THESIS

THE ROLE OF THE ERYTHROCYTE AND SUBSEQUENT ATP RELEASE IN BLOOD FLOW AND OXYGEN DELIVERY TO THE HUMAN FOREARM DURING HYPOXIC EXERCISE

Submitted by

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ABSTRACT

THE ROLE OF THE ERYTHROCYTE AND SUBSEQUENT ATP RELEASE IN BLOOD FLOW AND OXYGEN DELIVERY TO THE HUMAN FOREARM DURING HYPOXIC EXERCISE

Hypoxia and exercise each cause ATP to be released from the erythrocyte, increasing vasodilation to match the blood flow and oxygen demands of the exercising skeletal muscle tissue (Bergfeld & Forrester, 1992; Ellsworth, 2004; Gonzalez-Alonso, Olsen, & Saltin, 2002). However, few studies have examined the extent to which ATP can be released from the erythrocyte, especially due to hypoxic exercise. We hypothesized that hypoxic exercise would cause further augmentation in erythrocyte-derived ATP release, vasodilation, and blood flow. To test this hypothesis, in 10 healthy young adults, blood samples were taken from a deep venous catheter inserted into the experimental arm and analyzed to determine and compare the amount of ATP released under normoxic and then hypoxic exercise. Forearm blood flow (FBF; Doppler ultrasound) and vascular conductance (FVC) responses to submaximal rhythmic forearm handgrip exercise (15% maximal voluntary contraction) in normoxia and during systemic hypoxia (80% arterial oxygen saturation; pulse oximetry) were measured and calculated, respectively. Compared to normoxic rest, 3 minutes of normoxic exercise significantly increased plasma ATP (45±4 nmol/L *vs* 101±22 nmol/L; $P<0.05$). Plasma ATP with hypoxic exercise was only significantly greater than normoxic rest at 30 seconds (114±4 nmol/L; $P<0.05$) and 3 minutes (84±12 nmol/L; $P<0.05$) of exercise. ATP collected at any time point with 3 minutes of hypoxic exercise was not significantly greater than with 3 minutes of normoxic exercise (P=NS). Forearm blood flow 3 minutes of hypoxic exercise (250±26 ml min^{-1}; $P<0.05$)

were both greater than with normoxic exercise (201±21 ml min^{-1}; $P<0.05$) or normoxic rest (29±4 ml min^{-1}; $P<0.05$). Forearm vascular conductance was greater with hypoxic exercise (257±29 ml min^{1} $(100mmHg)^{-1}$; $P<0.05$) than with normoxic exercise (212±23 ml $min^{-1}(100mmHg)^{-1}$; $P<0.05$) or normoxic rest (33±4 ml $min^{-1}(100mmHg)^{-1}$; $P<0.05$). As plasma ATP did not continually increase with hypoxic exercise, we conclude that hypoxic exercise may not be a strong enough stimulus for erythrocyte-derived ATP release. Despite a lack in ATP release, FBF and FVC were still maintained, suggesting that ATP may not be as important for vasodilation, enhanced blood flow, and oxygen delivery to the skeletal muscle as previously thought. Other factors involved in vasodilation and blood flow augmentation during hypoxic exercise warrant further investigation.

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CHAPTER I

INTRODUCTION

The main function of the erythrocyte is to transport oxygen to the periphery and export carbon dioxide to the lungs (Gonzalez-Alonso, Richardson, & Saltin, 2001). Erythrocytes are also able to sense the amount of oxygen in the body and meet peripheral oxygen demand by dilating the vasculature to enhance blood flow and thus oxygen supply (M. L. Ellsworth, 2004a; Gonzalez-Alonso, Olsen, & Saltin, 2002; Gonzalez-Alonso et al., 2001; Kirby, Crecelius, Voyles, & Dinenno, 2012).

There are three pathways in which the erythrocyte acts as a vasodilator and propagates blood flow and oxygen delivery to demanding tissues: 1) nitrite reduction to nitric oxide (NO), 2) S-nitrosohemoglobin (SNO-Hb) bioactivity, and 3) adenosine 5'-triphosphate (ATP) release. Although the first two mechanisms have revealed important roles in vascular control, we wanted to continue exploring the necessity of ATP release because our research has demonstrated that ATP may be a stronger candidate for blood flow and oxygen regulation (Crecelius, Kirby, Luckasen, Larson, & Dinenno, 2012; Crecelius, Kirby, Richards, et al., 2011; Crecelius, Kirby, Voyles, & Dinenno, 2010, 2011; Dinenno & Joyner, 2003; Kirby, Crecelius, Richards, & Dinenno, 2013; Kirby et al., 2012; Kirby, Voyles, Carlson, & Dinenno, 2008).

ATP liberated by the erythrocyte is used to control blood flow to the tissues by inducing vasodilation after binding to purinergic (P_{2Y}) receptors on the endothelium (M. L. Ellsworth, Forrester, T., Ellis, C.G., and Dietrich, H.H., 1995). As a result of vasodilation, blood flow and oxygen supply are increased in a dose-dependent relationship (Gonzalez-Alonso et al., 2001). The amount of ATP released is heavily reliant on the oxygen saturation of the erythrocyte; the

more de-oxygenated the erythrocyte is, such as occurs with hypoxia and exercise, the greater the stimulus for ATP release (Kirby, Crecelius et al. 2012). The observed quantity of ATP released from the erythrocyte in this dose-dependent relationship appears to boost ATP concentrations in the venous circulation with exercise (Gonzalez-Alonso et al., 2002) and with hypoxia (Bergfeld & Forrester, 1992; Dufour et al., 2010). Even though multiple studies have examined the relationship between erythrocyte de-oxygenation and ATP release (M. L. Ellsworth, 2004a; Gonzalez-Alonso et al., 2002; Gonzalez-Alonso et al., 2001; Jagger, Bateman, Ellsworth, & Ellis, 2001; Kirby et al., 2012), and have established that ATP liberation from the erythrocyte is enhanced during hypoxic rest and with normoxic exercise, almost none have investigated the breadth to which ATP is released when changing from a normoxic environment to a hypoxic environment during exercise.

The purpose of this review is to discuss the current literature highlighting the significance of the erythrocyte in blood flow regulation as it relates to ATP release and vasodilation during normoxic rest, submaximal normoxic exercise, and most importantly, submaximal hypoxic exercise. The physiology, structure, and function of the erythrocyte will first be discussed, followed by an overview of ATP production and the relationship between the erythrocyte and ATP release. Lastly, the mechanisms behind the compensatory vasodilation that occurs with hypoxic exercise will be examined.

CHAPTER II

REVIEW OF THE LITERATURE

Formation of the Erythrocyte

Erythropoiesis is the formation of the erythrocyte that begins with hematopoiesis, which is the development of any kind of cell in the blood (Boron and Boulpaep 2012). Short-term hematopoietic stem cells (ST-HSCs) are stem cells found in the bone marrow and peripheral blood of adults that initiate the hematopoietic process by inducing the formation of various progenitor cells, the first of which is the common myeloid progenitor (CMP) (Boron and Boulpaep 2012). After CMP is formed, it is next transitioned into a megakaryocyte erythroid progenitor and then into a burst-forming unit-erythroid (BFU-E) (Boron and Boulpaep 2012). The conversion into a BFU-E is critical in forming proerythroblasts, which are the direct predecessor of the basic erythrocyte (Boron and Boulpaep 2012). Proerythroblasts are a type of progenitor cell referred to as colony-forming units (CFUs) that lead to the production of the large, nucleated basophilic erythroblast (Boron and Boulpaep 2012). From this step in the process, polychromatic erythroblasts are created and hemoglobin begins to materialize (Boron and Boulpaep 2012). Next, orthochromatic erythroblasts are produced and the presence of hemoglobin is obvious. The nucleus, ribosomes, and mitochondria of the erythroblasts are then expelled into the extracellular space to create either immature (reticulocytes) or fully developed erythrocytes, respectively (Boron and Boulpaep 2012). Once a mature erythrocyte is produced, it has a lifespan of approximately 120 days (Boron and Boulpaep 2012).

In the typical young adult male and female, 60% and 50% of their total body weight is made up of water, respectively (Boron and Boulpaep 2012). The discrepancy in the amount of water weight between males and females is due to the fact that males have more muscle and less fat

than females, and that muscle cells contain more water than fat (Boron and Boulpaep 2012). Sixty-percent of the average 70-kg male's body weight contains approximately 42 liters of total body water, in which 17 liters (40%) is extracellular fluid and 25 liters (60%) is intracellular fluid. For the purpose of this review, the most important component of total body water is the extracellular fluid, which contains the blood plasma. Within the extracellular fluid, 5.5 and 3 liters make up the intravascular and extracellular blood volume, respectively. The difference of 2.5 liters is made up of the essential cellular aspects of the blood: leukocytes, platelets, and erythrocytes, the last of which is the focal point of this review.

Structure of the Erythrocyte—Hematocrit

Hematocrit is the portion of blood volume filled by the erythrocyte, or the total amount of erythrocytes; quantified after centrifuging and pipetting a sample of blood (Boron and Boulpaep 2012). After extracting out the "buffy coat" or the top layer of the analysis tube, which is made up of platelets and white blood cells, the erythrocytes are what remain at the bottom of the column (Boron and Boulpaep 2012). Ordinarily, adult women have a hematocrit value of 40% and a total erythrocyte volume of 28 mL/kg of body weight, whereas the amount of hematocrit in adult men is 45% with a total erythrocyte volume of 36 mL/kg of body weight (Boron and Boulpaep 2012). Thus, the blood is heavily populated by erythrocytes.

Structure of the Erythrocyte—Hemoglobin

The most influential portion of the erythrocyte is the hemoglobin (Hb) molecule, which is a tetrameric protein containing 16 heme ions and 16 globin polypeptides (Boron and Boulpaep 2012). One erythrocyte contains approximately 280 million hemoglobin molecules (Greendyke,

Meriwether, Thomas, Flintjer, & Bayliss, 1962). The heme ions are composed of ferrous iron (Fe^{2+}) ions that when combined with a sufficient amount of oxygen turn the blood red (e.g., arterial blood) and when unable to adequately combine with oxygen turn the blood purple (e.g., venous blood) (Boron and Boulpaep 2012). Globin allows each hemoglobin molecule to bind to a maximum of four oxygen molecules. Oxygen more readily binds to hemoglobin when the molecule is in the relaxed (R) state, as opposed to the tensed (T) state. Specifically, the relaxed hemoglobin molecule has an attraction to oxygen that is approximately 150 times greater than when it is tense. In adult women and men, the typical blood hemoglobin content is 14.0g/dL and 15.5g/dL, respectively.

Function—Traditional Role

The three main functions of the erythrocyte are to: 1) distribute oxygen to the periphery from the lungs by way of the pulmonary capillaries, 2) remove carbon dioxide from the tissues back to the lungs by way of the systemic capillaries, and 3) ensure that the acidic and basic values of the body are normal (Jagger et al., 2001). Due to the fact that erythrocytes do not consist of organelles or a nucleus, they are not involved in either oxidative metabolism or protein synthesis (Boron and Boulpaep 2012). However, they do take part in glycolytic metabolism and the pentose shunt, which produces nicotinamide adenine dinucleotide phosphate (NADP) and pentoses, the most simplest and common form of carbohydrates (Boron and Boulpaep 2012). Through glycolysis, the erythrocyte is able to produce adenosine 5'-triphosphate (ATP), which will be discussed in further detail in a later section.

Traditional Role: Oxygen and Carbon Dioxide Transport

The hemoglobin-oxygen dissociation curve (Figure 1) explains the relationship between the hemoglobin molecule and the amount of oxygen released from the erythrocyte. On the x-axis of the curve is the partial pressure of oxygen (P_{O2}), measured in millimeters of mercury (mmHg). P_{O2} symbolizes the partial pressure of oxygen that ends up being dissolved in the plasma of the arterial blood, and is not dependent on the amount of hemoglobin. P_{O2} is also often referred to as oxygen tension and is commonly described as how much oxygen will be conveyed to the tissue from the erythrocyte (Sprague and Ellsworth 2012). The y-axis represents the oxygen saturation (S_{O2}), which is the percentage of oxygen that can bind to hemoglobin. In regards to oxygen administration, experimental evidence supports the erythrocyte as the primary regulator in oxygen distribution due to its oxygen content, as opposed to the oxygen tension of the environment (Sprague & Ellsworth, 2012). Oxygen content is a ratio that expresses the amount of oxygen that can be bound to hemoglobin; it is the oxygen carrying capacity of hemoglobin (Sprague and Ellsworth 2012). Typically, for every 100 molecules of hemoglobin, 98% of the binding sites are occupied by oxygen (Boron and Boulpaep 2012). The amount of dissolved O_2 into the blood (P_{O2}) is not enough to meet the body's demands because the average amount delivered to the tissues is 15mL of O_2 per minute (Boron and Boulpaep 2012). At rest, a 70-kg person consumes approximately 250mL of O_2 per minute, meaning that to depend on P_{O2} for O_2 supply one would have to increase their cardiac output at rest by at least 17 times (Boron and Boulpaep 2012). The fact that it is the oxygen content rather than the oxygen tension that is necessary for oxygen regulation reinforces the idea that the erythrocyte is essential for oxygen distribution because the amount of oxygen the tissue employs is directly associated with the oxygen content of the erythrocyte (Sprague and Ellsworth 2012).

