ABSTRACT

VASODILATORS PRODUCED DURING SKELETAL MUSCLE CONTRACTION
INFLUENCE ONE ANOTHER’S ABILITY TO ELICIT VASODILATION

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Pharmacological inhibitions of vasodilators produced during exercise suggest that no single vasodilator is obligatory in mediating active hyperaemia. Therefore, vasodilators may interact to compensate and mask the loss of one another. Thus, we aimed to investigate if interaction(s) exist between vasodilators potassium (K⁺), nitric oxide (NO) and adenosine (ADO). To identify interactions we applied vasodilators in the absence and presence of one another on the transverse arteriole of the hamster cremaster muscle in situ. By measuring change in vessel diameter we assessed the magnitude of vasodilation elicited by a combination of vasodilators compared to when each were applied alone. Results show physiological concentrations of K⁺ blunt the magnitude of dilation produced by NO and ADO by altering inwardly rectifying potassium channels and Na⁺/K⁺-ATPase activity. The significance is that vasodilators interact to influence one another’s dilatory ability offering a potential explanation as to why an obligatory vasodilatory has not been identified.
Acknowledgements

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When I stumble along my academic path you are always there to catch me, you never shy away from the ‘hard talks’ that I sometimes need and you do this with such understanding and kindness. You embody all the qualities I long for in myself, I look up to you (although not literally), the way you treat others, your approach to both science, and life. I will always remember, treasure, strive to be the type of educator and person you are and I can only hope that I can make as profound of an impact on someone else’s life comparable to the impact you have made on mine.

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5'NUC:</td>
<td>5' nucleotidase</td>
</tr>
<tr>
<td>AC:</td>
<td>Adenylyl cyclase</td>
</tr>
<tr>
<td>Ach:</td>
<td>Acetylcholine</td>
</tr>
<tr>
<td>ADO:</td>
<td>Adenosine</td>
</tr>
<tr>
<td>ADP:</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ANS:</td>
<td>Autonomic nervous system</td>
</tr>
<tr>
<td>ATP:</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BaCl:</td>
<td>Barium chloride</td>
</tr>
<tr>
<td>cAMP:</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>cpm:</td>
<td>Contractions per minute</td>
</tr>
<tr>
<td>COX:</td>
<td>Cyclooxygenase</td>
</tr>
<tr>
<td>Cx:</td>
<td>Connexin</td>
</tr>
<tr>
<td>DAP:</td>
<td>3,4-diaminopyridine</td>
</tr>
<tr>
<td>EC:</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>EDHF:</td>
<td>Endothelial-derived hyperpolarizing factor</td>
</tr>
<tr>
<td>EK+:</td>
<td>K+ equilibrium potential</td>
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<tr>
<td>FMD:</td>
<td>Flow-mediated dilation</td>
</tr>
<tr>
<td>GC:</td>
<td>Guanylyl cyclase</td>
</tr>
<tr>
<td>GTP:</td>
<td>Guanosine-5’-triphosphate</td>
</tr>
<tr>
<td>ISS:</td>
<td>Interstitial space</td>
</tr>
<tr>
<td>K+:</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>K_ATP:</td>
<td>ATP-sensitive potassium channels</td>
</tr>
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<td>Potassium chloride</td>
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<td>KIR+:</td>
<td>Inwardly rectifying potassium channel</td>
</tr>
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</tr>
<tr>
<td>L-NAME:</td>
<td>N’-nitro-L-arginine methyl ester</td>
</tr>
<tr>
<td>L-NMMA:</td>
<td>N-Methylarginine</td>
</tr>
<tr>
<td>L:</td>
<td>Tissue vascularization</td>
</tr>
<tr>
<td>Na+ / K+ -ATPase:</td>
<td>Sodium-potassium pump</td>
</tr>
<tr>
<td>NE:</td>
<td>Norepinephrine</td>
</tr>
<tr>
<td>NMJ:</td>
<td>Neuromuscular junction</td>
</tr>
<tr>
<td>nNOS:</td>
<td>Neuronal nitric oxide synthase</td>
</tr>
<tr>
<td>NO:</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS:</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>O2:</td>
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</tr>
<tr>
<td>Oua:</td>
<td>Ouabain</td>
</tr>
<tr>
<td>P:</td>
<td>Pressure</td>
</tr>
<tr>
<td>PG:</td>
<td>Prostaglandin</td>
</tr>
<tr>
<td>PGI2:</td>
<td>Prostacyclin</td>
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<tr>
<td>PKA:</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>PSS:</td>
<td>Physiological salt solution</td>
</tr>
<tr>
<td>Q:</td>
<td>Flow</td>
</tr>
<tr>
<td>r:</td>
<td>Radius</td>
</tr>
<tr>
<td>R:</td>
<td>Resistance</td>
</tr>
<tr>
<td>r2:</td>
<td>Correlation coefficient</td>
</tr>
<tr>
<td>RBC:</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>SNAP:</td>
<td>S-nitroso-n-acetylpenicillamine</td>
</tr>
<tr>
<td>SNS:</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>TA:</td>
<td>Transverse arteriole</td>
</tr>
<tr>
<td>VSM:</td>
<td>Vascular smooth muscle</td>
</tr>
<tr>
<td>XAC:</td>
<td>Xanthine amine congener</td>
</tr>
<tr>
<td>( \eta ):</td>
<td>Viscosity</td>
</tr>
<tr>
<td>( \mu )NOS:</td>
<td>Skeletal muscle specific nitric oxide synthase</td>
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Chapter 1. Introduction

1.1 Introduction of Active Hyperaemia

William Gaskell (1877) (Gaskell, 1877) first described the relationship between blood flow and skeletal muscle after observing that muscle contraction caused an increase in the magnitude of blood flow to stimulated muscle. This phenomena, in which increased tissue metabolism causes an increase in blood flow is now referred to as active hyperaemia. More than a century after Gaskell's initial observation, research has better characterized this relationship. By measuring the rate of blood flow in response to increases in various indices of metabolic rate (for example; contraction frequency (Goodman et al., 1978), O\textsubscript{2} uptake (Goodman et al., 1978; Kalliokoski et al., 2005) and running speed (Laughlin & Armstrong, 1982; Copp et al., 2010)) has revealed that rate of blood flow and skeletal muscle metabolism are linearly related (Figure 1).

Consequently, this evidence provides an indication that skeletal muscle vasculature has the capacity to mediate increases in flow to precisely match the heightened metabolic activity of a tissue. From a physiological standpoint, the hyperaemic response allows an increase in blood flow through the exchange vasculature in order to match O\textsubscript{2} demand of working muscle. The importance of which is demonstrated by the fact that an imbalance between O\textsubscript{2} supply and skeletal muscle demand results in a decrease in tissue function (Gorman et al., 1988; Stainsby et al., 1990; Hogan et al., 1994). Although initially observed in skeletal muscle, the active hyperaemic response has also been observed in, but not limited to, cerebral (Sciotti et al., 1993; Harder et al., 1998; Filosa & Iddings, 2013), cardiac (Knabb et al., 1984; Randall & Jones, 1985) and ocular (Riva et
al., 2005; Pournaras et al., 2008; Kur et al., 2012) tissue. While this response occurs in different tissue beds, active hyperaemia is exemplified in skeletal muscle as the rapid changes in the tissue’s metabolic rate, from rest to intense and prolonged periods of activity, can cause up to a 100-fold increase in the rate of flow (Andersen & Saltin, 1985; Casey & Joyner, 2011). Thus, the capacity to alter blood flow to match O₂ demand within a rapid, seconds, timeframe, highlights the highly responsive and dynamic nature of the skeletal muscle vasculature, therefore making it an ideal and widely used model to study active hyperaemia (Clifford & Hellsten, 2004; Korthuis, 2011). It is important to note the active hyperaemic response does not just allow for an increase in bulk blood flow within a tissue, as studies investigating the regional distribution of flow in the microvascular network have found that during contraction, increases in flow are coordinated to be specifically directed to the capillaries associated with working muscle fibers (Berg et al., 1997). This observation indicates that the microvasculature actively functions to coordinate and distribute flow specifically to metabolically active cells within a tissue. Collectively, as the vasculature is able to match blood flow in response to changes in tissue metabolism, coupled with its ability to specifically coordinate regional increases in flow underlines the high degree of regulation, precise control and coordination that exists between skeletal muscle and its associated vasculature.
Figure 1. Changes in blood flow in response to increases in various indices of metabolism. A) The rate of canine hind limb blood flow in response to heightened rate of muscle stimulation (Goodman et al., 1978)  B) Canine hind limb blood flow plotted as a function of \( O_2 \) uptake at rest (•), during muscle stimulation (x) and in response to cold temperatures (O) (Goodman et al., 1978). C) Rate of blood flow to rat thigh muscles in response to increases in running speed (Laughlin & Armstrong, 1982).

1.2 Control and coordination of blood flow to capillaries in skeletal muscle during increased metabolism.

During periods of both rest and activity, blood flow through the capillary beds needs to be precisely coordinated in order to match flow with the metabolic rate of skeletal muscle cells. During periods of quiescence, skeletal muscle demand for blood flow is low in comparison to periods of activity (Andersen & Saltin, 1985; Jasperse & Laughlin, 2005; Casey & Joyner, 2011). Thus, at rest, capillary perfusion density is minimal (with only a fraction of all skeletal muscle capillaries being actively perfused) and rate of blood flow through the capillaries is
relatively slow (Krogh, 1919; Honig et al., 1982; McDonagh et al., 1982; Dawson et al., 1987; Kindig et al., 2002; Krix et al., 2005). However, upon the onset of contraction, when skeletal muscle metabolism is heightened and the demand for O₂ increases there is an increase in capillary perfusion density as flow becomes directed to previously unperfused capillaries associated with active muscle fibers (Krogh, 1919; Honig et al., 1980, 1982; Lindbom & Arfors, 1985; Sweeney & Sarelius, 1989), in addition to a substantial increase in the rate of capillary blood flow (Hudlická et al., 1982; Hargreaves et al., 1990; Brown et al., 2005). These changes in capillary hemodynamics, collectively known as capillary recruitment function to 1) increase the magnitude of flow to active tissue(s) and 2) increase surface area, allowing for an enhanced O₂ exchange between vasculature and muscle (Klitzman et al., 1982; Weber et al., 2006).

Capillary recruitment, specifically the increase in capillary perfusion density during increased muscle activity, was initially characterized by August Krogh (1919). By contracting skeletal muscle after a dye had been intravenously injected, Krogh fixed tissue sections and counted the number of dye filled capillaries, which he presumed were actively perfused. Comparing stimulated muscle sections to unstimulated muscle sections, Krogh estimated that muscle contraction caused a 3-6-fold increase in the number of capillaries perfused. While Krogh himself admitted that his collected data was only semi-quantitative, the relationship between increased capillary perfusion and increased muscle metabolism he described has been confirmed by numerous studies (Honig et al., 1980, 1982; Hudlická et al., 1982; Sweeney & Sarelius, 1989; Klitzman et al.,
As mentioned, the rate of blood flow through capillaries of skeletal muscle is also correlated with increases in muscle metabolism (Hudlická et al., 1982; Hargreaves et al., 1990; Brown et al., 2005). However, the reported change in blood flow rate during rest and contraction has been shown to differ depending on muscle fiber type and study conditions (i.e. contraction parameters, methodology of measuring flow, species tested) (Laughlin & Armstrong, 1983; Laughlin, 1987; Hudlicka, 2011; Horiuchi et al., 2014).

With the increase in blood flow velocity and capillary perfusion during contraction, Delashaw & Duling (1988) aimed to better characterize the hemodynamic changes occurring during skeletal muscle activity. Using both pharmacological and physiological stimuli to alter capillary perfusion density as a means to identify potential perfusion patterns, it was found that in response to muscle contraction capillaries were recruited in groups or modules, rather than individually. Later research by Berg and Sarelius (1995), who investigated modular capillary recruitment in other skeletal muscle tissue beds, found that the number of modules perfused during contraction differed from what was reported by Delashaw and Duling (1988), suggesting that modular capillary recruitment may be dependent on the skeletal bed capillaries are embedded within. Together these studies demonstrate that heightened metabolic activity leads to the recruitment of capillaries in groups or modules rather than individually, which presumably functions as a means to rapidly increase regional blood flow to skeletal muscle during contraction.
Despite variations within the literature regarding hemodynamic alterations at the level of the capillary during contraction, be it the difference in reported rate of blood through the capillaries or the absolute number of capillary modules perfused during contraction, the increase in capillary recruitment ultimately work in conjunction to enhance tissue perfusion and adequately match \( O_2 \) delivery to skeletal muscle during changes in skeletal muscle activity.

Due to the lack of vascular smooth muscle (VSM), capillaries are unable to alter their luminal diameter, and therefore their state or magnitude of perfusion. Thus, it is the changes in the diameter of upstream resistance vasculature that both dictate and drive changes in flow at the level of the capillary during periods of rest and activity. With upstream resistance arterioles being largely responsible for the changes in capillary blood flow, it is important to understand the parameters that influence flow at the arteriolar level. Equation [1] describes the relationship between blood flow (Q), pressure (P) and resistance (R) showing that flow is a function of both pressure differential and resistance within an arteriole (Skimming et al., 1997). Under normal physiological conditions, perfusion pressure remains relatively constant, functioning within tight physiological limits, thus it is the changes in vascular resistance that determine alterations in flow (Skimming et al., 1997; Korthuis, 2011). Further, vascular resistance is dependent upon 3 variables 1) tissue vascularization (length of the vessel) 2) blood viscosity and 3) vascular radius (Pries et al., 1996). Normally, vascularization and viscosity remain relatively consistent over time, having a minimal impact on minute-to-minute changes to the resistance of blood flow. Thus, it is the changes
in luminal radius \( r \), via the contraction or relaxation of VSM that determine the degree of vascular resistance and therefore blood flow. Equation [2] describes how resistance is proportionally related to tissue vascularization \( L \), blood viscosity \( \eta \) and inversely proportional to radius.

\[
[1] \quad Q = \frac{P_1 - P_2}{R}
\]

\[
[2] \quad R \propto \frac{8\eta \cdot L}{\pi r^4}
\]

It is important to note that the denominator, radius, is to the fourth power, thus small changes in vessel radius can translate into large changes in vascular resistance, thereby having a significant influence in changing blood flow. As alterations of vascular resistance within the arterioles of the microcirculation are responsible for the coordination and delivery of blood to capillary modules it is important to discuss factors that influence arteriolar radius of skeletal muscle vasculature during both periods of rest and activity.

1.3. Influences of vascular tone at rest

In resting skeletal muscle the VSM of resistance microvasculature is in a partially constricted state, referred to as basal vascular tone. Vascular tone at rest is the product of multiple contributors, all of which influence the degree of VSM contraction. Currently it is thought that central (neural) input exerts a potent vasoconstriction contribution to basal tone where as factors such as sheer stress and the tonic production of metabolic factors from skeletal muscle contributes a vasodilatory influence. Additionally, factors such as the myogenic response are
unique in the fact that they can cause dilation or constriction depending on vascular wall pressure differentials. With all these factors having different effects on the vasculature it is ultimately a functional interplay between vasodilating and vasoconstricting factors that determines the state of VSM contraction and therefore luminal diameter at rest. The balance of each act in conjunction to provide arteriolar resistance and facilitate the delivery of minimal yet sufficient blood flow to the capillaries supplying resting muscle, while preserving the ability of the vasculature to alter its diameter in the presence of other vasoactive factors.

**Wall Sheer Stress** - Endothelial cells (EC) comprise the inner surface of all blood vessels and are the interface between red blood cells (RBCs) and the VSM. Due to the direct contact between EC and RBCs, EC’s experience a frictional force produced by the laminar flow of blood along the inner lumen of the vessel, which is known as shear stress (Koller et al., 1993; Davies, 1995; Stepp et al., 1999). Shear stress has been shown to trigger the release of vasoactive factors. (Koller & Kaley, 1990, 1991; Koller et al., 1993, 1994, 1995; Rodbard, 1966; Melkumyants et al., 1987) which has been observed in both the macro- and micro- circulation and is thought to aid in ensuring adequate delivery of blood flow during periods of skeletal muscle activity and quiescence (Bevan & Laher, 1991; Clifford et al., 2010). As the endothelium has been shown to be obligatory in the modulation of arteriolar diameter during changes in shear stress (Furchgott & Zawadzki, 1980; Hull et al., 1986; Pohl et al., 1986; Rubanyi et al., 1986; Koller & Kaley, 1991) it is currently believed the endothelium is the origin of vasoactive factors released during changes in shear stress, perhaps explaining why its
absence abolishes any changes in vascular diameter when shear stress is altered. This observation provides clear evidence, which implicates its necessity in facilitating and translating changes in shear stress into changes in vessel diameter (Messina et al., 1977; Campbell et al., 1996; Pohl & de Wit, 1999). Mechanistically, there is no generally accepted model of how the vasculature is able to sense changes and shear. However there is some evidence to suggest that the changes in shear are sensed by mechanoreceptors in the vessel wall which trigger a signaling cascade leading to the release (or increased release) of vasoactive factors from the endothelium, which diffuse and act on adjacent VSM causing vasodilation (Davies, 1995; Clifford et al., 2010).

