.

Reactive hyperemia is a commonly used technique to determine peak blood flow to an area of interest (in this case the upper arm). During occlusion, you may feel tightness and a tingling sensation within the arm that may be uncomfortable. These feelings disappear within a few seconds after cuff release.

The use of surface electromyography (EMG) is another commonly used non-invasive technique to determine muscle activation and muscle mass involvement during exercise. The use of electrodes (Ag-AgCl) may cause minor skin irritations, which usually disappear within 1-3 days post trial. As well, all areas exposed to electrodes will be cleaned with alcohol prior to and post exercise to reduce irritation of the skin.

Venipuncture is a common procedure with minimal risk. The use of sterile needles for each venous puncture and using proper sterile techniques will reduce the risk of infection at the site. Bruising around the site of insertion into the vein sometimes occurs because of blood sampling or when the needle is removed. Bruising and any soreness associated with the venipuncture generally fades within one day of the procedure.

**Acetazolamide:** The risks associated with Acetazolamide administration are very minimal. Drowsiness, tingling of the skin (paresthesias), headache, loss of appetite, and excessive urination (polyuria) may accompany large doses. Hypersensitivity
reactions are very rare and may consist of fever, skin reactions, bone-marrow depression, and renal lesions although these are more common in elderly individuals, who will not take part in this study. A metabolic acidosis will develop with chronic administration. Because acetazolamide is not metabolized, it is excreted in the urine within 24 hours (~99%), and therefore, symptoms revert to normal with discontinued treatment. If a severe acidosis develops, intravenous infusion of sodium bicarbonate or sodium lactate can correct the acidosis.

Ammonium Chloride: The risks associated with Ammonium Chloride administration are very minimal. Excessive doses may cause gastric upset, vomiting, excessive thirst, headache, hyperventilation, progressive drowsiness, confusion, and acidosis. Similar to Acetazolamide treatment, symptoms of ammonium chloride cease when treatment is stopped. As well, sodium bicarbonate and sodium lactate can be used intravenously to treat severe cases of acidosis.

POSSIBLE BENEFIT TO YOU IF YOU DECIDE TO TAKE PART IN THIS RESEARCH
We cannot promise that you will receive any benefits from this research since this is a basic physiology/biochemistry study, and as such, there may not be any direct health-related benefits to be gained by your participation. However, you will learn by observing how theory taught in the classroom is translated into research and the process of scientific inquiry. If you are interested, the rationale for doing the study as well as the theory and significance of each test and your results from each test will be explained. From this, you will better understand the physiological response to any exercise program.

COST TO YOU FOR TAKING PART IN THIS STUDY
There is no cost to you for participating in this study.

PAYMENT OR OTHER COMPENSATION TO YOU FOR TAKING PART IN THIS RESEARCH
If you decide to take part in this research you will not receive monetary compensation nor will you receive any “extra credit” for any courses that you are enrolled in at the University of Toledo.

PAYMENT OR OTHER COMPENSATION TO THE RESEARCH SITE
The University of Toledo is not receiving money or other benefits from the sponsor of this research as reimbursement for conducting the research.

ALTERNATIVE(S) TO TAKING PART IN THIS RESEARCH
Since this research will be conducted on normal, healthy individuals of The University of Toledo and surrounding community and involves perturbation of normal physiological conditions (through inhibition of carbonic anhydrase by Acetazolamide administration), no reasonable therapeutic alternatives will be available.

CONFIDENTIALITY - (USE AND DISCLOSURE OF YOUR PROTECTED HEALTH INFORMATION)
By agreeing to take part in this research study, you give to The University of Toledo (UT), the Principal Investigator and all personnel associated with this research study
your permission to use or disclose health information that can be identified with you that we obtain in connection with this study. We will use this information to for the purpose of conducting the research study as described in the research consent/authorization form.

The information that we will use or disclose includes the data collected as described in the procedures section. We will only use this information for ourselves as part of this research plan. Under some circumstances, the Institutional Review Board and Research and Sponsored Programs of the University of Toledo may review your information for compliance audits. We may also disclose your protected health information when required by law, such as in response to judicial orders.