Another important aspect of this curve is the P_{50}, which is a value that represents the P_{O2} in the blood when hemoglobin is 50% saturated; 26.6 mmHg for a healthy person. A right shift on the curve equates to a larger P_{50} value, decreasing hemoglobin's affinity for oxygen while making it easier for hemoglobin to release oxygen. Conversely, a leftward shift on the curve increases hemoglobin's affinity for oxygen but decreases its ability to release oxygen. Factors that affect whether the curve shifts left or right include body temperature, the pH of the blood, diphosphoglycerate (2,3-DPG), and the partial pressure of carbon dioxide (P_{CO2}). As the body increases in temperature, such as occurs with exercise, the curve will shift to the right, signifying that more oxygen can be released from the hemoglobin molecule to keep up with the metabolic demands. On the other hand, a leftward shift occurs when body temperature decreases. In cases of alkalosis (pH > 7.45), the hemoglobin has a higher affinity for oxygen, as opposed to acidosis (pH < 7.35), in which the hemoglobin molecule offloads oxygen to balance the acid-base environment and return the pH to normal. Diphosphoglycerate (2, 3-DPG) is an isomer containing three carbons that inhabits the erythrocyte at close to 5 mmol/L. The body increases the production of (2, 3-DPG) in situations of low oxygen states, as is the case in hypoxia. An increase in (2, 3-DPG) shifts the curve to the right to release oxygen to tissues that are experiencing hypoxia; it is part of a positive feedback loop to help prevent and control hypoxia. When the P_{CO2} is increased, the curve shifts to the right. The curve is sigmoidal in shape because more molecules bind as the maximum amount of oxygen that can be bound to hemoglobin is reached. In other words, the curve levels out as hemoglobin becomes more saturated with oxygen. Based on the Hb-O_2 Dissociation Curve, the erythrocyte plays an important role in oxygen transport in the blood based on how saturated oxygen is with the hemoglobin molecule. The more hemoglobin molecules that are available for oxygen to bind to them, the greater the

amount of oxygen the blood will encompass, and vice versa. The ability of the erythrocyte to dissociate oxygen from hemoglobin in times of demand is crucial to metabolic homeostasis.

Traditional Role: Gas Exchange

During exercise, both the temperature and the P_{CO2} of the skeletal muscle increase while the pH decreases, attenuating oxygen's ability to bind to hemoglobin and facilitate O_2 release (Boron and Boulpaep 2012). In the attempt to consistently maintain blood flow in the tissues, the exercising muscles demand hemoglobin molecules located in the systemic capillaries to offload oxygen at a faster rate (Boron and Boulpaep 2012). If the exercising tissues are sufficiently oxygenated, then hemoglobin will be alerted to provide less oxygen. In the pulmonary circulation, hemoglobin is triggered to supply more oxygen when the temperature and P_{CO2} drop while the pH rises. During respiratory acidosis, erythrocytes infiltrate the systemic capillaries, causing an increase in the amount of P_{CO2} in the extracellular space. The over-production of P_{CO2} results in CO_2 migrating into the erythrocytes, consequently reducing the intracellular pH (Boron and Boulpaep 2012). To restore the pH and P_{CO2} back to normal while increasing blood supply the Hb-O_2 Dissociation Curve shifts to the right, decreasing hemoglobin's affinity for oxygen; a mechanism referred to as the Bohr Effect (Boron and Boulpaep 2012).

Within the blood, CO_2 can be transported one of five ways: 1) dissolved in the blood plasma; 2) converted into carbonic acid (H_2CO_3); 3) transformed into bicarbonate (HCO_3^-); 4) changed into carbonate (CO_{2-3}); or 5) altered into carbamino hemoglobin (Hb-NH-COO^-) (Boron and Boulpaep 2012). Although a portion (11%) of CO_2 stays in the plasma of the blood, the majority (89%) of it infiltrates the erythrocyte. Inside the erythrocyte are two subunits of carbonic anhydrase, labeled CA I and CA II (Boron and Boulpaep 2012). These two isoforms are

enzymes that allow CO_2 to be transported from the tissues into the pulmonary capillaries and finally expelled into the atmosphere by converting approximately 64% of the 89% of CO_2 into bicarbonate ions (HCO_3^-) (Boron and Boulpaep 2012). The erythrocyte also contains a Cl-HCO_3 Anion Exchanger 1 (AE1) transport protein that replaces chloride (Cl^-) for HCO_3^- ions across the plasma membrane, which allows more CO_2 to be carried and taken up by the erythrocyte (Boron and Boulpaep 2012). At approximately 1 million copies per cell, the AE1 is the most bountiful protein within the erythrocyte and is thus extremely important for CO_2 transportation in the lungs; just one AE1 molecule alone can displace up to 50,000 ions per second (Boron and Boulpaep 2012). Behind AE1, the water channel aquaporin 1 (AQP1) is the most ample protein within the erythrocyte, and converts the remaining 21 and 4% of the CO_2 found in the erythrocyte into carbamino compounds with hemoglobin or simply dissolves within the cell, respectively (Boron and Boulpaep 2012).

As the amount of CO_2 produced accumulates with exercise or hypoxia, O_2 saturation drops, a mechanism known as the Haldane Effect (Boron and Boulpaep 2012). Oxygen is released from the hemoglobin molecule when blood passes into the systemic capillaries, which boosts hemoglobin's carrying capacity for CO_2; allowing blood to take up more CO_2 (Boron and Boulpaep 2012). On the other hand, when blood traverses the pulmonary capillaries, the CO_2-carrying capacity falls and any excess production of CO_2 is let go as hemoglobin binds to more oxygen (Boron and Boulpaep 2012). Hence, the hemoglobin molecule is an essential part of gas exchange and balancing the acid-base environment.

New Role—the Erythrocyte as the Primary Oxygen Sensor

In 1919, renowned physiologist August Krogh believed that each capillary served as the principal oxygen transporter to the nearby tissues that comprised a "cylindrical region" (the Krogh cylinder) (Sprague, Bowles, Achilleus, & Ellsworth, 2011b). However, he failed to understand or recognize just how elaborate the microvasculature and its need for oxygen is (Sprague, Bowles, Achilleus, & Ellsworth, 2011a). Krogh did not consider the possibility that somehow and somewhere within the microvasculature an entity is able to sense the oxygen demands of the skeletal muscle and then provide the appropriate amount of perfusion to that area (Sprague et al., 2011a).

In order for the metabolic needs of tissues to be met, the appropriate amount of nutrients and oxygen must be available and supplied to the tissue (M. L. Ellsworth, 2000). The proper regulation of blood flow is essential in order to increase the delivery of oxygen when needed, and decrease it when that supply has been met. Thus, there stands to be a mechanism which can detect the demand of oxygen and metabolites of the tissue and then supply the tissues with the correct amount. Once Krogh's model was ruled out, many hypotheses were generated to try and explain the mechanism behind oxygen sensing and distribution to maintain homeostasis. Previous research has suggested three mechanisms for oxygen sensing and transport to the tissues: 1) the arterioles can independently sense the amount of oxygen and blood in the tissues; 2) the tissues can self-produce an intermediary that alters the blood and oxygen levels; and 3) the tissues have a specified area for oxygen sensing and supply (M. L. Ellsworth, 2000). However, these three hypotheses have lacked enough substantial evidence to maintain credibility. The first hypothesis was refuted because it was discovered that PO_2 variability did not have any effect on arteriole diameter, and thus the arterioles could not be an independent sensor (Jackson, 1987). In

regards to the second hypothesis, local infusion of adenosine did not significantly increase the arteriole diameter to conclude that the tissues themselves were the appropriate mechanism (Hester, 1990). Similar to Krogh's model, the third hypothesis suggested that the tissues have a "localized sensor" which is responsible for oxygen and blood flow supply. Despite this idea, more contemporary experiments have discovered that the procedure of oxygen exchange is too complex to occur locally, and most likely takes place in the microvascular tree (M. L. Ellsworth, Popel, & Pittman, 1988). Based on the failure of the three previously mentioned hypotheses to establish the mechanism behind oxygen sensing, it is apparent that there must be another factor that is responsible for oxygen sensing and supply. Although still controversial and currently under further investigation, substantial concrete data have pointed to the erythrocyte as this one such factor.

In addition to its traditional role as the main oxygen carrier in the body, recent evidence has established a new role for the erythrocyte as the primary regulator of oxygen supply and demand (Jagger et al., 2001). The idea that the erythrocyte is the universal oxygen sensor is based on the fact that the erythrocyte is already known to possess the oxygen carrier, hemoglobin, and its ability to release ATP (Dietrich, Ellsworth, Sprague, & Dacey, 2000). Over the years, three considerable scientific phenomenon's have been discovered which prove the hypothesis that the erythrocyte is indeed responsible for oxygen and blood flow sensing and supply due to ATP production: 1) a decrease in S_{O2} results in ATP release from the erythrocyte; 2) ATP applied at various concentrations into the lumen of arterioles leads to conducted upstream vasodilation and substantially increases the diameter of arterioles; and 3) vessel diameter and ATP effluent only increase when red blood cells are intact and introduced during decreased oxygenated environments (M. L. Ellsworth, 2004b). Appointing the erythrocyte as the primary oxygen

sensor proves to be a very efficient mechanism because it does not require the recruitment of such a complex system to control the vasculature.

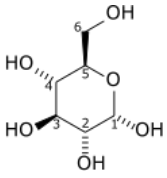
The amount of vasodilation and blood flow rely on how much ATP is created and discharged from the erythrocyte, which is based on the number of erythrocytes created and available (J. Edwards, Sprung, Sprague, & Spence, 2001; Sridharan et al., 2010). The more hypoxic the environment, the greater the stimulus for erythrocyte production and ATP generated through glycolysis. As vasodilation accelerates, erythrocyte distribution escalates, allowing for a continuous supply of ATP until exercise ceases (M. L. Ellsworth et al., 2009; Pries, Secomb, Gaehtgens, & Gross, 1990). This idea has been confirmed in our laboratory by occluding muscle blood flow to examine the impact on ATP release (Kirby et al., 2013). Inhibiting skeletal muscle perfusion was employed to determine if ATP release was derived from the erythrocyte and if adequate blood flow was in fact necessary for enhanced ATP release with exercise. Skeletal muscle perfusion was ultimately restrained before and during moderate intensity rhythmic handgrip exercise by inflating a blood pressure cuff placed on the upper arm to suprasystolic levels (200 mmHg) (Kirby et al., 2013). During exercise, venous plasma ATP supply indicated that ATP appreciably decreased and contrary to popular belief, was not elevated above resting values (Kirby et al., 2013). Exercise proved incapable of increasing ATP in the plasma when upper arm occlusion was induced before exercise began (Kirby et al., 2013). Thus, adequate perfusion in the skeletal muscle during exercise is critical for ATP release. Because decreased ATP release was a consequence of attenuated blood flow, these findings also support the idea that the erythrocyte is responsible for ATP supply. Lastly, continuous supply of erythrocytes must take place in order for ATP, vasodilation, and blood flow to be sustained.

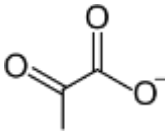
Determining and coordinating oxygen supply and demand to the periphery is a convoluted and intricate process that involves cooperation at the mechanical, metabolic, and cellular levels. Additionally, every level of the microvasculature must be sufficiently supplied. Interestingly, the erythrocyte is able to sense a change in the oxygen status within the body and deliver the appropriate amount of oxygen based on the demand (Jagger et al., 2001). Specifically, the erythrocyte controls oxygen delivery based on the rate of blood flow, which is influenced by multiple mechanisms that either constrict or dilate the vessel (Crawford, Chacko, Kevil, & Patel, 2004). The mechanisms that affect vasoconstriction or vasodilation are numerous and include ATP, prostacyclin, and nitric oxide, and will be expanded upon in the following sections. One prime example of oxygen sensing by the erythrocyte is during exercise, in which the metabolic activity of the skeletal muscle is enhanced and requires more oxygen to sustain performance and physiological function (Jagger et al., 2001). As exercise intensity and thus metabolic activity increase from rest to exercise, hemoglobin subsequently de-saturates, signaling the erythrocyte to release more ATP, dilate the vasculature, and upsurge blood flow to the exercising tissues (Jagger et al., 2001). The ability of the erythrocyte to successfully and continually match oxygen supply and demand is critical for physiological maintenance; without it, consequences such as tissue damage and muscle fatigue could occur (Jagger et al., 2001). Blood flow and oxygen supply and demand is an elaborate process, but the erythrocyte is able to sense tissue gradient changes and adapt to meet the body's demands across the entire microvascular tree (M. L. Ellsworth, Forrester, T., Ellis, C.G., and Dietrich, H.H., 1995).

ATP: Production

Adenosine 5'-triphosphate (ATP) is produced within the cytoplasm of the erythrocyte through the glycolytic pathway as a result of skeletal muscle contractions and hypoxia. The oxygen status of the hemoglobin molecule within the erythrocyte strongly influences and initiates the production and release of ATP. As hemoglobin becomes more de-oxygenated, glycolysis is up-regulated in order to keep up with the oxygen demand (Ellis, Jagger, & Sharpe, 2005). Glycolysis is the metabolic pathway that results in glucose ($C_6H_{12}O_6$) being broken down into pyruvate (CH_3COCOO^-), is a very profitable source of ATP production, and yields a net of 2 ATP molecules each time (Brooks et al. 2000). The glycolytic pathway is depicted in the figure below:

[Glucose] [Pyruvate]



+ 2 $[NAD]^+$ + 2 [ADP] + 2 $[P]_i$ →  + 2 [NADH] + 2 H^+ + 2 [ATP] + 2 H_2O

As glycolysis is initiated, glucose must first be phosphorylated by the hexokinase enzymes and transformed into glucose-6-phosphate (G6P) (Brooks et al. 2000). During this reaction, ATP is consumed, allowing glucose to be steadily transferred into the cell and produces one molecule of ATP (Brooks et al. 2000). The enzyme glucose phosphate phosphohexoisomerase then transforms G6P into fructose 6-phosphate (F6P), granting fructose to be phosphorylated in order to infiltrate the glycolytic pathway (Brooks et al. 2000). The enzyme phosphofructokinase (PFK) is then used to convert F6P into fructose 1, 6-diphosphate (F1, 6DP), producing the second molecule of ATP. The enzyme aldolase breaks down F1, 6DP into glyceraldehyde 3-

phosphate (G3P), which then is converted into 1, 3-diphosphoglycerate (1, 3-DPG) (Brooks et al. 2000). In order for the third ATP molecule to be generated, 1, 3-DPG must be changed into 3-phosphoglycerate (3-PG) by the 3-phosphoglycerate kinase. This glyceric acid is further broken down into 2-phosphoglycerate (2-PG) and then phosphoenolpyruvate (PEP) through the enzymes phosphoglyceromutase and enolase, respectively (Brooks et al. 2000). The fourth molecule of ATP is then created when the enzyme pyruvate kinase transforms PEP into pyruvate. The last step involves lactate dehydrogenase breaking down into lactate, which ends up in the muscle. Although four molecules of ATP are made during this process, the net yield is two because the other two molecules are used to produce ADP and H^+ during the production of G6P and F1, 6DP.