Despite the uncertainty of how the vascular wall can sense change in shear stress, investigations have identified and implicated nitric oxide (NO), prostacyclin (PGL2) and endothelial-derived hyperpolarizing factor (EDHF), as the major vasoactive factors released by the endothelium during shear stress (Messina et al., 1977; Pohl et al., 1987; Cooke et al., 1991; Campbell et al., 1996; Duffy et al., 1998; Miura & Gutterman, 1998; Pohl & de Wit, 1999; Stoner et al., 2012). NO has been shown to play a prominent role in mediating basal tone as studies antagonizing nitric oxide synthase (NOS), the enzyme responsible for NO’s synthesis, have observed a decrease vessel luminal diameter at rest (Wilson & Kapoor, 1993; Gilligan et al., 1994; Doshi et al., 2001). Thus, it stands to reason that NO is tonically released at rest, and involved in maintaining adequate blood flow to skeletal muscle during periods of non-contraction. PGL2 has also been shown to be an important component of the shear stress response as the
blockade of cyclooxygenase (COX), responsible for the formation of PGI$_2$, has been found to reduce resting blood flow in the brachial artery of the forearm in humans, however as blood flow was the primary parameter measured while the mechanism(s) behind how the change in flow occurred were not investigated we can only presume that the decrease arterial flow can be attributed to a decrease in luminal diameter (Duffy et al., 1998). Thus, like NO, PGI$_2$ seems to be tonically released and necessary in maintaining adequate blood flow during periods of non-contraction. EDHF has also been implicated in mediating basal tone, however due to the fact that the mechanism(s) or factor(s) responsible for eliciting EDHF dilation have not been identified makes it difficult to define its relative contribution in the regulation of resting blood flow (Stoner et al., 2012). Despite EDHF’s poor characterization, it is known that its relative role in the shear stress response is dependent on vascular branch order, playing a more prominent role in smaller blood vessels as opposed to larger vessels, as such its effect(s) may be an important factor in the regulation of microvascular tone while not being as prominent in the vessels of the macrovasculature (Urakami-Harasawa et al., 1997). Further research needs to be conducted to better understand EDHF’s influence and impact on basal vascular tone at each vascular branch order.

As blood vessels are constantly experiencing shear stress, there is presumably, a tonic release of NO, PGI$_2$ and EDHF at rest. As each are able to modulate vascular resistance it stands to reason they all likely contribute to basal tone during periods of non-contraction in skeletal muscle. As these 3 vasodilators are thought to be present contemporaneously during periods of rest, research has
investigated the possibility that each of them may interact, or influence one another’s vasodilatory ability. Currently there is evidence to suggest that NO may act to partially inhibit the release of PGI$_2$ (Osanai et al., 2000) and that the effect of EDHF on the vasculature becomes more prominent when NO release is antagonized, suggesting NO may act to inhibit or attenuate the contribution of EDHF to basal tone (Cohen & Vanhoutte, 1995). Taken together these observations suggest a redundant system may underlie shear stress regulation of tone in which flow can be preserved at rest, despite the compromised release of another vasodilator. However, redundancy does not address disparities in the literature, more specifically why some studies have found that both NO and PGI$_2$ are necessary for the maintenance of basal blood flow (Wilson & Kapoor, 1993; Duffy et al., 1998; Clifford et al., 2010; Koller & Kaley, 1990; Koller et al., 1994), while others have reported that NO inhibits the release of PGI$_2$ (Osanai et al., 2000; Stoner et al., 2012). Perhaps these contradictory observations can be explained by the fact that NO may not completely inhibit the release of PGI$_2$ and the limited release of PGI$_2$ may have a substantial effects on regulation of blood flow at rest, so much so that when blocked there is an observable decrease in vessel diameter. Alternatively, differences in the vascular bed or the vascular branch order studied may offer another plausible explanation for the discrepancies in the literature, as the effects of shear stress on the vasculature is dependent on vessel luminal diameter and tissue composition in which the vasculature is associated with (Clifford et al., 2010; McAllister, 2003). While shear stress occurs during muscle contraction its contribution to basal tone is far more
relevant in the regulation of flow during periods of non-contraction (Clifford et al., 2010).

**Figure 2:** Possible signaling cascade in which shear stress can signal for the release of NO, PG1\_2 and EDHF to cause vasodilation of the VSMC. However, this graphic does not illustrate the potential interactions that exist between the vasodilator released. Adapted from (Clifford, 2011).

*Myogenic Response* – The myogenic response is the inherent response of the VSM to changes in transmural pressure. More specifically in response to elevations in transmural pressure, the VSM contracts, causing vasoconstriction, while reduction in pressure leads to relaxation and subsequent vasodilation. This response was initially characterized by Bayliss (1902) (Bayliss, 1902) who noted large increases in volume of the canine hind limb vasculature following aortic occlusions. Bayliss reasoned that the increase in intravascular pressure followed by the observed vessel constriction was too rapid to be mediated by local metabolite production or any other proposed vascular mediator and therefore concluded vascular tone, was, in part, dependent on the vessels response to changes in intravascular pressure. Despite being described more than a century
ago further investigations continue to support the hypothesis put forth by Bayliss and currently the myogenic response is widely considered a major contributor to resting tone of skeletal muscle vasculature (Meininger & Davis, 1992; Davis & Hill, 1999; Schubert & Mulvany, 1999). Further investigations into the role of the myogenic response in the regulation of tissue blood flow have found that the magnitude of the myogenic response is dependent on vessel luminal diameter, being more pronounced in smaller sized vessels (such as; arterioles) thereby making it a more prominent regulator in the microvasculature as opposed to larger vessels of the macrovasculature (Schubert & Mulvany, 1999). The importance of transmural pressure (and the accompanying myogenic response) in the regulation of tone is illustrated by the fact that isolated arteriole preparations do not acquire vascular tone until they are pressurized to physiological levels, after which tone develops to the same degree as comparable vessels found in vivo (Davis, 1993). While the contribution of the myogenic response in the maintenance of tone is becoming better understood, the mechanism(s) in which the myogenic response elicits its effect is still unclear. Currently it is hypothesized that the myogenic response is reliant upon mechanotransduction in order to elicit its effects. More specifically, it is thought the VSM is deformed by changes in transmural pressure, this deformation is then sensed by structural components of the cell, causing the activation of mechanosensitive ion channels which facilitate the contraction or relaxation of the VSM to maintain tone at rest (Davis & Hill, 1999).
Central (neural) input – Central (or neural) vasculture control is predominately regulated by the sympathetic nervous system (SNS), an arm of the autonomic nervous system (ANS), as an expansive network of perivascular sympathetic nerves embedded in skeletal muscle surround the microvascular network (Fleming et al., 1987; Welsh & Segal, 1997). It has been shown that during periods of non-contraction there is a relatively high basal SNS activity in nerve fibers innervating skeletal muscle. As the SNS releases the vasoconstrictor norepinephrine (NE) along with other vasoactive factors such as neuropeptide Y (NPY) and ATP, it is thought that together they contribute to the maintenance of basal vascular tone (Hodges et al., 2009). In an attempt to better comprehend the role of high SNS activity in the regulation of blood flow at rest, Rowlands et al. (1968) (Rowlands & Donald, 1968) and Donald et al. (1970) (Donald et al., 1970) upregulated SNS activity, via stimulation of the lumbar sympathetic trunk (SNS nerve bed) and assessed the alterations of blood flow in non-exercising skeletal muscle observing that increases in SNS activity leads to a net decrease in the magnitude of blood flow. Presumably, the heightened SNS activity caused an increase in NE release and a subsequent vasoconstriction in the resistance vasculature, thus decreasing blood flow. From these experiments it appears that basal SNS activity functions in maintaining tone, by increasing vascular resistance via release of NE. While there is literature (Peterson et al., 1988) that has reported that the SNS denervation (sympathectomy) had no impact on the magnitude of resting blood flow, contrary to what was hypothesized. However, this counterintuitive observation was attributed to the fact that blood flow measurements were performed on active animals, thus skeletal muscle was not
truly at rest and therefore, the apparent lack of SNS contribution to resting blood flow could be explained by the fact that moderate increases in blood flow (via the active hyperemic response of working muscle) may have masked or superseded the effect of the sympathectomy in this study. Separate studies have also assessed the vascular response after SNS denervation in rats during rest and exercise, and have shown that when SNS input is removed there is an overall increase in pre-exercise and exercise blood flow in certain muscle fiber types. More specifically, denervation caused an increase in blood flow in skeletal muscle comprised mainly of glycolytic fibers while there was an unchanged rate in flow to muscle primarily comprised of oxidative fibers (Delp & Armstrong, 1988). It was hypothesized that the differences in blood flow observed upon SNS removal were attributable to different muscle fiber types having different α-adrenergic receptor density or sensitivity to NE. Previous studies lend support to this hypothesis as it has been shown that fast-twitch muscle (comprised primarily of glycolytic muscle fibers) is more sensitive to α-adrenergic stimulation compared to slow-twitch muscle (comprised primarily of oxidative muscle fibers) (Gray, 1971; Simoneau & Kelley, 1997). Furthermore, it was shown that during α-blockade blood flow increased to fast-twitch muscle while remaining unchanged in slow-twitch suggestive of a higher α-adrenergic contribution in the maintenance of tone in fast-twitch muscle (Laughlin, 1987). As the role of SNS activity is mediated differently between skeletal muscle beds, makes it difficult to make a definitive conclusion regarding the mechanism in which SNS mediates vascular control at rest in skeletal muscle. However, in general, the primary function of the SNS is to partially constrict VSM thereby contributing to vascular tone and ensuring the
minimal yet sufficient blood supply to muscle however the extent of its role is tissue dependent.

Metabolic vasodilators – Both in vitro and in vivo studies have provided evidence that metabolically-derived vasodilators are tonically released from quiescence skeletal muscle presumably as a byproduct of basal muscle metabolism. While there are numerous metabolic vasodilators released at rest the scope of this introduction cannot specifically address the role of each putative vasodilator on basal tone. However by discussing NO, ADO and K⁺, as their concentrations have all been quantified during periods of both contraction and non-contraction (Balon & Nadler, 1994; Hellsten et al., 1998; Juel et al., 2000) will provide a general understanding of metabolic vasodilator contribution to basal vascular tone in skeletal muscle.

Prior research has shown that NO, K⁺ and ADO are present during periods of non-contraction in skeletal muscle interstitial space (ISS) in relatively small concentrations. NO has been measured at 62±3pmol/mg in in vitro skeletal muscle cells (Balon & Nadler, 1994), while ADO and K⁺ have been measured from the skeletal muscle interstitial space of humans during rest at concentrations of 220±110nmol/L, 4.19±0.09mM, respectively (Juel et al., 2000; Hellsten et al., 1998). Although the concentration of each dilator released is minimal, all are widely accepted as potent vasodilators, thus, it stands to reason that the small concentrations released at rest may be sufficient to elicit a constant, modest vasodilation, which may act to contribute to basal vascular tone. Studies utilizing
pharmacological interventions to investigate the role of metabolic vasodilators released at rest have also provided convincing evidence of their involvement in tone regulation. More specifically, exposure to antagonists of NO have been shown to decrease baseline diameter in quiescent skeletal muscle, suggesting that the tonic release of NO has a substantial contribution to the maintenance of vascular tone during periods of non-contraction (Wilson & Kapoor, 1993a; Gilligan et al., 1994; Doshi et al., 2001; et al., 2012). Antagonism of ADO, on the other hand, seems to have no effect on resting vascular tone, and in some cases its antagonism increases the magnitude of resting blood flow indicating that ADO is not a major contributor to the maintenance of basal vascular tone (Rådegran & Calbet, 2001). The contribution of K⁺ also been investigated. Numerous studies have found that altering the activity of Kᵢᵣ (Edwards & Hirst, 1988; Nichols & Lopatin, 1997; Jackson, 2000), Kᵦᵥᵯ (Jackson, 1993, 2000; Vanelli & Hussain, 1994; et al., 1995), Kᵥ (Nelson & Quayle, 1995; Jackson et al., 1997; Jackson, 1998) channels during rest effect tone, and as each of these channels influence, or are influenced by the presence of K⁺, implicates its role in basal vascular tone however the degree and magnitude of the ion’s contribution still needs to be further characterized in different vascular branch orders.

As outlined above there are a multitude of different factors all of which contribute to basal tone, however, the relative influence of each has not been fully elucidated. While individually each of these factors can impact vascular resistance, basal vascular tone is ultimately a balance between multiple inputs all
of which function together to provide adequate arteriolar resistance, ensuring that blood flow is matched to the demand of quiescent skeletal muscle.

1.4 Influences of vascular tone during skeletal muscle contraction

While the factors prevalent in the maintenance of tone during quiescence, discussed above, still exert an influence upon the skeletal muscle vasculature during contraction, there is a shift in terms of their relative influence and contribution to vascular tone upon the onset of skeletal muscle contraction. Generally speaking, during muscle contraction there is an increase in the synthesis and release of local, metabolically derived vasodilators, while the factors such as; myogenic response and shear stress and central input (SNS-mediated central vascular control) are found not to be as predominant in the modulation of tone during contraction as they were during skeletal muscle quiescence.

Wall Shear Stress – As previously discussed shear stress is a prominent contributor in the regulation of basal vascular tone, however its influence during contraction is not as well characterized. Changes in shear stress are usually mediated by alterations in blood viscosity and/or the velocity of blood flow (Koller et al., 1993). As viscosity remains relatively constant over short periods of time, it is unlikely that it would alter the magnitude of shear stress exerted on the ECs of the vasculature during muscle contraction and therefore would not contribute to the modulation of vascular resistance. Changes in rate of blood flow would also not likely contribute to the initial dilation at the onset of contraction, as velocity
would only be altered preceding changes in vascular resistance. Therefore shear stress may become more of a contributor to vascular diameter once vessel diameter has increased and blood flow and metabolism are matched (steady-state of blood flow), helping to maintain a constant luminal diameter to facilitate the supply of blood to active muscle. Changes in shear stress have been shown to cause vasodilation, known as flow mediated dilation (FMD), however investigations into the its relative role in blood flow regulation during contraction have been conflicting, making it difficult to come to a definitive conclusion about its role during active hyperaemia. Investigation by Hester (1993) into NO’s involvement in FMD found that in the microvasculature of hamster cremaster muscle, NO’s effect seemed to be dependent on the vascular branch order with NO mediating the dilation of small arterioles of the microvasculature network while having no observable effect on larger branch segment during contraction. Separate studies have dismissed endothelial derived NO as a primary mediator in active hyperaemia as its blockade has been observed not to have an impact on the magnitude of dilation during active hyperaemia in the skeletal muscle vasculature of humans (Endo et al., 1994; Shoemaker et al., 1997; Rådegran & Saltin, 1999). Additionally a study investigating the role of endothelial-derived NO and PGI₂ in canine skeletal muscle vasculature by King-Vanvlack (King-Vanvlack et al., 1995) found that both the blockade of NO alone, and in the presence of an antagonist of PGI₂, had no impact on the magnitude of blood flow during muscle contraction. Similar conclusions, that neither NO or PGI₂ seem to have a role in mediating increases in blood flow during exercise, have also been reached by numerous other studies (Endo et al., 1994; O’Leary et al., 1994; Shoemaker et
al., 1996; Wilson & Kapoor, 1993a, 1993b; Brock et al., 1998; Rådegran & Saltin, 1999; Shoemaker et al., 1997). Taken together, these findings provide convincing evidence that the active hyperaemic response observed is not primarily mediated by endothelial-derived vasodilators arising from changes in shear stress. Thus, it is apparent that the stimuli, such as shear stress, that are predominate in the maintenance and regulation of basal vascular tone may not be as influential during muscle contraction, which may be indicative of an apparent shift in the primary regulators of vessel diameter between rest and activity.

Myogenic Response – While the myogenic response is thought to be an important regulator of basal tone, evidence suggests that it does not have an equally influential effect in the regulation of blood flow during muscle contraction (Bacchus et al., 1981). It has been shown that during skeletal muscle contraction transmural pressures acting on the walls of associated muscle vasculature decreases. As previously mentioned, unidentified mechanosensors sense the drop in pressure and elicits a signaling pathway that results in vasodilation. Although the myogenic response does contribute to contraction mediated increases in muscle blood flow, pressure-induced responses play a far more prominent and arguably important role in the maintenance of tone and the regulation of blood flow during periods of non-contraction.

