DISSERTATION

MECHANISMS OF IMPAIRED RED BLOOD CELL ATP RELEASE IN OLDER ADULTS: IMPLICATIONS FOR ALTERED VASCULAR CONTROL WITH AGE

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ABSTRACT

MECHANISMS OF IMPAIRED RED BLOOD CELL ATP RELEASE IN OLDER ADULTS: IMPLICATIONS FOR ALTERED VASCULAR CONTROL WITH AGE

The following dissertation is comprised of a series of experiments with the overall aim of determining the mechanisms of impaired ATP release from red blood cells (RBCs) of healthy older adults in response to hemoglobin deoxygenation and identifying a potential role of this impairment in the declines in vascular control of peripheral blood flow with advancing age. Advancing age is the primary risk factor for cardiovascular disease (CVD), which is the leading cause of death in societies today and is strongly associated with arterial dysfunction. Furthermore, impairments in vascular control and the subsequent regulation of tissue blood flow and oxygen delivery contribute to vascular pathologies such as atherosclerosis and ischemic disease, as well as the age-associated declines in functional capacity, exercise tolerance, and overall quality of life. Thus, understanding the mechanisms of the age-related impairments in vascular control and identifying potential therapeutic targets holds significant potential for reducing the healthcare burden associated with a rapidly aging population.

Accordingly, the ultimate goal of this dissertation is to determine if an *in vivo* pharmacological approach can be utilized to treat the age-related declines in RBC ATP release, thereby restoring circulating ATP responses and subsequent vascular control during the physiological stimuli of hypoxia and exercise in healthy older adults. The key novel findings of this dissertation are that (i) age-associated declines in RBC deformability are the primary mechanism of impaired deoxygenation-induced ATP release from RBCs of healthy older adults; (ii) primary (healthy) aging is not associated with a global decline in RBC function given that inhibition of cyclic AMP hydrolysis by phosphodiesterase 3 did not improve deoxygenation-induced ATP release from RBCs of older adults and that the cellular responses to G_i protein

activation remained intact with age; and (iii) that systemic Rho-kinase inhibition via administration of fasudil improves the age-related impairments in vascular control and circulating ATP during systemic hypoxia and exercise, which may be related to enhanced RBC ATP release and NO bioavailability. These findings are the first to identify a role for Rho-kinase inhibition in improving these physiological responses in healthy older adults and are therefore clinically significant for aging population in which impaired vascular control contributes to elevations in cardiovascular disease risk and declines in exercise tolerance, functional independence and overall quality of life.

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CHAPTER I – INTRODUCTION AND EXPERIMENTAL AIMS

Cardiovascular disease (CVD) is the leading cause of death in societies today, and the majority of CVD-related mortality is associated with arterial dysfunction. Advancing age is the primary risk factor for CVD, and it is estimated that over 90% of all deaths associated with CVD are observed in adults over 60 years of age. Furthermore, human aging is associated with a decline in functional capacity that leads to reductions in exercise tolerance, functional independence, and overall quality of life. All of these age-associated changes, as well as vascular pathologies like atherosclerosis and ischemic disease, involve impairments in vascular control and the subsequent regulation of tissue blood flow and oxygen delivery.

The local control and regulation of blood flow involves the integration of multiple signaling pathways and vascular responses, the end goal of which is the precise matching of oxygen supply to tissue metabolic demand. While the exact mechanisms of how the body senses this oxygen demand and stimulates the appropriate vascular responses to increase supply remain unclear, a growing body of evidence indicates that red blood cells (RBCs) may play a central role in this process. Specifically, RBCs can act as 'sensors' for oxygen demand in addition to their traditional role as oxygen 'carriers', in that they are able to stimulate vasodilation and increased oxygen delivery to the tissue through the release of the adenine nucleotide adenosine triphosphate (ATP) in direct proportion to the degree of hemoglobin deoxygenation. ATP release from RBCs is also stimulated by deformation of the RBC membrane, and more deformable cells have been shown to release more ATP in response to a given stimulus. Many components of the intracellular signaling cascade for ATP release from RBCs in response to stimuli such as hemoglobin deoxygenation or membrane deformation have been characterized (Figure 1.1). First, hemoglobin deoxygenation and membrane deformation both stimulate the heterotrimeric inhibitory G (G_i) protein. G_i stimulation is followed by activation of adenylyl cyclase (AC) and increases in intracellular cyclic AMP (cAMP), the overall level of

