## THESIS

# MECHANISMS OF EXERCISE HYPEREMIA DURING ELEVATED RESTING OXYGEN DELIVERY IN HUMANS

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## ABSTRACT

# MECHANISMS OF EXERCISE HYPEREMIA DURING ELEVATED RESTING OXYGEN DELIVERY IN HUMANS

The coupling between skeletal muscle oxygen delivery  $(O_2D)$  and metabolic demand is largely attributed to the integration of feedback, and feedforward vascular mechanisms. It has been demonstrated that blood flow responses remain intact despite pharmacological elevations in resting blood flow, suggesting the existence of a vasodilator capable of augmenting hyperemia independent of tissue oxygen demand. We hypothesized that the change in forearm blood flow (FBF) from rest to steady-state exercise is preserved independent of baseline  $O_2D$ , and that reciprocal reductions in oxygen extraction coincide with elevated O<sub>2</sub>D (Protocol 1). Additionally, pharmacological blockade of K<sub>ir</sub> channels and ATPase will reduce the change in FBF. In 10 young healthy adults, we quantified forearm blood flow (FBF; Doppler ultrasound), venous oxygen saturation ( $SvO_2$ ), oxygen extraction ( $O_2$  extraction; deep venous blood samples), and forearm oxygen consumption ( $mVO_2$ ) at rest and throughout 5 minutes of mildintensity (10% maximal voluntary contraction; MVC) rhythmic handgrip exercise under control (CON) conditions and following intra-arterial infusion of the vasodilator sodium nitroprusside (SNP) to elevate local FBF and  $O_2D$ . In Protocol 1, we elevated resting FBF and  $O_2D$  to levels that matched (MAT) and exceeded (EXC) steady-state FBF (FBF: MAT; 166 ± 25 ml/min, P=NS, EXC; 219 ± 27 ml/min, P<0.05) during control (CON) exercise trials (FBF: CON; 172 ± 24). Changes in blood flow remained intact ( $\Delta$ FBF: CON; 135 ± 20 ml/min vs. MAT; 132 ± 19 ml/min vs. EXC;  $167 \pm 26$  ml/min, P=NS across all conditions), despite elevations in resting FBF which were adequate to sustain steady-state contractile activity under CON conditions.

Reciprocal reductions in O<sub>2</sub> extraction were observed in the MAT (O<sub>2</sub> extraction: CON;  $63 \pm 3\%$ vs. MAT;  $43 \pm 5\%$ , P<0.05) and EXC trials (O<sub>2</sub> extraction: CON;  $63 \pm 3\%$  vs. EXC;  $35 \pm 5\%$ ., P < 0.05) compared to CON during exercise. Additionally, we measured venous K<sup>+</sup> in a subset of participants (N=6) to evaluate changes in K<sup>+</sup> efflux (venous [K<sup>+</sup>] x FBF/1000) as an index of K<sup>+</sup> release during exercise, alluding to K<sup>+</sup>-mediated activation of K<sub>ir</sub> channels and the ATPase. Five participants completed Protocol 2 which included control and elevated FBF trials (Saline + Block and SNP + Block) with the addition of intra-arterial infusion of barium chloride (BaCl<sub>2</sub>) and ouabain to inhibit K<sub>ir</sub> channels and the ATPase, respectively. Blockade of these pathways reduced the change in FBF that persisted during the Protocol 1 MAT trial ( $\Delta$ FBF: MAT; 148 ± 21 ml/min vs. SNP + Block; 96  $\pm$  13 ml/min, P<0.05). From this data, we are able to determine that changes in blood flow during exercise persist despite elevations in resting  $O_2D$  (via SNP) prior to the start of exercise and that trends in  $O_2$  extraction follows changes in total  $O_2D$ . We believe that local skeletal muscle K<sup>+</sup> release is capable of activating K<sub>ir</sub> channels and the ATPase in a feed-forward manner which initiates a hyperpolarizing signal, thus augmenting blood flow independent of tissue oxygen demand.

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## CHAPTER 1 – REVIEW OF LITERATURE

The regulation of skeletal muscle blood flow during exercise is precisely coupled to oxygen consumption. Several hallmark studies have demonstrated that across a variety of muscle beds and exercise modalities, work rate, or tissue metabolic demand, and blood flow share a linear relationship. Typically, this response involves integration of local feedforward mechanisms such as potassium-mediated vasodilation and mechanical effects on the vasculature, metabolic feedback mechanisms, and interactions with sympathetic nervous system mediated vasoconstriction. The net effect of this integration is the precise regulation of oxygen delivery (O<sub>2</sub>D) to match the metabolic demand of the active tissue (Andersen & Saltin 1985; Saltin et al. 1998; Hamann et al. 2005; Joyner & Casey 2015). In humans, small muscle mass contraction evokes a biphasic blood flow response characterized by an immediate increase in muscle blood flow, followed by a slower, progressive increase in muscle blood flow proportional to the degree of steady-state tissue oxygen consumption ( $mVO_2$ ) (Tschakovsky et al. 2004; Wray et al. 2005; Kirby et al. 2007; Crecelius et al. 2013). Thus, the immediate response must rely on a mechanism capable of rapidly increasing, and properly distributing blood flow to active muscle at the onset of contractile work. While several metabolically-derived candidate substances have been proposed, none have been demonstrated to be essential in the early hyperemic response. (Hester et al. 1982; Shephard et al. 2016; Ranadive et al. 2019; Dillon et al. 2020). Hence, we have attempted to divulge an obligatory vasoactive substance which is unique to the functional hyperemic response observed upon the initiation, and continuation of rhythmic dynamic exercise.

This review will address the primary mechanisms which govern skeletal muscle blood flow, evaluate contributors to immediate exercise hyperemia at the onset of exercise as well those that contribute to steady-state hyperemia, and highlight the effect of elevated resting O<sub>2</sub>D prior to the initiation of exercise in humans. As such, this review will build upon existing observations and present them in the context of the current study.

## **Determinants of Skeletal Muscle Blood Flow**

Blood flow and thus oxygen delivery to a given tissue, is determined primarily by the presence of an arteriovenous pressure gradient across the vascular bed ( $\Delta P = P_a - P_v$ ) and its vascular conductance (VC), ( $Q_a = \Delta P \times VC$ , where  $Q_a$  represents arterial inflow) (Andersen & Saltin 1985; Saltin et al. 1998; Joyner and Casey 2015). Indeed, alterations in perfusion pressure and the degree of vascular tone may act independently, or synergistically, to dictate tissue blood flow (Joyner & Casey 2015). In skeletal muscle, perfusion pressure remains largely unaffected during small muscle mass exercise. Thus, changes in vessel tone of arterioles supplying skeletal muscle are likely the primary determinant of blood flow during exercise. Vascular conductance is directly proportional to vessel radius raised to the fourth power; thus, small changes in vessel diameter lead to disproportionately large changes in O<sub>2</sub>D. During skeletal muscle contractile work, various metabolic, endothelial derived, and blood borne substances are released within the local tissue milieu and directly influence vessel radius (Joyner & Casey 2015). Vasodilating substances, along with sympathetic nervous system activity, act to modulate blood flow and  $O_2D$ during exercise by way of their direct effect on vessel radius (Rosenmeier et al. 2003; Hearon et al. 2016; Kruse et al. 2017).

## Influence of Local Vasoactive Substances

From a human health perspective, it is not ideal to develop a single process as the primary response to any variety of physiological stimuli. Often, dysfunction within a single step of any physiological mechanism renders it ineffective (Roux, 2014). For this reason, mechanisms which synergistically contribute to vascular control operate in a redundant manner. A well acknowledged observation, redundancy refers to the various pathways in place which ensure proper tissue O<sub>2</sub>D. Drawing upon this idea, studies focused on dissecting these pathways have identified a multitude of contributing substances which act to directly affect vascular tone and thus exercise hyperemia in humans (Rosenmeier et al. 2003; Schrage et al. 2004).

The complex nature of vascular control in humans remains to be fully elucidated. While many candidate substances likely contribute to blood flow regulation, only a portion of them have been pursued in recent research. In much of the research performed over the past several decades, nitric oxide (NO) and vasodilating prostaglandins (PG), in combination, play a substantial role in regulating basal vascular tone, but contribute minimally to exercise hyperemia. Pharmacological inhibition of NO synthase (NOS) and cyclooxygenase (COX), enzymes which generate NO and PG, respectively, minimally reduces skeletal muscle blood flow during exercise, including brief forearm contractions in humans (Crecelius et al. 2013, Casey et al. 2013). Indeed, the reduction in skeletal muscle blood flow is negligible throughout onset and steady-state during combined inhibition of NO and PG (Crecelius et al. 2014). Although reductions in blood flow at the onset of exercise are negligible, NO and PG synthesis may contribute significantly to basal tone in humans, and may also play a role during steady-state exercise (Ilkka et al. 2011). Wray et al. showed a 20-25% reduction in forearm blood flow when NO and PG antagonism during high-intensity steady-state exercise in healthy young humans

(Wray et al. 2011). Similar to these findings, reductions in leg blood flow during dynamic exercise have been regularly observed during combined NO and PG inhibition without any further reductions observed with additional blockade of endothelial-derived hyperpolarizing factors (EDHF). Interestingly, Mortensen et al. observed an increase in venous ATP concentrations during knee extensor exercise with concomitant EDHF blockade. This observation demonstrates that other vasodilating substances were upregulated in order to sustain O<sub>2</sub>D, thus generating a spillover of ATP into the venous circulation. Together, these outcomes reinforce the idea of system redundancy to ensure proper muscle O<sub>2</sub>D (Mortensen et al. 2007). It should be noted though, that differences in conclusions regarding the role of NO and PG may be attributable to the exercise model (forearm vs. thigh) studied, and/or the timing of pharmacological administration (Boushel et al. 2002; Schrage et al. 2004).