ATP: Stimulus for Release

It takes approximately 100 to 500ms for the erythrocyte to detect a decrease in oxygen, release ATP, and for vasodilation to occur (M. L. Ellsworth et al., 2009). ATP release from erythrocytes during times of decreased oxygen, which happens with exercise, is crucial to ensure adequate perfusion as the metabolic requirements continue to enhance. Typically, the erythrocyte delivers ATP when oxygen tension of the surrounding environment is approximately 26 ± 6 mmHg, also known as P_{50}; the value at which the hemoglobin molecule is 50% saturated (Sprague and Ellsworth 2012). The oxygen content of the hemoglobin molecule drastically affects oxygen sensing and delivery in that the less oxygen the hemoglobin molecule is exposed to, the greater the stimulus for ATP release from the erythrocyte. Oxygen sensing by the erythrocyte is primarily manipulated by the conformational change and/or the oxygen status of the hemoglobin molecule (Buehler & Alayash, 2004). Conformational change occurs as

erythrocytes traverse the vasculature, while the oxygen status is altered with hypoxia or exercise. A decrease in oxygen supply, such as occurs during hypoxia and exercise, causes the hemoglobin molecule to become de-saturated, affecting the oxygen and blood flow all the way from the arterioles down to the capillaries (Buehler & Alayash, 2004). There is a strong, positive relationship between the amount of ATP in the plasma and the degree of de-oxygenated hemoglobin (Buehler & Alayash, 2004). As oxygen levels drop, the hemoglobin molecule begins to de-saturate, stimulating the liberation of ATP from the erythrocyte (M. L. Ellsworth et al., 2009).

ATP: Signal Transduction Pathway for Release

Because ATP is such a giant molecule, it cannot just simply pass through the membrane of the erythrocyte, implying that there must be another way for it to escape the inside of the cell and travel into the extracellular space. Such a mechanism has been described as the Signal Transduction Pathway; a series of steps that occur inside the membrane in response to the mechanical deformation or reduced oxygen saturation of the cell (Olearczyk, Stephenson, Lonigro, & Sprague, 2001).

Once the hemoglobin molecule becomes de-saturated, which occurs with increases in metabolic demand or systemic and tissue hypoxia, the heterotrimeric G protein, G_i, is the first step in the signal transduction pathway leading to ATP release from erythrocytes (Goldman et al., 2012; Olearczyk, Stephenson, Lonigro, & Sprague, 2004). These heterotrimeric G proteins, also known as guanosine nucleotide-binding proteins, constitute a large group of proteins that are regulated by G protein-coupled receptors (GPCRs). At a population of 1,000, GPCRs are the

most abundant receptor family on the exterior of the cell and are critical in a multitude of cellular responses (Boron and Boulpaep 2012). GPCRs control the activity of G proteins by allowing them to bind to either guanosine triphosphate (GTP) or guanosine diphosphate (GDP) in which the G proteins are then either switched on or off, respectively. Once the hemoglobin molecule becomes de-saturated, stimulated G proteins have the option of binding to one of the three subunits of heterotrimeric G proteins, α, β, and γ, in which the subunit $G\alpha_s$ (stimulatory) binds to GTP and activates adenylyl cyclase (AC). If G proteins bind to $G\alpha_i$, adenylyl cyclase activity will be inhibited. In response to AC activation, 3'5'-cyclic adenosine monophosphate (cAMP) is amplified (Sprague et al., 2011a). cAMP is a second messenger found in all cells and is catalyzed within the cells from ATP by AC (Sprague and Ellsworth 2012). Human erythrocytes contain phosphodiesterases (PDEs) 2, 3A, 3B, 4 and 5, but it is PDE3 that plays the most important role in ATP transportation by hydrolyzing cAMP production in response to a low oxygenated environment for the stimulus of ATP release (Sprague and Ellsworth 2012). Within a matter of seconds, the amount of cAMP is enhanced 5-fold, prompting the stimulation of the enzyme protein kinase A (PKA) and afterwards binding to it (Boron and Boulpaep 2012). As a result of the increase in cAMP, the cystic fibrosis transmembrane regulator (CFTR) is also switched on. The CFTR is a glycoprotein located in most cells that moves Cl^- across the membrane of the cell through the Cl^- channel (Boron and Boulpaep 2012). The CFTR also contains two nucleotide-binding domains (NBD1 and NBD2) that have the ability to bind to other proteins and nucleotides. An area described as the cyotplasmic regulatory (R) domain divides the CFTR into two sides and has the capacity to phosphorylate protein kinase C and protein kinase A. Once PKA is phosphorylated, the CFTR is activated, and ATP can attach to the NBDs, allowing the CFTR to be either opened or closed.

When opened, ATP passes through the CFTR to be released from the cytoplasm of the erythrocyte and into the extracellular space by way of Pannexin-1, a protein related to invertebrate innexins that forms gap junctions to permit substances to travel between the intracellular and extracellular areas (Locovei, Bao, & Dahl, 2006b). Pannexin-1 is sensitive to mechanical stress, can easily be penetrated by ATP, and allows ATP to be effectively released from the erythrocyte in response to decreased O_2 tension (Locovei, Bao, & Dahl, 2006a). Once expelled into the extracellular space, ATP binds to purinergic receptors (P_{2Y1} and P_{2Y2}) on the endothelium and is then able to activate the influential vasodilators prostacyclin, nitric oxide, and endothelium-derived hyperpolarizing factor (EDHF) to directly relax the vascular smooth muscle and propagate upstream vasodilation (Sprague et al., 2011a; Sridharan et al., 2010).

ATP—Role as a Vasodilator and Oxygen Supplier

According to Poiseuille's Law, blood flow is a factor of the radius or diameter of the vessel to the fourth power (M. L. Ellsworth, Forrester, T., Ellis, C.G., and Dietrich, H.H., 1995). Therefore, the magnitude of blood flow is reliant on the change in vascular tone in that small changes in smooth muscle vessel diameter result in large fluctuations in blood flow (M. L. Ellsworth, Forrester, T., Ellis, C.G., and Dietrich, H.H., 1995). Once released from the erythrocyte through the Signal Transduction Pathway, ATP binds to P_{2Y1} and P_{2Y2} on the endothelium and stimulates two very dominant vasodilators, prostacyclin (PGI_2) and Nitric Oxide (NO) (Ellsworth 1995; Jagger, Bateman et al. 2001).

PGI_2 is created after the enzyme cyclooxygenase (COX) converts arachadonic acid into the prostacyclin precursor PGH_2. After PGI_2 is formed, it binds to prostaglandin I_2 (IP) receptors located on vascular smooth muscle cells, activating G_s proteins (Sprague & Ellsworth, 2012).

PGI_2 induces smooth muscle relaxation, vasodilation, and ATP release from the erythrocyte through the IP Receptor Pathway. Similar to the Signal Transduction Pathway, the IP Receptor Pathway involves the same chain of events that allows ATP to be released when oxygen levels fall; increases in AC, cAMP, PKA, and CFTR activity. However, the IP Receptor Pathway differs from the Signal Transduction Pathway in that it utilizes the PDE isoform PDE3A (as opposed to PDE3B) to regulate cAMP, and the final conduit for ATP release is by way of voltage-gated anion channels (VDAC) located on the mitochondrial membrane instead of the Pannexin-1 channel (Sprague & Ellsworth, 2012).

The enzyme nitric oxide synthase (NOS) forges NO when it converts the amino acid L-arginine into L-citrulline. Two types of NOS exist in the skeletal muscle and endothelial cells; neuronal NOS (nNOS) and endothelial NOS (eNOS), respectively (Dawes, Chowienczyk, & Ritter, 1997; Furchgott & Martin, 1985; Pohl, Holtz, Busse, & Bassenge, 1986). eNOS is the principal regulator of smooth muscle cell tone and can be triggered by mechanical force, shear stress, or β-adrenergic receptor (Dawes, Chowienczyk, & Ritter, 1997; Furchgott & Martin, 1985; Pohl, Holtz, Busse, & Bassenge, 1986). Following production and liberation from the endothelium, NO prompts relaxation of the vascular smooth muscle (vasodilation) by activating the enzyme guanylate cyclase, which escalates the activity of the second messenger cyclic guanosine monophosphate (cGMP) to attenuate the influx of calcium into the intracellular space of the cell (Murad 1986). Guanylate cyclase mobilized by NO also generates vasodilation by inactivating myosin light chains (enzymes that control skeletal muscle contractions) or by causing hyperpolarization through potassium (K^+) channel activation (Moncada & Higgs, 1993).

In addition to PGs and NO, K^+-mediated hyperpolarization has recently gained substantial attention for its role in ATP-induced vasodilation (Armstrong, Dua, & Murrant, 2007; Crecelius

et al., 2012; Crecelius, Richards, Luckasen, Larson, & Dinenno, 2013; Schrage, Dietz, & Joyner, 2006). Vascular smooth muscle cell tone is heavily dependent on intracellular calcium (Ca^{2+}) concentrations; vasoconstriction and contraction result from increases in Ca^{2+}, whereas vasodilation and relaxation are a product of decreases in Ca^{2+} (Crecelius et al., 2012). The quantity and extent to which Ca^{2+} enters the cell is based upon whether voltage-dependent Ca^{2+} channels are open or closed, which depends upon the cell's membrane potential (Crecelius et al., 2012). The membrane potential of a cell is how electrically charged a cell is; a cell is considered "depolarized" when it is positively charged and "hyperpolarized" when it is negatively charged. Hyperpolarization can be caused one of two ways.

The first mechanism for hyperpolarization involves increases in intracellular Ca^{2+} that occur with skeletal muscle contractions and stimulates small- and intermediate-conductance Ca^{2+}-activated K^+ (SK_{Ca} and IK_{Ca}) channels to open, whereas the second mechanism occurs when there is an increase in K^+ efflux from these channels into the myoendothelial space (Crecelius et al., 2012; G. Edwards, Dora, Gardener, Garland, & Weston, 1998; Segal, 2005). An increase in the amount of K^+ in the myoendothelial space due to skeletal muscle contractions activates Na^+/K^+-ATPase and inwardly-rectifying K^+ (K_{IR}) channels, both of which regulate the cell's membrane potential and result in hyperpolarization. When hyperpolarization does occur, it inhibits voltage-dependent Ca^{2+} channels, decreasing the amount of Ca^{2+} in the intracellular space, leading to vascular smooth muscle cell relaxation, and ultimately, vasodilation (Crecelius et al., 2012). Once initiated, this phenomenon is a conducted reaction that expands upstream through the microvasculature via gap junctions of neighboring smooth muscle and endothelial cells (Crecelius et al., 2012).

ATP infused into endothelial cells *in vitro* can enhance hyperpolarization through SK_{Ca} and IK_{Ca} channel activation and combined inhibition of these channels using the selective SK_{Ca} and IK_{Ca} blockers apamin and triarylmethane (TRAM-34) have notably attenuated vasodilation through these respective pathways (Sheng & Braun, 2007; Winter & Dora, 2007). Furthermore, studies in which the pharmacological agents barium ($BaCl_2$) and ouabain octahydrate have been used to block Na^+/K^+-ATPase and K_{IR} channels, respectively, have demonstrated a significant decrease in vasodilation (Crecelius et al., 2012; Winter & Dora, 2007). Our laboratory and others have clearly demonstrated that when these such pharmacological agents were utilized to measure hyperpolarized-induced vasodilation in the forearm, blood flow was considerably impaired (Crecelius et al., 2012; Ralevic, 2001; Smith et al., 2008; Winter & Dora, 2007). Even with simultaneous forearm ATP infusions, blood flow to the forearm (quantified as %Δ in forearm vascular conductance) decreased by at least 40% when either K_{IR} and Na^+/K^+ -ATPase or K_{IR} alone were blocked at rest (Crecelius, Kirby et al. 2012). Additionally, hyperpolarization caused exclusively through K_{IR} activation is significant enough that when this channel is blocked, ATP-mediated vasodilation is drastically reduced (Crecelius et al., 2012). The inability of such a potent vasodilator as ATP to maintain blood flow with these blockers on suggests that ATP induces vasodilation by working through hyperpolarization.

The Sympathetic Nervous System

Sympathetic nervous system activity greatly influences vascular tone and the resulting blood flow and oxygen delivery to the skeletal muscle at rest, during exercise, and with hypoxia. The adrenal medulla found in the adrenal gland is the major control center for sympathetic activity and relays information provided by the afferent pathways from the baroreceptors to the efferent

pathways in the periphery to regulate blood volume, blood pressure, and thus blood flow (Boron and Boulpaep 2012). The nerve endings and adrenal medulla possess the cathecholamines norepinephrine and epinephrine that when released into the bloodstream bind to α- and β-adrenergic receptors on the vascular smooth muscle, respectively (Boron and Boulpaep 2012). Binding to α-adrenergic receptors causes vasoconstriction whereas β-adrenergic receptor activation leads to vasodilation (Boron and Boulpaep 2012). The sympathetic nervous system has such a large influence on vascular tone that when α-adrenergic receptors are blocked in the forearm of young healthy adults, blood flow at rest multiplies at least two to three times (Dinenno, Dietz, & Joyner, 2002). Based on increased catecholamine levels and through direct measurement of muscle sympathetic nerve activity (MSNA) using microneurography, sympathetic nervous system activity is also enhanced during exercise and hypoxia, and is a dose-dependent response to both (Dinenno et al., 2002). Sympathetic vasoconstriction takes place during exercise and hypoxia to ensure that the arteries do not abnormally dilate, which would threaten blood pressure. Similar to rest, when α-adrenergic receptors are blocked during systemic hypoxia, an enhancement of vasodilation automatically occurs (Seals, Johnson, & Fregosi, 1991). When exercise takes place in the presence of hypoxia, sympathetic nervous system activity is even more enhanced than if each condition occurred individually (Seals et al., 1991). Importantly, recordings of MSNA during submaximal hypoxic exercise in the forearm are greater by at least two-fold than during normoxic exercise or hypoxia at rest (Seals et al., 1991). Combining the total amount of MSNA and plasma norepinephrine values during normoxic exercise or hypoxic rest are still appreciably less than what occurs during hypoxic exercise, implying that the combination of the two mechanisms is responsible for the heightened MSNA response in skeletal muscle (Seals et al., 1991). One explanation for the physiological

response to this synergistic interaction is that the metaboreflex activation and sensitivity that occurs during exercise could be further heightened with hypoxia. Decreases in pH that take place during both exercise and hypoxia could also stimulate a greater response of MSNA (Seals et al., 1991). Nonetheless, vasodilation and blood flow are still amplified in the presence of sympathetic activity so that oxygen can be properly supplied to the exercising tissue (Hartley, Vogel, & Landowne, 1973; Mazzeo et al., 1995; Rowell, Saltin, Kiens, & Christensen, 1986; Wilkins et al., 2008; Wilkins, Schrage, Liu, Hancock, & Joyner, 2006). Thus, sympathetic activity is a prominent and important mediator of blood flow regulation at rest, during exercise, with hypoxia, and throughout hypoxic exercise.