which is controlled by the balance between AC-mediated synthesis and phosphodiesterase 3- (PDE3) mediated hydrolysis of cAMP. Increases in intracellular cAMP then stimulate protein kinase A (PKA) followed by the cystic fibrosis transmembrane conductance regulator (CFTR) that, through a mechanism that remains to be determined, facilitates ATP release via pannexin 1 channels. Upon release, circulating ATP binds to purinergic P2Y receptors along the endothelium and evokes a vasodilatory response that acts locally and conducts along the vessel to increase the distribution of blood within the tissue as well as the overall delivery of blood to the tissue. Collectively, these findings demonstrate that RBCs are ideally positioned and able to both detect imbalances between oxygen delivery and metabolic need and initiate a vascular response to facilitate the matching of local tissue oxygen supply and demand.

With advancing age, skeletal muscle blood flow responses to hypoxia and exercise are impaired relative to healthy young adults. Our laboratory has demonstrated previously that the vasodilatory response to intra-arterial infusion of ATP is not different between young adults and healthy older adults exhibiting 'classic' endothelial dysfunction as evidenced by reduced acetylcholine-mediated vasodilation. Thus, if aging was to adversely affect the contribution of ATP to vascular tone and the control of peripheral blood flow, the impairment must be related to the source of ATP (i.e., RBC ATP release). Accordingly, our laboratory was the first to demonstrate age-related impairments in ATP release with primary (healthy) aging based on (i) blunted increases in plasma [ATP] in response to systemic isocapnic hypoxia (Sp_{O_2} ~80%) and graded-intensity rhythmic handgrip exercise, with the latter also being closely associated with impairments in vasodilation and forearm blood flow; and (ii) impairments in hemoglobin deoxygenation-induced ATP release from isolated RBCs of older adults. These age-associated declines in ATP release and the control of tissue blood flow and oxygen delivery may predispose this population to increased risk for CVD, ischemic disease, exercise intolerance, and a decline in overall quality of life. Moreover, circulating ATP has diverse effects beyond the control of vasomotor tone, including potent antiadhesive and anticoagulative properties, thus

further predisposing this aging population to increased CVD risk. Currently, the underlying mechanisms of this age-associated impairment in RBC ATP release and its contribution to impaired vascular responses to changes in oxygen supply or demand during physiological stimuli such as hypoxia or exercise are unknown.

Therefore, the overall goal of this dissertation is to identify the changes in RBC structure and function with advancing donor age that contribute to impaired ATP release and determine whether *in vivo* pharmacological treatment of these changes can restore RBC ATP release and improve vascular responses to hypoxia and exercise in older adults.

Specific Aims

Experiment 1: to determine if decreased membrane deformability of RBCs from healthy older adults contributes to impaired ATP release from isolated RBCs of older vs. young adults during hemoglobin deoxygenation.

Experiment 2: to determine if cAMP signaling within RBCs is altered in healthy older adults and contributes to impaired ATP release from isolated RBCs of older vs. young adults during hemoglobin deoxygenation.

Follow-up Experiment: to determine if responsiveness to G_i activation is impaired in isolated RBCs from older vs. young adults.

Experiment 3: to determine if systemic Rho-kinase inhibition improves the hemodynamic and circulating ATP responses to hypoxia and exercise in healthy older adults, as well as ATP release from isolated RBCs of older vs. young adults during hemoglobin deoxygenation.

This collection of work provides the first insight into mechanisms of impaired RBC ATP release with advancing age, demonstrating that age-associated decreases in RBC membrane deformability are the primary mechanism of blunted deoxygenation-induced ATP release from RBCs of healthy older adults, whereas the impairment is not due to changes in intracellular cAMP signaling or responsiveness to G_i activation. Furthermore, the translation of these novel findings from isolated RBCs to *in vivo* physiology provides the first experimental evidence that systemic administration of the Rho-kinase inhibitor fasudil improves both the hemodynamic and circulating ATP responses to hypoxia and exercise in healthy older adult humans. Although determining the precise mechanisms of these fasudil-mediated improvements is beyond the scope of this dissertation, they may be related to the concomitant improvements in circulating ATP based on the work herein that demonstrates the ability of Rho-kinase inhibition to restore deoxygenation-induced ATP release from isolated RBCs of healthy older adults; additional mechanisms could involve increases in the bioavailability of other vasodilators such as nitric oxide based on established cellular targets of Rho-kinase. These collective findings may hold significant therapeutic potential for aging populations in which alterations in vascular control and the regulation of blood flow and oxygen delivery contribute to increases in cardiovascular disease risk and declines in exercise tolerance, functional independence, and overall quality of life.