The discovery that particular vasoactive substances are capable of vascular smooth muscle (VSM) hyperpolarization has led to continued investigation of how this mechanism is able to augment resistance vessel vasodilation (VanTeefelen & Segal 2006; Moore et al. 2010; Hellsten et al. 2012; Behringer & Segal 2012). Adenosine triphosphate (ATP), K<sup>+</sup>, and adenosine (ADO) have been recognized as primary contributors to vessel hyperpolarization, and augmented O<sub>2</sub>D during exercise. Yet, it seems that their individual contributions depend heavily on experimental methodology. For this reason, it is difficult to cumulatively interpret the integrative mechanisms which regulate blood flow in humans during exercise.

Endogenous adenosine arises from the catabolism of ATP, spillover from accumulation within the local interstitial space, and directly from endothelium. During exercise, interstitial ADO rises as a function of duration and intensity (Martin et al. 2006). Intuitively, it seems that ADO may play a potential role in evoking increases in skeletal muscle blood flow at the onset of

exercise via vessel hyperpolarization. However, it has been repeatedly demonstrated that ADO is inconsistent in its ability to cause any significant vasodilation in humans. In fact, two similar investigations by Martin et al. categorized participants as either ADO "responders" or "non-responders" (Martin et al. 2006*a*; Martin et al. 2006*b*). For this reason, it is likely that while endogenous ADO formation is important in many other physiological scenarios, its contribution to functional hyperemia in humans is negligible.

#### Local Oxygen Sensing: Erythrocyte Dependent ATP Release

Erythrocytes (RBC; red blood cell) act as local oxygen (O<sub>2</sub>) sensors traversing the resistance vessels and capillaries which supply skeletal muscle (Gonzalez-Alonso et al. 2001; Jagger et al. 2001; Gonzalez-Alonso et al. 2002). During states of altered oxygen levels such as exercise and hypoxia, they are capable of releasing ATP; a potent vasodilator which increases local blood flow (Crecelius et al. 2012; Shepherd et al. 2016). To date, the conditions under which ATP elicits vasodilation has been observed during steady-state conditions (Gonzalez-Alonso et al. 2001; Crecelius et al. 2012; Hearon et al. 2017). As oxygen demand plateaus within 3-5 minutes, depending on the muscle mass being studied, so too does O<sub>2</sub>D. Indeed, repeated investigations have highlighted the role of RBC derived ATP release in coupling O<sub>2</sub>D to demand by way of the RBCs ability to sense the local oxygen levels during continuous skeletal muscle contraction.

The difference between arterial oxygen content (CaO<sub>2</sub>) and muscle oxygen content increases during skeletal muscle contractile work (Gonzalez-Alonso et al. 2001; Jagger et al. 2001; Roach et al. 2017). As such, erythrocytes traversing through the local microcirculation release oxygen from hemoglobin (Sprague & Ellsworth, 2012). Diffusion of oxygen into

skeletal muscle mitochondria then serves to sustain skeletal muscle contractile work (Kamga et al. 2012). Consequently, conformational changes of hemoglobin proteins due to oxygen release allows for efflux of ATP into the vessel lumen where it binds to purinergic receptors along the endothelium (Burnstock 1990; Rongen et al. 1994; Sluyter 2015). Purinergic receptor binding leads to synthesis of NO and PG, and hyperpolarization by activating membrane K<sub>ir</sub> channels and ATPase, ultimately leading to vasodilation and significant increases in O<sub>2</sub>D (Rongen et al. 1994; Hellsten et al. 2012; Hearon et al. 2017).

Partitioning the contribution between partial pressure of oxygen (PO<sub>2</sub>) and arterial oxygen content (CaO<sub>2</sub>) as the primary regulator of erythrocyte ATP release has proven difficult to decipher. While PO<sub>2</sub> is crucial for normal physiological function, ATP release is influenced by hemoglobin oxygenation state, which affects CaO<sub>2</sub> to a large extent (Gonzalez-Alonso et al. 2001; Roach et al. 2017). Manipulation of CaO<sub>2</sub> through carbon monoxide (CO) inhalation and decreased hematocrit (Hct) have proven effective in their ability to alter the degree of hemoglobin oxygenation. Indeed, because CO has an affinity for hemoglobin which is nearly 200-times greater than that of O<sub>2</sub> and remains bound to the heme protein, its administration directly impacts O<sub>2</sub> kinetics *in vivo* (Gonzalez-Alonso et al. 2001; Gonzalez-Alonso et al. 2002; Calbet et al. 2003).

In healthy humans, alterations in CaO<sub>2</sub> during CO inspiration is directly correlated with changes in vascular conductance and blood flow. In accordance, combined inspiration of CO + hyperoxic gas to decrease CaO<sub>2</sub> and increase PO<sub>2</sub>, respectively, results in a hyperemic response of the same magnitude as CO administration alone (Gonzalez-Alonso et al. 2001). Further, isovolumetric reductions in hemoglobin concentrations [Hb] and hematocrit to decrease CaO<sub>2</sub> produces similar hyperemic responses to hypoxia when CaO<sub>2</sub> is matched (Roach et al. 2017).

Collectively, these observations potentiate changes in CaO<sub>2</sub>, or more descriptively, in oxyhemoglobin, as the stimulus for erythrocyte-dependent ATP release.

Interpreting these responses together alludes to a potential explanation for ATP causing immediate increases in blood flow at the onset of exercise, assuming changes in CaO<sub>2</sub> occur in short time spans (< 5 sec). Thus, a gap in the literature exists which details the ability of RBC derived ATP to elicit vasodilation at the onset of exercise. Unfortunately, at this time, there is no well-established pharmacological approach to block ATP binding. Alternative approaches to determining the role of ATP in immediate hyperemia require further investigation through novel approaches *in vivo*.

### Muscle Blood Flow at the Onset of Exercise: Mechanical Forces

Following a brief contraction, immediate increases in blood flow occur (Wray et al. 2005; Kirby et al. 2007; Crecelius et al 2013). The hyperemic response is proportional to the strength of contraction and occurs in a monophasic fashion in humans (Tschakovsky et al. 2004). Initial observations of immediate hyperemia following a contraction were theorized to be a result of skeletal muscle pump activity (Pollack et al. 1949; Laughlin et al. 1987). Upon contraction, skeletal muscle shortening compresses the veins and displaces the hydrostatic column of blood (Pollack et al. 1949). In turn, increases in the arteriovenous gradient allows for larger differences in pressure which augment muscle blood flow that occur after the first post-contraction cardiac cycle independent of metabolic vasodilation.

Attempts to mimic mechanical distortion of the vasculature in a similar manner to the skeletal muscle pump through external compressive forces have proven to be effective in evoking hyperemia (Kirby et al. 2007). Single and sustained compressions evoke a pressure

dependent, monophasic hyperemic response similar to that of a single voluntary contraction. However, while responses are similar in trend, the magnitude of peak and total hyperemia during voluntary contractions exceeds that observed during sustained and single external compressions (Kirby et al. 2007).

Despite observations which support a role for the muscle pump to immediate exercise hyperemia, multiple arguments have been demonstrated against its significance (Hamann et al. 2003; Hamann et al. 2004; Saunders et al. 2004). Specifically, treadmill exercise failed to augment canine hind limb blood flow any further following pharmacological infusion of ADO to elicit maximal vasodilation. Implying a reduction in venous pressure at the onset of exercise, it can be assumed that this mechanism does not contribute significantly to exercise hyperemia. Furthermore, intra-arterial hind limb infusion of K<sup>+</sup> to arrest smooth muscle hyperpolarization abolishes hyperemia following a brief tetanic contraction, suggesting that production of a vasodilator acting on the smooth muscle is obligatory for rapid increases in blood flow (Hamann et al. 2004). Most compelling of all, reducing contribution of the muscle pump in the human forearm displays no change to rapid blood flow increases compared to conditions when the muscle pump is left intact (Wray et al. 2005). Collectively, these data do not dismiss the muscle pump altogether; rather they suggest that its contribution is not obligatory for rapid changes in hyperemia at the onset of exercise and that an active dilation mechanism must be in place.

#### Muscle Blood Flow at the During Exercise: Potassium-Mediated Vasodilation

Skeletal muscle resistance vessels modulate the magnitude of O<sub>2</sub>D during exercise through changes in resistance vessel diameter (VanTeeffelen et al. 2006; Moore et al. 2010). In

animal and isolated vessel models, the significance of K<sup>+</sup>- mediated vasodilation in initiating vasodilation is prominent (Dora et al. 2001; Burns et al. 2004; Ahn et al. 2017). Release of K<sup>+</sup> during skeletal muscle fiber repolarization is uniquely suited to stimulate rapid hyperemia at the onset of exercise. Increases in K<sup>+</sup> within the interstitial space stimulates endothelial and VSM K<sub>ir</sub> channels and ATPase (Mohrman & Sparks, 1974; Nordsborg et al. 2003; Sonkusare et al. 2016). Concomitant activation of these structures augment endothelial cell hyperpolarization, and subsequent VSM relaxation. As a result, resistance vessels within close proximity dilate, and propagate the hyperpolarizing signal upstream to larger feed arteries to increase bulk blood flow delivery which can be detected immediately following the first contraction (Behringer & Segal, 2012).