Neural/Systemic Control Mechanisms – As previously mentioned, in skeletal muscle vasculature the role of central vascular control is primarily mediated by sympathetic nerve activity. In order to gain insight into its role on blood flow regulation during exercise Peterson et al. (1988) (Peterson et al.,
1988) examined the influence of SNS activity on hind limb blood flow in sympathectomized rats during exercise. By ligating lumbar sympathetic nerves and observing blood flow during running exercise Peterson et al. (1988) observed there was no difference in the magnitude of blood flow between control group and sympathectomized rats with-in the first 2 minutes of exercise. However after 2 minutes, once steady-state blood flow was reached, sympathectomized rats had a sustained increase in the magnitude of muscle blood flow compared to the control group. Current research would suggest that the effects of sympathetic nerve activity are involved in the initial increases in blood flow but are overcome by local mechanisms. Observations made by Peterson (1988) provide an indication that while sympathetic nerve activity is not overly influential in mediating the initial hyperaemic response, its effects soon become apparent when steady-state blood flow is reached. The fact that removal of SNS input increases the magnitude of vasodilation in skeletal muscle vasculature may provide an indication that during exercise there are competing inputs between SNS-elicited vasoconstriction, via NE, NPY, ATP, and vasodilatory factors released during exercise. However the ability of sympathetic activation to cause vasoconstriction, via NE, is diminished in metabolically active muscle. This phenomenon, in which the vasculature, during contraction can escape the vasoconstriction produced by the SNS, is termed functional sympatholysis and presumably functions to optimize blood flow in order to meet $O_2$ demands of working muscle despite increases in sympathetic vasoconstriction during exercise. While functional sympatholysis is influenced by numerous mechanisms, local products released during increased muscle activity have been shown to
dampen the vasoconstrictive effects of the SNS during exercise. For example, a study by Victor & Thomas (1998) found that NO attenuates α-adrenergic vasoconstriction in exercising skeletal muscle, thereby decreasing the vascular resistance produced by the SNS during exercise. As NO concentration is positively correlated with increases in skeletal muscle activity, it stands to reason that NO plays an important role in blood flow regulation as heightened NO release would blunt any increase in sympathetic vasoconstriction occurring during exercise-induced increases in SNS activity. The same study also investigated if PGI₂ and ADO participate in attenuating SNS constriction during heightened muscle activity, yet findings indicate neither had an apparent role in functional sympatholysis. With NE functioning through α-receptors and the decoration of α₁-adrenoceptor and α₂-adrenoceptor on the vasculature differing depending on the vessel branch order (large resistance arterioles possessing both α₁- and α₂-adrenoceptors whereas smaller, terminal arterioles predominately being decorated with α₂-adrenoceptors) Faber & Anderson (1991) found that sensitivity of arteriolar α₁-adrenoceptor and α₂-adrenoceptor receptors differ depending on contraction parameters. The study by Faber & Anderson (1991) found that during moderate exercise intensities the metabolites released preferentially blunted α₂-mediated constriction allowing for vasodilation in smaller vasculature. At higher intensities the products released blunt α₁-mediated constriction ultimately allowing for a much larger increase in bulk blood flow in order to match the larger metabolic demand of the muscle. This study taken together with the data presented by Victor & Thomas (1998) suggests that during exercise the metabolic byproducts released can attenuate SNS-mediated vasoconstriction to not only
allow the skeletal muscle vasculature to ‘escape’ the vasoconstriction but, through preferential blunting of $\alpha_1$ and $\alpha_2$ receptors, metabolites can also cause attenuation of vasoconstriction to specifically allow for a precise increase in blood flow depending on the intensity of skeletal muscle activity.

*Metabolic Vasodilators* – Similar to the regulation of basal vascular tone, control of blood flow during skeletal muscle contraction a multitude of locally produced stimuli arising from muscle contraction, exert their action and influence vessel diameter. It has been demonstrated that active hyperaemia occurs despite being isolated from central/neural vascular control influences. Gorczynski (1978) was among the first to provide evidence to suggest that a local response may be the primary mediator in eliciting an increase in blood flow during contraction. By stimulating small bundles of skeletal muscle fibers overlying arterioles in the hamster cremaster muscle, such that the influence of FMD, myogenic response and central input were negligible, caused an increase in arteriolar diameter in the vessel segment directly associated with the stimulated muscle. This observation suggests that factors produced in the local environment during contraction can alter the skeletal muscle vasculature. Termed the metabolic hypothesis, it is currently thought that of the numerous substances released during increased skeletal muscle metabolism, some act to increase diameter in resistance vasculature providing a mechanism in which tissue metabolic state (an indices of $O_2$ requirement) can be directly communicated to supplying vasculature. Currently it is thought that metabolites from active skeletal muscle diffuse (or are released) into the interstitial space (ISS) and are able to act
on the VSM causing dilation. Furthermore, research has also identified that some vasodilators released during contraction such as, ADO can not only produce a local vasodilation but also stimulate the spread of vasodilation along the vascular tree (known as a remote or conducted response) by the conduction of hyperpolarization along the vessel wall through gap junctions (Duza & Sarelius, 2003; Tallini et al., 2007; Rivers & Frame, 1999). NO has also been shown to inconsistently elicit conducted responses, whereas conducted responses to dilatory concentrations of KCl have not yet been investigated (Kurjiaka & Segal, 1995; Doyle & Duling, 1997; Hoepfl et al., 2002; Chen & Rivers, 2001; Budel et al., 2003, Tallini et al., 2007). Thus, during muscle contraction the release of metabolic vasodilators act on multiple vessel branch orders causing both a local and conducted dilation. Together this creates an integrated vascular response in which the different stimulated vascular branch orders create specific pathways of decreased vascular resistance leading to an increase in blood flow and ultimately an increase in capillary recruitment associated with working muscle.

Further evidence has lent support to the metabolic hypothesis demonstrating that as skeletal muscle metabolism increases, there is a stepwise increase in vasoactive metabolic byproducts released into the ISS. During prolonged periods of heightened metabolism, vasodilators are constantly cycling between release and inactivation, reabsorption or wash out after diffusing into the bloodstream. Upon the cessation of skeletal muscle contraction the tissue’s metabolic activity returns to resting conditions, dilator release is decreased or stopped, the vasoactive effects of dilators action are washed out or neutralized.
and the blood vessel returns to basal diameter. This relationship, between metabolic vasodilators and the vasculature provides a local mechanism in which $O_2$ needs of skeletal muscle can be directly communicated to the vasculature, and as increases in metabolism cause the stepwise increase in vasodilator released, this allows for the precise increase in vessel diameter allowing blood flow to specifically match metabolic demands of the muscle. Additionally, when contracting muscle transitions to periods of rest the effects of vasodilators released quickly diminishes, vessel diameter decreases and blood flow matches basal metabolic rates.

There has been a long history of investigations attempting to identify a single dilator that is responsible (or necessary) in mediating the active hyperaemic response. While many different metabolites such as; potassium ($K^+$), adenosine (ADO), nitric oxide (NO), prostanoids (PG), adenosine triphosphate (ATP) among others have been hypothesized to contribute to the hyperaemic response, studies have demonstrated that despite blocking the release or synthesis of these individual vasodilators, the hyperaemic response remains largely intact. These observations suggest that there is not one specific metabolic vasodilator necessary to facilitate hyperaemic response during contraction. However, the inhibition of a dilator having no impact on the magnitude of vasodilation does not infer its lack of importance, but may hint that upon inhibition, or loss of effect of a vasodilator, the release of other dilators may be up-regulated, or a new dilator may be released in order to compensate (Hellsten et al., 2012). Therefore, it stands to reason there may be multiple dilators and
dilatory pathways that converge, or interact to create a compensatory mechanism during skeletal muscle activity in order to preserve blood flow upon the loss or reduced release of another dilator. However, despite the lack of any one vasodilator being necessary in mediating active hyperaemia the increased presence of some vasodilators during muscle contraction may be indicative of their significance in flow regulation during contraction.

Potassium (K⁺) – K⁺ has been implicated in mediating active hyperemia during exercise (Hirche et al., 1980; Wilkerson et al., 1982; Kiens et al., 1989; Lott et al., 2001). Investigations into K⁺’s role in blood flow regulation found that the ion can elicit both vasodilation and vasoconstriction depending upon its concentration. Dawes (1941) (Dawes, 1941) was one of the first to describe the duality of K⁺, after observing that the perfusion of small concentrations of KCl in the canine hind limb vasculature caused vasodilation, whereas higher concentrations elicited substantial vasoconstriction. The primary origin of extracellular K⁺ is thought to be from the opening of voltage-dependent potassium (Kᵥ) channels in active muscle fibers during the repolarization stage of the action potential, in which the K⁺ fluxes outward into the ISS (Armstrong et al., 2007). Once in the ISS it elicits its effects via the alteration in inwardly rectifier potassium (Kᵢᵣ) channels and Na⁺/K⁺-ATPase (also known as the Na⁺/K⁺ pump) activity on the VSM. Low K⁺ concentrations (10-20mM) cause an increase in the activity of the Na⁺/K⁺-ATPase of VSM. As the pump causes a net extrusion of K⁺ ions this leads to a net negative charge within the cell, thus the increase in activity causes a substantial hyperpolarization relaxing the VSM and consequently eliciting a
vasodilation (Nelson & Quayle, 1995; Quayle et al., 1997; Haddy, 1983; Burns et al., 2004). K⁺ also alters the gating properties of Kᵢᵣ channels. More specifically, the presence of relatively small increases in [K⁺]ₒ leads to changes in the current-voltage relationship relative to K⁺ equilibrium potential (E_K), translating into an increase in outward current of K⁺ through Kᵢᵣ channels, leading to VSM hyperpolarization and relaxation followed by a vasodilation (Nelson & Quayle, 1995; Quayle et al., 1997; Loeb et al., 2000; Burns et al., 2004). Since the effects of K⁺ on the vasculature are concentration dependent, studies by Juel et al. (Juel et al., 2000), investigated the concentrations of K⁺ present in the skeletal muscle ISS in humans during exercise. By measuring interstitial K⁺ concentration via microdialysis, after different intensities of one-legged knee extensor exercises, it was found that K⁺ concentrations are correlated with intensity of muscle contraction, with up to 9mM K⁺ accumulating in the ISS of skeletal muscle after intense bouts of exercise. Further studies characterizing the role of K⁺ found that upon the initiation of exercise there is an extremely rapid efflux of K⁺ that accumulates in the ISS, perhaps suggesting the ion could be a key initiator of active hyperaemia at the onset of exercise (Scott et al., 1970; Hník et al., 1973; Mohrman & Sparks, 1974; Vyskocil et al., 1983; Naik et al., 1999; Tschakovsky et al., 1996). As dilatory concentrations of K⁺ are present in the ISS during exercise, it provides support of potassium’s role as a vasodilator in active hyperaemia (Vyskocil et al., 1983; Juel et al., 2000; Lott et al., 2001). Upon the cessation of contraction there is an increased K⁺ concentration in venous blood indicating that K⁺ is primarily removed by diffusion from the ISS into the blood stream (Hník et al., 1973).
Adenosine (ADO) – ADO is widely regarded as a potent metabolically derived vasodilator shown to be involved in the modulation of cardiac (Knabb et al., 1984; Edlund & Sollevi, 1995; Mustafa et al., 2009; Wilson & Mustafa, 2009) and cerebral (Dirnagl et al., 1994; Phillis, 2004; O'Regan, 2005) vasculature and is viewed as one of the primary regulators of skeletal muscle blood flow. Evidence for ADO’s role in active hyperaemia comes in the form of numerous observations that implicate its involvement such as; 1) ADO receptors have been identified on VSM and ECs (Proctor & Duling, 1982; Lynge & Hellsten, 2000; Wunsch et al., 2000; Lynge et al., 2001), 2) ISS ADO concentration are correlated with exercise (Hellsten et al., 1998; Costa et al., 2000), 3) enzymes responsible for ADO synthesis are up-regulated during contraction (Rådegran & Hellsten, 2000), 4) ADO antagonists attenuate the magnitude of vasodilation during exercise (Tabaie et al., 1977; Proctor, 1984; Poucher et al., 1990) and 5) perfusion of ADO and its precursors, AMP, have been shown to increase blood flow in skeletal muscle (Patterson & Shepherd, 1954). Taken together this provides convincing evidence for ADO as a prominent mediator of the hyperaemic response. The predominant sources of extracellular ADO have been identified as vascular ECs (Nees et al., 1985; Deussen et al., 1986; Hellsten & Frandsen, 1997) and skeletal muscle (for review see (Marshall, 2007)), however, there is also evidence suggesting that nerves may also be a potential source of ADO (Cunha & Sebastião, 1993). ADO is not directly released into the ISS but instead it is thought that ADO precursors (ATP, AMP, cAMP and ADP) are released during muscle contraction and then synthesized into ADO (Lo et al., 2001; Cheng et al., 2000; Mo & Ballard et al., 2001; Hellsten et al., 1998). More specifically ATP is degraded by ecto-ATPases
and ADPases into adenosine monophosphate (AMP), leading to an increase in $[\text{AMP}]_0$. There is also evidence to suggest cyclic adenosine monophosphate (cAMP) is released from skeletal muscle and is degraded into AMP, contributing to the rise in $[\text{AMP}]_0$. Once in the ISS AMP is converted into ADO via 5’-ecto-nucleotidase (5’-NUC). There has been debate over the location of ADO synthesis (is it formed intracellularly and translocated into the ISS or is it formed in the interstitium). However infusion of ADO precursor, AMP into muscle ISS was observed to cause an increased accumulation of ADO, providing evidence of an extracellular pathway of ADO synthesis (Frandsen et al., 1998). There is also evidence of an intracellular pathway for the production of ADO, which is then translocated extracellularly, however it is thought that the vast majority of extracellular ADO is synthesized extracellularly (Radegran et al., 2000, Deussen et al., 1999). Evidence has shown that ADO is involved in eliciting vasodilation almost immediately upon the onset of contraction (Ross et al., 2013). Thus the extracellular synthesis of ADO offers a plausible explanation of how ADO concentrations can rapidly increase, if ADO synthesis was primarily intracellular there would presumably be a lag between onset of contraction and the dilatory action of ADO, as it would need to be synthesized and translocated to the ISS in sufficient concentrations before its effects eliciting its effects. Once present in the ISS of skeletal muscle, ADO elicits vasodilation by binding onto purinergic receptors on VSM, specifically binding onto receptor subtypes $A_1$, $A_{2A}$ and $A_{2B}$, in humans (Lynge & Hellsten, 2000). This binding initiates a G-coupled signaling cascade, stimulating adenylyl cyclase (AC) and increasing cAMP, causing the increase in protein kinase A (PKA). This in turn, causes a decrease in calcium-
calmodulin kinase needed for the phosphorylation of myosin vascular smooth muscle contraction, resulting in the relaxation of the VSM and subsequent vasodilation (Herlihy et al., 1976; Silver et al., 1984; Olah, 1997; Sato et al., 2005; Olsson & Perason, 1990).

Like K⁺, ADO concentrations in skeletal muscle ISS are correlated with muscle metabolism, however it was not until recently that relative concentrations of ADO in the ISS at rest and during exercise were quantified. Hellsten (1998) (Hellsten et al., 1998) used microdialysis to measure ADO concentrations and observed that graded intensities of knee extensor exercise caused a step-wise increase of ADO concentration in the ISS, indicating a correlation between ISS ADO concentration and exercise intensity. More specifically, resting concentrations of ADO have been measured at 2.2x10⁻⁷ mol/L rising to 1.14x10⁻⁶ mol/L upon initiation of light exercise. Furthermore, ADO precursors ADP, ATP and AMP have been measured in the ISS both at rest and during exercise and ISS concentrations of the precursors also increased as a resultant of heightened exercise, further supporting the assertion that ADO synthesis occurs in the interstitium (Morstensen et al., 2009; Li et al., 2003; Hellsten et al., 1998). Similar concentrations of ISS ADO to what was reported by Hellsten (1998) have also been found in the ISS of different skeletal muscle beds with resting concentrations of 2.3x10⁻⁷ mol/L rising to 8.2x10⁻⁷ mol/L during mild intensity forearm exercise (Costa et al., 2000). Despite evidence suggesting that ADO is a prominent putative vasodilator responsible for the regulation of blood flow (Tabaie
et al., 1977; Proctor & Duling, 1982; Metting et al., 1986; Poucher et al., 1990; Rådegran & Calbet, 2001), there has been evidence suggesting that it is not necessary in the regulation of blood flow (Honig & Frierson, 1980; Klabunde et al., 1988; Koch et al., 1990; Schwartz & McKenzie, 1990). These contradictory results suggest that there is insufficient evidence that any one vasodilator necessary in mediating the active hyperaemic response

**Nitric Oxide (NO)** – While there is substantial evidence implicating NO as a key player in the maintenance of basal vascular tone, its role during active hyperaemia is not as clearly defined. In skeletal muscle NO is formed by the hydrolysis of L-arginine into NO and L-citrulline by the catalytic enzyme nitric oxide synthase (NOS). While there are different isoforms of NOS, both neural NOS (nNOS) and skeletal muscle specific NOS (µNOS) are present within the cytosol and sarcolemma of skeletal muscle (Walford & Loscalzo, 2003; Stamler & Meissner, 2001). After the synthesis, NO rapidly diffuses into the extracellular space then into vascular smooth muscle cells. NO then binds to and activates guanylyl cyclase (GC), which catalyzes the dephosphorylation of guanine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP). The increase in activity of GC translates into an intracellular increase in cGMP, which serves as a secondary messenger signaling for smooth muscle relaxation (Cohen, 1995; Walford, 2003; Persson; 1991). Specifically cGMP induces vascular smooth muscle relaxation by 1) activating K⁺ channels causing hyperpolarization, 2) stimulating cGMP-dependent kinase that activates myosin light chain kinase phosphatase which dephosphorylates myosin light chain kinase leading to
relaxation and 3) increasing cGMP inhibiting calcium entry into the cell and decreasing intracellular calcium concentration (Cohen & Vanhoutte, 1995; Walford & Loscalzo, 2003; Persson, 1991; Archer et al., 1994).

There is considerable evidence suggesting that muscle contraction promotes increased synthesis of skeletal muscle derived NO, which then diffuses into the extracellular space (Tidball et al., 1998; Silveira et al., 2003). However, despite these findings, there have been conflicting studies as to the relevance of NO’s release during heightened metabolism. Some studies have reported that pharmacologic NOS antagonists such as L-NMMA and L-NAME have significantly reduced muscle blood flow at rest and post exercise (Wilson & Kapoor, 1993b; Shoemaker et al., 1997; Endo et al., 1994; Rådegran & Saltin, 1999), but had no affect during exercise, while other studies have shown that blood flow is affected by NOS antagonism during exercise (Gilligan et al., 1994; Duffy et al., 1999; Doshi et al., 2001). These differences in findings are thought to be due in part to the methodology used for measurement of blood flow (Rådegran & Hellsten, 2000; Clifford & Hellsten, 2004). The attenuation of active hyperaemic response during exercise is able to be achieved when NOS is applied along with COX, suggesting that perhaps a different NO-independent pathway is able to account for the blockade of the NO-pathway suggesting that a redundant system is active during situations in which NO release is compromised.
**Figure 3.** Simplified schematic of how vasodilators potassium, nitric oxide and adenosine elicit vasorelaxation. A) nNOS catalyzes the conversion of L-arginine (not shown) to NO, which diffuses into VSM, increasing the activity of GC through different signaling pathways (not shown) decreases the intracellular concentrations of calcium, causing vasodilation. Potassium is released from the skeletal muscle, the increase of extracellular concentration changes channel properties of K\(_i\)R channel and Na/K ATPase, causing potassium to flux out of the cell causing a more negative membrane potential, cellular hyperpolarization, which causes vasodilation. Membrane bound 5'NUC catalyzes the conversion of AMP into ADO, ADO then activates AC and through different signaling pathways decreases intracellular calcium causing vasodilation. Adapted from (Clifford & Hellsten, 2004.)