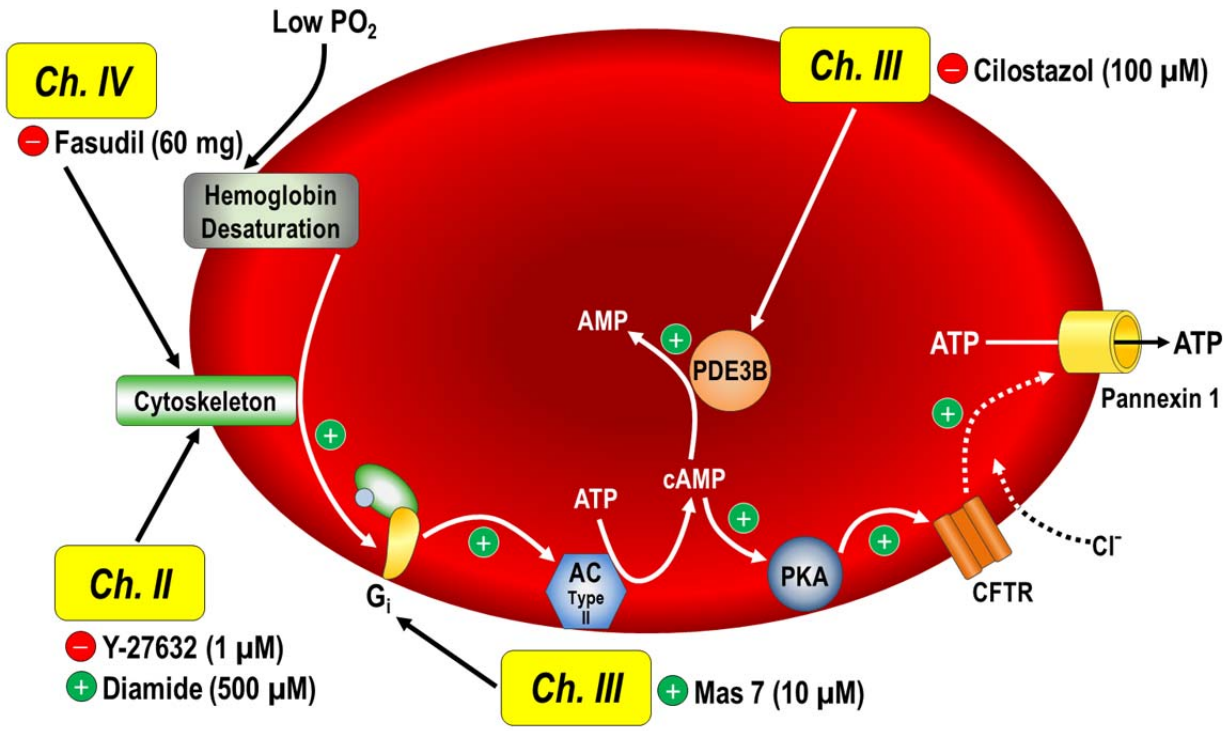

Figure 1.1. Experimental targets of the signaling cascade for deoxygenation-induced ATP release from red blood cells (RBCs)
The work described in Chapter II targeted the RBC cytoskeleton, utilizing the Rho-kinase inhibitor Y-27632 to increase membrane deformability and the cell-stiffening agent diamide to decrease membrane deformability in isolated RBCs. The work described in Chapter III utilized the phosphodiesterase 3 (PDE3) inhibitor cilostazol to limit the hydrolysis of cAMP and the G_i activator mastoparan 7 (Mas 7) to evaluate the effects of age on the subsequent cellular responses in isolated RBCs. The work described in Chapter IV utilized the Rho-kinase inhibitor fasudil to target RBC deformability in order to improve ATP release and subsequent hemodynamic responses *in vivo*.