Although human studies cannot fully elucidate the kinetics of molecular mechanisms, they do provide insight towards their relative *in vivo* contribution in midst of a variety of pharmacological and physiological stimuli. In humans, much of the current research regarding the role of K<sup>+</sup> induced hyperemia has been performed using the forearm model with the intent of limiting any confounding influences from central changes in cardiac output and sympathetic outflow (Crecelius et al. 2013; Crecelius et al. 2014). This assures that any change in O<sub>2</sub>D supplying the forearm vasculature is a result of local vasodilation, specifically. Under these conditions, pharmacological inhibition of K<sub>ir</sub> channels and ATPase in the human forearm vasculature reduces blood flow during a single dynamic contraction at various intensities (Crecelius et al. 2013) when metabolic demand is presumably unchanged. Based on this observation, we question whether a substance capable of stimulating these structures is capable of evoking vasodilation independent of oxygen demand. It is clear that K<sub>ir</sub> channels and the ATPase are necessary for increases in blood flow following a single contraction. In addition, our

lab has shown that while their relative contribution to single contractions is prominent, blood flow is reduced by ~ 50% and ~ 30% through onset (0 – 180 sec) and steady-state (>180 sec) handgrip exercise, respectively. This provides evidence that throughout the hyperemic response to exercise, proper activation of these structures is crucial (Crecelius et al. 2014).

At this time the major barrier to allocating  $K^+$  as the primary contributor to rapid vasodilation in humans is the ability to safely, and accurately, measure interstitial K<sup>+</sup> kinetics during exercise (Terwoord et al. 2018). During exercise, increases in interstitial [K<sup>+</sup>] generates a gradient which drives K<sup>+</sup> into the blood stream of the venous circulation draining the muscle bed. At moderate exercise intensities, increases in [K<sup>+</sup>] within the muscle interstitium are sufficient to evoke observable changes in plasma venous [K<sup>+</sup>]. Although in vivo measurements of interstitial K<sup>+</sup> in humans is limited, it does lend insight into the role of endogenous K<sup>+</sup> during steady-state hemodynamics. Sinoway et al. used micro-dialysis to measure various interstitial metabolites at rest, during moderate and heavy-intensity exercise, and recovery. Results from this study demonstrated that interstitial K<sup>+</sup> increases in parallel with exercise intensity, reflecting an origin from muscle fiber recruitment (Sinoway et al. 2001). Thus, given the in vivo diffusion gradient between the interstitium and vessel lumen, we are able to make the generalization that changes in venous K<sup>+</sup> concentrations are reflective of K<sup>+</sup> ions moving from an area of high concentration within the interstitium, into the plasma. Clearly, K<sup>+</sup> ions serve as a potent stimulus for dilation throughout exercise.

#### Exercise Hyperemia during Conditions of Elevated Resting Oxygen Delivery

Metabolic demand of active tissue dictates blood flow and O<sub>2</sub>D in a precise manner. Skeletal muscle contraction generates signals to increase blood flow through the production of locally acting metabolic vasodilators (Andersen & Saltin, 1985; Saltin et al. 1998). Typically, within 2-3 minutes, blood flow achieves steady-state in which O<sub>2</sub>D is equal to oxygen (metabolic) demand of skeletal muscle. In healthy humans, small muscle mass exercise can continue for up to three hours as a result of constant vasodilator production to preserve oxygen consumption (Joyner & Casey, 2015). Yet, attempts to identify any obligatory substance in sustained hyperemia have proven difficult.

Several vasodilator substances have been identified as possible contributors to sustained O<sub>2</sub>D. One of the first experiments to employ pharmacological intervention infused ADO into dog hind limbs to elevate resting O<sub>2</sub>D. Ideally, infusion of a selected vasodilator will result in; 1) prolonged increases in resting O<sub>2</sub>D which match the rate of O<sub>2</sub>D observed during steady-state exercise under control conditions, and 2) no further increase in hyperemia during contractile activity. Vasodilation in response to ADO tapered over the course of infusion as a result of decreased sensitivity to ADO (tachyphylaxis). More notably, electrically stimulated contractions during ADO infusion cause increases in blood flow proportional to contractions under control conditions (Hester et al. 1982). This methodological approach has become a popular method to evaluate the role of a variety of known vasodilation substances in humans.

Following this initial study, a small number of investigations have used prolonged infusion of ADO, sodium nitroprusside (SNP), and ATP in humans (Shepherd et al. 2016; Ranadive et al. 2019; Dillon et al. 2020). ADO, SNP, and ATP all cause vasodilation through distinct pathways with minimal overlap between one another. While all three compounds are capable of elevating  $O_2D$  in humans, changes in blood flow during superimposed moderate handgrip exercise are similar to changes observed during saline infusion. This argues the notion that a set tissue metabolic demand is the only regulator of  $O_2D$  in humans. Although the

elevations in resting O<sub>2</sub>D are pharmacologically generated, there should be no further changes in blood flow during superimposed contraction, assuming metabolic regulation is the only variable which dictates blood flow to skeletal muscle. In light of the observation that changes in blood flow persisted despite adequate O<sub>2</sub>D to skeletal muscle prior to exercise, primary investigators hypothesized that, independent of baseline O<sub>2</sub>D, rest to exercise transitions are likely reliant on a feed-forward mechanism which is capable of augmenting blood flow at the start of exercise, despite no change in tissue metabolic demand. (Shepherd et al. 2016; Ranadive et al. 2019; Poole et al. 2020).

In the discussion section, Shepherd et al. hypothesize that skeletal muscle derived K<sup>+</sup> may likely contribute to feed-forward hyperemia which occurs independent of metabolic demand. Unfortunately, at this time, no reliable pharmacological antagonist for voltage-gated K<sup>+</sup> (VGK) channels, the primary channel through which K<sup>+</sup> exits the myocyte, exists for reliable human application. However, moderate concentrations of arterial K<sup>+</sup> infusion can limit endogenous effects during exercise by altering membrane potential of skeletal muscle. Utilizing this approach allows for direct insight into how skeletal muscle derived K<sup>+</sup> is capable of achieving vasodilation during exercise. Terwoord et al. demonstrated that brachial artery infusion of KCl attenuated the change in hyperemia compared to control conditions, and vasodilator infusion. Infusion of low concentrations of KCl limits endogenous skeletal muscle K<sup>+</sup> production, thus allowing for comparison among other exercise conditions. We can assume that a reduction in hyperemia is owed to blunted K<sup>+</sup> production. This observation demonstrates that normal conditions elicit a reproducible change in blood flow during exercise as a result of a feed-forward mechanism which must be directly linked to skeletal muscle K<sup>+</sup> release, and occurs independent of tissue metabolic demand (Terwoord et al. 2018).

## **Rationale for Current Proposed Study**

Based on accumulating evidence, it seems that elevating resting O<sub>2</sub>D does not affectively abolish further increases in vasodilation at the initiation of exercise, nor the sustained vasodilation observed during steady-state exercise. Direct support of the notion that the degree of vasodilation is proportional to skeletal muscle fiber recruitment exists and lends further insight into how K<sup>+</sup> may augment vasodilation independent of satisfactory O<sub>2</sub>D. In conjunction with this outcome, rest to exercise transitions which manipulate fiber recruitment in humans augments vasodilation. Administration of BaCl<sub>2</sub> reduces this response, establishing an intimate link between muscle fiber recruitment, K<sup>+</sup>-mediated K<sub>ir</sub> channel activation, and vasodilation during exercise intensity transitions in humans (Terwoord et al. 2020).

## *Hypothesis*

We hypothesize that changes in blood flow and  $O_2D$  will persist through the onset, and steadystate phase of exercise, despite elevations in resting  $O_2D$  to match and exceed skeletal muscle oxygen requirements in young healthy humans. Furthermore, reciprocal reductions in oxygen extraction will occur as a result of elevated  $O_2D$ . Finally, pharmacological application of BaCl<sub>2</sub> and OUA will attenuate changes in blood flow which occur through K<sup>+</sup>-mediated feed-forward activation of K<sub>ir</sub> channels and the ATPase, independent of elevated  $O_2D$  and metabolic feedback.