The University of Toledo is required by law to protect the privacy of your health information, and to use or disclose the information we obtain about you in connection with this research study only as authorized by you in this form. There is a possibility that the information we disclose may be re-disclosed by the persons we give it to, and no longer protected. However, we will encourage any person who receives your information from us to continue to protect and not re-disclose the information.

Your permission for us to use or disclose your protected health information as described in this section is voluntary. However, you will not be allowed to participate in the research study unless you give us your permission to use or disclose your protected health information by signing this document.

Your access to your own protected health information may be denied during the term of the research study, but you can access your information once the research study is completed.

You have the right to revoke (cancel) the permission you have given to us to use or disclose your protected health information at any time by giving written notice to John Thistlethwaite or Dr. Scheuermann. However, a cancellation will not apply if we have acted with your permission, for example, information that already has been used or disclosed prior to the cancellation. Also, a cancellation will not prevent us from continuing to use and disclose information that was obtained prior to the cancellation as necessary to maintain the integrity of the research study.

Except as noted in the above paragraph, your permission for us to use and disclose your protected health information has no expiration date.

A more complete statement of University of Toledo’s Privacy Practices is set forth in its Joint Notice of Privacy Practices. If you have not already received this Notice, a member of the research team will provide this to you. If you have any further questions concerning privacy, you may contact the University of Toledo’s Privacy Officer at 419-383-3413.

**IN THE EVENT OF A RESEARCH-RELATED INJURY**

In the event of injury resulting from your taking part in this study, treatment can be obtained at a health care facility of your choice. You should understand that the costs of such treatment will be your responsibility. Financial compensation is not available
IRB # _______________________

ICF Version Date: __________

through The University of Toledo or The University of Toledo Medical Center. By signing this form you are not giving up any of your legal rights as a research subject.

In the event of an injury, contact:

John Thistlethwaite at 419.302.9660 or 419.530.2058
or
Dr. Barry Scheuermann at 419.530.2692

VOLUNTARY PARTICIPATION
Taking part in this study is voluntary. You may refuse to participate or discontinue participation at any time without penalty or a loss of benefits to which you are otherwise entitled. If you decide not to participate or to discontinue participation, your decision will not affect your future relations with the University of Toledo or The University of Toledo Medical Center.

NEW FINDINGS
You will be notified of new information that might change your decision to be in this study if any becomes available.

OTHER IMPORTANT INFORMATION
It is important that you are the only one that takes the study drug that you are given as part of this research. It is very important that you keep it (these) out of the reach of children and persons who may not be able to read or understand the label.

ADDITIONAL ELEMENTS
You listened to a summary of the purposes and procedures of this research project, and you may ask more questions about the study at any time. Your refusal to participate or withdraw from the study will have no effect on your status in The University of Toledo or the Toledo, Ohio community.
OFFER TO ANSWER QUESTIONS
Before you sign this form, please ask any questions on any aspect of this study that is unclear to you. You may take as much time as necessary to think it over. If you have questions regarding the research at any time before, during or after the study, you may contact John Thistlethwaite at 419.530.2058 or Dr. Barry Scheuermann at 419.530.2692.

If you have questions beyond those answered by the research team or your rights as a research subject or research-related injuries, please feel free to contact the Chairperson of the University of Toledo Biomedical Institutional Review Board at 419-383-6796.

SIGNATURE SECTION (Please read carefully)

YOU ARE MAKING A DECISION WHETHER OR NOT TO PARTICIPATE IN THIS RESEARCH STUDY. YOUR SIGNATURE INDICATES THAT YOU HAVE READ THE INFORMATION PROVIDED ABOVE, YOU HAVE HAD ALL YOUR QUESTIONS ANSWERED, AND YOU HAVE DECIDED TO TAKE PART IN THIS RESEARCH.

BY SIGNING THIS DOCUMENT YOU AUTHORIZE US TO USE OR DISCLOSE YOUR PROTECTED HEALTH INFORMATION AS DESCRIBED IN THIS FORM.