1.5 Multiple metabolic vasodilators are released under different muscle contraction parameters

As skeletal muscle activity causes the release (or increased release) of metabolic vasodilators that act on the vasculature to proportionally match blood flow with metabolic rate, it would be logical to presume that changes in metabolism would alter the amount and type of vasodilator produced and released. In order to gain insight into the relationship between skeletal muscle metabolism and metabolic vasodilators released, Dua et al. (2009) used different stimulation parameters to alter muscle metabolism and determine the contribution of NO, ADO and K\(^+\) to arteriolar dilation under different stimulus and contraction...
frequencies. Results indicated that, while numerous vasodilators impact arteriolar
diameter, their relative importance and contribution in eliciting dilation is
dependent upon the parameters in which muscle is stimulated.

Further, the data showed that NO is an active vasodilator at lower
contraction frequencies but not at higher frequencies. However, since NO has
been shown to be positively correlated with increases in metabolism, we would
expect NO to be released at all contraction frequencies. Therefore, between low
and high contraction frequencies it seems that the vasodilatory role of NO
changes.

When the same experiment was repeated, but a source of K+ was
inhibited via application of 3,4-diaminopyridine (DAP), an antagonist of voltage-
gated K+ channels, there was no significant alteration in the magnitude of dilation
at lower contraction frequencies; however there appeared to be an attenuation of
dilation higher frequencies. These findings suggest that K\textsuperscript{+} release via K\textsubscript{v}
channels does not play a role in dilation at low contraction frequencies, but
becomes a key dilator at higher frequencies.

Alternatively, when stimulus frequencies were altered as contraction
frequency was held constant, ADO was shown to be an active vasodilator at
lower stimulation frequencies but not at high. ADO in the ISS has been shown to
be positively correlated with an increase in metabolism, thus we would expect
ADO to be released at all stimulus frequencies. Therefore, ADO acts as a
significant vasodilator at lower stimulus frequencies but becomes less effective at higher frequencies.

During different contraction and stimulation parameters, only some of the tested vasodilators appear to play a role in significantly contributing to vasodilation even though they should all be present under all contraction conditions. One hypothesis explaining this observation is the increased concentration of one vasodilator during contraction may blunt or attenuate the effect of the other vasodilators.

In the field of active hyperaemia research there has been a significant focus on trying to identify a single vasoactive compound that is obligatory in mediating the hyperaemic response. Pharmacological interventions to block the effects of different putative dilators during heightened metabolism have mostly been used to assess the relative role of a particular vasodilator during active hyperaemia. However, there has been no substantial evidence to implicate any dilator required in mediating active hyperaemia. With a distinct lack of evidence of an obligatory vasodilator, an alternative hypothesis would be that during heightened tissue metabolism, active hyperaemia is the result of multiple dilators interacting with one another in such a way that vasodilators are able to compensate for one another. The new focus is the idea of redundancy among metabolic vasodilators whereby, when the release of one vasodilator is blunted or inhibited, another vasodilator action can be elicited, which would explain why little to no change is seen during the blockade of individual vasodilators. Thus it is
possible that, under normal physiological conditions, within the milieu of vasodilators one or more are actively contributing to vasodilation while also exerting an inhibitory effect on released dilator(s) present. This type of interaction between vasodilators could function in such a way that in the event that a primary dilator that contributes to vasodilation and attenuation of other dilators is either inhibited or blunted, could allow the dilator being attenuated to exert its action to compensate for the loss of the other’s effect. From a physiological standpoint the principle of redundancy would ensure adequate blood flow to tissue even during the event that the release of another vasodilators is inhibited, impaired or blunted.

Therefore, we sought to test whether redundancy occurs between vasodilators. Since it is known that K\(^+\), ADO and NO are contemporaneously released during muscle contraction. Coupled with the data presented in Dua (2009), which provides evidence of potential interactions between these three vasodilators, make them the ideal candidates to test whether metabolic vasodilators produced during skeletal muscle contraction influence one another’s ability to elicit vasodilation. Additionally this may also provide preliminary evidence for redundancy within the active hyperaemic response.

1.6 Aims

**Aim 1:** To determine if the vasodilators; K\(^+\), ADO and NO affect one another’s vasodilatory ability.

To address this aim 6 separate experimental protocols were designed. The aim of each was to identify if the magnitude of vessel dilation caused by the
application of NO, ADO and K\textsuperscript{+} alone was altered when applied in the presence of one another. The results would provide an indication a) if interactions occur between the 3 tested dilators and b) which combination of vasodilators interact with one another.

Protocols of Aim 1:

**Aim 1.1:** Assessing if NO, ADO and K\textsuperscript{+} affect one another’s dilatory potential when vasodilators are added sequentially.

1. Quantifying K\textsuperscript{+}’s effect on the vasodilatory ability of NO
2. Quantifying NO’s effect on the vasodilatory ability of K\textsuperscript{+}
3. Quantifying ADO’s effect on the vasodilatory ability of NO
4. Quantifying NO’s effect on the vasodilatory ability of ADO
5. Quantifying K\textsuperscript{+}’s effect on the vasodilatory ability of ADO
6. Quantifying ADO’s effect on the vasodilatory ability of K\textsuperscript{+}

**Aim 1.2:** Assessing if NO, ADO and K\textsuperscript{+} affect one another’s vasodilatory potential when added simultaneously

7. Determining if the attenuation of NO in the presence of 10mM KCl occur upon simultaneous addition of one another
8. Determining if the attenuation of ADO in the presence of 10mM KCl occur upon simultaneous addition of one another
9. Determining attenuation of ADO in the presence of NO occurs upon simultaneous addition of one another

**Aim 2:** To determine if antagonism of inward-rectifier potassium ion channel (K\textsubscript{IR}) and sodium/potassium pump (Na\textsuperscript{+}/K\textsuperscript{+}-ATPase), amplifies, or rescues, the attenuated dilatory effects of NO and ADO when added in the presence of KCl.
Protocols of Aim 2:

Aim 2.1: Determining if $K_{IR}$ channels and $Na^+/K^+$-ATPase are involved in the dilatory pathway of $K^+$
10. Quantifying the impact of a $K_{IR}$ channel antagonist, barium chloride ($BaCl$), on the vasodilatory effect of 10mM KCl
11. Quantifying the impact of a $Na^+/K^+$-ATPase antagonist, ouabain (Oua) on the vasodilatory effect of 10mM KCl

Aim 2.2: Assessing how the antagonism of $K_{IR}$ and $Na^+/K^+$-ATPase effect the vasomotor response elicited by NO and ADO when added in the presence of 10mM KCl
12. Quantifying how antagonism of $K_{IR}$ channel with BaCl effects the magnitude of attenuation of NO in the presence of 10mM KCl
13. Quantifying how antagonism of $Na^+/K^+$-ATPase with Oua effects the magnitude of attenuation of NO in the presence of 10mM KCl
14. Quantifying how antagonism of $K_{IR}$ channel with BaCl effects the magnitude of attenuation of ADO in the presence of 10mM KCl
15. Quantifying how antagonism of $Na^+/K^+$-ATPase channel with Oua effects the magnitude of attenuation of ADO in the presence of 10mM KCl
Chapter 2. Methods

2.1 Ethical Approval

All experiments were approved by the Institutional Animal Care Committee Review Board at the University of Guelph and were conducted in accordance with the guidelines of the Canadian Council on Animal Care (CCAC).

2.2 General Protocol: Preparing the cremaster muscle for experimentation

Stock sodium pentobarbital (54.7mg/ml) (Ceva Stané Animale, France) was diluted in 0.9% saline and a weight-dependent (70mg/kg) loading dose was administered to adult male golden Syrian hamsters (88g – 168g) via intraperitoneal injection. Once in the surgical plane of anesthesia a tracheotomy was performed. Subsequently, a polyethylene catheter was inserted into the left femoral vein allowing for infusion of supplemental pentobarbital anesthetic throughout the surgery. The animal was placed on an acrylic glass platform and convective heat transfer via the circulation of warmed water through tubing secured under the animal helped to maintain a constant esophageal temperature of approximately 37.0°C throughout each experimental protocol. The right cremaster was prepared for in situ microscopy as described in Baez (1973) and Murrant (2000). Briefly, a lateral longitudinal cut was made in the scrotum, fascia and connective tissue were removed from the cremaster, the muscle was then cut longitudinally and separated from both the epididymis and testis. After separation, the testis was pushed into the abdominal cavity. The cremaster muscle was spread over a semicircular silicon plate embedded in the acrylic platform and secured by insect pins in order to maintain muscle tension. Throughout the
cremaster isolation surgery, the muscle was constantly superfused with a physiological salt solution (PSS, also known as; bicarbonate-buffered salt solution) containing (in mMol/L) 131.9 NaCl, 4.7 KCl, 2.0 CaCl₂, 1.2 MgSO₄, 30 NaHCO₃ and 0.3mg/L tubocurarine hydrochloride pentahydrate. A physiological pH (7.35±1.00) of PSS was attained and maintained by aeration with 5% CO₂ and 95% N₂ gas. During the cremaster isolation surgery PSS was warmed by heat convection via a warming coil to maintain a physiological temperature of 34.0±0.5°C.

After isolating the cremaster muscle the hamster and acrylic platform were transferred onto the microscope stage for experimentation. A maintenance dose of sodium pentobarbital was infused via femoral vein catheter using an anesthetic pump (KDS model 100, kdScientific, USA) at a constant rate (10mg/ml/hr) for the duration of the experiment. Once the animal was placed on the microscope, the temperature of PSS was maintained using a warmed tissue-organ bath attached to a dripper which superfused PSS over the cremaster muscle. An aerator, bubbling 5% CO₂ and 95% N₂ gas was placed in the tissue-organ bath in order to maintain physiological pH of the PSS solution throughout each experimental protocol. After successfully transferring the animal onto the microscope there was an equilibration period of 45-minute prior to experimentation and data collection.

Visualization of the microvasculature of the cremaster muscle was done via transillumination using an Olympus BX51WI microscope (Olympus, Japan), and a x40 water immersion objective (Olympus, Japan) with a numerical aperture
of 0.8. The microscope image of the cremaster muscle was displayed using a Dage DC-220 video camera (Dage-mti, USA) on a monitor and recorded with EZ Grabber (Geniatech, China) image recording software. The arteriole of interest; the transverse arteriole (TA), measuring ~40um, was identified using methods previously outlined Sweeney (1989) and Murrant (2000).

All in situ experiments were digitized and analyzed offline. Still images from video recording were captured every 10±1 seconds using FrameShot software (EoF Productions, USA) and arteriolar diameter was measured using ImageJ software (U.S National Institute of Health, USA). ‘n’ values denote the number of animals used per data set, only one TA from each animal was used for experimentation. Data was reported as mean ± standard error. Control, experimental baseline and maximal diameters were compared using a student t-test. Data analysis was conducted using 2-way repeated measures ANOVA. When the ANOVA identified significant difference a student t-test was used to determine whether diameter changes were statistically significant. Differences were considered statistically significant when p<0.05.

2.3 Protocols Addressing Aim 1.1 – Assessing if NO, ADO and KCl, effect one another’s dilatory potential

Aim 1.1 protocols shared a similar experimental design. A range of concentrations of a single dilator were superfused over the cremaster in increasing concentrations at 2-minute intervals creating a dose response (referred to as control condition). PSS was then superfused over the preparation
for 20 minutes to washout any residual effects of the dilator. Immediately following washout a single concentration of a 2nd dilator was added for 5 minutes and then the same concentrations of the first vasodilator used in the initial dose response were repeated but in the presence of the 2nd dilator (referred to as experimental condition). Video images of the site of interest were taken 1 minute prior to the dose response of each dilator, during application of each concentration and for 2 minutes during dilator washout. Similarly video images 1 minute prior to the application of both vasodilators, during application of both dilators and 2 minutes during washout were also recorded. Below lists the 6 different experimental protocols conducted along with the specific dilators and the concentrations that were tested (see appendix for timeline of each of the protocols of aim 1.1 listed below).

Protocol 1: To assess if NO alters the dilatory ability of K⁺. A dose response of KCl (10mM and 20mM) was conducted and then repeated in the presence of 10⁻⁷M, NO-donor, S-nitroso-N-acetylpenicillamine (SNAP).

Protocol 2: To assess if K⁺ alters the dilatory ability of NO. A dose response of SNAP (10⁻⁸M to 10⁻⁴M) was conducted and then repeated in the presence of 10mM KCl.

Protocol 3: To assess if ADO alters the dilatory ability of K⁺. A dose response of KCl (10mM and 20mM) was conducted and then repeated in the presence of 10⁻⁷M ADO.
Protocol 4: To assess if K⁺ alters the dilatory ability of ADO. A dose response of ADO (10⁻⁸M to 10⁻⁵M) was conducted and repeated in the presence of 10mM KCl.

Protocol 5: To assess if NO alters the dilatory ability of ADO. A dose response of ADO (10⁻⁸M to 10⁻⁵M) was conducted and repeated in the presence of 10⁻⁷M SNAP.

Protocol 6: To assess if ADO alters the dilatory ability of NO. A dose response of SNAP (10⁻⁸M to 10⁻⁵M) was conducted and repeated in the presence of 10⁻⁷M ADO.

2.4 Protocols Addressing Aim 1.2 – Assessing if NO, ADO and KCl, effect one another’s dilatory potential when added simultaneously.

Protocol 7: Assessing if NO is attenuated when simultaneously added with KCl.

To test the hypothesis that K⁺ can alter the magnitude of vasodilation elicited by NO when both vasoactive factors are added simultaneously, the cremaster was exposed to a single concentration (10⁻⁷M) of SNAP for 10 minutes (referred to as control condition) followed by superfusion of PSS for 20 minutes to washout any residual effects of the dilator. Immediately following washout, 10⁻⁷M SNAP was dissolved in 10mM KCl-buffered PSS and applied for 10 minutes (referred to as experimental condition). Video images of the site of interest were taken 1 minute prior to the application of SNAP, during application and for 2 minutes during dilator washout. Similarly video images 1 minute prior to the
application of SNAP and KCl, during application and 2 minutes during washout were also recorded (see appendix for timeline of the protocol).

**Protocol 8: Assessing if ADO is attenuated when simultaneously added with KCl.**

To test whether K\(^+\) can alter the magnitude of vasodilation elicited by ADO when both are added simultaneously, the cremaster was exposed to a single concentration (10\(^{-7}\)M) of ADO for 10 minutes (referred to as control condition) followed by the application of PSS for 20 minutes to washout residual effects of the dilator. Immediately following washout, 10\(^{-7}\)M ADO was dissolved in 10mM KCl-buffered PSS and applied for 10 minutes (referred to as experimental condition). Video images of the site of interest were taken 1 minute prior to the application of the ADO, during application and for 2 minutes during dilator washout. Similarly video images 1 minute prior to the application of both KCl and ADO, during application and 2 minutes during washout were also recorded (see appendix for timeline of the protocol).

**Protocol 9: Assessing if NO and ADO effect one another’s vasodilatory ability when applied simultaneously.**

To test if NO and ADO effect one another’s vasodilatory ability when both are added simultaneously, the cremaster was exposed to a single concentration (10\(^{-7}\)M) of either SNAP or ADO for 10 minutes (referred to as control condition) followed by superfusion with PSS for 20 minutes to washout any residual effects of each dilator. Immediately following washout, 10\(^{-7}\)M of SNAP and 10\(^{-7}\)M ADO were dissolved in PSS and applied for 10 minutes (referred to as experimental condition). Video images of the site of interest were taken 1 minute prior to the
application of SNAP or ADO, during application of each and for 2 minutes during dilator washout. Similarly video images 1 minute prior to the application of SNAP AND KCL, during application of both dilators and 2 minutes during washout were also recorded (see appendix for timeline of the protocol).

2.5 Protocols addressing Aim 2.1 – Determining if $K_{IR}$ channels and $\text{Na}^+$/\text{K}^+-ATPase are involved in the dilatory pathway of KCl.

**Protocol 10: Assessing antagonism of $K_{IR}$ using Barium Chloride on the vasodilation produced by 10mM KCl**

To test the hypothesis that $K_{IR}$ channel antagonist BaCl can attenuate the magnitude of vasodilation elicited by 10mM KCl the cremaster was exposed to a single concentration of KCl (10mM) for 10 minutes (referred to as control condition). After KCl exposure, PSS was superfused over the cremaster for 20 minutes to washout any residual effect of KCl. Immediately following washout, 50x10^{-6} M BaCl dissolved in PSS was superfused over the cremaster for 30 minutes to allow for BaCl’s antagonistic effects to ‘wash-in’. Subsequently, BaCl dissolved in 10mM PSS was superfused over the cremaster for 10 minutes (referred to as experimental condition). Video images of the site of interest were taken 1 minute prior to KCl application, during application and for 2 minutes following KCl washout. Similarly video images 1 minute prior to the application of BaCl in KCl, during application and for 2 minutes during washout were also recorded (see appendix for timeline of the protocol).
Protocol 11: Assessing the antagonism of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase using Ouabain on the vasodilation produced by 10mM K\textsuperscript{+}

To test the hypothesis that Na\textsuperscript{+}/K\textsuperscript{+}-ATPase antagonist Oua can attenuate the magnitude of vasodilation elicited by 10mM KCl the cremaster was exposed to a single concentration (10mM) of KCl for 10 minutes (referred to as control condition). After KCl exposure, PSS was superfused over the cremaster for 20 minutes to washout any residual effect of the dilator. Immediately following washout 10\textsuperscript{-4}M Oua dissolved in PSS was superfused over the cremaster for 30 minutes to allow for its antagonistic effects to ‘wash-in’. Subsequently, Oua dissolved in 10mM PSS was superfused over the cremaster for 10 minutes (referred to as experimental condition). Video images of the site of interest were taken 1 minute prior to 10mM KCl application, during application of KCl and for 2 minutes during washout. Similarly video images 1 minute prior to the application of 10\textsuperscript{-4}M Oua in 10mM KCl, during application and 2 minutes after washout were also recorded (see appendix for timeline of the protocol).