Baroreceptors are mechanoreceptors located on the carotid sinus and aortic arch and are a significant component of a neural feedback mechanism that responds to stretch in the blood vessel to control blood pressure (Boron and Boulpaep 2012). These receptors modulate vascular tone through a negative feedback loop, known as the baroreceptor control of arterial pressure, in which modifications in blood pressure alert the baroreceptors to either stimulate vasodilation or vasoconstriction and adjust heart rate accordingly (Boron and Boulpaep 2012). If blood pressure drops then the stretch on the blood vessels is reduced; decreasing the firing rate of baroreceptors, accelerating sympathetic activity, and attenuating parasympathetic activity through vasoconstriction. As a result of sympathetic nervous system enhancement, heart rate is increased in order to boost blood pressure back up to normal values (Boron and Boulpaep 2012). Conversely, a rise in blood pressure stretches the vessels and stimulates the baroceptors to inhibit sympathethetic activity and intensify parasympathetic activity; the outcome of which is vasodilation and decreased heart rate to prevent blood pressure from reaching harmful values (Boron and Boulpaep 2012).

Peripheral chemoreceptors also contribute to arterial blood pressure regulation through sympathetic tone by controlling ventilation and identifying fluctuations in the pH, P_{O2}, and P_{CO2} in the blood (Boron and Boulpaep 2012). Similar to the baroreceptors, these receptors are located on the carotid and aortic arches. If either the pH or arterial P_{O2} drop, or P_{CO2} elevates, the peripheral chemoreceptors and thus the sympathetic nervous system will be activated to alert the adrenal medulla to slow down heart rate and constrict the blood vessels; and vise versa if the opposite fluctations occur. A fall in P_{O2} (less than 100 mmHg) most commonly occurs during hypoxia in which an increased ventilation rate is the most influential stimulus for these receptors. Amplified ventilation due to hypoxia expands the lungs, signalling these pulmonary stretch receptors and increasing heart rate by inhibiting the cardioinhibitory center. An upsurge in P_{CO2} as a result of hypoxia is another result of increased ventilation and also leads to tachycardia. There is an inverse relationship between P_{CO2} and pH levels, and the metabolic acidosis that occurs during hypoxia once the pH decreases (below 7.25) signals the peripheral chemoreceptors in the carotid body to be activated.

Sympatholysis

Although substantial sympathetic nerve activity is prevelant during rest, exercise, hypoxia, and hypoxic exercise, blood flow supply to the tissues is still maintained. Fortunately, not only does ATP aid in vasodilation, but it can also blunt vasoconstriction by inhibiting postjunctional α-adrenergic sympathetic activity of both receptor α_1 and α_2 subtypes, a term referred to as "functional sympatholysis" (Kirby et al., 2008; Rosenmeier, Hansen, & Gonzalez-Alonso, 2004). During exercise, this sympatholytic phenomenom is augmented and linear with increasing intensity; a greater amount of ATP is released with a higher intensity of exercise and has a more

profound sympatholytic effect (Kirby et al., 2012; Kirby et al., 2008; Rosenmeier et al., 2004). Specifically, minimal ATP levels that appear with moderate muscular contractions are not as sympatholytic as those that occur at higher, more frequent, muscular contractions (Kirby et al., 2012). Such a theory has been extensively studied and proven true using rhythmic handgrip exercise to measure ATP production and its effects on postjunctional α-adrenergic vasoconstrictor responsivenes (Kirby et al., 2008; Rosenmeier et al., 2004). Precisely, small amounts of infused ATP (500 nmol L^{-1}) into the human forearm do not significantly inhibit α-adrenergic vasoconstriction at rest, whereas moderate (1000 nmol L^{-1}) and high (2000 nmol L^{-1}) levels do (Kirby et al., 2008). This observation is similar to graded exercise intensity, in which mild rhytmic handgrip exercise contractions (5% MVC) are not as sympatholytic as moderate exercise (15% MVC) is (Kirby et al., 2008). Although MSNA and plasma norepinpehrine levels tend to increase when ATP is infused into the exercising femoral artery, vasodilation and leg blood flow are still enhanced (Rosenmeier et al., 2004). Nor does blood flow decrease when tyramine, an α-adrenergic receptor stimulator, is infused (Rosenmeier et al., 2004). Even when MSNA increased two to four times from rest, forearm and leg blood flow have been sustained with ATP infusion (Hanada, Sander, & Gonzalez-Alonso, 2003; Rosenmeier et al., 2004). Despite the fact that it is not solidified as to the exact process in which ATP is able to blunt sympathetic activity, release of vasodilators such as NO, PG, and EDHF from the endothelium has been the focus (Rosenmeier et al., 2004). The importance of this sympatholytic paradox is that blood flow and oxygen delivery to the exercising muscle can be maintained in the presence of sympathetic nerve activity.

Compensatory Vasodilation with Hypoxic Exercise

Exercise and hypoxia are individual stimuli for ATP release. In contrast to rest and under normoxia, one-legged knee extensor exercise has demonstrated extraordinary femoral arterial and venous plasma ATP release that is graded with exercise intensity (Gonzalez-Alonso et al., 2002; Mortensen, Thaning, Nyberg, Saltin, & Hellsten, 2011). Notably, a 3-fold escalation in ATP release has been revealed in both the femoral arterial and venous plasma as participants performed 10 to 45% of their maximal workload during one-leg extensor exercise (Mortensen et al., 2011). Steady-state handgrip exercise (15% MVC) also provokes noteworthy rises in venous plasma ATP values in the human forearm from rest (Kirby et al., 2013). The enhanced ATP release with exercise seems to be systemic, as non-exercising limbs have also experienced rises in venous plasma ATP concentrations from rest to during exercise (Gonzalez-Alonso et al., 2002). Additionally, plasma ATP levels remain elevated even 10 minutes after the cessation of exercise (Gonzalez-Alonso et al., 2002).

As opposed to breathing room-air, the amount of plasma ATP measured in the femoral vein increases when participants breathe hypoxic air at rest (Mortensen et al., 2011). When samples of isolated human erythrocytes have been subjected to a hypoxic environment within tonometer bulbs for 50 seconds, the erythrocytes released appreciably more ATP than when placed in normoxic bulbs (Bergfeld & Forrester, 1992). Our laboratory has demonstrated a 205±57% progression in ATP release from de-oxygenated erythrocytes *in vitro* compared to cells that remained in normoxic tonometer gas bulbs (Kirby et al., 2012).

Multiple studies from our laboratory and others have identified that it is the oxygenated state of the hemoglobin molecule within the erythrocyte that is the specific stimulus for ATP release and vasodilation during exercise and hypoxia; the more de-oxygenated the hemoglobin molecule

becomes, the greater the response for ATP release and vasodilation (Crecelius et al., 2010; Crecelius, Kirby, Voyles, et al., 2011; M. L. Ellsworth, Forrester, T., Ellis, C.G., and Dietrich, H.H., 1995; Kirby et al., 2012; Kirby et al., 2008; Kirby et al., 2009; Seals et al., 1991). Indeed, there is a strong inverse relationship between the amount of ATP in the venous plasma and the extent of hemoglobin de-saturation, both in exercising and non-exercising limbs (Gonzalez-Alonso et al., 2002).

As a result of hemoglobin de-oxygenation from exercise or hypoxia, erythrocyte-derived ATP release yields profound enhancements in blood flow. Specifically, there is a tight coupling between ATP release and blood flow in the human skeletal muscle. Leg blood flow linearly increases above resting values with single knee-extensor exercise, while hypoxia substantially contributes to leg blood flow augmentation at rest, throughout exercise, and after exercise (Mortensen et al., 2011). Compared to normoxia, hypoxic rest increases leg vascular conductance upwards of 35% (Gonzalez-Alonso et al., 2001).

Based on the establishment that both exercise and hypoxia individually stimulate ATP release, it seems highly promising that the combination of the two (hypoxic exercise) would further heighten ATP release from the erythrocyte. Importantly, with hypoxic exercise a greater de-oxygenated stimulus for ATP could contribute to augmented blood flow and oxygen delivery, as the dramatic elevation of ATP values with hypoxic exercise have been strongly correlated with significant surges in both vascular conductance and leg blood flow (Mortensen et al., 2011). Furthermore, blood flow and vascular conductance augmentation is graded with exercise intensity during hypoxia, and is inversely associated with hemoglobin de-oxygenation (Gonzalez-Alonso et al., 2002). As a result of severe hypoxic exercise, leg blood flow increases

by approximately 23±5% (Dufour et al., 2010). Moreover, systemic hypoxia during exercise produces elevations in leg blood flow within the first minute (Gonzalez-Alonso et al., 2001).

Further support for compensatory vasodilation with hypoxic exercise based on erythrocyte-derived ATP release comes from sympatholysis. As previously discussed, ATP is a potent sympatholytic agent that can blunt the sympathetic nervous system activity that occurs during exercise and hypoxia. Thus, it would seem possible that hypoxic exercise would further augment this sympatholytic response and contribute to even greater rates of ATP release, vasodilation, and forearm blood flow. Indeed, it has been shown in multiple studies that this sympatholytic phenomenon occurs simultaneously with graded muscle sympathetic nervous system activity during hypoxic exercise (Dinenno & Joyner, 2003; Rosenmeier, Dinenno, Fritzlar, & Joyner, 2003; Tschakovsky, Sujirattanawimol, Ruble, Valic, & Joyner, 2002). There is substantial legitimate evidence that ATP is able to attenuate the sympathetic activity to allow for increased blood flow with hypoxic exercise, especially at higher exercise intensities or decreasing arterial oxygen content values, and compared to normoxic exercise (Rosenmeier et al., 2003; Rosenmeier et al., 2004; Tschakovsky et al., 2002).

The fact that upsurges in blood flow in the exercising tissue under hypoxia are associated with hemoglobin desaturation and ATP release highlights the erythrocyte's ability to adapt to mechanically induced stress in order to control blood flow. Additionally, the fact that blood flow during hypoxic exercise is still able to be maintained despite high values of sympathetic activity suggests that erythrocyte-derived ATP release is even more intensified when the two stimuli for release are combined. Moreover, it solidifies the idea that hypoxic exercise is a powerful stimulus for ATP release.

STATEMENT OF PROBLEM

Although previous research has established that there is a concrete relationship between hypoxia, exercise, and ATP release, little work has been done to examine the extent to which the oxygen content of the erythrocyte can affect the vasculature, both at rest and during submaximal hypoxic exercise. Whether or not ATP released from the erythrocyte into the circulation is bi-directionally changing warrants further investigation. Precisely how much ATP can be released from the erythrocyte during normoxic rest, submaximal normoxic exercise, and submaximal hypoxic exercise, and if there is a limit to this release has not been identified. Lastly, understanding if the cardiovascular and circulatory systems can be further manipulated to augment blood flow remains unknown.

HYPOTHESIS

As hypoxia and exercise individually augment both blood flow and ATP release, we hypothesize that hypoxia in combination with rhythmic handgrip exercise will evoke an even greater escalation in forearm blood flow due to local vasodilation, and that this response will be related to a further increase in ATP release from the erythrocyte.

CHAPTER III

METHODS AND PROCEDURES

Subjects

With written informed consent and after approval from the Institutional Review Board, 10 young healthy adults (5 men, 5 women; age = 21.5 ± 1 years; weight = 74.4 ± 2.9 kg; height = 172.2 ± 1.9 cm; body mass index = 25.2 ± 0.7 kg m^{-2}; means ± S.E.M.) took part in the present study. All subjects were recreationally physically active and free from any diseases or comorbidities. Specifically, our subjects were normotensive (<140/90 mmHg), non-smokers, non-diabetic, non-obese (BMI <29.9 kg/m^2), and not on any medications. Subjects were not excluded based on race or ethnicity. Female subjects were in the initial follicular phase of their menstrual cycle, or if they were taking oral contraceptives, they must have been in the placebo phase of the medication. Timing of the menstrual cycle was important to ensure that endothelial function and the sympathoadrenal system were not impacted hormonally. After a 4-hour fast and without having exercised or consumed caffeine, medications, or supplements for at least 24 hours before the experimental trial, all subjects were studied in the early morning and placed in the supine position for the remainder of the study. In addition to the climate controlled laboratory (20-22ºC), a small fan was aimed at the experimental forearm of all subjects to attenuate the influence of skin blood flow on forearm hemodynamics. The following experimental design was in agreement with the *Declaration of Helsinki*.

Systemic hypoxia

To maintain consistent pH values while manipulating oxygen saturation levels and inducing variable ventilation rates, all subjects breathed through a pneumotachograph (model VMM-2a, Interface Associates, Laguna Niguel, CA, USA) connected to the partial-rebreathe technique constructed by Banzett *et al*. (Banzett, Garcia, & Moosavi, 2000). A nose clip was worn to prevent any nasal breathing. After baseline measurements during normoxic conditions were collected, subjects first experienced oxygen levels representative of normoxia (21% room air) and then hypoxia (80% SaO_2) through a spirometer tube connected to a medical gas blender. Oxygen saturation was monitored using a pulse oximeter attached to one earlobe and connected to a monitor (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA). The system described above has been successfully used previously in our laboratory to elicit hypoxia (80% SaO_2) and has demonstrated significant vasodilation in the forearm (Kirby et al., 2012).

Rhythmic handgrip exercise

Using their non-dominant arm, all subjects were instructed to place their hand around a handgrip dynamometer (Stoelting, Chicago, IL, USA) and to squeeze their hand while contracting only muscles in their forearm in time with a metronome. After determining each individual subjects' maximum voluntary contraction (MVC; mean 34.4 ± 3.3 kg, range 18-51 kg), weight representative of submaximal exercise and corresponding to 15% MVC (mean 5.5 ± 0.6 kg, range 2.7-9.5 kg) was lifted 4-5 cm over the pulley system attached to the dynamometer. The subjects exercised at a duty cycle of 1 second contraction-2 seconds relaxation (20 contractions per minute) for a total of 6 minutes (3 minutes normoxic exercise, 3 minutes

hypoxic exercise). Both audio and visual cues were used to ensure that the subjects were maintaining correct timing of contractions as described previously (Dinenno & Joyner, 2003). This workload was chosen in order to attenuate any surges in sympathetic activity and to elicit vasodilation during exercise (Seals et al., 1991; Wilkins et al., 2008).