The date you sign this document to enroll in this study, that is, today’s date, MUST fall between the dates indicated on the approval stamp affixed to the bottom of each page. These dates indicate that this form is valid when you enroll in the study but do not reflect how long you may participate in the study. Each page of this Consent/Authorization Form is stamped to indicate the form’s validity as approved by the UT Biomedical Institutional Review Board (IRB).

<table>
<thead>
<tr>
<th>Name of Subject (please print)</th>
<th>Signature of Subject or Person Authorized to Consent</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relationship to the Subject (Healthcare Power of Attorney authority or Legal Guardian)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Person Obtaining Consent (please print)</th>
<th>Signature of Person Obtaining Consent</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Name of Witness to Consent Process (when required by ICH Guidelines) (please print)</th>
<th>Signature of Witness to Consent Process (when required by ICH Guidelines)</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

This space for IRB Approval Date Stamps
IRB # ________________________
ICF Version Date: ________

YOU WILL BE GIVEN A SIGNED COPY OF THIS FORM TO KEEP.
Appendix B

Medical History Questionnaire…………………………………………..……………..110
Do you have any of the following health conditions?*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Family history of heart disease?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.e. Heart attack, bypass, stroke, or sudden death before age 55 in 1st degree male relative (father, brother, son) or before age 65 in 1st degree female relative (mother, sister, daughter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking habit?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.e. Current cigarette smoker or one who has quit within the previous 6 months</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High blood pressure?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.e. &gt;140/90 on two separate occasions or currently on antihypertensive medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal cholesterol levels?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.e. Total Cholesterol &gt;200mg/dL, or LDL &gt;130 mg/dL, or HDL &lt;35 mg/dL, or currently on lipid lowering medication</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High fasting glucose?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.e. Fasting blood glucose &gt;110 on two separate occasions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Are you inactive?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>i.e. Accumulate &lt;30 minutes of moderate physical activity on most days of the week</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BMI ___________ For Office Use Only</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If you can answer yes to 2 or more above please obtain medical clearance for exercise from your personal physician.

Do you currently have any of the following?*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain in the chest, neck, jaw, or arms?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shortness of breath at rest or with mild exertion?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dizziness or fainting?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Difficulty breathing while lying down, relieved by sitting up?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Awakened by shortness of breath?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Swelling in your ankles?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rapid heart rate while at rest?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Leg pain or cramping while walking, relieved with rest?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart murmur?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unusual fatigue or shortness of breath with usual activities?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If you can answer yes to any of the above please obtain medical clearance for exercise from your personal physician.

Do you have a history of the following?*

<table>
<thead>
<tr>
<th>Condition</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart attack or stroke?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart surgery (CABG, angioplasty)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metabolic disorder (diabetes, kidney, thyroid)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Respiratory problems (asthma, COPD)?</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hospitalization or surgery within the last 6 months?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

If you can answer yes to any of the above please obtain medical clearance for exercise from your personal physician.

---

* Adapted from ACSM’s Guidelines for Exercise Testing and Prescription Sixth Edition

---

<table>
<thead>
<tr>
<th>Contact Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name:</td>
</tr>
<tr>
<td>City:</td>
</tr>
<tr>
<td>Zip:</td>
</tr>
<tr>
<td>Home Phone:</td>
</tr>
<tr>
<td>Work:</td>
</tr>
<tr>
<td>Cell:</td>
</tr>
<tr>
<td>E-mail:</td>
</tr>
</tbody>
</table>

Preferred contact method:

Emergency Contact Information

<table>
<thead>
<tr>
<th>Name:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relationship:</td>
</tr>
<tr>
<td>Home:</td>
</tr>
<tr>
<td>Work:</td>
</tr>
<tr>
<td>Cell:</td>
</tr>
</tbody>
</table>

Prescribed Medications:

---

Allergies:

Supplements (nutritional/athletic):

Do you have an orthopedic condition/arthritis that may limit your activity?

Are you pregnant? If so, how many weeks?

Do you have any other problems or medical conditions not addressed on this form?

How long have you had your medical condition(s)?

Signature: ____________________________

Date: ___________