CHAPTER II – MANUSCRIPT I

Role of red blood cell deformability in impaired deoxygenation-induced ATP release with age

Summary

Red blood cells (RBCs) release adenosine triphosphate (ATP) upon deoxygenation, which binds to endothelial purinergic receptors and stimulates conducted vasodilation. As local tissue metabolic demand increases, ATP release increases in direct proportion to the degree of hemoglobin deoxygenation, thus allowing RBCs to both detect and initiate a vascular response to imbalances between oxygen supply and demand. However, RBCs from older adults have an impaired ability to release ATP in response to deoxygenation compared to RBCs from young adults, yet the underlying cause of this remains unknown. RBC deformability has been shown to decrease with advancing donor age, thus we hypothesized that increasing membrane deformability (via the Rho-kinase inhibitor Y-27632) would restore ATP release from RBCs of older adults, while decreasing membrane deformability (via the cell-stiffening agent diamide) would impair ATP release from RBCs of young adults. Blood filtrometry was used to measure red (blood) cell transit time (RCTT) as an index of deformability in RBCs from young (24 ± 1 years; n = 9) and older adults (64 ± 2 years; n = 9), with the higher RCTT in RBCs from older adults in control conditions indicating lower deformability compared to young (RCTT: 8.541 ± 0.050 vs. 8.234 ± 0.098 (a.u.), respectively, $P < 0.05$). Isolated RBC ATP release during normoxia (PO_2 ~112 mmHg) and hypoxia (PO_2 ~18 mmHg) was quantified in RBCs from young (23 ± 1 years; n = 13 for Y-27632 and diamide) and older (65 ± 1 years; n = 14 and 10 for Y-27632 and diamide, respectively) adults using the luciferin-luciferase technique following RBC incubation with saline (vehicle control), Y-27632, or diamide. On average, the relative change in ATP release from normoxia to hypoxia in saline conditions was significantly less in RBCs from older compared with young adults (~50% vs. ~125%; $P < 0.05$). Y-27632 improved RBC ATP

release to 111.7 ± 17.2% and deformability (RCTT) to 8.228 ± 0.083 in older adults such that neither were different from the young control ($P > 0.05$), whereas diamide decreased RBC ATP release to 67.4 ± 11.8% and impaired deformability (RCTT = 8.955 ± 0.114) in young adults such that they were similar to the older control. Our findings indicate that decreased RBC deformability is a primary contributor to age-related impairments in RBC ATP release, and that this may have implications for altered vascular control with advancing age.

Introduction

The local control and regulation of blood flow involves the integration of multiple signaling pathways and vascular responses, the end goal of which is the precise matching of oxygen supply to tissue metabolic demand (Clifford & Hellsten, 2004; Mortensen & Saltin, 2014; Joyner & Casey, 2015). Of these signaling pathways, ATP is among the most unique in that it can stimulate vasodilation that acts both locally to help distribute blood flow within a tissue and conducts upstream to facilitate increased blood flow and oxygen delivery to the tissue (Collins *et al.*, 1998; Winter & Dora, 2007; Dora, 2017), it is the only molecule that has been shown to have the intrinsic ability to blunt sympathetically-meditated vasoconstriction when administered exogenously (Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008; Hearon Jr. *et al.*, 2017), and it has potent antiadhesive and anticoagulative properties (Hrafnkelsdóttir *et al.*, 2001; Zhu *et al.*, 2011; Kirby *et al.*, 2014). Importantly, circulating concentrations of ATP increase during hypoxia and exercise in healthy young adults, and are closely correlated with skeletal muscle blood flow during exercise (Mortensen *et al.*, 2011; Kirby *et al.*, 2012). Advancing age in humans is associated with impairments in vasodilation and regulation of blood flow to the skeletal muscle during exercise, which can contribute to increases in cardiovascular disease morbidity and mortality, as well as declines in functional capacity, exercise tolerance, functional independence, and overall quality of life (WHO, 1993; Go *et al.*, 2014; Hearon Jr. & Dinenno, 2016; Mozaffarian *et al.*, 2016). Interestingly, advancing age is also accompanied by an

attenuation in circulating ATP during hypoxia and exercise (Kirby *et al.*, 2012), but not an impaired responsiveness to ATP as determined by measuring vasodilation in the forearm to brachial artery infusion of ATP (Kirby *et al.*, 2010). Although there is evidence that the vasodilatory responsiveness to ATP may differ with age in the leg and that this can be modulated by physical activity status (Mortensen *et al.*, 2012), the collective evidence suggests that if aging adversely affects the contribution of ATP to vascular control and regulation of skeletal muscle blood flow, the impairment must be related to the source of ATP.