## CHAPTER 2 – INTRODUCTION

Oxygen delivery  $(O_2D)$  to skeletal muscle increases immediately at the onset of contractile activity due in large part to vasodilation of the resistance vasculature (Marshall & Tandon, 1984; Naik et al, 1999; VanTeefellen & Segal, 2000; VanTeefellen & Segal, 2006). Typically, redundant feedback from a multitude of vasodilating substances act to increase O<sub>2</sub>D in proportion to tissue metabolic demand in order to sustain contractile activity (Bockman, 1983; Andersen & Saltin, 1985; Mohrman & Regal, 1988; Blomstrand et al. 1997). Indeed, investigations in animal models have demonstrated that across various skeletal muscle fiber types, and exercise intensities, the magnitude of tissue oxygen consumption is met with a proportional rise in O<sub>2</sub>D (Bockman, 1983; Mohrman & Regal, 1988). Critical to the coupling of  $O_2D$  to tissue oxygen demand is the endothelium, which serves as a focal point for the integration of numerous vasoactive stimuli. Local vasodilating substances such as nitric oxide (NO), prostaglandins (PG), and hyperpolarizing factors like erythrocyte-derived ATP act through the endothelium as a result of feedback from metabolically active tissue (Boushel et al. 2002; Schrage et al. 2004; Crecelius et al. 2012). In addition, metabolic factors produced solely from skeletal muscle are also capable of producing robust vasodilation in humans during exercise (Boushel et al. 2003; Clifford & Hellsten, 2004).

Several primary vasoactive substances have been hypothesized to contribute to exercise hyperemia in humans (Joyner & Casey, 2015; Clifford & Hellsten, 2004). To better understand the relative role of various substances which may contribute to functional hyperemia, an approach which utilizes pharmacological manipulation to elevate resting O<sub>2</sub>D has commonly been employed. This method intends to elevate resting O<sub>2</sub>D via infusion of a particular

vasodilator, increasing the concentration to a degree which attenuates the effect of any endogenous production (Hester et al. 1982; Shepherd et al. 2016; Ranadive et al. 2019). Assuming any contribution of the substance being investigated, hyperemic responses to exercise or other physiological manipulation should be completely abolished, or, at the very least, reduced. Hester al. conducted a classic experiment in which they infused adenosine into the dog hind limb at a rate which increased plasma adenosine levels to more than 1,000 times the normal resting concentration to produce robust vasodilation. Electrically stimulated contractions superimposed upon adenosine infusion elicited a hyperemic response which was consistent across multiple preparations. Furthermore, the magnitude of the hyperemic responses was nearly identical to the hyperemic responses observed under control conditions prior to adenosine infusion. Interestingly, from this data they established that, due to the minimal quantity of adenosine produced endogenously, the exercise and reactive hyperemic responses must be owed entirely, or almost entirely, to a factor other than adenosine (Hester et al. 1982). Utilizing a similar approach, Shepherd et al. infused ATP into the human forearm which evoked a sustained vasodilation that persisted for 180 minutes. The addition of voluntary rhythmic contractions at 20% of maximal voluntary contraction produced a hyperemic response that remained intact throughout the infusion despite blood flow being elevated to a level which matched steady-state oxygen demand under control conditions (Shepherd et al 2016). Building upon these observations, we employed ourselves to further examine changes in forearm blood flow when resting O<sub>2</sub>D exceeds the metabolic cost of contraction, and the vasoactive substance responsible for these observations.

It seems that a mechanism capable of producing vasodilation, despite adequate O<sub>2</sub>D to sustain tissue metabolic demand, is likely present in humans. In animals, attempts to dissociate

O<sub>2</sub>D from metabolic demand have demonstrated that locally released products derived from skeletal muscle such as potassium (K<sup>+</sup>) ions, which are capable of generating vasodilation independent of metabolic feedback, contribute to functional hyperemia throughout the hyperemic response to exercise (Hazeyama & Sparks, 1979; Wilson et al. 1994; Juel et al. 2007). Interstitial  $[K^+]$  rises as a result of skeletal muscle fiber recruitment (Mohrman & Sparks, 1974). As interstitial [K<sup>+</sup>] accumulates, hyperpolarization of resistance vessels ensues, allowing for substantial increases in vasodilation and sequential increases in O<sub>2</sub>D in murine models (Weidelt et al. 1997; Behringer & Segal, 2012; Ahn et al. 2017). Mohrman and Sparks showed that the temporal rise in  $K^+$  release within the local skeletal muscle milieu in response to a brief (< 1s) tetanic contraction make it a uniquely suited candidate for the initiation of rapid hyperemia at the onset of exercise. More recently, Armstrong et al. demonstrated in the hamster cremaster muscle that  $K^+$  initiates vasodilation following a single contraction and is reliant on the activation of inwardly-rectifying potassium ( $K_{ir}$ ) channels and the Na<sup>+</sup>/K<sup>+</sup>-ATPase (ATPase) to initiate vasodilation. Furthermore, in young healthy humans, pharmacological blockade of K<sub>ir</sub> channels and the ATPase reduces onset and steady-state hyperemia by ~50% and ~30%, respectively, during mild-intensity forearm handgrip exercise (Crecelius et al. 2014). Lastly, K<sub>ir</sub> channel activation is obligatory for proportional changes in hyperemia during transitions between mildto-moderate forearm exercise intensities (Terwoord et al. 2020).

Given this information as background, it is clear that  $K^+$  mediated activation of  $K_{ir}$  channels and the ATPase evoke vasodilation to some extent in every phase of the hyperemic response to exercise. Thus, we sought to elucidate whether changes in blood flow persist during elevations in resting O<sub>2</sub>D and if any alterations in O<sub>2</sub> extraction occur as a result (Protocol 1). We also sought to determine the role of K+-mediated activation of K<sub>ir</sub> channels and the ATPase

play in feed-forward augmentation of blood flow during during exercise when resting O<sub>2</sub>D is pharmacologically manipulated (Protocol 2). We tested the hypothesis that changes in hyperemia during exercise are preserved during elevations in resting O<sub>2</sub>D through alterations in O<sub>2</sub> extraction when forearm mVO<sub>2</sub> is not different (Protocol 1) and, that feed-forward K<sup>+</sup>mediated activation of K<sub>ir</sub> channel and ATPase contributes substantially to the hyperemic response observed during exercise (Protocol 2).

## CHAPTER 3 – METHODS

## **Subjects**

After providing written informed consent, a total of 10 young, healthy adults (6 male, 4 female; age,  $23 \pm 1$ ; body mass index,  $25 \pm 1$ ) participated in the present study with approval from the Colorado State University Institutional Review Board (IRB Protocol #19-9344H). Participants were sedentary to moderately active, normotensive, nonsmokers, and, for the majority, not taking any medication aside from oral contraceptives (N=1 female taking Meloxicam; anti-inflammatory). All studies were performed in the Human Cardiovascular Physiology Lab located at Colorado State University following a 12-hour fast and 12-hour abstention from caffeine and exercise in the supine position and in accordance with the *Declaration of Helsinki*. Participants were studied in the supine position in a cool (~20°C) temperature-controlled room with a fan directed toward the skin to minimize the contribution of forearm skin blood flow to overall forearm hemodynamics.

## Body composition and forearm volume

Whole body dual-energy X-ray absorptiometry (DEXA; Hologic, Bedford, MA, USA) was used to determine body composition and forearm volume (FAV) of the experimental arm for normalization of drug doses.

#### Arterial and venous catheterization, blood pressure and heart rate

Following local anesthesia (2% lidocaine), an 18-gauge, 7.6 cm catheter was placed into the brachial artery of the non-dominant forearm under aseptic conditions for local administration of study drugs through a three-port connector. The catheter was continuously flushed with 3 ml/h with heparinized saline throughout the study and connected to a pressure transducer for beat-by-beat measurements of arterial pressure waveforms and mean arterial pressure (MAP). Additionally, for Protocols 1 and 2, a 20-gauge, 5.1 cm was inserted in a retrograde fashion into the antecubital vein of the experimental forearm for deep venous blood samples (Racine et al 2018). Saline was continuously infused through the venous catheter at a rate of ~ 2 ml/min for the duration of the study. Heart rate was determined using a three-lead echocardiogram (ECG) (Cardiocap/5; Datex-Ohmeda, Louisville, CO, USA).

## Forearm blood flow

Brachial artery diameter and mean blood velocity (MBV) were measured proximal to the catheter insertion site with a 12-MHz linear array ultrasound probe (Vivid 7; General Electric, Milwaukee, WI, USA). Blood velocity was measured using a probe with an insonation angle  $<60^{\circ}$  at a frequency of 5 MHz, and MBV was determined as a weighted mean of the spectrum of Doppler shift frequencies analyzed by a Multigon 500 M TCD spectral analyzer (Multigon Industries, Mount Vernon, NY, USA). Brachial artery diameter measurements were made in triplicate in duplex mode at end-diastole at rest and between contractions during onset and steady-state exercise. Forearm blood flow (FBF) was calculated in ml/min as FBF= MBV x  $\pi$  (brachial artery diameter/2)<sup>2</sup> x 60.

## Rhythmic handgrip exercise

Prior to the experiment, maximal voluntary contraction (MVC) was determined as the average of three squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) within 3%

deviation from one another (MVC 43.0  $\pm$  3.8 kg; value range: 30-56). Participants performed dynamic forearm contractions using a handgrip pulley system to lift a weight corresponding to 10% MVC. The weight was lifted 4 –5 cm over the pulley with a duty cycle of 1 s contraction to 2 s relaxation (20 contractions/min) using visual (alternating light colors) and auditory feedback (metronome) to ensure correct timing. This mild work intensity was chosen to facilitate comparison with previous work from our laboratory (Crecelius et al. 2013; Crecelius et al. 2014; Terwoord et al. 2018) and to limit any changes in cardiac output or reflexive activation of sympathetic nervous system activity (Kirby et al. 2008). Therefore, our experimental model isolates the effects of skeletal muscle contraction on local vascular mechanisms in humans.