2.6 Protocols addressing Aim 2.2 – Assessing how the antagonism of K\textsubscript{IR} and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase effect the vasomotor response elicited by NO and ADO when added in the presence of 10mM KCl

Protocol 12: Quantifying the effects of antagonism of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase on NO and ADO dilation in the presence of 10mM KCl

To test the hypothesis that antagonism of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase can rescue, or restore, the attenuated vasomotor response when NO and separately, ADO, each are added in the presence 10mM KCl, the cremaster was exposed to a 5-minute application of 10mM KCl-buffered PSS followed by a range of SNAP (10\textsuperscript{-8}M-10\textsuperscript{-5}M) or ADO concentrations (10\textsuperscript{-8}M-10\textsuperscript{-5}M) each dissolved in 10mM KCl-buffered
PSS, added in sequential order from lowest to highest concentration at 2-minute intervals (referred to as control condition). PSS was then superfused over the cremaster muscle for 20 minutes to wash out any residual effects of either dilator. Immediately following washout, 10^{-4}M Oua dissolved in PSS was superfused over the cremaster for 30 minutes to allow its antagonistic effects to ‘wash-in’.

Subsequently, 10^{-4}M Oua in 10mM KCl-buffered superfusate was applied for 5 minutes followed by the application of the same concentrations of SNAP or ADO dissolved in 10mM KCl-buffered and in the presence of 10^{-4}M Oua, in increasing concentrations for 2 minutes each (referred to as experimental condition). Video images of the site of interest were taken 1 minute prior to application of SNAP + 10mM KCl or ADO + 10mM KCl, during application of each concentration and for 2 minutes during washout. Similarly video images 1 minute prior to the application of Oua and KCl, during application of SNAP and Oua or ADO and Oua in the presence of 10mM KCl and 2 minutes during washout were also recorded (see appendix for timeline of the protocol).

Protocol 13: Quantifying the effects of antagonism of K_{ir} on NO or ADO dilation in the presence of 10mM KCl

To test the hypothesis that antagonism of K_{ir} can rescue, or restore, the attenuated vasomotor response when NO and separately, ADO, each are added in the presence 10mM KCl, the cremaster was exposed to a 5-minute application of 10mM KCl-buffered PSS followed by a range of SNAP (10^{-8}M-10^{-5}M) or ADO concentrations (10^{-8}M-10^{-5}M) each dissolved in 10mM KCl-buffered PSS, added in sequential order from lowest to highest concentration at 2-minute intervals (referred to as control condition). PSS was then superfused over the cremaster
muscle for 20 minutes to wash out any residual effects of either vasodilator. Immediately following washout, 50x10^-6 M BaCl dissolved in PSS was superfused over the cremaster for 30 minutes to allow its antagonistic effects to ‘wash-in’. Subsequently, 50x10^-6 M BaCl, in 10mM KCl-buffered superfusate was applied for 5 minutes followed by the application of the same concentrations of SNAP or ADO dissolved 10mM KCl-buffered PSS and in the presence of 50x10^-6 M Oua, in increasing concentrations for 2 minutes each (referred to as experimental condition). Video images of the site of interest were taken 1 minute prior to application of SNAP + 10mM KCl or ADO + 10mM KCl, during application of each concentration and for 2 minutes during washout. Similarly video images 1 minute prior to the application of BaCl and KCl, during application of SNAP or ADO and BaCl dissolved in 10mM KCl and 2 minutes during washout were also recorded (see appendix for timeline of the protocol).
Chapter 3. Results

**Aim 1:** To determine if the vasodilators $K^+$, ADO and NO affect one another’s vasodilatory ability.

3.1 *The dilatory profile of single concentrations of KCl, NO and ADO*

We have shown that the application of a physiologically relevant concentration (10mM) of KCl produces a transient vasodilation of the TA when superfused over the cremaster muscle. More specifically, over a 10-minute application KCl caused a rapid increase in luminal diameter, peaking around the 3rd minute of exposure followed by a constant decline in diameter until about the 5th minute where luminal diameter returned to pre-exposure diameter for the remainder of the exposure period (see appendix for vasodilatory response of 10mM KCl over 10 minute application). Separately, we have also shown that the individual application of NO ($10^{-7}$M) and ADO ($10^{-7}$M) produced a relatively modest but constant increase in luminal diameter of the TA, this observed change in diameter was sustained throughout each of the vasodilator’s 10 minute exposure (see Fig 7A,B and C for vasodilatory profile of each dilator).
Table 1: Measurements of average baseline and maximum arteriolar diameter. Control baseline refers to the average luminal diameter before exposure to any of the tested vasodilators while experimental baseline refers to the average luminal diameter preceding the application of experimental combination of vasodilators.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Baseline Diameter (µm)</th>
<th>Maximum Diameter (µm)</th>
<th>n</th>
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<tbody>
<tr>
<td></td>
<td>Protocol</td>
<td>Control</td>
<td>Experimental</td>
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<tr>
<td>NO effect on K⁺</td>
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<tr>
<td>K⁺ effect on ADO</td>
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<td>38.4±2.9</td>
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<tr>
<td>ADO effect on K⁺</td>
<td>13.6±2.0</td>
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<td>NO effect on ADO</td>
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<td>41.0±2.2</td>
</tr>
</tbody>
</table>

Values are mean ± SE.

3.2 Assessing the effect that K⁺ and NO have on one another

As shown in Figure 4A peak changes in diameter over a 2-minute exposure to 10mM and 20mM KCl caused a dose dependent increase in vessel diameter. After the washout period, both concentrations of KCl were reapplied in the presence of a single concentration (10⁻⁷M) of, the NO-donor, SNAP. The presence of NO caused no observable change in the magnitude of vessel response when compared to the dilation produced by KCl alone. Thus, the vasomotor response of the TA to dilatory concentration of KCl is seemingly
unaffected by the presence of lower, physiologically relevant concentrations of NO.

Increasing SNAP concentrations, ranging from $10^{-8}$M-$10^{-5}$M, produced an expected stepwise increase in the magnitude of dilation of the TA (Fig 4B). During the SNAP dose response the overall variability in peak change in diameter is relatively small and consistent between each of the SNAP concentrations indicative of a relatively consistent vasomotor response between vessels to each of the tested concentrations of SNAP. When the same range of SNAP concentrations were reapplied in the presence of a single dilatory concentration (10mM) of KCl a significant attenuation of NO’s dilatory effects occurred at all tested concentrations. The presence of KCl was able to attenuate the magnitude of dilation produced by $10^{-8}$M, $10^{-7}$M, $10^{-6}$M, $10^{-5}$M SNAP by approximately 68.1%, 77.6%, 67.8% and 50.0%, respectively. Variability of the vasomotor response during SNAP and KCl exposure differed from SNAP alone. Variability of peak change in diameter during SNAP and KCl seemed to be correlated with SNAP concentration, more specifically as SNAP concentrations increased there seemed to be a dose dependent increase in the variability of the vessel response, suggesting that higher NO concentrations were not as consistent, in terms of their response to the attenuative effects of KCl compared to lower concentrations. Overall, we have demonstrated physiologically relevant dilatory concentrations of KCl are able to attenuate NO over a wide range of concentrations. These data provide evidence that vasoactive factors implicated in mediating the active hyperaemic response are able to interact and influence one another’s response, and in this specific case, one vasodilator, KCl, is able to blunt the vasodilatory
effect of another. The fact that KCl was able to facilitate the attenuation of NO is also surprising as each are potent vasodilators, yet when added together instead of a summation of the magnitude of dilation produced by each there was an overall attenuation of SNAP’s dilatory ability, thereby indicating that the presence of KCl interferes with the pathway in which NO elicits its dilatory effects.

Figure 4

A. NO effect on KCl-elicited Dilation

B. KCl effect on NO-elicited Dilation
Figure 4: NO did not affect the magnitude of KCl-induced vasodilation (A) while KCl attenuated the magnitude of vasodilation produced by NO (B). A) The change in diameter in response to incremental doses of KCl in the absence (■) and presence (□) of a $10^{-7}$M SNAP. B) The change in diameter in response to incremental concentrations of SNAP in the absence (■) and presence (□) of 10mM KCl. * Indicates experimental groups significant differed (p<0.05) from control.

3.3 Assessing the effects that $K^+$ and ADO have on one another.

The assessment of KCl’s effect on ADO closely paralleled the finding of KCl’s effect on NO. As shown in Figure 5A the application of 10mM and 20mM KCl caused an expected dose-dependent increase in vessel diameter of the TA. After washout, when both concentrations of KCl were reapplied in the presence of a single concentration ($10^{-7}$M) of ADO, there was no observable change in the magnitude of dilation with the peak change in diameter being no different than the dilation produced upon the application of KCl alone, thus indicating that the vasomotor response of the TA to dilatory concentration of KCl is unaffected by the presence of ADO.

Figure 5B outlines the effects of KCl on ADO, which also closely mirrors a similar dilatory profile to KCl’s effect on NO. When applied alone, a stepwise increase in ADO concentrations, ranging from $10^{-8}$M-$10^{-5}$M, produced an expected dose-dependent increase in vessel diameter. Similar to the observed variability of the vasomotor response during NO dose response, the variability of ADO applied alone also appears to be relatively small and consistent between each of the concentrations tested, again, indicative of a relatively consistent response between vessels to each of the tested concentrations of ADO. When the same range of ADO concentrations were reapplied in the presence of a single concentration (10mM) of KCl, there was a significant attenuation of the magnitude
of dilation elicited by ADO at all concentrations tested barring $10^{-5}\text{M}$, when compared to the application of ADO alone. More specifically, the presence of KCl was able to attenuate the magnitude of dilation produced by $10^{-8}\text{M}$, $10^{-7}\text{M}$ and $10^{-6}\text{M}$ by approximately 80.6%, 75.3% and 62.5%, respectively. Variability of the vessel response to ADO and KCl exposure seemed to be correlated with ADO concentration, as increasing concentrations of ADO seemed to cause a dose dependent increase in the variability of the vasomotor response suggesting that the vessel response to higher ADO concentrations was not as consistent to the attenuative effects of KCl compared to lower concentrations. As KCl is able to attenuate the response of a wide range of ADO concentrations tested, taken with the fact KCl was able to attenuate NO's dilatory response provides convincing evidence that physiologically relevant concentrations of $K^+$ have a potent inhibitory influence on the dilatory response of two prominent metabolic vasodilators both of which are involved in facilitating the active hyperaemic response. Furthermore, this observation provides further evidence to support that the addition of certain vasodilators together can produce blunted vasomotor response compared to when added alone, again supporting the notion that the addition of multiple vasodilators do not necessarily cause an additive increase in vessel diameter.
Figure 5: ADO did not affect the magnitude of KCl-induced vasodilation (A) while KCl attenuated the magnitude of vasodilation produced by ADO (B). A) The change in diameter in response to incremental doses of KCl in the absence (■) and presence (□) of $10^{-7}$M ADO. B) The change in diameter in response to incremental doses of ADO in the absence (■) and presence (□) of 10mM KCl. * Indicates experimental groups significant differed (p<0.05) from control.
3.4 Assessing the effects that NO and ADO have on one another.

Application ADO, ranging from $10^{-8}$M-$10^{-5}$M, produced an expected dose-dependent increase in vessel diameter (Fig 6A). The range of ADO concentrations tested produced relatively small variability and taken together with the fact that prior ADO dose response shared a small variability in vessel diameter between concentrations of ADO (Figure 5B) provides further evidence that the magnitude of dilation elicited by each tested concentration of ADO is consistent between vessels. When the dose response of ADO was repeated in the presence of a single concentration ($10^{-7}$M) of SNAP there were no apparent differences in vessel diameter compared to the dose response of ADO alone. This observation suggests that NO does not influence or impact the vasodilatory response of ADO. Furthermore the variability of vessel diameter when ADO was applied alone was comparable to when ADO was applied in the presence of NO suggests the vasomotor response to ADO was relatively consistent between concentrations despite the presence of NO.

Application of a range of SNAP concentrations ($10^{-8}$M-$10^{-5}$M) caused an expected stepwise increase in the vascular diameter of the TA (Fig 6B). Peak changes in vessel diameter during the NO dose response had a relatively small variability between the different concentrations of NO tested. Together with the fact that prior NO dose response shared a small variability in vessel diameter between concentrations of SNAP (Fig 5A) provides evidence that the magnitude of dilation elicited by each tested concentration of SNAP is consistent between vessels. When the dose response of SNAP was repeated in the presence of a single concentration ($10^{-7}$M) of ADO there were no differences in vessel diameter
compared to SNAP alone. This observation suggests that ADO does not influence or impact the vasodilatory response of NO. Compared to when SNAP was applied alone the presence of ADO had little impact on the variability of the vasomotor response elicited by the range of NO concentrations tested again implying that the presence of ADO had little impact on the magnitude of dilation elicited by NO.

**Figure 6:**

**A. NO effect on ADO-elicited Dilation**

**B. ADO effect on NO-elicited Dilation**
Figure 6: Neither NO (A) or ADO (B) affected the vasodilatory abilities of one another. A) The change in diameter in response to incremental doses of ADO in the absence (■) and presence (□) of 10^{-7}M SNAP. B) The change in diameter in response to incremental doses of SNAP in the absence (■) and presence (□) of a single concentration of ADO. * Indicates experimental groups significantly differed (p<0.05) from control.

3.5 Assessing the effect KCl, ADO and NO have on one another when added simultaneously.

Table 2 outlines the average baseline diameter prior to and preceding exposures to control and experimental vasodilators along with average maximal diameter. Baseline diameters did not differ during each of the protocols therefore does not alter the interpretation of the results presented below.

Figure 7A shows the time course of vasodilation when the TA is exposed to a single concentration (10^{-7}M) of SNAP in the absence and presence of a single concentration (10mM) of KCl. NO alone produced a sustained increase in vessel diameter of approximately 9.4µm throughout the 10-minute exposure. Yet, when added in the presence of KCl the dilatory response changed when compared to the application of SNAP alone. More specifically there was an increase in vascular diameter peaking 2-minutes post-exposure followed by a steady decline in vessel diameter returning to approximately baseline diameter around the 6th minute of application. These data show that when added together there is no apparent additive effect of each vasodilator early in the exposure, that is to say the magnitude of dilation elicited by each NO and KCl did not summate. However, over the exposure period, after 5 minutes, an inhibitory effect of NO’s dilatory ability became apparent. As dilatory profile of KCl in the presence of NO was similar to 10mM KCl response when applied alone, suggests that when added together KCl elicits a dilation as if NO was not present, perhaps indicating
that KCl is inhibiting the effect of NO immediately and completely upon exposure to the vessel. Furthermore, these data lend support to our initial observation that KCl is able to attenuate the effects on NO’s vasodilatory ability (Fig 4B).

Figure 7B shows the time course of vasodilation when the TA is exposed to a single concentration (10^{-7}M) of ADO in the absence and presence of a single concentration (10mM) of KCl. ADO alone produced a sustained increase in vessel diameter of approximately 4.2µm throughout the 10-minute exposure. Yet, when added in the presence of KCl ADO’s dilatory response is changed compared to when applied alone. More specifically in the presence of KCl there was an initial increase in vascular diameter peaking at 2-minutes post-exposure followed by a steady decline in diameter returning to baseline around the 5^{th} minute of application. These data show that when added together there is no apparent additive effect of each vasodilator early in the exposure, as the magnitude of dilation elicited by each ADO and KCl did not summate. However, over the exposure period, after 6 minutes an inhibitory effect of ADO’s dilatory ability became apparent. The dilatory profile of KCl in the presence of ADO was similar to 10mM KCl response when applied alone, thus suggesting that like the simultaneous addition of NO and KCl, when added together KCl acts as though ADO was not present, indicating that KCl is inhibiting the effect of ADO immediately and entirely. Again, these data lend support to our initial observation of KCl’s attenuative effects on ADO elicited vasodilation (Fig 5B).