While the exact mechanisms of how the body senses local changes in metabolic demand and stimulates the appropriate vascular responses to match the oxygen supply remain unclear, a growing body of evidence indicates that red blood cells (RBCs) may play a central role in this process (Bergfeld & Forrester, 1992; Ellsworth *et al.*, 1995; Ellsworth, 2000; Jagger *et al.*, 2001; Jensen, 2009; Ellsworth & Sprague, 2012). Specifically, RBCs can act as a 'sensor' for oxygen demand in addition to their traditional role as an oxygen 'carrier', in that they are able to stimulate vasodilation and increased oxygen delivery to the tissue through the release of ATP in direct proportion to the degree of hemoglobin deoxygenation (Dietrich *et al.*, 2000; Jagger *et al.*, 2001; González-Alonso *et al.*, 2002; Sprague *et al.*, 2009). RBCs also release ATP in response to cell deformation, with more deformable cells releasing more ATP in response to a given stimulus (Sprague *et al.*, 1998; Faris & Spence, 2008; Sridharan *et al.*, 2010*b*; Thuet *et al.*, 2011). Importantly, the aforementioned increase in circulating ATP during exercise has been shown to be dependent on skeletal muscle perfusion, which indicates that intravascular sources such as RBCs play an essential role in this response (Kirby *et al.*, 2013). Consistent with the evidence that RBCs are a primary source of circulating ATP and that circulating ATP responses to hypoxia and exercise are impaired with age, our laboratory was the first to demonstrate that deoxygenation-induced ATP release is impaired in RBCs isolated from healthy older adults compared to RBCs from young adults (Kirby *et al.*, 2012).

Although the underlying mechanisms of this age-associated impairment in RBC ATP release are unknown, one likely candidate is the age-associated decrease in RBC membrane fluidity and deformability (Reid *et al.*, 1976; Hegner *et al.*, 1979; Gelmini *et al.*, 1987, 1989) given that acute, pharmacologically-induced increases or decreases in RBC deformability produce parallel changes in ATP release from RBCs of young healthy donors. Accordingly, the primary purpose of the present study was to determine if age-related declines in RBC deformability contribute to impaired deoxygenation-induced ATP release in RBCs from healthy older adults. Specifically, we tested the hypothesis that increasing RBC deformability would improve deoxygenation-induced ATP release in RBCs from older adults, and conversely, that decreasing RBC deformability would attenuate deoxygenation-induced ATP release in RBCs from young adults.

Methods

Ethical approval and subjects

With Institutional Review Board approval and after written informed consent, a total of 18 young and 15 older healthy adults participated in the present investigation. Of those, 12 young and 10 older subjects participated in multiple experiments. All subjects were free from overt cardiovascular disease as assessed from a medical history, free of cardiovascular medications, non-smokers, non-obese, normotensive, and sedentary to moderately active. Young female subjects were studied during the early follicular phase of their menstrual cycle to minimize any potential cardiovascular effects of sex-specific hormones, whereas older female subjects were post-menopausal and not taking hormone replacement therapy. Additionally, older subjects were further evaluated for clinical evidence of cardiopulmonary disease with a physical examination and resting and exercise (Balke protocol) electrocardiograms. Body composition was determined by whole-body dual-energy X-ray absorptiometry scans (QDR series software,

Hologic, Inc., USA). Whole blood lipid panels were run using a Piccolo Xpress chemistry analyzer (Abaxis, USA). All studies were performed according to the *Declaration of Helsinki*.

Isolation of red blood cells

Blood was obtained by either catheterization of the brachial artery (if the subject was participating in another study in the laboratory) or venipuncture of the antecubital vein and collected into Vacutainer tubes containing sodium heparin (158 USP units) after a 4 hour fast and 12 hour abstention from caffeine, alcohol, and exercise. RBCs were isolated by centrifugation of the collected whole blood (500*g*, 4°C, 10 min) followed by removal of the plasma and buffy coat by aspiration. Packed RBCs were resuspended and washed three times in a cell wash buffer containing (in mM) 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0 Tris-base, 5.5 glucose, and 0.5% BSA, with pH adjusted to 7.4 at room temperature (Sridharan *et al.*, 2010*b*; Thuet *et al.*, 2011; Kirby *et al.*, 2012). All studies were performed immediately after blood collection and RBC isolation.