Forearm blood flow and vascular conductance

Doppler ultrasound using a 12 MHz probe (GE Vingmed Ultrasound Vivid 7, Horten, Norway) was placed on the brachial artery of the experimental arm and was employed to determine arterial mean blood velocity (MBV) and brachial artery diameter. Using a technique previously described by our laboratory, the probe was securely placed on skin at the location of the brachial artery at <60 degrees and directly near the catheter site to analyze and measure the MBV during baseline, normoxic exercise, and hypoxic exercise at a frequency of 5 MHz (Dinenno & Joyner, 2003). The Doppler shift frequency spectrum was analyzed via a Multigon 500V TCD (Multigon Industries, Mt Vernon NY, USA) spectral analyzer from which mean velocity was determined as a weighted mean of the spectrum of Doppler shift frequencies. Brachial artery diameter measurements were made in duplex mode at end-diastole and between contractions during steady-state conditions. Forearm blood flow (FBF) was derived from the equation:

$$FBF = MBV\ (cm/s) \times \pi\ (\text{brachial artery diameter}/2)^2 \times 60,$$

where FBF is in ml min^{-1}, the MBV is in cm s^{-1}, the brachial diameter is in cm, and 60 was used to convert from ml s^{-1} to ml min^{-1}. Forearm blood flow values were then used to determine

forearm vascular conductance (FVC), which was represented in ml min^{-1} $(100mmHg)^{-1}$ and was calculated using the equation: (FBF/MAP) x 100, where MAP represents mean arterial pressure.

Forearm volume and fat-free mass

All subjects underwent a dual energy x-ray absorptiometry scan (DEXA; Hologic, Inc; Bedford, MA, USA) to determine forearm volume and forearm fat-free mass.

Non-Invasive determination of arterial blood pressure and heart rate

Arterial blood pressure values were collected at baseline by placing a blood pressure cuff at the level of the brachial artery around the non-experimental arm which was connected to a monitor (Cardiocap/5 Datex-Ohmeda, Louisville, CO, USA). To continuously monitor blood pressure at the level of the heart throughout the experiment, a finger photoplethysmography (Finometer, Finapres Medical Systems, Amsterdam, The Netherlands) was attached to the middle finger of the non-experimental hand. A 3-lead Electrocardiogram (Cardiocap/5 Datex-Ohmeda, Louisville, CO, USA) attached to the skin of the subject at the locations of leads I, II, and III was employed for continuous Heart Rate (HR) measurements.

Venous catheterization

Based on the size of each subjects' vein, an 18-22 gauge, 1-2 in catheter was placed into the antecubital vein of the experimental arm in a retrograde direction to allow blood gas and ATP

samples to be drawn from. Saline was continuously infused through the catheter to prevent backup.

Blood gases

2 mL of blood drawn from the venous catheter using a pre-heparinized 3cc syringe was inserted into a blood gas machine (Siemens Rapid Point 405 Series Automatic Blood Gas System, Los Angeles, CA, USA) to analyze in pH, partial pressures of oxygen and carbon dioxide (PCO_2 and PO_2), total hemoglobin (tHb), oxygen saturation (SO_2), and hematocrit (Hct) at rest, with steady-state exercise, and then every 30 seconds during exercise under both oxygen saturations.

ATP measurements

2 mL of blood was drawn from venous catheter using a 10cc heparinized syringe and then expelled into another tube storing 2.7 mL of an ATP "stop solution" which inhibits ATP degradation. From there, ATP was centrifuged as soon as possible for 3 minutes at a rate of 4,000 rpm and at 22°C. After removing 100uL of supernatant, the luciferin-luciferase technique previously described in our laboratory (Kirby et al., 2012) was used to measure plasma ATP in Relative Light Units (RLU) using a luminometer (Turner Biosystems 20/20n, Sunnyvale, CA, USA). RLUs were derived from an average of 10 seconds. The standard curve for ATP was also constructed before each experiment by spiking a baseline blood:diluent sample of 90uL of plasma with 10uL of varying ATP concentration. Any values for ATP or plasma greater than

15% variation were not used. After calculating the final RLUs and by using the blood:diluent sample, final venous plasma ATP was calculated as:

$$[ATP] = (ATP_{blood:diluent} - ATP_{hemolysis}) \times (1.35+1-HCT)/(1-HCT).$$

The values from this equation represent the cumulative amount of circulating vasoactive ATP in the skeletal muscle (Kirby et al., 2012). Final ATP effluent was expressed in (nmol/L) and was calculated using the following equation:

$$ATP\ effluent = (FBF \times ATP)/1000.$$

Theses value exhibit the absolute amount of ATP draining the skeletal muscle and that which induces the observed degree of vasodilation (Kirby et al., 2012). Forearm blood flow was taken into consideration as it can vary with and without hypoxia and exercise, and has a positive relationship with ATP production.

Hemolysis

The amount of hemolysis was analyzed to ensure that ATP was being released from the erythrocyte solely because of the low oxygen environment, and not due to the erythrocyte breaking open during blood handling. Using the same blood collected for the ATP measurements, 1 mL of supernatant was collected for plasma Hb analysis using a spectrometer (Molecular Devices SpectraMax) at three different wavelengths; 380, 415, and 450. The plasma Hb value was used to express the percentage of hemolysis using the equation:

% Hemolysis = {(100-HCT) x plasma [Hb]/total [Hb]} x 100. Values greater than 2 standard deviations from the mean were not considered.

The percentage of hemolysis was determined for normoxic rest, normoxic steady-state exercise, and then every 30 seconds from initiation of hypoxic exercise to cessation of the trial (minute 6).

Experimental protocol

A detailed timeline for the entire protocol is shown in Figure 2. Subjects rested for at least 20 minutes after the venous catheter was inserted into the antecubital space of the experimental arm. Thereafter, baseline data was collected immediately before the subject began continuous rhythmic handgrip exercise (15% MVC) in time with the metronome under normoxia for a total of 3 minutes. After the first 3 minutes and for an additional 3 minutes of exercise, arterial oxygen saturation was decreased to 80% (hypoxia) by way of the partial re-breathe system. Blood gas and ATP samples were drawn from the venous catheter immediately after baseline measurements and right before exercise began, prior to oxygen alteration, 3 minutes into exercise, and then every 30 seconds throughout the last 3 minutes of exercise in which the subject was in a state of hypoxia (Fig. 1).

Data acquisition and analysis

During the experimental trials, all data was continuously recorded and saved to a computer at a frequency of 250 Hz. After data collection and using a different computer, signal processing software (Windaq, DATAQ Instruments, Akron, OH, USA) was used for analysis. Mean Arterial Pressure (MAP) was computed from the arterial pressure waveform. An average of 30 seconds was used to determine oxygen saturations (pulse oximeter), FBF, FVC, HR, and

MAP for each trial. End-tidal CO_2 and minute ventilation were derived using data from the last minute of each trial. Blood samples collected at each time point were used to determine blood gas values. Sigmaplot (Systat Software) was used for all statistical comparisons.

Statistics

Analysis of Variance (ANOVA) was used to determine any differences between subjects. Statistical significance was set at $P<0.05$ and values were expressed as means ± SEM. One-tailed, paired t-tests were used to compare the amount of ATP collected at the pre-determined time points and within each condition, respectively.

CHAPTER IV

RESULTS

We studied 10 (5 male, 5 female) young health adults. Subject characteristics are presented in Table 1.

Systemic hemodynamic and respiratory responses

Systemic hemodynamic and respiratory responses are presented in Table 2 and Table 3. Heart rate significantly increased from rest at 30 seconds of hypoxic exercise until 3 minutes of hypoxic exercise ($P<0.05$). Heart rate between minute 1 and minute 3 of hypoxic exercise were significantly increased from steady-state normoxic exercise ($P<0.05$). Mean arterial pressure significantly increased from rest once steady-state normoxic exercise began, and this augmentation remained by 3 minutes of hypoxic exercise ($P<0.05$). Ventilation and end-tidal CO_2 were significantly greater than rest with steady-state exercise, and at 3 minutes of hypoxic exercise ($P<0.05$). The targeted oxygen saturation of ~80% was achieved during hypoxic exercise, and SpO_2 during hypoxic exercise was significantly decreased from rest and steady-state exercise ($P<0.05$).

Forearm blood flow and vascular conductance responses

Absolute values of FBF and FVC are presented in Table 2 and Table 3. Forearm blood flow and vascular conductance were significantly increased from rest with steady-state normoxic

exercise and across all time points during hypoxic exercise ($P<0.05$). Hypoxic exercise also significantly increased FVC and FBF above steady-state values at 30 seconds and 3 minutes, respectively ($P<0.05$).

Blood gases

Blood gas data are presented in Table 4. With the onset and until the end of hypoxic exercise, the pH was significantly increased from rest ($P<0.05$). PCO_2 significantly increased from rest with steady-state normoxic exercise, but then decreased from steady-state with and during hypoxic exercise ($P<0.05$). PO_2 decreased from rest and was significantly decreased at minute 2:30 and minute 3 during hypoxic exercise ($P<0.05$). PO_2 at minute 3 in hypoxic exercise was significantly less than steady-state PO_2 and 30 seconds of hypoxic exercise ($P<0.05$). As expected with hypoxia, ctO_2 and SO_2 decreased from normoxic rest to the end of hypoxic exercise, and between minute 2 and minute 3 of hypoxic exercise were significantly less than rest, steady-state normoxic exercise, and 30 seconds of hypoxic exercise ($P<0.05$). FHHb values were significantly increased from rest, steady-state normoxic exercise, and 30 seconds of hypoxic exercise at minutes 2, 2:30, and 3 during hypoxic exercise ($P<0.05$). No significant differences were observed in the p50 ($P=0.074$).

Plasma ATP and ATP effluent responses

Trends in venous plasma ATP and ATP effluent are presented in Figure 5 and Figure 6. Plasma ATP was significantly different from rest with steady-state normoxic exercise, and at 30

seconds and 3 minutes of hypoxic exercise, whereas the augmentation in ATP effluent was maintained throughout the entire protocol ($P<0.05$).

CHAPTER V

DISCUSSION

The purpose of the present study was to examine the extent to which the oxygen content of the erythrocyte, by way of ATP release, can affect the vasculature, both at rest and during submaximal exercise. Specifically, the present study focused on determining how much ATP could be released from the erythrocyte during normoxic rest, submaximal normoxic exercise, and submaximal hypoxic exercise, and if there was a limit to ATP release from the erythrocyte.

The primary novel findings from this study are as follows. First, forearm blood flow and vascular conductance both increased from normoxic rest with steady-state normoxic exercise, and were further augmented with hypoxic exercise (Table 2; Figure 4). Secondly, venous plasma ATP and ATP effluent were both significantly greater than rest with steady-state normoxic exercise and by the third minute of hypoxic exercise (Figure 6). Finally, although we hypothesized that hypoxic exercise would promote enhanced ATP release from the erythrocyte, data from the present study demonstrate that there was not a significant amount of ATP release between 30 seconds and 3 minutes of hypoxic exercise (Figure 5).

No further stimulus

Based on previous data demonstrating that hypoxia and exercise individually augment ATP release (Bergfeld & Forrester, 1992; Gonzalez-Alonso, Olsen, & Saltin, 2002; Gonzalez-Alonso, Richardson, & Saltin, 2001; Kirby, Crecelius, Richards, & Dinenno, 2013; Kirby, Crecelius, Voyles, & Dinenno, 2012; Mortensen, Thaning, Nyberg, Saltin, & Hellsten, 2011), it was expected that the combination of the two stimuli in the current study would elicit an even greater

release of ATP. However, although ATP values at 30 seconds and 3 minutes of hypoxic exercise were statistically significantly different from normoxic rest, the time points in between were not (Figure 5). It appears that the greatest change in ATP values occurred when subjects transitioned from normoxic rest to steady-state normoxic exercise (Figure 5). Absolute values of ATP revealed that between normoxic rest and steady-state normoxic exercise, there was an increase of 56 nmol/L of ATP, whereas ATP release only increased by 13 nmol/L as subjects began hypoxic exercise, and did not continue in a linear fashion (Figure 5). By minute 3 of hypoxic exercise, ATP had surprisingly *decreased* by 30 nmol/L (Figure 5).

Blood gas analysis from the subjects in the present study demonstrated a similar trend in ATP release, as it did not continue to increase, even when hypoxia (80% SpO_2) was achieved. Aside from inducing mechanical stress, hypoxic exercise also causes reductions in certain blood gas parameters (pH, PCO_2, PO_2, ctO_2, SO_2) and SpO_2 values, while enlarging others (FHHb and the p50). Whereas pH, PCO_2, PO_2, ctO_2, SO_2 and SpO_2 are all negatively related to hemoglobin de-oxygenation and inversely related to ATP release, FHHb and the p50 are positively related to hemoglobin de-oxygenation and inversely related to ATP release. Indeed, every pertinent value in the present study trended in the correct direction in response to hypoxic exercise, and should have prompted considerable ATP release from the erythrocyte and into the microvasculature (Table 4). It is possible that the erythrocyte is not as sensitive or as correlated to changes in the oxygenated state of the hemoglobin molecule as originally thought.

Our hypothesis and contradicting results are in-line with other studies that have examined erythrocyte-derived ATP release (Crecelius, Kirby, Richards, & Dinenno, 2013; Kirby et al., 2012). Despite the fact that absolute values of ATP increased in a graded manner when de-oxygenated in tonometer bulbs *in vitro,* ATP with exercise did not produce linear results (Kirby

et al., 2012). Plasma measurements of ATP during graded rhythmic handgrip exercise (5, 15, and 25% MVC) revealed that the greatest change in ATP release occurred between the first and last bout of exercise, whereas there was not a noticeable change between the first and second adjustment in exercise intensity; 30 nmol/L *vs.* 9 nmol/L, respectively (Kirby et al., 2012). Likewise, when rhythmic forearm compressions were combined with rhythmic handgrip exercise to induce hemoglobin de-oxygenation and mechanical stress, venous plasma ATP was not significantly different throughout the trial in that ATP values tripled from rest with mild compressions (50 mmHg) but were not significantly elevated thereafter with greater levels of compression (100 and 200 mmHg) (Crecelius et al., 2013). Similar to the present study, the previous studies also showed significant and graded increases in FBF, FVC, and ATP effluent.