#### Vasoactive drug infusions

#### Protocol 1 (elevated resting oxygen delivery)

All vasoactive drugs were infused via the brachial artery to evoke a localized effect within the experimental forearm vasculature. Saline was used a control infusate. Each participant (N=10) completed Protocol 1, which was constructed to evaluate mechanisms of vascular control during pharmacological elevations in resting oxygen delivery ( $O_2D$ ;  $C_aO_2 x$ FBF). Sodium nitroprusside (SNP; Nexus Pharmaceuticals, Lincolnshire, IL) was infused at rest and throughout the duration of exercise. Our goal was to both match (MAT), and exceed (EXC) forearm skeletal muscle oxygen requirements (mVO<sub>2</sub>) observed during steady-state exercise under control (CON) conditions via SNP infusion at rest before beginning contractions. SNP was chosen for its ability to act as an endothelium-independent, smooth muscle nitric oxide donor that does not impact rapid vasodilation or the rest to steady-state blood flow response during forearm handgrip exercise in humans (Hearon et al. 2019; Terwoord et al. 2018). SNP was initially infused at a rate which elicited a dose of  $2.0 \ \mu g/dl \ FAV/min$  or  $4.0 \ \mu g/dl \ FAV/min$  depending on the experimental condition. The infusion rate was intentionally adjusted to match (MAT; SNP  $2.0 \ \mu g/dl \ FAV/min$ ), and exceed (EXC: SNP  $4.0 \ \mu g/dl \ FAV/min$ ), the level of hyperemia observed during steady-state exercise under control (CON; saline) conditions, at rest.

## <u>Protocol 2 (local K<sub>ir</sub> channel and Na<sup>±</sup>/K<sup>±</sup>-ATPase inhibition)</u>

To evaluate the role of K<sub>ir</sub> channel and ATPase activation on exercise hyperemia, (N=5) participants performed control exercise (saline + blockade) and exercise following elevations in resting O<sub>2</sub>D (SNP + blockade) with the addition of barium chloride (BaCl<sub>2</sub>; K<sub>ir</sub> channel inhibitor, Pencol Compounding Pharmacy, Denver, CO) and ouabain octahydrate (Na<sup>+</sup>/K<sup>+</sup>-ATPase inhibitor, Pencol Compunding Pharmacy, Denver, CO). BaCl<sub>2</sub> was infused at 0.9  $\mu$ mol/dl FAV/min with an absolute dose of 8-10  $\mu$ mol/min in combination with ouabain which was infused at 2.7 nmol/min (Crecelius et al. 2013; Crecelius et al. 2014) throughout the duration of exercise.

In Protocol 2, we attempted to elevate resting  $O_2D$  above baseline resting values to a level which as closely paralleled CON steady-state FBF as possible. Because infusion rates for BaCl<sub>2</sub> and ouabain, and thus the effective concentration present within the forearm vasculature, are limited for concerns related to participant safety, we had to adjust the degree to which we elevated resting FBF as to not dilute either antagonist, rendering them ineffective. With the addition of BaCl<sub>2</sub> and ouabain our goal was to reduce blood flow during the onset (0-180 sec) and steady-state (>180 sec) phases of exercise.

## Experimental protocol

### Protocol 1

Participants arrived at the laboratory following an overnight fast 12-hour fast and 12-hour abstention from caffeine, alcohol, and exercise. Participants lay supine with the experimental arm abducted to 90° and elevated slightly above heart level on a tilt-adjustable table throughout the study visit. All experiments were performed in a cool, temperature-controlled laboratory  $(20-22^{\circ}C)$  with a fan directed toward the experimental arm to minimize the contribution of skin blood flow to overall forearm hemodynamics. The primary experimental layout for Protocol 1 is illustrated in Fig. 1. In 10 young healthy subjects, we aimed to determine if changes in blood flow from rest to steady-state remain intact during pharmacological elevations in resting O<sub>2</sub>D. Shown in Fig. 1, resting FBF was elevated to a level which matched (MAT) and exceeded (EXC) steady-state FBF under control (CON) conditions, via intrarterial SNP infusion. Furthermore, we sought to elucidate how each condition affected oxygen extraction (O<sub>2</sub> extraction %; C<sub>a</sub>O<sub>2</sub> –  $C_vO_2/C_aO_2 \ge 100$  and forearm oxygen demand (mVO<sub>2</sub> ml O<sub>2</sub>/min; FBF x  $C_aO_2 - C_vO_2 \ge 100$ 0.001), and if venous plasma K<sup>+</sup> concentrations rise during exercise (K+ effluent; mmol/min). Thus, rhythmic handgrip exercise was performed during 1) control conditions (CON), 2) conditions in which resting  $O_2D$  was elevated to a level which *matched* steady-state exercise under control conditions via SNP (MAT), and 3) conditions in which resting O<sub>2</sub>D was elevated to a level which exceeded steady-state exercise under control conditions via SNP (EXC). The order of drug conditions in Protocol 1 was pseudorandomized and counterbalanced across participants. Study visits in which either MAT or EXC trials were performed before the CON trial, practice handgrip exercise was performed at 10% MVC to determine consistent landmarks and FBF values to replicate MBV and brachial artery diameter for comparison. Venous blood

gas measurements were taken at rest, through the initial phase of exercise (1 sec, 15 sec, 30 sec, and 45 sec), and during steady-state (> 180 sec) exercise in each condition. In addition, arterial oxygen saturation was monitored via pulse oximeter throughout the entire study, and a single arterial blood gas measurement was taken prior to the experimental protocol at rest to determine arterial oxygen content (CaO<sub>2</sub>).

#### Protocol 2

Following the completion of Protocol 1, a subset of participants (N=5) performed two more rhythmic handgrip exercise trials. The first consisted of saline infusion plus additional administration of BaCl<sub>2</sub> and ouabain  $(BaCl_2 + ouabain)$  to block Kir channels and the ATPase, respectively. The second consisted of SNP infusion plus additional administration of BaCl<sub>2</sub> and ouabain  $(SNP + BaCl_2 + ouabain)$ . Thus, handgrip exercise was performed under both conditions to evaluate the role of Kir channel and ATPase inhibition on the normal FBF response to mild-intensity exercise. During the second trial, we elevated resting FBF above resting values to isolate the effects of feed-forward mechanisms during exercise and determine if Kir channel and ATPase inhibition attenuates changes in FBF from rest to steady-state exercise. The order of drug conditions in Protocol 2 was pseudorandomized and counterbalanced across participants. During the loading period of drug infusions at rest, participants performed three single dynamic forearm contractions to facilitate delivery of the infusate to the vasculature of the muscle fibers recruited for this type of contraction (Terwoord et al. 2018). For each condition, baseline hemodynamics were allowed to steady (~5 min) before participants began rhythmic handgrip exercise at 10% MVC to evaluate onset and steady-state exercise hyperemia and vasodilation. Each trial was separated by >10 min rest to allow for adequate washout of drugs.

## Data acquisition and analysis

Throughout the duration of each trial, MBV, arterial pressure, and heart rate data were collected and stored at 250 Hz and were analyzed offline with signal-processing software (Windag; DATAQ Instruments, Akron, OH). MAP was determined from the arterial pressure waveform. Prior to the start of each trial, two minutes of resting data was collected to ensure baseline hemodynamics were similar between trials and determined as a 30s average. Following two minutes of resting data collection, vasoactive drug infusion was initiated. Baseline for MBV, arterial pressure and HR data was collected again for proper comparison between conditions. For both Protocols 1 and 2, FBF and MAP were analyzed in 3s averages that corresponded to each contraction:relaxation (1:2s) cycle throughout the duration of exercise. 15s averages for FBF were also determined for both protocols using the same method. In the case that the MBV signal quality obtained during a 3-s cycle was altered due to operator error, a mathematical average of the preceding and the MBV of the subsequent bins was used. This occurred in <1% of all bins analyzed. HR was determined at rest and steady-state exercise. Steady-state FBF was used to calculate K<sup>+</sup> efflux at rest and during mild intensity exercise. The vasodilatory response to administration of SNP before exercise represents a 30s average once blood flow remained steady for >1 min.

#### **Statistics**

Data are presented as means ± SE. In Protocol 1, rest-to-steady state FBF and oxygen extraction were assessed using two-way (time x drug) repeated measures ANOVA. Absolute changes in FBF and K<sup>+</sup> measures were assessed using one-way repeated measures ANOVA. In Protocol 2, onset-FBF was assessed using two-way (time x drug) repeated measures ANOVA. In Protocols

1 and 2, resting hemodynamics were compared using paired *t* tests. Tukey's *post hos analysis* testing was utilized as appropriate. All statistical analyses were performed using GraphPad Prism (Prism; GraphPad Software, San Diego, CA) and significance was set *a priori* at P<0.05.

## CHAPTER 4 – RESULTS

### **Resting hemodynamics**

Resting hemodynamics (FBF, MAP and HR) are presented in Table 1. MAP and HR changed during the EXC condition compared to CON in Protocol 1 (P<0.05). This response is likely due to the large dose of SNP necessary to evoke elevations in resting O<sub>2</sub>D. Resting FBF was higher in the MAT condition, and even greater in the (P<0.05). In Protocol 2, SNP caused substantial changes in resting FBF (P<0.05).