Measurement of red blood cell deformability

RBC deformability was measured using the St. George's blood filtrometer (Carri-Med, Dorking, UK) (Sprague, 1996; Sprague *et al.*, 1998, 2001*b*, 2001*a*; Olearczyk *et al.*, 2004*a*; Sridharan *et al.*, 2010*b*; Thuet *et al.*, 2011; Clapp *et al.*, 2013; Richards *et al.*, 2014). This device develops a calibrated 3 cm H_2O pressure gradient across a vertically mounted, 13 mm diameter polycarbonate filter (Nucleopore) with 9.53 mm exposed surface diameter and average pore size of 5 μm. Distal to the filter, the outflow channel was filled with CWB and flow was prevented by a tap. Proximal to the filter, the chamber and an open-ended capillary tube were filled with either CWB (as described above for RBC isolation, but with pH adjusted to 7.4 at 37°C) alone or a 10% hematocrit solution of RBCs and CWB, both warmed to 37°C. For calibration, the time required for CWB alone to pass through the filter was measured by four

fiber optic detectors and recorded digitally, with this process being repeated until the coefficient of variance between runs was 1% or less. The RBC suspension was then passed through the calibrated filter, and red (blood) cell transit time (RCTT) was calculated based on the rate at which the RBC suspension traversed the filter relative to the rate of CWB alone as described previously (Sprague, 1996). If the average filter pore size and hematocrit are kept constant, then RCTT is a unitless index of RBC deformability, with lower RCTT indicating greater RBC deformability. Measurements of RBC deformability were made after a 30-min incubation with either saline (vehicle control), the Rho kinase inhibitor Y-27632 (1 μM; Sigma), or the thiol cross-linking agent diamide (500 μM; Sigma). This dose of Y-27632 has been shown to increase deformability and hypoxia-induced ATP release in RBCs from healthy humans (Thuet *et al.*, 2011), whereas diamide has been shown to decrease RBC deformability and hypoxia-induced ATP release (Sridharan *et al.*, 2010*b*; Thuet *et al.*, 2011) without significantly altering hemoglobin or normal cell function (Kosower *et al.*, 1969; Maeda *et al.*, 1983). RBC deformability was measured on the same day and in triplicate for each condition, with the treatment order randomized and counterbalanced between subjects. RBC deformability was measured on a different day than the measurements of deoxygenation-induced ATP release in order to ensure that RBCs were studied within ~4 hours of isolation, which would not be possible if both measurements were made on the same day.

Red blood cell deoxygenation and measurement of extracellular ATP

As described previously by our laboratory (Kirby *et al.*, 2012), washed RBCs were diluted to 20% hematocrit with a bicarbonate-based buffer containing (in mM) 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 11.1 glucose, 23.8 $NaHCO_3$, and 0.5% BSA warmed to 37°C. This 20% hematocrit RBC suspension was placed in a rotating bulb tonometer (Eschweiler GmbH & Co. KG, Germany) and warmed to 37°C. On separate days, RBCs were incubated in the tonometer bulbs with either 1 μM Y-27632 or 500 μM diamide, each paired with a saline-

treated sample (vehicle control), for 30 min in normoxia (16% O_2, 6% CO_2, balanced nitrogen; PO_2 = 111.8 ± 0.7 mmHg and FO_2Hb = 94.9 ± 0.1% across all age groups and conditions). A sample of drug- and saline-treated RBCs were removed from each tonometer bulb for measurement of extracellular and intracellular ATP in normoxia (details below). RBCs were then deoxygenated by exposure to hypoxia (1% O_2, 6% CO_2, balanced nitrogen; PO_2 = 18.4 ± 0.5 mmHg and FO_2Hb = 21.7 ± 1.0% across all age groups and conditions) for 15 min and RBC samples were taken for measurement of ATP as in normoxia. Normoxic and hypoxic gases were blended via gas blender (MCQ Gas Blender Series 100, Italy) and humidified before introduction into the tonometer bulbs. Blood gases were confirmed by blood gas analysis (Siemens Rapid Point 405 Series Automatic Blood Gas System, Los Angeles, CA) (Kirby *et al.*, 2012).