Figure 7C shows the time course of vasodilation when the TA is exposed to a single concentration of ADO (10^{-7}M) and SNAP (10^{-7}M) in the absence and presence of one another. The addition of SNAP alone produced a sustained
increase in vessel diameter of approximately 8.2µm throughout the 10-minute exposure similar to the magnitude of dilation in which previous application of 10^{-7} M ADO produced (Fig 7A). Similarly to the application of SNAP, the exposure of ADO alone also produced a sustained increase in vessel diameter of approximately 4.5µm throughout the 10-minute exposure, again comparable to the magnitude of dilation produced by previous applications of 10^{-7} M ADO (Fig 7B). When both 10^{-7} M SNAP and 10^{-7} M ADO were added simultaneously there was an initial peak in dilation occurring 3 minutes post exposure followed by a decline in diameter and by the 5th minute of exposure vessel diameter reached a steady state for the remainder of the application. When compared to the vasodilatory pattern of ADO and NO alone there is evidence to suggest the contemporaneous presence of both dilators affect one another’s vasodilatory ability. It seems as though NO is responsible for the initial dilation for the first 4-minutes of exposure and over time its vasodilatory effects become less influential with ADO’s effects becoming more pronounced later in the application period, suggesting NO may play a more prominent role in the initial increase in vasodilation whereas the effects of ADO are prominent in sustaining longer term dilation. This interaction was not observed when NO and ADO were applied sequentially when addressing Aim 1.1 (Fig 6A and B.) this is likely due to the 5 minute exposure of each dilator before the combination of each were added, which may have masked this observed relationship between the 2 vasodilators.
Table 2: Measurements of average baseline and maximum arteriolar diameter. Control baseline refers to the average luminal diameter before exposure to any of the tested vasodilators while experimental baseline refers to the average luminal diameter preceding the application of experimental combination of vasodilators.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Baseline Diameter (µm)</th>
<th>Maximum Diameter (µm)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simultaneous Application of 10⁻⁷M SNAP + 10mM KCl</td>
<td>19.0±2.1</td>
<td>22.0±2.2</td>
<td>22</td>
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<tr>
<td>Simultaneous Application of 10⁻⁷M ADO + 10mM KCl</td>
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<tr>
<td>Simultaneous Application of 10⁻⁷M ADO + 10⁻⁷M SNAP</td>
<td>16.8±0.9</td>
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</tbody>
</table>

Values are mean ± SE.

Figure 7:

A. KCl effect on NO-elicited Dilation when Applied Simultaneously
Figure 7: KCl attenuated the magnitude of vasodilation of both NO (A) and ADO (B) when applied simultaneously. NO and ADO were responsible for different phases of the vasodilatory response when added simultaneously together (C). A) The change in diameter over 10 minute exposure to $10^{-7}$M SNAP in the absence (■) and presence (□) of 10mM KCl compared to 10mM KCl alone (▲). B) The change in diameter over 10 minute exposure to $10^{-7}$M ADO in the absence (■) and presence (□) of 10mM KCl compared to 10mM KCl alone (▲). C) The change in diameter over 10 minute exposure to $10^{-7}$M SNAP (□) and $10^{-7}$M ADO applied alone (■) and in the presence (▲) one another. *Indicates experimental (SNAP or ADO in the presence of KCl) groups significantly differ (p<0.05) from control (SNAP or ADO alone).
**Aim 2:** To determine if antagonism of inward-rectifier potassium ion channel (K\(_{ir}\)) and sodium/potassium pump (Na\(^+\)/K\(^+\)-ATPase), amplifies, or rescues, the attenuated dilatory effects of NO and ADO when added in the presence of KCl.

3.6 Baseline and maximal diameter when assessing BaCl’s and Oua’s effect on the KCl’s vasodilatory ability.

Average baseline and maximum diameters are shown in Table 3. Differences between baseline diameters before and after exposure to vasodilators or antagonists can influence the vessel’s dilatory potential (defined as the amount of dilation a vessel can undergo from baseline diameter until maximal diameter is reached, calculated as the difference between maximal and baseline diameter). In our set of experiment to address Aim 2.1, baseline diameter between control and experimental conditions did not statistically differ from one another except when quantifying BaCl’s effect on KCl elicited dilation. In order to test that vessel responsiveness was not influenced by difference in baseline diameter during the assessment of BaCl’s effect on KCl dilation we graphed peak change in diameter elicited by 10mM KCl as a function of both baseline diameter and dilatory potential. As outlined in Figure 8A baseline diameters showed a very low correlation with the peak change in diameter of 10mM KCl (r\(^2\) = 0.06). Furthermore, Figure 8B outlines that dilatory potential is not significantly correlated with peak change in diameter of 10mM KCl (r=0.0002). Thus as the difference between baseline diameters after exposure to experimental vasodilators does not have a significant influence on the TA’s vasomotor response thus the difference between control and experimental baseline diameters does not alter the interpretation of the results.
Table 3: Measurements of average baseline and maximal arteriolar diameter. Control baseline refers to the average luminal diameter before exposure to any vasodilator. Experimental baseline refers to the average luminal diameter preceding the application of experimental combination of vasodilator and antagonist.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Baseline Diameter (µm)</th>
<th>Maximum Diameter (µm)</th>
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<tbody>
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<td>10mM KCl + BaCl application</td>
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<tr>
<td>10mM KCl + Oua application</td>
<td>14.7±0.7</td>
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Values are mean ± SE. * Experimental baseline diameter significantly differs (p<0.05) from initial baseline diameter.

Figure 8:
A.

Figure 8: Baseline diameter (A) and dilatory potential (B) did not influence the peak changes in vessel diameter elicited by 10mM KCl. A) Comparison of baseline diameter and peak change in diameter elicited by KCl. B) Comparison of baseline diameter and dilatory potential. Line of best fit was calculated to determine the correlation coefficient values between baseline diameter and dilatory potential and the peak change in diameter.
3.7 Quantifying the contribution of $K_{\text{IR}}$ channels and $\text{Na}^+/\text{K}^+$-ATPase in the dilations produced by KCl.

Figure 9 illustrates the effect of $K_{\text{IR}}$ (Fig 9A) and $\text{Na}^+/\text{K}^+$-ATPase (Fig 9B) antagonist on the peak change in diameter elicited by 10mM KCl compared to when KCl was applied alone. Application of $50 \times 10^{-6}$M of $K_{\text{IR}}$ antagonist BaCl significantly blunted the peak dilatory response of KCl by 43.5%, from $11.1 \pm 1.6\mu$m to $6.3 \pm 1.6\mu$m while exposure to $10^{-4}$M of $\text{Na}^+/\text{K}^+$-ATPase antagonist Oua significantly blunted the peak dilatory response of KCl by 40.1% from $9.3 \pm 1.5\mu$m to $5.5 \pm 1.8\mu$m. Taken together these data demonstrates that both $K_{\text{IR}}$ channels and $\text{Na}^+/\text{K}^+$-ATPase are involved in the dilatory pathway of KCl. Furthermore, it also demonstrates that the chosen concentrations of each antagonist were sufficient in antagonizing each channel as KCl-elicited dilation was significantly blunted. We did not expect KCl dilation to be abolished as each antagonist was applied separately, leaving the other unantagonized therefore allowing it to partially mediate KCl dilation. Therefore, we only expected a blunted response during the application of each antagonist which was sufficient enough to implicate their involvement in mediating KCl dilation.
Figure 9: The antagonism of both $K_{ir}$ and $Na^+/K^+$-ATPase, by BaCl (A) and Oua (B), respectively attenuate the vasodilatory action of KCl. A) The average peak change in diameter to the application of KCl in the absence (■) and presence (■) of BaCl. B) The average peak change in diameter to the application of 10mM KCl in the absence (■) and presence (■) of Oua. * Indicates experimental groups significantly differed (p<0.05) from control.
3.8 Effect of BaCl on the magnitude of dilation produced by NO and ADO when in the presence of KCl

Table 4 outlines the average baseline diameter prior to and preceding exposures to control and experimental vasodilators along with average maximal diameter. Baseline diameters did not differ during each of the protocols therefore it does not alter the interpretation of the results presented below.

Figure 10A shows that increasing SNAP concentrations ranging from $10^{-8}$M-10$^{-5}$M in the presence of 10mM KCl lead to a stepwise increase in the diameter of the TA. As was expected, the magnitude of dilation during KCl + SNAP application was blunted when compared to prior dose response of SNAP alone (Fig 4B). Similar to other experimental protocols that exposed the cremaster to SNAP and KCl together (Fig 4B), the variability of the vasomotor response also seemed to be correlated with SNAP concentration, as concentrations of SNAP increased there seemed to be a dose dependent increase in the variability of the vasomotor response. This further indicates the response to higher NO concentrations was not as consistent to the attenuative effects of KCl when compared to lower concentrations. When the same range of SNAP + KCl was applied in the presence of 50x10$^{-6}$M of KIR antagonist, BaCl, the magnitude of dilation elicited by $10^{-8}$M or $10^{-7}$M SNAP were not significantly different compared to when SNAP and KCl were applied together. However, the vasomotor response elicited by higher concentrations, $10^{-6}$M and $10^{-5}$M SNAP displayed an amplified magnitude of dilation by approximately 68% and 89% respectively in the presence of BaCl and KCl compared to when with KCl alone. Taken together this indicates that while lower concentrations of SNAP are
seemingly unaffected by the blockade of $K_{IR}$ channels the response of higher concentrations of SNAP are amplified or rescued from KCl's inhibitory effects. As previously discussed the presence of dilatory concentrations KCl alter $K_{IR}$ channel activity, thus perhaps the altered $K_{IR}$ channel activity, is, in part, responsible for the attenuation of high concentration NO-elicited dilation. Furthermore, the observed response also suggests that the lower concentrations of NO, unaffected by the $K_{IR}$ blockade, are attenuated independently of $K_{IR}$ channels blockade, while at higher concentration of NO attenuation seems to be dependent on $K_{IR}$ channel activity, implying that KCl may attenuate NO via two different mechanisms depending on NO concentration.

Figure 10B shows that increasing ADO concentrations from $10^{-8}$M-$10^{-5}$M in the presence of 10mM KCl lead to a stepwise increase in the diameter of the TA. As expected, the magnitude of response of KCl + ADO application was blunted when compared to prior dose responses of ADO alone (Fig 5B). Furthermore the response of KCl and ADO was comparable to the vasomotor response observed in prior exposures of the two dilators (Fig 5B). Similar to other experimental protocols that exposed the cremaster to ADO with KCl together (Fig 5B) the variability of the vasomotor response seemed to be correlated with ADO concentration; as concentrations of ADO increased there seemed to be a dose dependent increase in the variability of the response, again indicating the response to higher ADO concentrations were not as consistent to the attenuative effects of KCl compared to lower concentrations. When the same range of ADO and KCl were applied in the presence of $50 \times 10^{-6}$M BaCl there was a significant amplification in the magnitude of dilation elicited by $10^{-8}$M or $10^{-7}$M ADO by
approximately 166% and 639%, respectively compared to when KCl + ADO were in the absence of BaCl. However, higher concentrations of ADO, $10^{-6}$M and $10^{-5}$M, displayed no change in the magnitude of dilation compared to the application of both KCl and ADO. Taken together this indicates that while higher concentrations of ADO are seemingly unaffected by antagonism of K$_{ir}$ channels, the response of lower concentrations of SNAP are rescued from the attenuative effects of KCl. The observed response also suggests that as higher concentrations of ADO are unaffected by the blockade of Na$^+$/K$^+$-ATPase attenuation by KCl occurs independently of Na$^+$/K$^+$-ATPase channel activity, while the KCl mediated attenuation of lower concentration of ADO are clearly, in part, dependent on Na$^+$/K$^+$-ATPase channel activity. Again suggesting that KCl may attenuate ADO via two different mechanisms depending on ADO concentration.

Figure 10C shows the difference in diameter elicited by the control combinations of vasodilators (ADO + KCl) and experimental (ADO + KCl + BaCl). As the differential between the magnitudes of dilation is different than 0, implies that the antagonist BaCl was able to amplify the response of ADO in the presence of KCl at all concentrations. If BaCl did not amplify the response of SNAP we would expect the difference between control and experimental dilation to be ≤0. The extent of BaCl’s effect on ADO dilation in the presence of KCl was not fully conveyed in Figure 9B, due to the fact the graphs is of peak average change in dilation, yet upon statistical analysis, it was found that $10^{-6}$M and $10^{-7}$M ADO + KCl in the presence of BaCl was significantly amplified compared to when KCl and ADO were applied together. Thus, the presence of BaCl ultimately amplifies (or rescues) ADO from KCl attenuation at all concentrations tested and therefore
implying that KCl attenuation of ADO is dependent upon the alteration of KIR activity.

**Table 4:** Measurements of average baseline arteriolar and maximum diameter. Control baseline refers to the average luminal diameter before exposure to any vasodilator. Experimental baseline refers to the average luminal diameter preceding the application of experimental combination of vasodilators and antagonist.

<table>
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<th>Protocol</th>
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<th>Maximum Diameter (um)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Initial</td>
<td>Experimental</td>
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<tr>
<td>SNAP + KCl + BaCl application</td>
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<td>18.9±2.3</td>
<td>16.1±2.6</td>
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</tbody>
</table>

Values are mean ± SE. * Experimental baseline diameter significantly differs (p<0.05) from initial baseline diameter.

**Figure 10:**

A. **BaCl effect on NO-elicited Dilation in the Presence of KCl**

![Graph showing BaCl effect on NO-elicited Dilation in the Presence of KCl]
**Figure 10:** The application of a BaCl in the presence of both KCl and ADO (A) and KCl and SNAP (B) causes an amplification in ADO and SNAP’s dilatory ability effect at certain concentrations A) The change in diameter in response to KCl and SNAP in the absence (■) and presence (□) of BaCl. B) The change in diameter in response to KCl and ADO in the absence (■) and presence (□) BaCl. C) The difference between experimental and control peak changes in diameter reveal that BaCl in the presence of KCl and ADO did amplify the vasodilation at all tested concentrations compared to when ADO was applied in the presence of KCl. * Indicates experimental groups significant differed (p<0.05) from control.
3.9 Baseline and maximal diameter when assessing of NO, ADO and KCl effect one another’s vasodilatory potential

Average baseline and maximum diameters are shown in Table 5. As previously stated differences in baseline diameter influence the vessel’s dilatory potential and therefore may impact the vessel’s responsiveness to stimuli. In order to show that the difference between average baseline diameters during the assessment of Oua’s effect on ADO’s dilation in the presence of KCl did not effect the vessel responsiveness, peak change in diameter elicited by ADO was graphed as a function of both baseline diameter and dilatory potential. However, as outlined in Figure 11A baseline diameters also showed a very low correlation with the peak change in diameter of elicited by $10^{-8}$M ($r^2=0.04$) $10^{-7}$M ($r^2=0.16$) $10^{-6}$M ($r^2=0.02$) $10^{-5}$M ($r^2=0.08$) ADO. Furthermore, Figure 11B outlines that dilatory potential does not seem to be significantly correlated with peak change in diameter of $10^{-8}$M ($r^2=0.48$) $10^{-7}$M ($r^2=0.54$) $10^{-6}$M ($r^2=0.67$) $10^{-5}$M ($r^2=0.68$) ADO. Thus, from this analysis, we can conclude that the significant differences between control and experimental baseline diameters, during the assessment on BaCl’s effect on ADO in the presence of KCl, did not have a significant influence on the vasomotor response of the TA and therefore does not alter the interpretation of the results.
Table 5: Measurements of average baseline and maximum arteriolar diameter. Control baseline refers to the average luminal diameter before exposure to any vasodilator. Experimental baseline refers to the average luminal diameter preceding the application of experimental combination of vasodilators and antagonist.

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Baseline Diameter (um)</th>
<th>Maximum Diameter (um)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNAP + KCl + Oua application</td>
<td>16.8±1.4</td>
<td>14.8±2.4</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>38.2±2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADO + KCl + Oua application</td>
<td>16.3±2.1</td>
<td>12.2±1.1*</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>36.7±2.0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are mean ± SE. * Experimental baseline diameter significantly differs (p<0.05) from initial baseline diameter.

Figure 11:

A. Baseline Diameter v. ADO Peak Change in Diameter

B. Dilatory Potential v. ADO Peak Change in Diameter

Figure 11: Baseline diameter (A) and dilatory potential (B) did not influence the peak changes in vessel diameter elicited by $10^{-8}$M (■), $10^{-7}$M (○), $10^{-6}$M (▲) and $10^{-5}$M (x) ADO in the presence of 10mM KCl and $50\times10^{-6}$M BaCl. A) Comparison of baseline diameter and peak change in diameter elicited by ADO in the presence of BaCl and KCl. B) Comparison of dilatory potential and peak change in diameter elicited by ADO in the presence of BaCl and KCl. Line of best fit was calculated to determine the correlation coefficient values between baseline diameter and dilatory potential and the peak change in diameter.
3.10 Effect of Oua on the magnitude of dilation produced by NO and ADO when in the presence of KCl

Figure 12A shows an increase in SNAP concentrations from $10^{-8}$M-$10^{-5}$M in the presence of 10mM KCl lead to a stepwise increase in the diameter of the TA. As was expected, the magnitude of dilation during KCl + SNAP application was blunted compared to the dose response of SNAP alone (Fig 4B). Similar to prior experimental protocols that exposed the cremaster muscle to KCl and SNAP together (Fig. 4B and 10A) the variability of the vasomotor response also was roughly correlated with SNAP concentrations, as concentrations of SNAP increased there seemed to be a dose dependent increase in the variability of the vasomotor response. When the same range of SNAP and KCl were applied in the presence of $10^{-4}$M Oua there was no significant change in the magnitude of dilation at any of the SNAP concentrations tested when compared to the dilatory response of SNAP and KCl exposure. These data show that Na$^+$/K$^+$-ATPase is not involved in KCl's attenuation of NO dilatory ability. Taken together with the dilatory pattern of SNAP and KCl in the presence of BaCl (Fig. 10A) it appears as though high concentrations of NO seem to be attenuated by KCl via a $K_{IR}$-dependent mechanism (such as a change in channel activity) but independently of Na$^+$/K$^+$-ATPase, lower concentration are attenuated by KCl independent of $K_{IR}$ or Na$^+$/K$^+$-ATPase suggesting another mechanism functions to attenuate low NO dilations in the presence of KCl.