#### Blood gas variables

Blood gas measurements are presented in Table. 3 for each condition during rest and exercise in Protocol 1. Regardless of condition, exercise caused a decrease in venous oxygen saturation (SvO<sub>2</sub>) and venous oxygen content (CvO<sub>2</sub>) relative to rest (P<0.05). This was reflected by an increase in oxygen extraction (O<sub>2</sub> extraction) during exercise (P<0.05). Forearm muscle oxygen consumption (VO<sub>2</sub>) increased during exercise relative to rest (P<0.05); however, no differences were present when comparing rest and exercise between conditions, signifying that metabolic rate was unchanged across conditions (P=NS).

#### Protocol 1

#### Rest to steady-state exercise hyperemia

Under CON conditions, FBF increased from rest (FBF;  $37 \pm 5$  ml/min) to steady-state (FBF;  $172 \pm 24$  ml/min, P < 0.01) in response to rhythmic exercise at 10% MVC (Fig. 2A). In the MAT condition, SNP (2.0 µg/dl FAV/min) was continuously infused at a rate which increased

resting FBF to match steady-state FBF under CON conditions (FBF: CON;  $172 \pm 24$  ml/min vs. MAT;  $166 \pm 25$  ml/min, *P*=NS, Fig. 2A). We continuously infused SNP (4.0 µg/dl FAV/min) at rest (FBF;  $219 \pm 27$  ml/min) which markedly exceeded (EXC) steady-state FBF during CON conditions (FBF: CON;  $172 \pm 24$  ml/min vs. EXC;  $219 \pm 27$  ml/min, *P*<0.01). Similar to Fig. 2A, O<sub>2</sub>D was not different from the outcomes of FBF (Fig. 2B). Absolute changes in FBF (Fig. 2C) were not different between conditions ( $\Delta$ FBF: CON;  $135 \pm 20$  ml/min vs. MAT;  $132 \pm 19$ ml/min vs. EXC;  $167 \pm 26$  ml/min, *P*=NS across all conditions). From this data, it is evident that changes in blood flow remain intact despite adequate O<sub>2</sub>D to sustain contractile activity prior to the start of exercise.

#### Forearm oxygen saturation, extraction, and consumption

Absolute SvO<sub>2</sub> was elevated in the MAT and EXC condition (Fig. 3A) relative to CON at rest (SvO<sub>2</sub>: CON; 55 ± 6 % vs. MAT; 87 ± 2 % & EXC; 87 ± 2%, P<0.01). Although resting SvO<sub>2</sub> were elevated in the MAT and EXC condition, exercise caused a marked decrease in each condition compared to rest (Fig. 3A, P<0.01), reflected by an increase in O<sub>2</sub> extraction during steady-state exercise compared to rest (Fig. 3B, P<0.01). As expected, forearm VO<sub>2</sub> significantly increased from rest to steady-state exercise (Fig. 3C, P<0.01), yet was not different at rest (mVO<sub>2</sub>: CON; 2.9 ± 0.4 ml O<sub>2</sub>/min, MAT; 3.7 ± 1.5 ml O<sub>2</sub>/min, EXC; 3.3 ± 1.6 ml O<sub>2</sub>/min) or during steady-state exercise (mVO<sub>2</sub>: CON; 22 ± 2.5 ml O<sub>2</sub>/min, MAT; 26 ± 3.4 ml O<sub>2</sub>/min, EXC; 26 ± 3.9 ml O<sub>2</sub>/min) between conditions (Fig. 3C, P=NS).

#### Venous potassium measures

Skeletal muscle  $K^+$  release is believed to play a role in exercise hyperemia in humans and may evoke vasodilation independent of tissue oxygen demand. We measured  $K^+$  efflux as an index of plasma [ $K^+$ ] which accounts for elevations in FBF. Total  $K^+$  efflux increased from rest

to steady-state exercise in each condition (Fig. 4A, P < 0.05). EXC was greater than MAT, which, in turn, was greater than CON (Fig. 4A, P < 0.05). However, when we present the absolute change in K<sup>+</sup> efflux from rest to exercise (K<sup>+</sup> efflux: CON; 0.6 ± 0.1 mmol/min, MAT; 0.6 ± 0.1 mmol/min, EXC; 0.76 ± 0.2 mmol/min) differences are abolished (Fig. 4B, P=NS)

## **Protocol 2**

## <u>Role of K<sub>ir</sub> channels and the ATPase</u>

A portion of the participants (N=5) from Protocol 1 completed an additional subset of trials in which we blocked K<sub>ir</sub> channels and the ATPase during normal exercise and when resting FBF was elevated via SNP. Steady-state FBF during CON trials of the five participants was not different from FBF during the elevated trial (SNP + Blockers) with the addition of blockers at rest (FBF: CON; 181 ± 39 ml/min vs. SNP + Blockers; 147 ± 20 ml/min, *P*=NS). Exercise elicited a significant increase in FBF in each condition (Fig. 5A, *P*<0.01). Interestingly, absolute steady-state FBF was lower when SNP + Blockers were administered compared to absolute FBF during the MAT condition from Protocol 1 despite a similar starting resting blood flow value (FBF: MAT; 186 ± 43 ml/min vs. SNP + Blockers; 147 ± 20 ml/min, *P*<0.05). Again, when we presented the absolute change in FBF, SNP + Blockers significantly reduced the absolute change (Fig. 5B) compared to the MAT condition ( $\Delta$ FBF: MAT; 148 ± 21 vs. SNP + Blockers; 96 ± 13 ml/min, *P*<0.05).

## CHAPTER 5 – DISCUSSION

The results from the current investigation are the first to demonstrate that exercise hyperemia in humans is not altered when baseline O<sub>2</sub>D exceeds that which is normally necessary to sustain contractile activity, prior to the onset of exercise. Furthermore, although not statistically significant, we demonstrate that Kir channel and ATPase activation is a requisite for augmenting blood flow, likely through feedforward activation via local skeletal muscle K<sup>+</sup> release. Several conclusions can be drawn from this data. Changes in blood flow during the onset phase of exercise through steady-state remain intact despite pharmacological elevations in resting O<sub>2</sub>D which met, and exceeded forearm skeletal muscle oxygen demands to sustain contractile work during mild-intensity forearm handgrip exercise. Next, proportional reductions in skeletal muscle oxygen extraction occur as a result of elevated O<sub>2</sub>D when oxygen demand is not different between conditions. Lastly, pharmacological inhibition of Kir channels and the ATPase blunt the hyperemic response during elevated baseline  $O_2D$  throughout the entirety of exercise, suggesting to the role of feedforward K<sup>+</sup>-mediated activation of these structures during exercise. Taken together, these findings cumulatively demonstrate that activation of K<sub>ir</sub> channels and the ATPase are capable of augmenting blood flow in a manner which is independent of normal feedback mechanisms which couple  $O_2D$  to oxygen demand during exercise in healthy humans.

#### Skeletal muscle oxygen demand during exercise

During exercise,  $O_2D$  ( $C_aO_2$  x blood flow) to skeletal muscle is a consequence of oxygen demand (VO<sub>2</sub>) (Andersen & Saltin, 1985; Blomstrand et al. 1997). Thus, changes in skeletal

muscle oxygen demand necessitate a proportional rise in  $O_2D$  in order to sustain contractile activity (Andersen & Saltin, 1985; Joyner & Casey, 2015). This occurs as a result of a balancing act between local skeletal muscle vasodilator production which serve to increase blood flow to the region of active muscle fibers, and simultaneous sympathetic outflow to evoke vasoconstriction of blood vessels in non-active tissue, with the ultimate goal of maintaining adequate blood pressure (Rowell 1993). Through our pharmacological approach in the current investigation, we were able to successfully dissect the aforementioned feedback mechanisms. By elevating resting O<sub>2</sub>D via local vasodilator infusion (SNP) into the brachial artery to increase forearm blood flow, we effectively dissected the mechanisms, feed-back and feed-forward, which produce exercise hyperemia. Evidence for our success is demonstrated in Fig. 3C which depicts the absolute change in blood flow from rest to steady-state exercise under CON, MAT, and EXC conditions. Importantly, no differences were observed between conditions, meaning a feedforward mechanism must exist which is capable of augmenting blood flow independent of oxygen demand. Furthermore, the magnitude of the absolute change in blood flow must be in direct proportion to exercise intensity (Kirby et al. 2012). This fundamental observation led us to further investigate the vasodilatory mechanism capable of producing such coordinated changes in blood flow despite adequate O<sub>2</sub>D to skeletal muscle at rest.

## Determining the role of particular vasodilators using elevated flow states

Our rationale for the current investigation centers on previous observations which have attempted to identify substances that are obligatory for exercise hyperemia (Hester et al. 1982; Shepherd et al. 2016; Ranadive et al. 2019; Dillon et al. 2020). These studies utilized elevated resting flow states which matched values observed under control conditions so that, assuming the substance of choice is necessary for the typical hyperemic response, any further changes in blood flow during superimposed contractions would be abolished. Numerous putative vasodilating substances have been classified with a non-obligatory role in the hyperemic response to exercise including ADO and ATP through this approach (Schrage et al. 2004; Shepherd et al. 2016; Dillon et al. 2020). Infusion of adenosine and ATP substantially increases resting forearm blood flow. However, during superimposed contractions at 10%, and 20%, maximal voluntary contraction, changes in blood flow are identical to control conditions (Shepherd et al. 2016; Ranadive et al. 2019; Dillon et al. 2020).