Thus, after the first 30 seconds of hypoxic exercise, there may not be a powerful enough stimulus to arouse the erythrocyte to release ATP. Or, an additional stimulus does not result in enhanced ATP release; the erythrocyte only needs modest changes in hemoglobin de-oxygenation for sizable amounts of ATP to be released and detected.

ATP degradation in the vasculature during hypoxic exercise

ATP in the microvasculature could potentially be degraded over time. A multitude of literature has proposed that exercise amplifies the production and activity of ecto-nucleosides to degrade nucleotides (ATP, ADP, AMP) in the blood (Coade & Pearson, 1989; Mortensen et al., 2011; Robson, Sevigny, & Zimmermann, 2006; Yegutkin, Samburski, Mortensen, Jalkanen, & Gonzalez-Alonso, 2007). Ecto-nucleosides are enzymes located on the surface of virtually every cell in the human body, including endothelial cells, and are responsible for metabolizing

nucleotides into nucleosides (Robson et al., 2006). These extracellular enzymes facilitate vascular control as they mediate the association and activity between nucleotides and their corresponding receptors (Robson et al., 2006). For example, when erythrocytes release ATP in response to hypoxia or exercise, ATP binds to purinergic receptors on endothelial cells. Furthermore, ecto-nucleosides control the availability of nucleotides by hydrolyzing them once they are released into the microcirculation, dismissing the functionality of purinergic receptors (Robson et al., 2006). The ecto-nucleosides specific to ATP degradation are ecto-nucleoside triphosphate diphosphohydrolase 1 and 2, termed ENTPDase1 and ENTPDase2, respectively (Robson et al., 2006). ENTPDase1 directly hydrolyzes ATP into AMP, whereas ENTPDase2 requires two steps that involve hydrolyzing ATP into ADP + P_i and then converting ADP into AMP and P_i (Coade & Pearson, 1989; Robson et al., 2006; Yegutkin et al., 2007). AMP can be further broken down into adenosine by way of ecto-5'-nucleotidase (Yegutkin, 2008). The rapid degradation of ATP results in a half-life that is barely a few seconds in duration (Mortensen et al., 2011).

In parallel with the current study, other studies have also noticed a lack of ATP augmentation during hypoxic exercise (Mortensen et al., 2011; Yegutkin et al., 2007). Whereas both hypoxic rest and normoxic exercise increased femoral venous plasma ATP, rhythmic knee-extensor exercise under hypoxia did not (Mortensen et al., 2011). Additionally, ATP infused into the femoral artery at rest increased total arterial plasma ATP, but no change was detected in the femoral vein (Mortensen et al., 2011). Additionally, only half of the infused ATP was identified within 20 cm downstream of femoral artery (Mortensen et al., 2011). Moreover, direct plasma NTPDase measurements during both submaximal and maximal exercise have revealed a two-fold increase in enzymatic activity that is maintained even after exercise subsides (Yegutkin et

al., 2007). Increases in plasma ADP values with exercise clarified that ATP was being broken down and was not significantly elevated during exercise (Yegutkin et al., 2007).

In relation to the current study, data from previous research suggest that although femoral venous ATP is increased with hypoxia and exercise alone, the combination of the two does not provide enough of a stimulus to further augment erythrocyte-derived ATP release, and suggests that degradation of ATP by ENTDPase 1 and 2 could explain this phenomenon. In the present investigation, we found a significant and linear increase in blood flow with hypoxic exercise duration which could have potentially enhanced ecto-nucleotidase activity. If ATP is being degraded as it traverses the microvasculature, this could be why we saw decreased values contrary to our hypothesis. The fact that ATP release dwindled also begs the question of whether or not hypoxia enhances greater enzymatic degradation. ATP may have increased with hypoxic exercise, but by the time the blood navigated the microcirculation and we took our blood samples, it could have already been degraded. Thus, our blood samples could be *underestimating* what is actually taking place *in vivo*.

Nitric Oxide

If hypoxic exercise is in-fact a poor stimulus for ATP release and ATP is being degraded as it traverses the microvasculature, this does not explain how or why forearm blood flow, vascular conductance, and ATP effluent were all significantly increased and maintained throughout the study. Thus, some other mechanism or pathway is causing vasodilation, and a considerable amount of research has proposed that another potent vasodilator, nitric oxide (NO), could be the source (Blitzer, Lee, & Creager, 1996; Blitzer, Loh, Roddy, Stamler, & Creager, 1996;

Olearczyk, Ellsworth, Stephenson, Lonigro, & Sprague, 2004; Olearczyk, Stephenson, Lonigro, & Sprague, 2004; Sprague & Ellsworth, 2012). Importantly, nitric oxide has been indicated to inhibit ATP release (Olearczyk, Ellsworth, et al., 2004). As previously discussed in the Literature Review, once ATP is released from the erythrocyte by way of the Signal Transduction Pathway, it binds to P_{2Y1} and P_{2Y2} receptors on the endothelium, prompting nitric oxide to be released from the endothelium and into the extracellular space, inducing relaxation of the vascular smooth muscle and ultimately, vasodilation.

ATP release and pulmonary vascular resistance have been shown to be attenuated as a result of decreased oxygen tension when isolated rabbit and human erythrocytes were pre-incubated with the NO donor *N*-(2-aminoethyl)-*N*-(2-hydroxy-2-nitrosohydrazino)-1,2-ethylenediamine (spermine NONOate) (Olearczyk, Ellsworth, et al., 2004). Deformation-induced ATP release was also prevented when erythrocytes were pre-incubated with spermine NONOate *in vitro* and mechanically deformed as they passed through a blood filtrometer (Olearczyk, Ellsworth, et al., 2004). Incubation of rabbit erythrocytes deficient of spermine NONOate confirmed that ATP inhibition was indeed a result of NO, as ATP values did not significantly rise without the ability of NO to be donated (Olearczyk, Ellsworth, et al., 2004). Infusions of the selective nitric oxide synthase inhibitor, L-NG-Nitroarginine Methyl Esther (L-NAME) into smooth muscle cells has proven to inhibit endothelium-derived ATP release (Collins, McCullough, & Ellsworth, 1998; McCullough, Collins, & Ellsworth, 1997). L-NG-Monomethylarginine (L-NMMA), a non-selective inhibitor of nitric oxide synthase, also reverts the hemodynamic and vascular responses that hypoxia causes by significantly increasing mean arterial blood pressure, decreasing cardiac output, and most importantly, increasing systemic vascular resistance by almost two-fold (Blitzer, Loh, et al., 1996). A closer look into NO's vascular contribution verified that NO levels

had been subsequently decreased by approximately 65% with L-NMMA blockade (Stamler, Loh, Roddy, Currie, & Creager, 1994).

The multitude of previous explorations support the idea that NO is working through a "negative feedback mechanism" along the Signal Transduction Pathway to inhibit ATP release from the erythrocyte. When NO accumulates and is expelled intraluminally, it can discriminatorily prevent any extra ATP to be released during states of low O_2 tension, such as with hypoxic exercise (Sprague & Ellsworth, 2012). Specifically, any further accrual of ATP cannot occur if erythrocytes move into an area of tissue in which values of NO are already raised (Sprague & Ellsworth, 2012). Although the precise mechanism by which NO is inhibiting ATP release has yet to be solidified, it is postulated that once it is released into the vascular lumen, NO can indirectly inhibit the heterotrimeric G protein, G_i (Olearczyk, Ellsworth, et al., 2004). Activation of G_i is the first step in the Signal Transduction Pathway and is thus extremely important for erythrocyte-derived ATP release. Nitric oxide has the ability to specifically target this initial step in the Signal Transduction Pathway by exciting mono-ADP-ribosyltransferase, an endogenous enzyme that prohibits the α-subunit of G proteins to separate from their βγ-subunit complex (Brune & Lapetina, 1989; Moss, Stanley, & Watkins, 1980; Olearczyk, Stephenson, et al., 2004; Schuman, Meffert, Schulman, & Madison, 1994). If the α-subunit cannot separate from the βγ-subunit complex, then G_i cannot be activated, which prevents the remaining steps in the Signal Transduction Pathway from being stimulated and ultimately blocks ATP release out of the erythrocyte and into the extracellular space. As opposed to the other phases along the Signal Transduction Pathway (i.e. adenylyl cyclase, cyclic AMP, phosphokinase a, and cystic fibrosis transmembrane receptor, and pannexin-1 activity) confirmation of NO's capability to objectively block G_i is supported by investigations in which washed rabbit erythrocytes were incubated with

spermine NONOate, followed by incubation with mastoparan, a peptide toxin that triggers G_i by spurring the α-subunit to separate from the βγ-subunit complex (Olearczyk, Stephenson, et al., 2004). Spermine NONOate successfully inhibited ATP to be released from washed rabbit erythrocytes when they were incubated with mastoparan (Olearczyk, Stephenson, et al., 2004). Conversely, spermine NONOate did not inhibit ATP release in erythrocytes incubated with iloprost or forskolin, which arouse G_s protein and adenylyl cyclase activity, respectively (Olearczyk, Stephenson, et al., 2004). Thus, it seems likely that ATP inhibition by NO is specifically targeted at G_i blockade.

Another way in which NO can be formed is through the reduction of the precursor ion, nitrite (NO2-), a process that is independent of erythrocyte-derived ATP release and is represented in the following equation: $Hb^{2+} + NO_{2-} \rightarrow Hb^{3+} + NO + OH^-$, in which nitrite is reduced to NO when combined with the ferrous state hemoglobin (Hb^{2+}) (Crawford, Chacko, Kevil, & Patel, 2004; Crawford et al., 2006). Plasma and erythrocytes contain roughly 120 and 290 nM of nitrite, respectively, but this ion can also be derived exogenously via cured meat (Dejam et al., 2005). Nitrite is strongly associated with endothelial nitric oxide synthase (eNOS) activity, and has been shown to play a very influential role in vasodilation during hypoxia, mechanical stress, and metabolic acidosis (Bryan et al., 2004; Cannon et al., 2001; Gladwin et al., 2000; Lauer et al., 2001). Nitrite reduction is the most responsive as hemoglobin undergoes a conformational change during increased de-oxygenation (i.e. hypoxia or exercise) and the p50 is reached (Crawford et al., 2006). Specifically, nitrite reductase activity is magnified at hemoglobin's p50 for two main reasons: 1) hemoglobin undergoes an allosteric conformation (relaxation), allowing for the molecule to favor and have a greater attraction for nitrite reduction, and 2) de-oxygenated hemoglobin opens up more room for nitrite to bind because a greater

number of de-oxygenated heme substrates become available (Gladwin et al., 2006). Once nitrite is reduced, NO attaches to its sole receptor on the hemoglobin molecule, soluble guanylyl cyclase (sGC), prompting synthesis of the nucleotide cyclic guanosine monophosphate (cGMP), stimulation of protein kinases specific to cGMP, and consequential relaxation of vascular smooth muscle (Gladwin et al., 2006). Erythrocytes are dependent upon nitrite reduction into NO to induce vasodilation because without the two coupled together, vasodilation could not occur, whereas the combination of the two appreciably induced vasodilation in a manner that was inversely proportional to decreases in hemoglobin de-saturation and strongly associated with the p50 of hemoglobin (Crawford et al., 2006). De-oxygenated hemoglobin depends on nitrite reduction, where this reaction is enhanced when hemoglobin is 50% saturated, but has little activity at 100% saturation (Crawford et al., 2006). Therefore, the stimulus for nitrite activity is enhanced at lower oxygen tensions and a lower threshold of NO is required to elicit profound effects on vasodilation and blood flow (Crawford et al., 2006).

Decreased levels of nitrite measured in the arteries and veins of the human forearm during rhythmic handgrip exercise imply that exercise heightens nitrite metabolism in the periphery and causes vasodilation (Gladwin et al., 2006). Even at rest, inhaled NO or infused nitrite into the brachial artery decrease systemic vascular resistance, prevent vasoconstriction during NO blockade via co-committal L-NMMA administration, considerably dilating the vasculature, and intensifying forearm blood flow (Gladwin et al., 2006). The addition of a nitric oxide synthase inhibitor, carboxy-PTIO, to rat thoracic aortas substantially averts vasorelaxation caused by nitrite reduction during hypoxia (Crawford et al., 2006). Carboxy-PTIO also prevents de-oxygenated erythrocytes from production cyclic guanosine monophosphate (cGMP), a crucial nucleotide and regulator of vascular smooth muscle relaxation (Crawford et al., 2006). Nitrite-

deficient aorta of rats significantly vasoconstrict, regardless of NOS blockade (Crawford et al., 2006).

Nitrite reduction to NO provides another mechanism in which vasodilation, blood flow, and oxygen delivery to the skeletal muscle can be achieved without relying on ATP release from the erythrocyte. Nitric oxide is another avenue in which blood flow and oxygen delivery can be regulated that refutes the necessity of ATP as a modulator of vascular tone.

Experimental considerations and limitations

The half-life of ATP has been estimated to only be a few seconds (Mortensen et al., 2011). Although venous blood samples are a better portrayal of the microcirculation than arterial blood samples, our blood samples only provide a "snapshot" of what is occurring within the microvasculature *in vivo*; if ATP release is in-fact increasing with hypoxic exercise we may not be able to detect it in-time (Crecelius et al., 2013). As we only measured ATP at one small area of the skeletal muscle, perhaps we should have also examined ATP values at the large feed arteries, as these have proven very influential in the hyperemic response of the vasculature during exercise (Lash, 1994). Due to the fact that the total peripheral resistance to flow is greatly comprised of arteries, the response of these feed vessels to muscle contractions dictates blood flow control to the skeletal muscle tissues (Lash, 1994). Vasodilation due to exercise is a propagated response that recruits more vessels throughout the vasculature, travels upstream from the site of initiation, and is transmitted from perfused to non-perfused tissues, allowing for increased oxygen delivery (Lash, 1994). The ability of the feed vessels to initiate a propagated vasomotor response contributes to a 40% reduction in overall vascular tone (Lash, 1994).