ATP was measured via the luciferin-luciferase technique as described previously (Sprague *et al.*, 2001*a*; Sridharan *et al.*, 2010*b*, 2010*a*; Thuet *et al.*, 2011; Kirby *et al.*, 2012; Richards *et al.*, 2013), with light emission during the reaction detected by a luminometer (TD 20/20, Turner Designs). For extracellular ATP (i.e., ATP release) measurements, a 10 µL sample of the 20% hematocrit suspension was taken from each tonometer bulb and diluted 500-fold (0.04% hematocrit), from which a 200 µL sample was taken and injected into a cuvette containing 100 µL of firefly tail extract (10 mg/mL DI water; Sigma) and 100 µL of D-luciferin (0.5 mg/mL DI water; Research Products International). Peak light output was measured at least in triplicate for each experimental condition and the mean was used for determination of ATP levels by comparison to a standard curve for ATP (Calbiochem) generated on the day of the experiment. Cell counts were obtained from each 0.04% RBC suspension and extracellular ATP was normalized to 4 x 10^8 cells. To confirm that ATP release was not due to hemolysis, the 0.04% RBC suspensions from which samples for ATP analysis and cell counting were taken were analyzed for free hemoglobin by measuring absorbance at 405 nm similar to previous

reports, and samples with significant lysis were excluded (Sprague *et al.*, 1998, 2011; Sridharan *et al.*, 2010*a*; Thuet *et al.*, 2011; Kirby *et al.*, 2012, 2014; Richards *et al.*, 2013).

Measurement of red blood cell total intracellular ATP

To confirm that the effects of donor age and pharmacological agents on RBC ATP release were not due to differences in total intracellular ATP or the increase in RBC glycolytic activity during hypoxia (Messana *et al.*, 1996; Campanella *et al.*, 2005; Lewis *et al.*, 2009), 50 µL samples of drug- and saline-treated RBCs (20% hematocrit) were taken from the tonometer bulbs in normoxia and hypoxia following measurement of extracellular ATP and lysed in DI water at room temperature (a 20-fold dilution). This lysate was diluted an additional 400-fold (8000-fold total) and ATP was measured using the same ATP assay used for determination of extracellular ATP (Sridharan *et al.*, 2010*b*, 2010*a*; Sprague *et al.*, 2011; Thuet *et al.*, 2011; Kirby *et al.*, 2012, 2014). Values were normalized to ATP concentration per RBC.

Statistics

All values are reported as mean ± SEM. Statistical analyses of absolute ATP values (intracellular and extracellular) were performed using R (R Core Team 2016, R Foundation for Statistical Computing, Vienna, Austria). Absolute ATP values were tested using a 3-way repeated measures ANOVA, with age as the between subjects factor (young vs. older) and drug/gas conditions as the within subject factors (control vs. drug and normoxia vs. hypoxia, respectively). When an interaction or main effect was found, appropriate pairwise comparisons were made. For statistical analyses of blood gases and the relative (%) change in ATP release from normoxia to hypoxia and RBC deformability (RCTT), SigmaPlot (Systat Software, San Jose, CA, USA) was used to perform a 2-way repeated measures ANOVA. In the event of a main effect of or interaction between age and drug condition, post hoc comparisons were made with Tukey's HSD test. Significance was set at $P < 0.05$.

Results

Subjects and blood gases

Subject characteristics are reported in Table 2.1. Compared to the young adults, older adults had either trending or significant elevations in body mass index (BMI), body fat percentage, and blood lipids, although all values were still within the normal healthy range. Blood gases for isolated RBCs are reported in Table 2.2. Most importantly, there were no significant differences in the fraction of oxygenated hemoglobin (FO_2Hb) between age groups or pharmacological treatments in normoxia or hypoxia.

Effect of donor age, Y-27632, and diamide on red blood cell deformability

RBC deformability was lower in RBCs from older adults as indicated by the significantly higher RCTT in the saline condition compared to young adults (8.541 ± 0.050 vs. 8.234 ± 0.098, respectively; $P < 0.05$) (Fig. 2.1). Incubation with the Rho-kinase inhibitor Y-27632 improved RBC deformability relative to the saline condition only in the older adults (RCTT: 8.228 ± 0.083; $P < 0.05$), such that there was no longer a difference between the age groups (Fig. 2.1). In contrast, incubation with diamide significantly decreased RBC deformability compared to saline in young and older adults (RCTT: 8.955 ± 0.114 and 9.242 ± 0.154, respectively; $P < 0.05$) (Fig. 2.1).