In contrast to studies which did not observe changes in hyperemia, Terwoord et al. infused KCl via the brachial artery to diminish any further release of endogenous forearm muscle fiber K<sup>+</sup> during superimposed mild-intensity exercise. The result was a significant reduction in onset and steady-state forearm blood flow (Terwoord et al. 2018). From this data, along with outcomes from Hearon et al. and Crecelius et al., we can confidently presume that local K<sup>+</sup> release from skeletal muscle plays a substantial role in exercise hyperemia, stimulates endothelial and vascular smooth muscle K<sub>ir</sub> channels; additionally, activation of K<sub>ir</sub> channels is necessary for adequate blood flow at the immediate start of, and through the onset and steadystate phase of exercise. To our knowledge, we are the first to demonstrate a reduction in blood flow during elevated resting flow conditions via inhibition of K<sub>ir</sub> channels and the ATPase, highlighting an obligatory feedforward role for these structures during exercise.

#### Role of K<sub>ir</sub> channels and the ATPase

Activation of K<sub>ir</sub> channels and the ATPase in humans is a primary contributor to vasodilation during exercise via hyperpolarization of resistance vessels (Dawes et al. 2002;

Burns et al. 2004). Kir channels, in particular, possess a unique ability to augment intracellular  $K^+$  efflux from endothelial and vascular smooth muscle cells, thereby evoking a more pronounced hyperpolarization, whereas the ATPase contributes to tonic regulation of intracellular ion concentrations (Sonkusare et al. 2016; Hearon et al. 2019). Indeed, isolated vessel studies have demonstrated the intrinsic capability of K<sub>ir</sub> channels to "amplify" endothelium-dependent hyperpolarization during both application of extracellular K<sup>+</sup>, and in response to vasoactive hyperpolarizing factors. The former of which is mimicking what is likely occurring in the local skeletal muscle environment in vivo (Nordsborg et al. 2003; Burns et al. 2004; Sonkusare et al. 2016). Hearon et al. elegantly constructed a pharmacological approach to testing this hypothesis in humans. Infusion of acetylcholine, an endothelium-dependent agonist which facilitates an increase in endothelial cell calcium to evoke vascular hyperpolarization, with superimposed light-intensity exercise elicited an augmented vasodilation which could not be replicated during infusion of the endothelium-independent dilator SNP (Hearon et al. 2019). Furthermore, application of BaCl<sub>2</sub> to block K<sub>ir</sub> channels resulted in a blunted vasodilatory response when repeated. The authors speculated that K<sup>+</sup> efflux from contracting skeletal muscle may contribute to feedforward vasodilatory signal which acts to rapidly activate K<sub>ir</sub> channels and augment vasodilation during exercise. These observations alluded to a role for K<sup>+</sup>-mediated activation of K<sub>ir</sub> channels as a mediator of feed-forward hyperemia and provided insight into the approach for the current investigation.

In Protocol 2, we repeated two of the three trials (CONb & MATb) as seen Protocol 1 trials with the addition of BaCl<sub>2</sub> and ouabain infusion to inhibit  $K_{ir}$  channels and the ATPase, respectively. Under conditions which elevated resting O<sub>2</sub>D via SNP infusion, concomitant blocker infusion reduced the change in blood flow observed during Protocol 1 by ~ 30% and

~40% through onset and steady-state exercise, respectively. The reductions we observed in the current study are similar to previous investigations in our lab (Crecelius et al. 2014). Though the values may differ slightly, the reduction in blood flow through onset and steady-state exercise support the findings of Crecelius et al. whom demonstrated that K<sub>ir</sub> channels contribute to nearly 50% and 30% of the hyperemic response during onset and steady-state exercise, respectively, in young healthy humans (Crecelius et al. 2014). Our current results may differ from these findings due to a "washout" effect of vasodilator infusion. In humans, the upper limit of BaCl<sub>2</sub> infusion is fixed for reasons related to human participant health and safety. For this reason, most pharmacological investigations utilize a stimulus which does not evoke large changes in hyperemia to avoid systemic effects. In the current investigation, it is likely that during the MATb condition, the substantial increase in resting blood flow diluted BaCl<sub>2</sub> concentrations within the local muscle vasculature. We were careful in our initial approach to avoid this problem, hence we decided early on to forego an exceeded blood flow condition with simultaneous BaCl<sub>2</sub> infusion. If the increase in resting blood flow did dilute or "washout" a portion of BaCl<sub>2</sub>, this would explain the less pronounced reduction observed in the current study compared to those observed by Crecelius et al. Thus, we may be underestimating the role of K<sub>ir</sub> channels and the ATPase in this response.

#### Experimental considerations

It appears that although K<sup>+</sup>-mediated activation of K<sub>ir</sub> channels and the ATPase are required for hyperemia, we were only able to reduce the change in FBF from rest to exercise by  $\sim$ 35%; thus, other factors must be present to account for the residual increase in FBF. The results from Protocol 1 distinguish the necessity of skeletal muscle to meet oxygen demand

through two principle mechanisms; O<sub>2</sub>D or O<sub>2</sub> extraction (Fick principle). Many recent studies have solidified the importance of oxygen sensing within the resistance vasculature to couple O<sub>2</sub>D to oxygen demand (Gonzalez-Alonso et al. 2001; Gonzalez-Alonso et al. 2002; Calbet et al. 2003; Kirby et al. 2012; Roach et al. 2017). Red blood cell ATP release occurs in response to the offloading of oxygen from hemoglobin to sustain oxidative ATP-generating pathways (Jagger et al. 2001; Sprague & Ellsworth, 2012). In the current investigation, venous oxygen saturation decreased markedly from rest to exercise (Fig. 4A). It is likely that despite differences in absolute SvO<sub>2</sub>, the required oxygen extraction evoked needed to sustain a red blood cell derived release of ATP which caused vasodilation. ATP acts as a hyperpolarizing factor once it binds to P<sub>2</sub>Y receptors along the endothelium and causes a propagated hyperpolarizing signal which increase local blood flow. Furthermore, one of the primary ways in which ATP facilitates vasodilation is through the activation of K<sub>ir</sub> channels and the ATPase, further demonstrating the necessity of these pathways to augment hyperemia during exercise (Crecelius et al. 2012).Our decision to highlight K<sub>ir</sub> channels and the ATPase in this investigation stems from a few key studies from our lab and others. First, it has been shown that K<sup>+</sup> is released, and more importantly, is capable of evoking vasodilation of resistance vessels, after a single electrically evoked tetanic contraction (Armstrong et al. 2007). Furthermore, this response was blunted during BaCl<sub>2</sub> application. Second, findings from several animal studies have demonstrated that  $K^+$  release is a consequence of skeletal muscle contraction and it a potent stimulator of  $K_{ir}$ channel and ATPase activation. Thus, K<sup>+</sup> is able to evoke vasodilation immediately at the start, and throughout the entirety of exercise despite elevations in resting oxygen delivery which are adequate to sustain contractile activity. Finally, our laboratory recently demonstrated that Kir channels are necessary for augmenting blood flow during transitions between varying exercise

intensities. Although only a relative workload was used throughout the duration of the current investigation, the outcomes is similar when transitioning from rest-to-exercise, further supporting the notion that K<sub>ir</sub> channel activation is capable of augmenting blood flow.

## Limitations

In the current investigation we were unable to reliably show changes in plasma potassium throughout the onset of exercise. We presume that activation of K<sub>ir</sub> channels and the ATPase is due to local skeletal muscle K<sup>+</sup> release several reasons. One is that numerous studies in animals and isolated vessels have shown that local interstitial [K<sup>+</sup>] increase immediately at the start of exercise and activate K<sub>ir</sub> channels and the ATPase at a range of extracellular concentrations (5-10 mM). It is possible that changes in oxygen demand at the level of the active forearm muscle caused erythrocyte-mediated ATP release as a consequence of hemoglobin deoxygenation. Our laboratory has demonstrated that ATP-mediated vasodilation is reliant on the activation of K<sub>ir</sub> channels and the ATPase to elicit vessel hyperpolarization. However, Shepherd et al. found that infusing ATP to elevate blood flow to a degree which matched blood flow under control conditions did not reduce changes in hyperemia at all. In contrast, it is likely that changes in venous oxygen content from rest to exercise reflect a necessary role for ATP-mediated vasodilation in the current investigation.

We must acknowledge a prominent role for small and intermediate conductance calciumactivated (sK<sub>Ca</sub>/IK<sub>Ca</sub>) potassium channels in evoking vessel hyperpolarization. Although no direct endothelium-dependent agonists were infused in the current investigation. Preliminary evidence from isolated cell studies suggest that high levels of shear-stress, a consequence of high blood flow generating a frictional force along the endothelial lining of blood vessels, may

generate acetylcholine (Ach), an endothelium-dependent agonist. It is theorized that Ach is generated and released from endothelial cells, acting in an auto/paracrine manner to elicit dilation in resistance vessels. Were this the case, it may help to explain the augmented change in blood flow observed higher in the EXC condition compared to CON and MAT (P=0.09). Nevertheless, we believe the current data accurately depict a K<sup>+</sup>-mediated feedforward mechanism which preserves and increase in blood flow throughout exercise via activation of K<sub>ir</sub> channels and the ATPase, independent of tissue metabolic demand.