Figure 12B shows that increase in ADO concentrations from $10^{-8}$M-$10^{-5}$M in the presence of 10mM KCl leads to a stepwise increase in the diameter of the TA. As was expected the magnitude of response of KCl and ADO application
blunted when compared to prior ADO dose responses. Furthermore the response of KCl + ADO was comparable to the vasomotor response observed in prior exposures of the two dilators (Fig 5B and 10B.). Similar to other experimental protocols that exposed the cremaster to ADO with KCl together (Fig 5B and 10B) the variability of the vasomotor response seemed to be correlated with ADO concentration; as concentrations of ADO increased there seemed to be a dose dependent increase in the variability of the response, again indicating the response to higher ADO concentrations was not as consistent between animals to the attenuative effects of KCl when compared to lower concentrations. When the same range of ADO and KCl were applied in the presence of Oua there was a significant amplification in the magnitude of dilation elicited by $10^{-8}$M or $10^{-7}$M ADO by approximately 129% and 211%, respectively. However, higher concentrations of ADO, $10^{-6}$M and $10^{-5}$M, displayed no change in dilation compared to when KCl and ADO were applied together. These data indicates that KCl attenuates higher concentrations of ADO independently of Na$^+/K^+$-ATPase, yet the attenuation of lower concentrations of ADO is rescued from KCl's attenuation perhaps indicating Na$^+/K^+$-ATPase is responsible, in part, for the observed attenuation of ADO-elicited dilation when in the presence of KCl. Taken together with dilatory pattern of ADO and KCl in the presence of BaCl (Fig 10B) it appears as though K$_{IR}$ channels are obligatory for KCl to elicit its attenuative effects while the involvement of Na$^+/K^+$-ATPase seems to be concentration dependent. This observation supports that changes when in the presence of KCl in activity of K$_{IR}$ and Na$^+/K^+$-ATPase are involved in the attenuation of low
concentration ADO, while attenuation at higher concentrations are solely dependent on changes in $K_{IR}$ channel activity.

**Figure 12**

A. **Oua effect on NO-elicited Dilation in the Presence of KCl**

B. **Oua effect on ADO-elicited Dilation in the Presence of KCl**

**Figure 12**: The application of a Oua in the presence of both KCl and SNAP (A) and KCl and ADO (B) causes an amplification in SNAP’s and ADO’s dilatory ability effect at certain concentrations A) The change in diameter in response to KCl and SNAP in the absence (■) and presence (□) of Oua. B) The change in diameter in response to KCl and ADO in the absence (■) and presence (□) Oua. Indicates experimental groups significant differed (p<0.05) from control.
Chapter 4. Discussion

4.1 Summary of Results

The present study demonstrates that vasodilators relevant in mediating active hyperaemia, interact with one another, such that, when applied to skeletal muscle vasculature together they influence one another’s vasodilatory ability compared to when applied alone. Our data indicate that dilatory concentrations of KCl exert a potent inhibitory effect on the dilatory abilities of both NO and ADO. More specifically, when the vasculature is pre-exposed to KCl for 5 minutes followed by the addition of NO and ADO (in the presence of KCl), the magnitude of dilation elicited by each is significantly attenuated compared to when applied in the absence of KCl. We have further demonstrated that pre-exposure to low concentration NO or ADO for 5-minute period followed by the addition of KCl (in the presence of NO or ADO) did not effect the magnitude of dilation produced by dilatory concentration of KCl, compared to when applied alone. Additionally, pre-exposure of the vessel to NO or ADO had no impact the dilatory ability of one another compared to when added alone. The conclusions that can be drawn from these experiments are two-fold 1) metabolic vasodilators have the ability to influence one another’s dilatory ability and 2) K⁺ is a prominent modulator of the dilatory ability of both NO and ADO.

In order to elucidate if the observed interactions were a product of their sequential application (if the 5-minute pre-exposure influenced vessel responsiveness to the addition on other vasodilators) each combination of
vasodilators were simultaneously added together and dilatory effects on one another were observed over a 10-minute exposure period. Results indicated that regardless of when KCl was applied with NO or ADO (sequentially or simultaneously) KCl caused a significant attenuation of each dilators ability to elicit vasodilation. Furthermore, from this experiment we concluded that the addition of two dilators do not necessarily cause an additive response, and in fact can cause less vasodilation compared to when each vasodilator is added alone. We also found evidence that during the simultaneous addition of NO and ADO, NO appeared to exert its dilatory action upon application, eliciting and mediating the initial vasodilation (for approximately 3 minutes). Yet, over time the magnitude of dilation elicited by NO diminished and the less potent dilatory effect of ADO became apparent, facilitating dilation throughout the remainder of application. Thus, it appears that when added together NO acts to cause initial increases in dilation while ADO acts in maintain a constant luminal diameter once the effects of NO have diminished.

After demonstrating that KCl’s attenuation of ADO and NO vasodilation are not dependent on the sequence in which they are added we aimed to further understand, mechanistically, how KCl is able to attenuate NO and ADO dilation. We have demonstrated that both KIR channels and Na⁺/K⁺-ATPase are involved in the dilatory pathway of KCl. From these observations we aimed to assess if the mechanisms involved in mediating KCl dilation were also responsible for the observed attenuation of NO and ADO dilation. We hypothesized that by
antagonizing channels involved in KCl dilation would rescue (or amplify) the dilatory ability of NO and ADO when applied in the presence of KCl. To test this hypothesis we observed the dilatory response of NO and ADO when applied in the presence of KCl and in the presence of a $K_{\text{IR}}$ and Na$^{+}$/K$^{+}$-ATPase antagonist. As the dilatory response of both NO and ADO in the presence of KCl were amplified when added in the presence of each antagonist, we conclude that the KCl-mediated attenuation of NO and ADO was mediated by the changes in of $K_{\text{IR}}$ channel and Na$^{+}$/K$^{+}$-ATPase activity that occur when exposed to KCl. More specifically the attenuation of high concentration NO in the presence of KCl seem to involve the altered activity of $K_{\text{IR}}$ channels, while lower concentrations seem to dilate independently of $K_{\text{IR}}$ channel activity. Further, Na$^{+}$/K$^{+}$-ATPase does not appear to be involved in the KCl mediated attenuation of NO. The attenuation of ADO by KCl, on the other hand, appears to be mediated by the changes in activity of $K_{\text{IR}}$ channels that occur when added in the presence of KCl, attenuation of low concentrations also seemed to involve the altered activity of Na$^{+}$/K$^{+}$-ATPase.

4.2 Experimental considerations

It is important to note that there are numerous different vessel branch levels that comprise the vascular tree within skeletal muscle. While each level of vasculature works in conjunction with others to regulate blood flow within skeletal muscle, they do not all share the same function with respect to their contribution in mediating increases in flow, during rest and activity (Renkin, 1984; Dodd & Johnson, 1991). Consequently, changes in luminal diameter at one branch order
may not reflect 1) the behavior of all vessels or 2) changes of flow in other parts of the vascular tree. However, the level of the vasculature studied, the TA, has a unique importance in regulating blood flow as it aids in the distribution (Klitzman et al., 1982; Sweeney & Sarelius 1989; Dodd & Johnsnon, 1991) and coordination of blood flow within active skeletal muscle and is recognized as one of the most responsive regions of the arteriolar network during skeletal muscle contraction (Dodd & Johnson 1991; VanTeeffelen & Segal, 2006, Marshall and Tandon, 1984) making it an appropriate assay to assess potential interactions among metabolic vasodilators.

As the cremaster was used as the muscle bed of interest it is important to address the fact that this muscle bed comprises of ~17% glycolytic ~83% oxidative muscle fibers (Murrant et al., 2014). Thus, the vasomotor response observed may not necessarily reflect the behavior of the microvasculature of muscles with drastically different fiber composition as studies have shown that vasculature associated with certain muscle fiber types react differently to metabolic vasodilators (McAllister, 2003). However, our laboratory has investigated the vasomotor response to metabolic vasodilators in the gluteus maximus of hamsters and found the vascular response is similar to that observed in the cremaster, thus vasomotor response between muscle types is comparable (Murrant et al., 2014). Therefore, the response of microvasculature in the cremaster muscle is reflective of other muscle beds of the body. Another drawback of the cremaster is that it is not a postural muscle, while the
implications of this, in terms of regulation of blood flow, is widely unknown, the ideal muscle for experimentation would be one used in locomotion, that is highly metabolically active, experiencing periods of rest and high intensity activity. However, as discussed it has been shown the vascular of the cremaster is comparable to postural muscles. Despite the limitation of the cremaster muscle there are numerous strengths in using this muscle. First and foremost the vessels of the microvasculature can be visualized with remarkable detail. As the cremaster is only several cell layers thick vessels are almost always in focus and are rarely obscured by fascia, connective tissue or muscle. This provides for very precise measurements of vessel luminal diameter, therefore variability and experimenter error, when measuring diameter, is likely reduced compared to measuring vessels in thicker tissue, that tend to be commonly out of focus and who’s luminal borders are difficult to visualize. Another strength of using the cremaster muscle is that multiple levels of the microcirculation can be visualized (from capillaries to feed arterioles). While in this study we only assessed the vasomotor response of one vascular level, the TA, multiple other branch order could have also been assessed. The benefit of this is that we are able to assess if our observations regarding vasodilatory interaction occur at different levels of the cremaster microvasculature, and we are able to do this without having to change experimental models, thereby reducing the number of variables that would have to be considered.
Differences were found between baseline diameters before and after drug exposures during the protocols assessing the impact of BaCl on KCl-elicited dilation and BaCl's effect on ADO dilation in the presence of KCl. Differences in baseline diameter indicate that smooth muscle length (an index of state of contraction of the VSM) differed before and after application of vasodilators. Given that VSM force generation is dependent upon length (or contractile state), it is possible the dilatory effects elicited by each vasodilator may impact its state of contraction, and therefore may influence their ability to constrict or dilate in the presence of a vasoactive factor (Davis & Gore, 1989; Seow, 2000). Thus, it stands to reason if baseline diameter influences magnitude of dilation elicited by the vasodilators tested, then the dilatory response of the blood vessel to vasodilators should be correlated with the vessels baseline diameter. As such, when a significant difference between initial and experimental baseline diameters was found we plotted change in diameter elicited by the study vasodilators tested as a function of both baseline diameter and dilatory potential. We found no strong correlation between baseline diameter, dilatory potential and change in diameter during the assessment of BaCl's effect on KCl dilation (Figure 8A and B) or BaCl's effect on ADO in the presence of KCl (Figure 11A and B). Therefore, the differences in baseline diameter did not significantly alter the vessel responsiveness and consequently does not alter the interpretation of the data.

The vasodilators investigated in this study were chosen due to their involvement in mediating the active hyperaemic response. Concentrations of K⁺
investigated were based on previous literature that has measured peak K$^+$ concentrations of $\sim$9mM in the ISS of skeletal muscle of humans during intense periods of exercise (Hnik, 1973; Juel, 2000). Ranges of both ADO (10$^{-9}$M-10$^{-5}$M) and NO (10$^{-8}$M-10$^{-4}$M) concentrations were investigated since it has been previously shown that the production of both ADO and NO concentration in the ISS is correlated with muscle metabolism. Therefore, there is the possibility of a wide range of concentrations of both ADO and NO accumulating extracellularly depending on intensity and duration of exercise. It has been reported that resting values of extracellular ADO in humans have been measured at $\sim$2.0x10$^{-7}$M (Hellsten, 1998; Costa, 2000) with increases up to 1.14x10$^{-6}$M during light intensity exercise (Hellsten, 1998). In vitro investigations of NO have reported resting values of 6.2x10$^{-11}$M and increasing upwards of 10x10$^{-9}$M during muscle stimulation (Balon & Nadler, 1994), however these values are from in vitro studies and therefore are likely underestimating values found in vivo during rest and exercise. Although there are a multitude of metabolic vasodilators reported to be involved in contraction induced vasodilation it was not necessary to investigate them all as we aimed to address if vasodilators interact with one another in the resistance microvasculature of skeletal muscle. Thus, based on previous literature we chose dilators that were established in mediating active hyperaemia and that previous evidence indicated may interaction with one another.
Interactions between K+, NO and ADO were investigated at the level of the TA, however, as the entire cremaster muscle was exposed to each vasodilator, all levels of vasculature were exposed to the effects of each vasodilator. It has been shown that NO and ADO (inconsistently) are able to elicit a conducted response (Cohen & Sarelius, 2002; Duza & Sarelius, 2003; Tallini, 2007; Rivers & Frame, 1999). Thus, it stands to reason that the observed dilation of the TA in the presence any of the vasodilators studied was not solely the local response of the vessel segment of interest, but rather the product of both the local response and the propagated dilation arising from different vascular branches such as the capillaries. Ultimately, it was not the purpose of this study to attempt to define whether the local or conducted response was affected during vasodilator interaction, but to establish whether vasodilator interactions exist. However, the fact that vasodilation observed in the TA in our studies is likely a product of local and conducted response raises a key question; does KCl cause diminution of NO and ADO elicited vasodilation by blunting the magnitude of the local response, or by blunting the magnitude of the conducted response they elicit. Future research is necessary to delineate which response is attenuated by KCl. Additionally, by investigating KCl’s impact on the magnitude of conducted response initiated by NO and ADO, we will be able to further characterize the impact of KCl-mediated interactions on skeletal muscle blood flow.

SNAP is a commonly used NO donor (Clifford & Hellsten, 2004; Dua et al., 2009; Murrant et al., 2014; Yang & Iadecola 1997; Toda et al., 2009) as it
spontaneously releases NO over time. Although the exact concentration of NO released with each concentration of SNAP is not known, it is to be expected that an incremental increase in NO concentration occurs with incremental increases in SNAP. Data presented displays a classic dose response behavior of increases in vasodilation to increases in SNAP concentration (Figure 6B and 8B.), which we would not expect if SNAP release of NO was sporadic. As the half-life of SNAP in aqueous solution exposed to air is ~4 hours (Terwel et al., 2000) steps to ensure that the handling of SNAP was consistent within each protocol for example, time between creation of the solution and exposure to the cremaster was kept as consistent as possible, (approximately 1 hour). Thus, the consistent time between SNAP synthesis and exposure, the expected vascular response to exposure of SNAP and the wide use of it in literature as a NO donor lends support to the use of SNAP as the appropriate NO-donor.

BaCl and Oua, were applied to antagonize K$_{IR}$ channels and Na$^+$/K$^+$-ATPase, respectively (Burns et al., 2004; Armstrong et al, 2007). As we blocked each channel individually, we expected only a blunted KCl dilation, as the other unblocked channel could still facilitate KCl dilation. Furthermore, as we could not ensure a complete antagonism of either K$_{IR}$ channel or Na$^+$/K$^+$-ATPase, we are unable to rule out the possibility that dilation produced during antagonism was solely by the unblocked mechanism, as unblocked channels may in part be facilitating KCl dilation. However, as we observed a reduced magnitude of 10mM KCl dilation in the presence of both 50µM BaCl and 10$^{-4}$M Oua, this indicates that
adequate concentrations of both agonists were applied to induce a change. Additionally, as we were only looking for the involvement of $K_{IR}$ and $Na^+ / K^+$-ATPase 100% inhibition of each was not necessary. However, since 100% inhibition was likely not achieved, it is probable that we are underestimating the role of $K_{IR}$ and $Na^+ / K^+$-ATPase in facilitating the KCl-elicited attenuation of NO and ADO by KCl. Lastly, the antagonism of both channels in the presence of KCl was not tested since, in our hands, the application of both Oua and BaCl caused inconsistent changes in vasculature luminal diameter, therefore we could not confidently measure the change in vessel diameter in the presence of both antagonists.

4.3 KCl attenuates the vasodilatory response of NO and ADO

The aim of NO and ADO application in the presence of KCl, was to assess if the vasodilatory response of NO or ADO differed when added in the presence of KCl as opposed to when added alone. Our data show that pre-exposure to single dilatory concentration of KCl, followed by exposure to a wide range of NO and ADO (in the presence of KCl) attenuates their dilatory response, compared to when each were added alone (Figure 6B and 7B.). As KCl has a transient dilation lasting approximately 5 minutes before returning to pre-exposure baseline we exposed the vessel for 5-minute to KCl letting it return to near baseline diameter before adding NO and ADO, allowing us to assess NO and ADO dilation in the presence of KCl at a stable baseline diameter. To our knowledge we are the first to demonstrate the vasodilatory pathways of $K^+$ and
NO and ADO interact, with K⁺ being able to attenuate the vasodilatory response elicited by a wide range of NO and ADO.

As it is presumed each of the vasodilators are released contemporaneously during contraction we would not expect that KCl would be present 5 minutes prior to ADO and NO as our initial protocols tested. Thus, there is the possibility that the observed interaction could be due to nature in which the vessel was exposed to each vasodilator. Therefore, to ensure the pre-exposure of the vessel did not cause the observed attenuation of either NO or ADO we simultaneously exposed the vessel to KCl and NO, and separately, KCl and ADO, thus mimicking the simultaneous release of each pair of vasodilators. When added alone both NO and ADO produced a relatively constant vasodilation that persisted throughout the 10-minute exposure. However, when simultaneously added with KCl we observed an initial peak in vasodilation followed by a constant decline in vessel diameter to pre-exposure, baseline levels (Figure 7A and 7B). The observed initial peak change in dilation was likely due to the vasodilatory effect of KCl, as the magnitude of peak dilation was comparable the vasodilatory profile of KCl added alone. This indicates that the addition of KCl to NO or ADO is not additive, if it were we would expect a peak dilation to be the sum of KCl-elicited dilation and the dilation produced by either NO or ADO. As the exposure of vasodilator combinations tested does not produce an additive dilation suggests that the attenuative interaction between KCl, ADO and NO occurs immediately upon exposure, with the transient dilation
produced by KCl masking ADO and NO dilation, therefore KCl completely and immediately inhibits the vasodilatory ability of both vasodilators.