Infusions of the neurotransmitter acetylcholine (ACh) into smaller, terminal arterioles results in a rapid and propagated response (due to a greater number of erythrocytes) that spreads upstream and increases blood flow at the site of feed arteries (Segal, 1991). Conversely, ACh application at the site of terminal feed arterioles causes blood to flow into non-perfused terminal arterioles (Segal, 1991). The fact that erythrocyte production increases in response to a dilatory catalyst and results in enhanced blood flow throughout the microcirculation suggests that the vasculature acts in a coordinated effort to ensure that adequate blood flow and oxygen delivery to skeletal muscle. This coordinated effort also implies that vasoactive metabolites, such as ATP, are transported away from the site of initiation as blood flow is conducted upwards and traverses the microvasculature. Thus, capturing the amount of ATP at one localized spot may not be an effective mechanism, as it does not correctly exemplify how the vasculature is responding to a vasodilatory stimulus (in this case, hypoxic exercise) *in vivo*. Specifically, the response of the vasculature to a dilatory stimulus is a "global" reaction, and attempting to quantify ATP in only one area of the microcirculation is not sufficient (Segal, 1991). In the current study, insertion of an arterial catheter for blood gas samples and ATP measurements may have provided a more in-depth analysis of erythrocyte-derived ATP release during hypoxic exercise. It would have been interesting to measure and compare the change in plasma ATP, flow, ATP effluent, and FVC between arterial and venous side. Unfortunately, we could not directly test ATP's influence on the vasculature during hypoxic exercise because explicit pharmacological purinergic receptor inhibitors do not currently exist for human use (Crecelius et al., 2013).

CHAPTER VI

CONCLUSION

As previously mentioned in the Literature Review, in addition to erythrocyte-derived ATP release, there are a multitude of other mechanisms and pathways that can contribute to vasodilation, such as nitric oxide, prostaglandins, and hyperpolarization. Thus, it is possible that erythrocyte-derived ATP release via the Signal Transduction Pathway is only one such mediator of hypoxic vasodilation, and that there could be overlap between these mechanisms and pathways. The lack of enhanced ATP release in the current study suggests that ATP during hypoxic exercise might only be playing a modest role in vasodilation, blood flow, and oxygen delivery to the skeletal muscles. Lastly, the fact that we did not see an increase with ATP between 30 seconds and 3 minutes of hypoxic exercise indicates that exercise hyperemia is a multifaceted issue and that its effects on the vasculature vary over time (Segal, 1991).

Table 1: Subject characteristics.

Male:Female	5:5
Age (years)	21.5±1.0
Body mass index (kg/m^2)	25.2±0.7
Body fat (%)	25.2±2.6
Forearm volume (ml)	937.8±54.8
Forearm fat-free mass (g)	787.2±59.8
MVC (kg)	34.9±3.3

Table 2: Systemic Hemodynamic and Ventilatory Responses.

	Normoxic Rest	**SS Normoxic Exercise**	**Hypoxic Exercise (3 min)**
HR (beats min^{-1})	59±4	57±3	77±4*†
MAP (mmHg)	88±3	95±4*	97±5*
FBF (ml/min^{-1})	29±4	201±21*	250±26*†
FVC (ml min^{-1} (100mmHg)$^{-1}$)	33±4	212±23*	257±29*†
Minute Vent. (l min^{-1}; BTPS)	7±0.5	10±0.7*	21±2*†
End Tidal CO_2 (%)	6.5±0.1	6.34±0.1*	6.3±1.3*
SpO_2 (%)	98±0.4	97±1	79±1.3*†

*P < 0.05 *vs* rest; † P < 0.05 *vs* steady-state exercise

Table 3: Systemic Hemodynamic and Ventilatory Responses (all time points).

	Normoxic Rest	SS Normoxic Exercise	Hypoxic Exercise (30 sec)	Hypoxic Exercise (1 min)	Hypoxic Exercise (1:30)	Hypoxic Exercise (2 min)	Hypoxic Exercise (2:30)	Hypoxic Exercise (3 min)
HR (beats min^{-1})	59±4	57±3	75±4*	76±4*†	80±4*†	79±5*†	81±4*†	77±4*†
MAP (mmHg)	88±3	95±4*	98±4*	99±5*	98±5*	98±5*	98±5*	97 ±5*
FBF (ml/min^{-1})	29±4	201±21*	226±23*	224±21*	237±21*	230±23*	237±23*	250±26*†◊
FVC ($ml\ min^{-1}\ (100mmHg)^{-1}$)	33±4	212±23*	229±25*†	226±24*	242±25*◊	235±25*	241±25*	257 ±29*†◊
SpO_2 (%)	98±0.4	97±1	89±2*†	84±2*†◊	80±1.4*†◊	80±1.4*†◊	79±1.4*†◊	79±1.3*†◊

*$P < 0.05$ *vs* rest; † $P < 0.05$ *vs* steady-state exercise; ◊ $P < 0.05$ *vs* 30 seconds hypoxic exercise

Table 4: Venous Blood Gases.

	pH	PCO_2 (mmHg)	PO_2 (mmHg)	ctO_2 (ml/dl)	SO_2 (%)	FHHb (%)	p50 (mmHg)
Normoxic Rest	7.36±0.03	48.4±5.7	24.7±5.4	8.9±2.9	43.6±14.1	55.5±13.8	22 ±2.1
SS Normoxic Exercise	7.30±0.003*	58.1±7.2*	23±3.1	6.8±1.4	32.8±6.1	66±5.8	27 ±0.8
Hypoxic Exercise (30 sec)	7.31±0.03*	57±6*	22.5±2.2	6.7±1.3	32±4.5	67±4.3	27±0.9
Hypoxic Exercise (1 min)	7.31±0.03*	56±6.4*‡	22±2.2	6.2±1.2	30±4.4	69±4.1	27.4±1.2
Hypoxic Exercise (1:30)	7.32±0.04*	53.4±6	21.2±2.6	6.1±1.5	29.5±6	69.2±5.6	30.2±9.1
Hypoxic Exercise (2 min)	7.32±0.04*†	50±4.3†◊	21±2.9	5.6±1.2*	28±5.1*	71±4.8*	27.4±1.2
Hypoxic Exercise (2:30)	7.32±0.04*†◊	49±8.3†◊	20.1±2.9*	5.5±1.2*†◊	27±5*◊	72±4.4*†◊	27.4±1.3
Hypoxic Exercise (3 min)	7.33±0.03*†◊	47.2±8.5†◊	20±3.2*†◊	5.4±1.4*†◊	27±5.4*†◊	72±5*†◊	28±1.7

*P < 0.05 *vs* rest; † P < 0.05 *vs* steady-state exercise; ◊ P < 0.05 *vs* 30 seconds hypoxic exercise; ‡ P < 0.05 *vs* 3 minutes hypoxic exercise

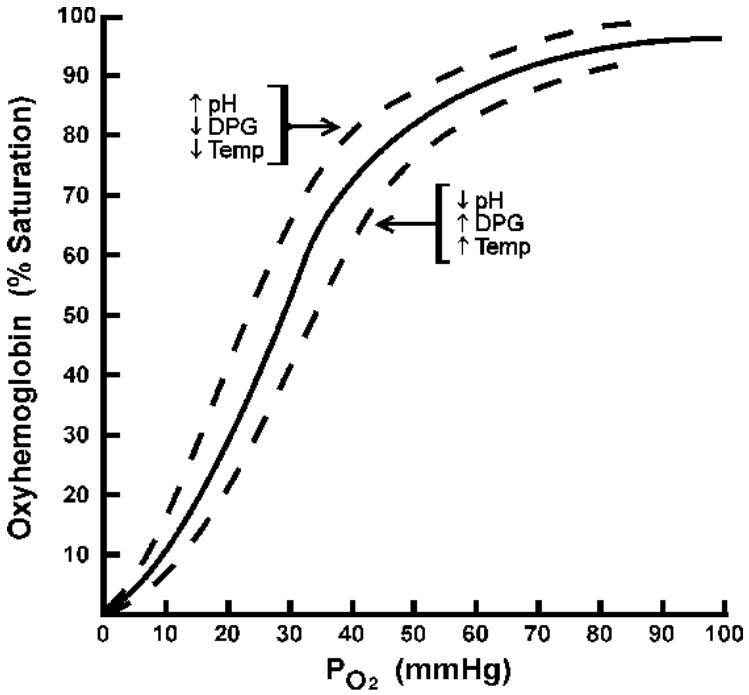


Figure 1. The Hb-O_2 dissociation curve.

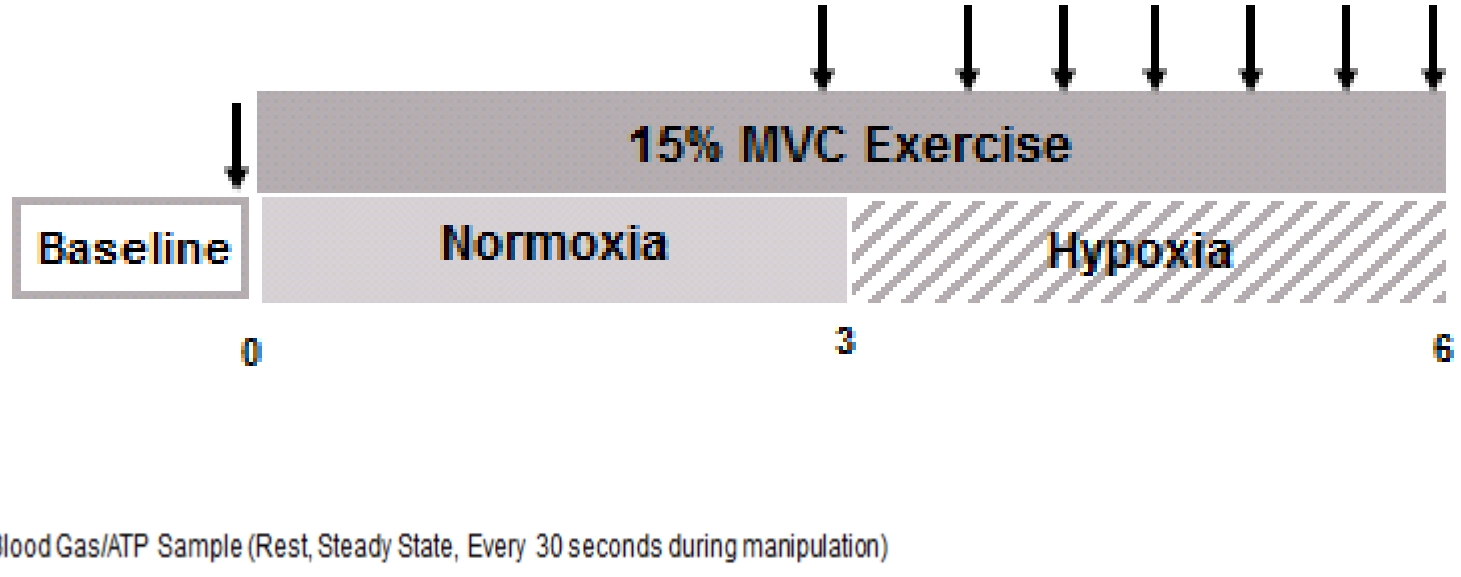


Figure 2. Timeline and experimental protocol.
Overall experimental protocol; subjects' non-dominant arm was instrumented with a deep venous catheter. After baseline measurements were collected, subjects performed 3 minutes of 15% MVC rhythmic handgrip exercise breathing normal room-air. Oxygen saturations were reduced to 80% (within first 2 minutes) and kept constant for the remainder of the trial while subjects continued 15% MVC rhythmic handgrip exercise breathing hypoxic air.

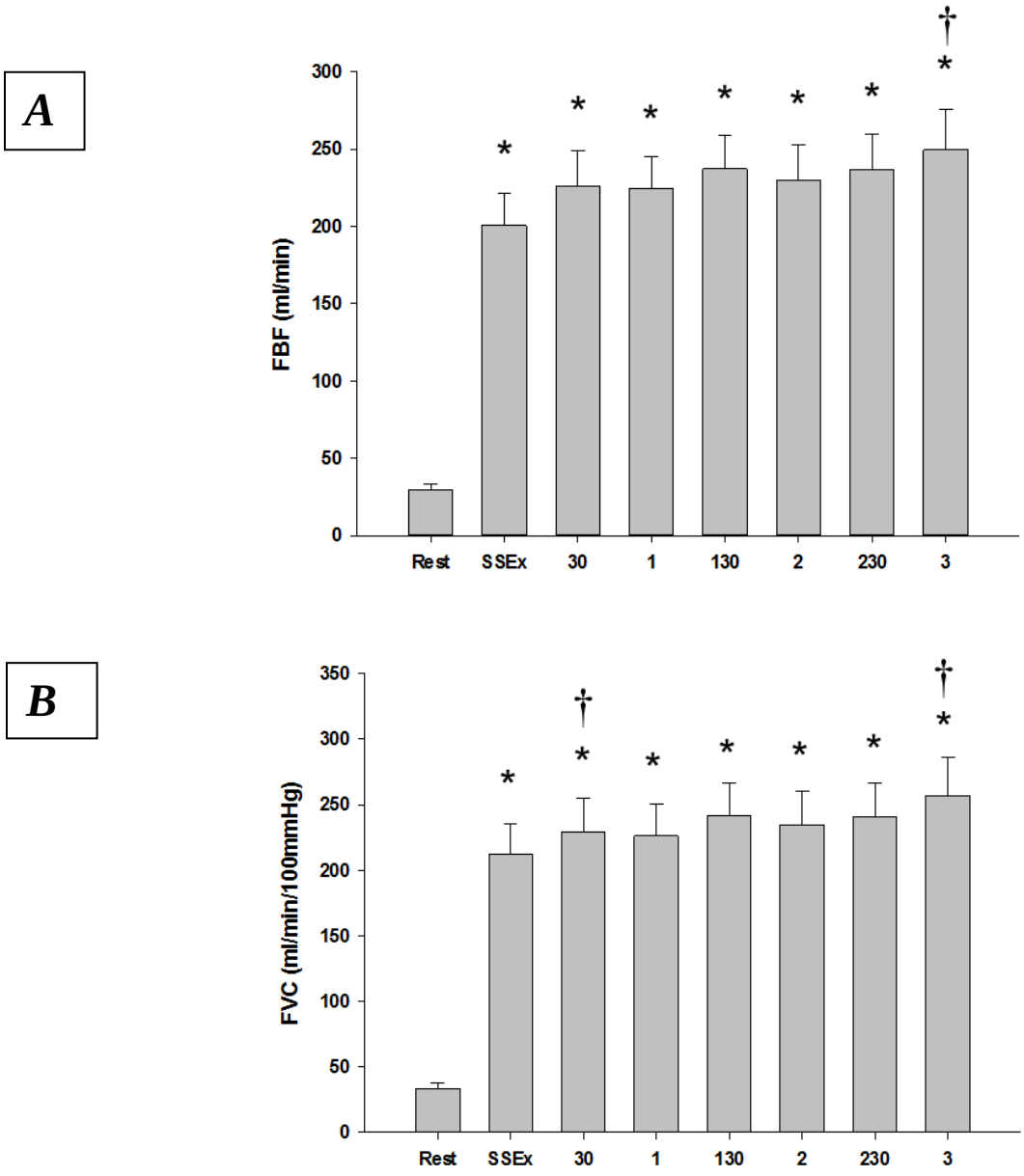


Figure 3. Forearm hemodynamics at rest, during steady-state normoxic exercise, and every 30 seconds of hypoxic exercise. Forearm blood flow (*A*) and forearm vascular conductance (*B*) across all trials. Steady-state exercise (SSEx) significantly increased FBF and FVC from rest, and this augmentation remained with hypoxic exercise (HypEx). Forearm blood flow at 3 minutes of hypoxic exercise was significantly greater than SSEx FBF. Forearm vascular conductance at 30 seconds and 3 minutes of hypoxic exercise were significantly greater than SSEx FVC. **P*<0.05 *vs* rest; † *P* < 0.05 *vs* SSex.