## Perspectives

The ability to rapidly increase blood flow during transition from rest to exercise helps to provide a surplus of oxygen which precedes the relatively slower onset of elevations in skeletal muscle metabolism (Behnke & Delp, 2010; Poole et al. 2020). Thus, a reduced capability to rapidly augment blood flow during transitions may reduce exercise capacity and overall quality of life (Poole et al. 2020). Healthy aging results in an attenuated vasodilatory response at the onset of exercise, and reduces skeletal muscle oxygen delivery (Jackson et al. 2010; Carlson et al. 2011; Behringer et al. 2013; J & Segal, 2018). Coupled with an elevation in sympathetic outflow and signal for vasoconstriction, it is important to understand how activation of K<sub>ir</sub> channels and the ATPase via local K<sup>+</sup> release may be related to reduced hyperemia (Balasubramanian et al. 2019).

Data from our lab has demonstrated that rapid and sustained vasodilation is reduced in older adults (Kirby et al. 2012). In support of this observation, experiments conducted in animal and isolated vessel models have demonstrated that hyperpolarization of both endothelial and vascular smooth muscle, a crucial vasodilatory mechanism which involves both K<sub>ir</sub> channels and the ATPase, is significantly reduced in both temporal and spatial magnitude (Behringer et al.

2013; Socha & Segal, 2018). A substantial portion of the reductions is vasodilatory capacity in older adults is owed to decreased activation of slow and intermediate calcium-activated potassium (SK<sub>Ca</sub>/IK<sub>Ca</sub>) channels. Mounting evidence suggests that activation of these channels is crucial to initiating hyperpolarization in response to vasodilatory stimuli; whereas K<sub>ir</sub> channels are particularly important in augmenting hyperpolarization. Taken together, one could surmise that the compounding effects of these outcomes is a reduced sustained and rapid vasodilation, respectively. Some have suggested that, with aging, the resting membrane potential of endothelial cells become more basally hyperpolarized due to "leaky" potassium channels. Were this the case, responsive changes in vessel tone may be diminished, especially due to local hyperpolarizing stimuli such as increases in local K<sup>+</sup>. More research is necessary to elucidate the factors which contribute to reduced responsiveness to hyperpolarizing factors so to ameliorate reductions in blood flow observed with aging.

## **Conclusions**

During small muscle mass exercise, blood flow to skeletal muscle relies on numerous vasodilatory pathways to match oxygen delivery to tissue oxygen demand. We have successfully shown that a feedforward mechanism exists that is capable of increasing blood flow independent of tissue oxygen demand. The findings indicate that during pharmacologically elevated resting oxygen delivery, changes in blood flow during exercise remain intact, and the magnitude of muscle oxygen extraction decreases in proportion to the absolute degree of oxygen delivery. Furthermore, the change in blood flow throughout exercise is reduced during pharmacological blockade of K<sub>ir</sub> channels and the ATPase. We speculate that local release of K<sup>+</sup> from contracting muscle fiber stimulates K<sub>ir</sub> channels and the ATPase to increase blood flow throughout exercise

in a feed-forward manner. Results from this study and others indicate that K<sup>+</sup>-mediated activation of K<sub>ir</sub> channels and the ATPase is obligatory for adequate hyperemia in humans to preserve active muscle oxygen consumption.

## TABLES AND FIGURES



**Figure 1. Experimental Protocols 1 & 2.** In Protocol 1, participants performed mild-intensity handgrip exercise (10% MVC) during saline infusion (CON), and during SNP infusion to either match (MAT) or exceed (EXC) blood flow measured during steady-state in the CON trial. In Protocol 2, participants repeated two exercise trials during 1) infusion of saline + blockers, and 2) infusion of SNP + blockers. Venous blood draws were performed at rest, after the first contraction, and at 15 sec, 30 sec, 45 sec, and >180 sec of exercise during Protocol 1.

Table 1. Protocol 1 resting hemodynamics

	CON	MAT	EXC	
FBF (ml min <sup>-1</sup> )	37 ± 5	$166 \pm 25^*$	$219 \pm 27^{*\dagger}$	
MAP (mmHg)	91 ± 2	85 ± 2	$83 \pm 2^*$	
HR (bpm)	$53 \pm 2$	$59 \pm 2$	$63 \pm 3^*$	

Values are means  $\pm$  SE for N = 10 participants. Statistical comparisons were performed using paired *t* tests. Abbreviations: FBF, forearm blood flow; MAP, mean arterial pressure; HR, heart rate. \**P*<0.05 compared to CON. <sup>†</sup>*P*<0.05 compared to MAT.

#### Table 2. Protocol 2 resting hemodynamics

	Saline + Blockers	SNP + Blockers
FBF (ml min <sup>-1</sup> )	$24 \pm 3$	$147 \pm 20^{*}$
MAP (mmHg)	$90 \pm 2$	$90 \pm 1$
HR (bpm)	$58 \pm 2$	$58 \pm 2$

Values are means  $\pm$  SE for N = 5 participants. Statistical comparisons were performed using paired *t* tests. Abbreviations: FBF, forearm blood flow; MAP, mean arterial pressure; HR, heart rate. \**P*<0.05 compared to CON.

	Rest	Exercise
Control		
SvO2 (%)	$55.0 \pm 5.6$	$34.9 \pm 2.5$
CaO2 (ml L-1)	$200 \pm 3.7$	$200 \pm 3.7$
CvO2 (ml L-1)	$115 \pm 12$	$74.0 \pm 6.0$
O2 extraction (%)	$42.2 \pm 6.7$	$63.0 \pm 3.2$
VO2 (ml min-1)	$2.92 \pm 0.4$	$21.8 \pm 2.5$
Matched		
SvO2 (%)	$86.9 \pm 2.1$	$52.1 \pm 4.4$
CaO2 (ml L-1)	$200 \pm 3.7$	$200 \pm 3.7$
CvO2 (ml L-1)	$189 \pm 7.0$	$114 \pm 9.0$
O2 extraction (%)	$5.30 \pm 4.7$	$43.1 \pm 4.5$
VO2 (ml min-1)	$3.66 \pm 1.5$	$25.6 \pm 3.4$
Exceeded		
SvO2 (%)	$87.0 \pm 1.7$	$59.8 \pm 4.2$
CaO2 (ml L-1)	$200 \pm 3.7$	$200 \pm 3.7$
CvO2 (ml L-1)	$193 \pm 7.0$	$129 \pm 10$
O2 extraction (%)	$3.50 \pm 4.0$	$35.2 \pm 5.1$
VO2 (ml min-1)	$3.34 \pm 1.6$	$26.2 \pm 3.9$



Figure 2. Rest to steady-state forearm blood flow (A), forearm oxygen delivery (B), and calculated absolute changes in forearm blood flow (C) during Protocol 1 (*N*=10). In Protocol 1, our attempt was to match (MAT) FBF at rest to FBF measured during steady-state CON conditions. Thereafter, we attempted to exceed (EXC) FBF during steady-state CON conditions at rest, *A*., Same as panel A, but displaying O<sub>2</sub>D, *B*. Changes in absolute FBF were calculated as the difference between resting FBF and steady-state exercise FBF, *C*. Changes in FBF remain intact despite significant differences in resting FBF. \**P*<0.05 steady-state exercise FBF compared to within condition resting FBF. †*P*<0.05 compared to CON steady-state exercise. \**P*<0.05 compared to MAT rest.



Figure 3. Rest to steady-state forearm venous oxygen saturation (A), forearm oxygen extraction (B), and forearm oxygen consumption (C) during Protocol 1 (N=10). At steady-state exercise, venous oxygen saturation is reduced in all three conditions compared to rest. In the MAT and EXC conditions, resting oxygen saturation is greater than CON, but follows a similar decline, A. Oxygen extraction increases during steady-state exercise in parallel with a decrease in venous oxygen saturation, B. Forearm oxygen demand is unchanged during exercise between conditions, signifying that metabolic demand, and feedback, are not different among conditions, C.\*P<0.05 compared to rest within condition.  $^{+}P<0.05$  compared to CON exercise.  $^{\$}P<0.05$  compared to CON rest.



Figure 4. Rest to steady-state skeletal muscle K<sup>+</sup> efflux (A), and calculated absolute changes in K<sup>+</sup> efflux (B) during Protocol 1 conditions (*N*=6). When FBF is accounted for, there is a distinct increase in skeletal muscle K+ release during exercise compared to rest, *A*. Furthermore, the magnitude of change between conditions is not different, *B*. \**P*<0.05 compared to rest within conditions. †*P*<0.05 compared to CON steady-state exercise. \$*P*<0.05 compared to MAT exercise.



Figure 5. Rest to steady-state forearm blood flow (A) and calculated absolute changes in forearm blood flow (B) during Protocol 2 (N=5). Rest to steady-state forearm blood flow followed a similar pattern during Protocol 2 compared to Protocol 1, A. Interestingly, when the absolute change in forearm blood flow from rest to steady-state is calculated, there is an apparent reduction in the *saline* + *blocker* and *SNP* + *blocker* trials, though only one was statistically significant, B. \*P<0.05 compared to MAT trial from Protocol 1. †P<0.05 compared to CON exercise. \*P<0.05 compared to MAT exercise.

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