4.4 Effect of ADO and NO’s vasodilatory ability on one another

We have also shown that the sequential addition of NO, and separately ADO, does not impact the dilatory magnitude of a range of KCl concentrations. More specifically a 5-minute exposure to ADO or NO followed by the addition of 10mM and 20mM KCl (in the presence of either NO or ADO) did not significantly alter the magnitude of dilation compared to when KCl was applied alone (Figure 6A and 7A). The 5-minute exposure period was to ensure a steady change in diameter was achieved before the range of concentration of the other vasodilator was applied. Further, the sequential addition of NO and ADO demonstrated that neither of the two vasodilators affected one another’s dilatory ability. The observed response to the sequential addition of NO and ADO provided evidence that NO does not appear to effect the dilatory ability of ADO and furthermore ADO also has no affect on the magnitude of NO’s vasodilation (Figure 9C.). These observations ultimately demonstrate that while there are instances in which vasodilators are able to interact there are also vasodilators that have no apparent influence on one another.

In order to ensure that our observations regarding the vasodilatory interaction was not dependent on the sequential addition of each vasodilator we added a single concentration of NO and ADO simultaneously and observed the
vessel response over a 10-minute exposure. Alone both NO and ADO produced a relatively constant vasodilation throughout the exposure period. However, the dilatory pattern observed when added simultaneously differed from what was observed when the two dilators were added sequentially. The addition of NO and ADO caused a rapid increase in vessel magnitude, similar to that when NO was applied alone and differing from the initial dilation elicited by ADO. The fact that the response elicited by the simultaneous application of ADO and NO did not differ from the dilation produced by NO alone suggests that the response of NO and ADO when applied together are not additive. The first 3 minutes of exposure to NO and ADO closely followed the dilatory profile of NO when applied alone, however after 3 minutes, while the vasodilation of NO alone persists throughout the 10 minute exposure, NO and ADO application produced a decrease in the change in diameter to a magnitude similar to that produced by ADO when applied alone. Taken together it seems that the simultaneous exposure of NO and ADO affect one another’s vasodilatory ability, more specifically it appears as though upon the onset of exposure NO’s effects are predominant, causing the initial dilation within the first 3 minutes, however as time progresses the effect of NO becomes less apparent, with ADO becoming a more influential dilator, maintaining the dilation for the remainder of exposure. These data indicate that interactions between vasodilators may have a temporal component

4.5 Effect of $K_{ir}$ channel antagonist $BaCl$ on the dilatory ability of NO and ADO when in the presence of $KCl$
We observed that blocking the primary mechanisms by which $K^+$ induces vasodilator via $K_{IR}$ channel and $\text{Na}^+$/\text{K}^+-\text{ATPase} we were able to rescue the vasodilation induced by NO and ADO. We added KCl and SNAP, and separately KCl and ADO alone and in the presence of $50 \times 10^{-6}$M BaCl (the same concentration we have shown to blunt KCl dilation). As expected the dose response of NO and separately ADO in the presence of a single dilatory concentration of KCl, caused a blunted dilatory response at each concentration tested compared to prior dose response of SNAP and ADO when each were applied alone. However when the dose response of both NO and ADO, in the presence of KCl was repeated in the presence of BaCl we observed an increase in the magnitude of dilation elicited by NO and ADO. In general the amplified, or restored, response of each vasodilators tested in the presence of BaCl provides an indication that the $K_{IR}$ channel are involved in $K^+$ attenuation of both NO and ADO.

When BaCl was applied in the presence of KCl and NO the dilatory response elicited by $10^{-6}$M and $10^{-5}$M SNAP were significantly amplified, compared to when NO and KCl were applied alone. With the blockade and suspected decrease in channel activity causing the amplification in NO response, it provides an indication that KCl’s attenuative effect at high concentrations of NO are dependent upon the alteration of $K_{IR}$ channel activity in the presence of KCl, while the attenuation at lower concentrations appears to be seemingly unaffected by the $K_{IR}$ channel blockade. Thus, the attenuation of NO dilation by $K_{IR}$ channels
are concentration dependent and K⁺ inhibition of NO at lower concentrations involves a different mechanism. The application of BaCl in the presence of KCl and ADO caused an amplified response of ADO at all concentrations tested, compared to when KCl and ADO were applied alone. This observation suggests that the alteration of Kᵢᵣ channel activity when in the presence of dilatory concentration of KCl is obligatory for attenuating the vasodilatory ability of ADO.

4.6 Effect of Na⁺/K⁺-ATPase channel antagonist BaCl on the dilatory ability of NO and ADO when in the presence of KCl

Similarly, we observed that blocking the second mechanism, Na⁺/K⁺-ATPase, by which K⁺ induces vasodilation we were able to restore the vasodilation induced by NO and ADO. As expected the application of NO, and separately ADO in the presence of KCl caused an expected decrease in the vasodilatory ability of NO and ADO compared to prior protocols when each were applied alone. However when applied in the presence of ouabain there was an observed amplification of ADO dilatory ability in the presence of KCl, whilst NO was not amplified at any of the concentrations tested.

When Oua was applied in the presence of KCl and NO there was no significant amplification of NO’s dilatory response at any concentration tested, compared to when NO and KCl were applied together. This lack of amplification at the concentrations tested provides an indication that KCl’s attenuative effect of NO act independently of Na⁺/K⁺-ATPase, that is to say the alteration of the channel activity in the presence of KCl does not mediate the attenuation,
suggesting a different pathway, independent of Na\(^{+}/K^{+}\)-ATPase, may mediate the observed attenuation.

When Oua was applied in the presence of KCl and ADO there was significant amplification of ADO’s dilatory response at lower concentrations tested (10\(^{-8}\)M and 10\(^{-7}\)M), but not higher concentrations, compared to when ADO and KCl were applied alone. This amplification at the concentrations tested provides an indication that KCl’s attenuative effect of ADO are dependent on Na\(^{+}/K^{+}\)-ATPase activity, at lower concentrations of ADO while the higher concentrations are unaffected by Na\(^{+}/K^{+}\)-ATPase antagonism, thus indicating the mechanism of their attenuation by KCl functions independently of Na\(^{+}/K^{+}\)-ATPase activity.

4.7 Redundancy among vasodilators of active hyperaemia

We show that the addition of two vasodilators does not always cause a net additive response, that is to say the magnitude of dilation elicited by one vasodilator does not compound or summate with the effect of another and can actually produce less vasodilation compared to when added individually. The fact that two dilators can produce a decrease in vascular diameter compared to when added alone may seem counterintuitive but the implications of this may actually provide insight into the interactions during active hyperaemia. As all 3 vasodilators tested are contemporaneously present, it is likely that during muscle contraction there is a constant interaction occurring between NO, ADO and K\(^{+}\), thus K\(^{+}\) is attenuating NO and ADO throughout skeletal muscle activity. Initially, this may seem to be counterintuitive to the purpose of the active hyperaemic
response, why would we want to reduce the ability of vasodilators to elicit their effects when during activity a net increase in blood flow is required? But upon closer analysis this type of interaction may in fact be suggestive of a compensatory or redundant system that underlies the active hyperaemic response. As KCl is able to reduce the dilatory ability of both NO and ADO this may act as a compensatory mechanism as the effects of NO and ADO can be amplified in order to preserve blood flow if the release of K\(^+\) is inhibited or hindered.

Potential interactions between vasodilators have recently been characterized. Boushel et al. (2002) and Engelke et al. (1996) found that the inhibition of both COX and NOS (enzymes responsible in the synthesis of PGI\(_2\) and NO, respectively) lead to a drastic decrease in blood flow during exercise. Investigations into the dilatory pathways of prostanoids and NO found that the inhibition of prostanoid formation is thought to increase NO synthesis via a cAMP-dependent mechanism (Bolz & Pohl, 1997). More specifically, the inhibition of prostanoids would lead to an increase in intracellular Ca\(^{2+}\) which would cause an increase in NO formation, thereby providing a compensatory mechanism to account for the loss of prostanoids dilatory effects (Frandsen, 2000; Karamouzis, 2001). This interaction may also provide a potential explanation as to why during the increase in skeletal muscle activity there is no change in blood flow during the antagonism of prostanoid synthesis as NO release would be amplified and therefore compensate for the loss of prostanoid’s vasodilatory effect. Thus this
interaction would make it seem as though prostanoids has little to no effect during active hyperaemia (Mortensen et al. 2009b, 2007). This suggests that the active hyperaemic response may not solely rely on the additive action of vasoactive factors released during muscle contraction, but instead may be the result of the interaction between vasoactive factors.

As discussed, prior research has begun to investigated potential vasodilator interactions underlying the active hyperaemic response, however to our knowledge there has been little to no research examining the potential interactions between K\(^+\), NO and ADO in the skeletal muscle vasculature. A study by Foley (1979) has assessed the interactions between ADO and elevations in K\(^+\) concentration in coronary artery smooth muscle in vitro found that the magnitude of relaxation elicited by ADO was inversely related to concentrations of K\(^+\), with dilatory concentrations of K\(^+\) (10mM) reducing the magnitude of vascular relaxation among elicited by ADO at all concentrations tested (10\(^{-5}\)-10\(^{-6}\)M). Foley’s findings parallel our own, showing that elevations in K\(^+\) cause an attenuation of ADO dilation in the vasculature. The fact that others have shown the same interaction between KCl and ADO may provide an indication that interactions between K\(^+\) and ADO are not specific to skeletal muscle tissue. Thus, interactions between K\(^+\) and ADO may be conserved within the vasculature of different muscle beds.
Given that vasodilatory factors are able to influence one another dilatory ability and the observations that pharmacological interventions blocking a single vasodilator are unable to abolish the hyperaemic response strongly indicate that redundant or compensatory mechanisms underlie skeletal muscle blood flow regulation. A redundant mechanism during active hyperaemia would allow for blood flow to be preserved when the release and/or synthesis of another vasodilator is impeded. As we have found that KCl is able to attenuate a wide range of NO and ADO, regardless of when it is exposed vasculature (sequentially or simultaneously) may be a novel redundant pathway within skeletal muscle vasculature. As concentrations of K$^+$ relevant to skeletal muscle contraction cause dilation, while actively attenuating both NO and ADO, it stands to reason that if K$^+$ was not released during skeletal muscle activity its absence would cause an amplified response of both NO and ADO. In other words, during active hyperaemia the presence of K$^+$ is constantly attenuating the vasodilatory effects of NO and ADO, however in situations where K$^+$ release is dampened or inhibited NO and ADO’s effects would be amplified, therefore, preserving the increase in blood flow despite the inhibition of K$^+$’s dilatory effect. It is important to note that the concentrations of K$^+$ used in our protocols are similar to concentrations found in skeletal muscle ISS after high intensity exercise, more research is needed to confirm if the relationship between KCl, NO and ADO holds true when lower K$^+$ concentrations are present in the ISS.
4.8 Summary of results and their implications on skeletal blood flow regulation

Given the critical nature of matching blood flow to O\textsubscript{2} requirements skeletal muscle it is not surprising to find redundancy built into the system such that if one component fails, another is there to keep the system from failing. This means that studies into active hyperaemia needs to shift focus from trying to determine the importance of single vasodilators to studying vasodilation interactions and the redundancy between them.

The present experiments have indicated that vasodilators produced and released during muscle contraction possess the ability to influence one another’s vasodilatory ability. More specifically we have shown that KCl is able to attenuate the magnitude of vasodilation elicited by NO and ADO regardless of how they were applied (sequentially or simultaneously). We have found that this attenuation is facilitated by change in K\textsubscript{IR} channel and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity that occurs when in the presence of KCl. Furthermore, we have also demonstrated that when simultaneously present NO and ADO appear to interact with one another, with NO acting to facilitate the initial increase in vasodilation early on in exposure, whereas ADO’s effects are more prominent later on in the application period.

Taken together these data provides convincing evidence that there may be a redundant, or compensatory, system that underlies the active hyperaemic response. While there have been limited investigations into vasodilator interaction and redundancy we are the first to our knowledge to demonstrate that
interactions occur between K⁺, ADO and NO. The major implications of these findings, in terms of their relevance to skeletal blood flow regulation are interactions between vasodilators provide evidence of a redundant or compensatory mechanism underlying hyperaemic response, where the effects of certain vasodilators are able to be amplified if another is inhibited. From a physiological stand point redundancy provides a mechanism in which blood flow can be preserved upon the inhibited or impeded release of another vasodilator. However, redundancy within the regulation of skeletal muscle blood flow is a relatively new area of investigation, with only a few known dilator interactions being characterized, the apparent lack literature in this area is in part, due to the complexity of investigating this system, as trying to identify potential interaction between dilators can be difficult as a compensatory pathway may be triggered upon blockade of a dilator. Additionally redundancy may also provide an explanation as to why pharmacological intervention have not been able to abolish the active hyperaemic response with blockades of individual vasodilators, as during the blockade another compensatory pathway may become active therefore masking the true effect of the dilator antagonism.
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Appendices

Experimental Timelines of Aim 1.1

Experimental Timeline: Protocol 1 – Quantifying NO’s effect in the vasodilatory ability of K⁺
Experimental Timeline: Protocol 2 – Quantifying K⁺'s effect in the vasodilatory ability of NO
Experimental Timeline: Protocol 3 – Quantifying ADO’s effect in the vasodilatory ability of K⁺
Experimental Timeline: Protocol 4 – Quantifying K⁺’s effect in the vasodilatory ability of ADO
Experimental Timeline: Protocol 5 – Quantifying ADO’s effect in the vasodilatory ability of NO
Experimental Timeline: Protocol 6 – Quantifying NO’s effect in the vasodilatory ability of ADO
Experimental Timelines of Aim 1.2

Experimental Timeline: Protocol 7 – Quantifying KCl’s effect on the vasodilatory ability of NO when added simultaneously
Experimental Timeline: Protocol 8 – Quantifying KCl’s effect on the vasodilatory ability of ADO when added simultaneously

1 minute of baseline

10^{-7} M ADO

Recovery

2 minutes

Washout

10^{-7} M ADO

1 minute of baseline

10 minutes

10^{-7} M ADO + 10 mM KCl

Max Diameter

2 minutes

10 minutes

Recovery

2 minutes
Experimental Timeline: Protocol 9 – Quantifying NO’s and ADO effect on the vasodilatory ability of one another

- 1 minute of baseline
- 10^{-7} M SNAP or 10^{-7} M ADO
- Recovery
- 2 minutes
- Washout
- 10^{-7} M SNAP
- 10^{-7} M ADO
- Recovery
- Max Diameter
- 2 minutes
- 10^{-7} M SNAP
- 2 minutes
- Washout
- 10^{-7} M ADO
Experimental Timelines of Aim 2.1

Experimental Timeline: Protocol 10 – Quantifying $K_{IR}$ channels involvement in the dilatory pathway of KCl.

1 minute of baseline

10 minutes

10mM KCl

2 minutes

Recovery

20 minutes

Washout

30 minutes

50x$10^{-6}$M BaCl Wash-in

10 minutes

10mM KCl + 50x$10^{-6}$M BaCl

Max Diameter

2 minutes

Recovery
Experimental Timelines of Aim 2.2

Experimental Timeline: Protocol 12 - Quantifying the effects of antagonism of $K_{IR}$ channel on NO dilation in the presence of 10mM KCl

1 minutes baseline

5 minutes 10mM KCl

2 minutes $10^{-8}$M SNAP + 10mM KCl

2 minutes $10^{-7}$M SNAP + 10mM KCl

2 minutes $10^{-6}$M SNAP + 10mM KCl

2 minutes Recovery

2 minutes 5 minutes 10mM KCl + BaCl

30 minutes 50x$10^{-6}$ M BaCl

20 minutes Washout

2 minutes 10mM KCl + BaCl

30 minutes $10^{-5}$M SNAP + 10mM KCl + BaCl

5 minutes $10^{-4}$M SNAP + 10mM KCl + BaCl

2 minutes Recovery

2 minutes 2 minutes 2 minutes 2 minutes 2 minutes Max Diameter
Experimental Timeline: Protocol 13 - Quantifying the effects of antagonism of Na⁺/K⁺-ATPase on ADO dilation in the presence of 10mM KCl.
Experimental Timeline: Protocol 14 - Quantifying the effects of antagonism of K\text{IR} channel on NO dilation in the presence of 10mM KCl

1 minutes baseline

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<thead>
<tr>
<th>5 minutes</th>
<th>2 minutes</th>
<th>2 minutes</th>
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<tbody>
<tr>
<td>10mM KCl</td>
<td>10^{-8}M ADO + 10mM KCl</td>
<td>10^{-7}M ADO + 10mM KCl</td>
<td>10^{-6}M ADO + 10mM KCl</td>
<td>10^{-5}M ADO + 10mM KCl</td>
<td>Recovery</td>
<td>1 minutes baseline</td>
<td>Washout</td>
<td>50x10^{-6}M BaCl</td>
<td>10mM KCl + 10mM KCl + 50x10^{-6}M BaCl</td>
<td>10^{-8}M ADO + 10mM KCl + 10mM KCl + 10^{-6}M ADO + 10mM KCl</td>
<td>10^{-7}M ADO + 10mM KCl + 10mM KCl + 10^{-6}M ADO + 10mM KCl</td>
<td>10^{-5}M ADO + 10mM KCl + 10mM KCl + 10^{-6}M ADO + 10mM KCl</td>
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10^{-6}M ADO
Experimental Timeline: Protocol 15 - Quantifying the effects of antagonism of Na⁺/K⁺-ATPase channel on ADO dilation in the presence of 10mM KCl