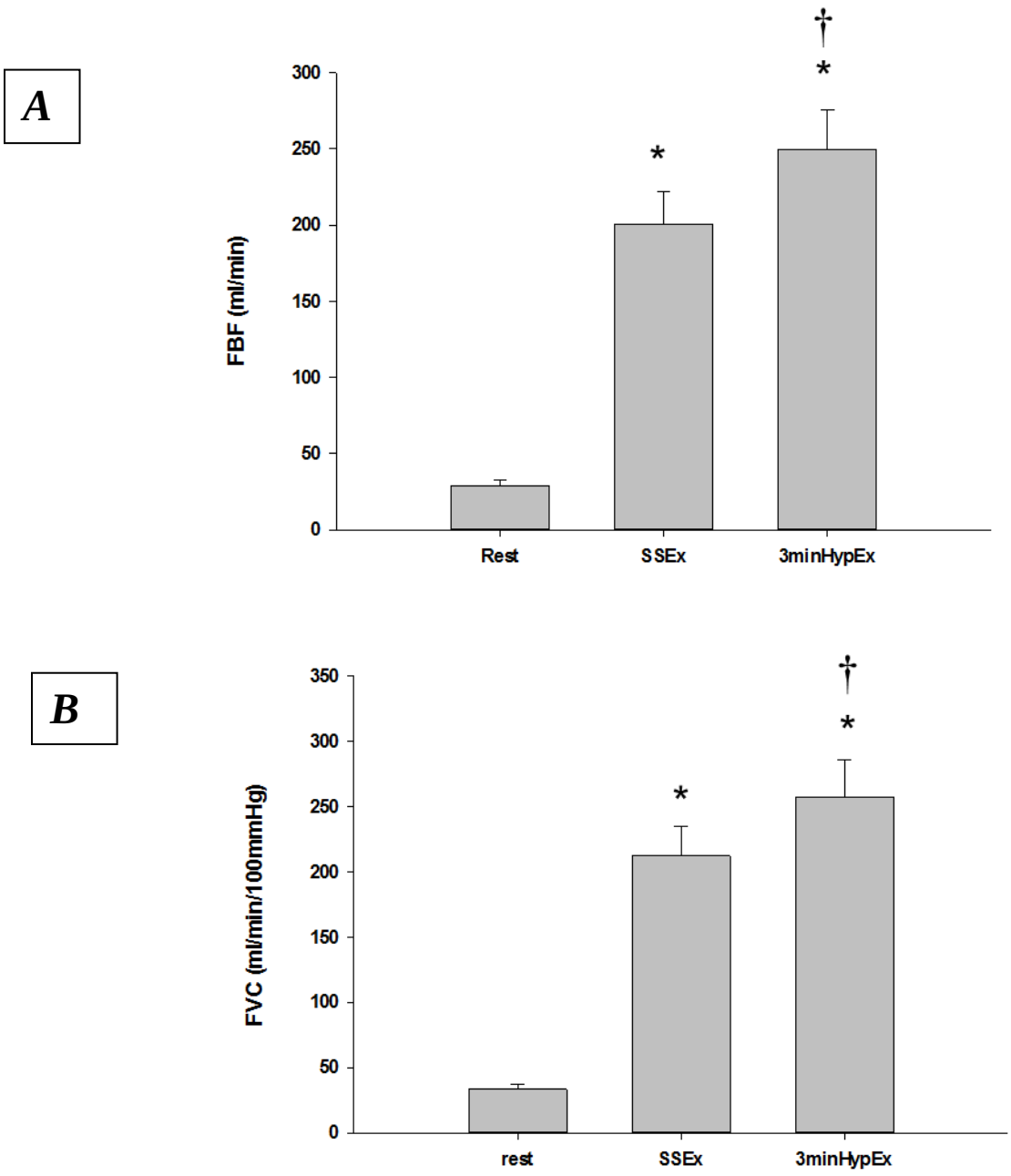


Figure 4. Forearm hemodynamics at rest, during steady-state normoxic exercise, and at 3 minutes of hypoxic exercise. Forearm blood flow (*A*) and forearm vascular conductance (*B*) at rest, during steady-state exercise (SSex), and at 3 minutes of hypoxic exercise (3minHypEx). Steady-state exercise and 3minHypEx FBF and FVC were significantly greater than rest. Forearm blood flow and FVC at 3minHypEx were significantly greater than SSex FBF and FVC. *P<0.05 *vs* rest; † $P < 0.05$ *vs* SSex.

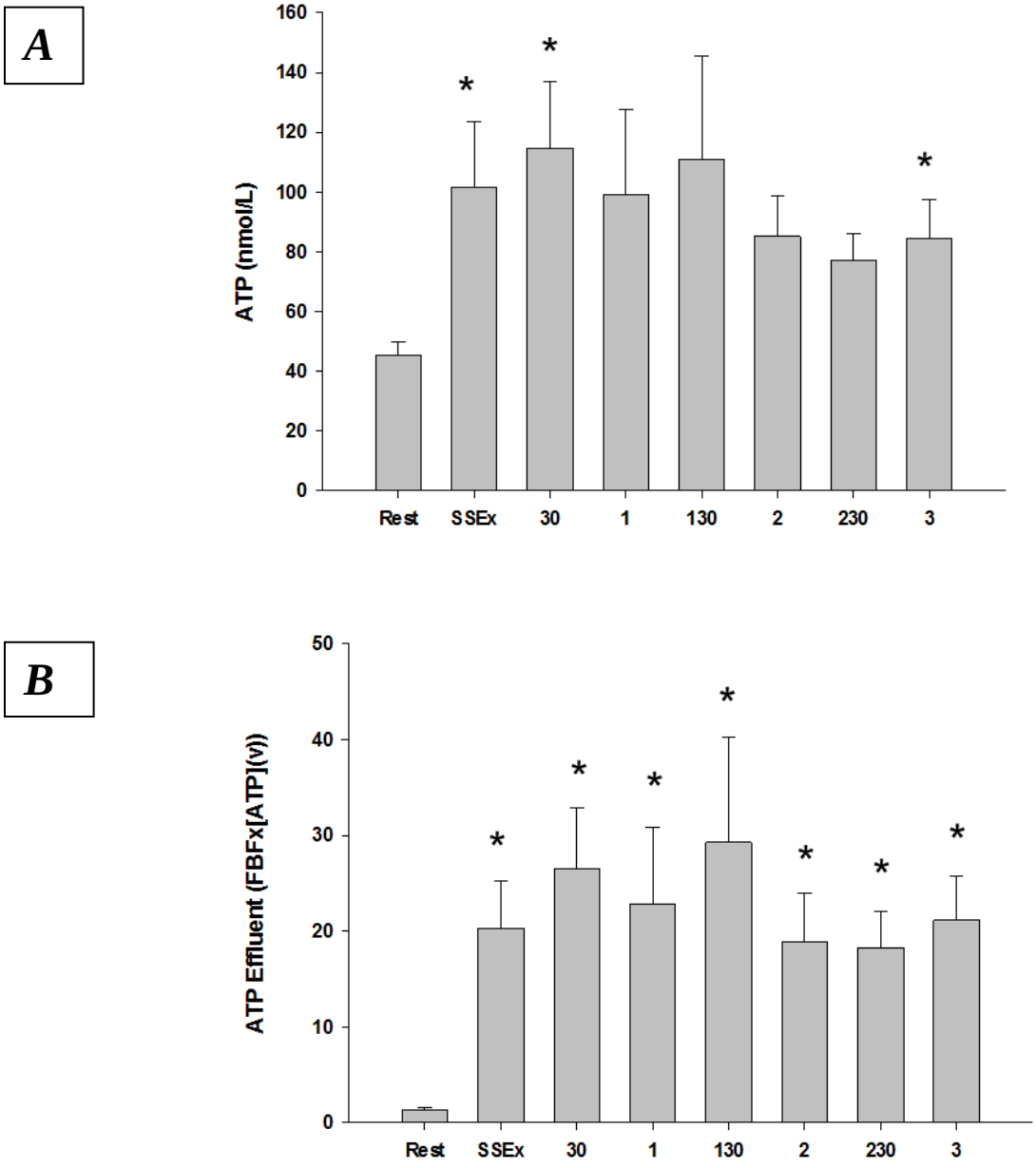


Figure 5. Plasma ATP and ATP effluent during steady-state normoxic exercise, and every 30 seconds of hypoxic exercise. Plasma ATP (*A*) and ATP effluent *(B)* across all trials. Steady-state exercise (SSex) significantly increased plasma ATP and ATP effluent from rest. Hypoxic exercise significantly increased plasma ATP from rest at 30 seconds and 3 minutes, whereas ATP effluent significantly increased from rest with hypoxic exercise at all time points. *$P<0.05$ *vs* rest.

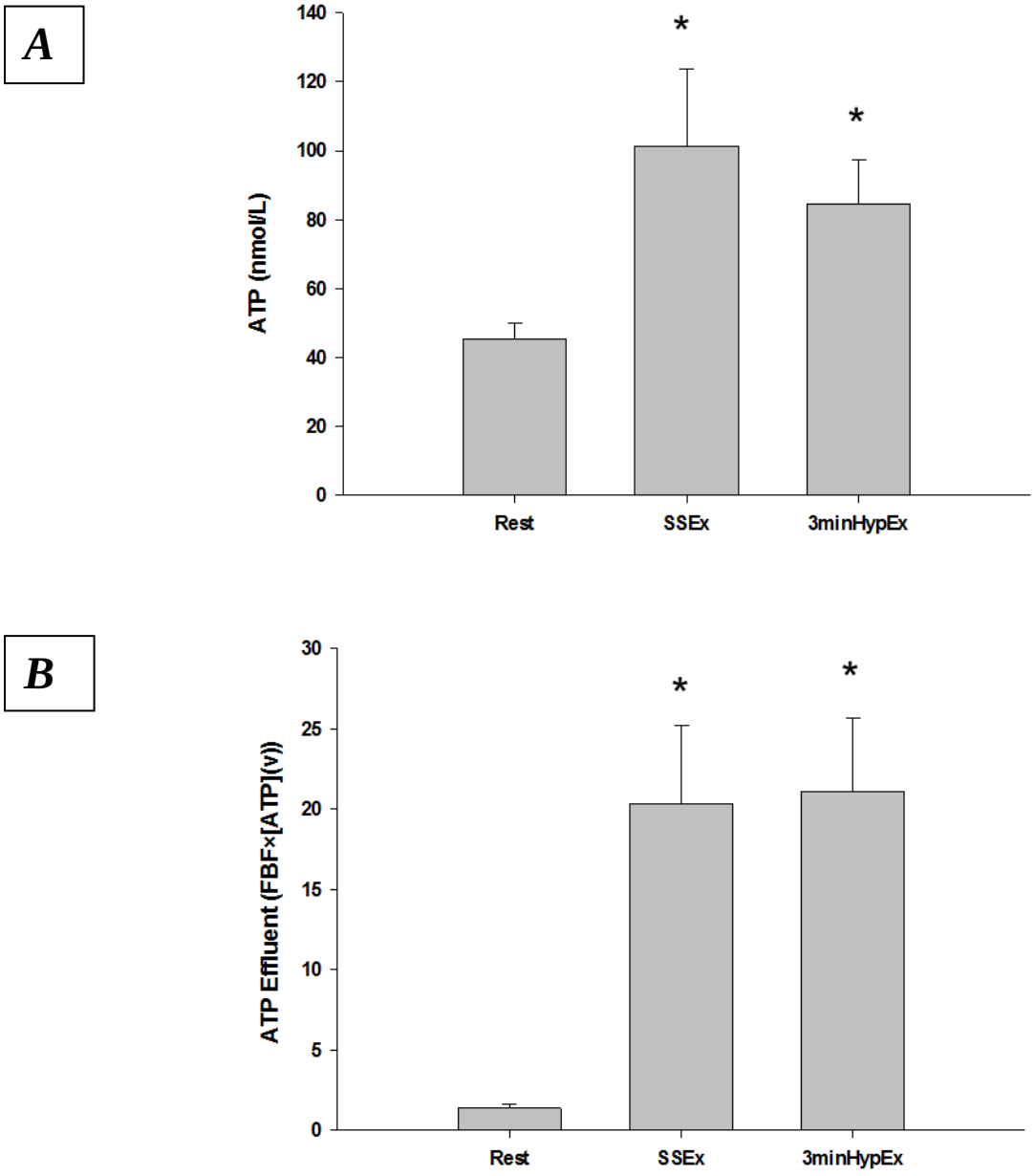


Figure 6. Plasma ATP and ATP effluent at rest, during steady-state normoxic exercise, and at 3 minutes of hypoxic exercise. Plasma ATP (*A*) and ATP effluent *(B)* at rest, during steady-state exercise (SSex), and at 3 minutes of hypoxic exercise (3minHypEx). Steady-state exercise and hypoxic exercise (HypEx) plasma ATP and ATP effluent were significantly greater than rest. **P*<0.05 *vs* rest.

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