Effect of donor age, Y-27632, and diamide on deoxygenation-induced ATP release from red blood cells

In the Y-27632 experiment, ATP release in normoxia was not different between age groups or drug condition (Fig. 2.2A). With saline, extracellular ATP from RBCs of older adults in hypoxia tended to be lower compared to young adults (19.7 ± 3.1 nmol/4 x 10^8 RBCs vs. 29.7 ± 4.3 nmol/4 x 10^8 RBCs, respectively; $P = 0.15$) (Fig. 2.2A) and the mean percent increase in RBC ATP release from normoxia to hypoxia was significantly impaired in the older vs. young

adults (35.8 ± 11.1% vs. 114.7 ± 11.0%, respectively; $P < 0.05$) (Fig. 2.2B). However, incubation of RBCs with Y-27632 completely reversed this age-related impairment in ATP release during hypoxia such that the 111.7 ± 17.2% increase in extracellular ATP from normoxia to hypoxia was no longer different from the young saline control (Fig. 2.2B; $P < 0.05$ vs. saline). Incubation of RBCs from young adults with Y-27632 also increased deoxygenation-induced ATP release to 159.7 ± 22.5% (Fig. 2.2B; $P < 0.05$ vs. saline).

In the diamide experiment, ATP release in normoxia was not different between age groups and was only decreased by diamide incubation in RBCs from young adults compared to saline (10.1 ± 1.9 nmol/4 x 10^8 RBCs vs. 12.3 ± 1.7 nmol/4 x 10^8 RBCs, respectively; $P < 0.05$) (Fig. 2.3A). Similar to the Y-27632 experiment, extracellular ATP from RBCs of older adults in the saline hypoxia condition trended towards being lower compared to young adults (18.0 ± 3.0 nmol/4 x 10^8 RBCs vs. 25.8 ± 2.5 nmol/4 x 10^8 RBCs, respectively; $P = 0.097$) (Fig. 2.3A) and the mean percent increase in RBC ATP release from normoxia to hypoxia was significantly impaired vs. young adults (57.7 ± 14.2% vs. 137.9 ± 25.3%, respectively; $P < 0.05$) (Fig. 2.3B). Relative to the saline condition, incubation of RBCs with diamide attenuated ATP release during hypoxia in young adults to 67.4 ± 11.8% (Fig. 2.3B; $P < 0.05$) such that it was not different from the older adults, but the effect on ATP release during hypoxia in the older adults was not significant (26.8 ± 20.2% increase in extracellular ATP from normoxia to hypoxia; $P = 0.241$ vs. saline) (Fig. 2.3B).

Effect of donor age, Y-27632, and diamide on red blood cell intracellular ATP

In both the Y-27632 and diamide experiments (Figs. 2.4A and 2.4B, respectively), there were no differences in intracellular ATP between the age groups ($P > 0.05$) and intracellular ATP was significantly higher in hypoxia vs. normoxia in all conditions (Fig. 2.4; $P < 0.05$). Incubation with Y-27632 had no effect on intracellular ATP compared to saline (Fig. 2.4A; $P >$

0.05), whereas diamide significantly decreased intracellular ATP vs. saline in both normoxia and hypoxia (Fig. 2.4B; $P < 0.05$).

Discussion

The primary novel finding from the present study is that the age-related decrease in RBC deformability is a significant mechanism of impaired deoxygenation-induced ATP release from RBCs of healthy older adult humans. Specifically, this is the first study to demonstrate that improving RBC deformability in older adults abolishes the impairment in deoxygenation-induced ATP release (Fig. 2.2), whereas decreasing RBC deformability in young adults impairs deoxygenation-induced ATP release to the same degree as occurs with advancing donor age (Fig. 2.3). To the best of our knowledge, this is also the first study to demonstrate that the age-related impairment in deoxygenation-induced ATP release is not due to changes in RBC metabolism, as intracellular ATP in normoxia and its increase during hypoxia were unaffected by donor age (Fig. 2.4). These collective findings provide the first insight into a key underlying mechanism of impaired RBC ATP release in healthy older adults, and indicate that targeting RBC deformability may be an effective therapeutic strategy to improve the decline in vasodilation and regulation of skeletal muscle blood flow and the increase in cardiovascular disease risk that occur with advancing age.

Determinants and pharmacological manipulation of red blood cell deformability

The fundamental structure of the RBC membrane is a phospholipid bilayer and an underlying cytoskeleton, which interact at cytoskeletal-integral protein complexes to form anchor points between the two layers. Although there are hundreds of proteins associated with the RBC membrane, the ones that are the primary determinants of RBC deformability are band 3, glycophorin C, and Rh-associated glycoprotein (RhAG) in the lipid bilayer and spectrin, actin, adducin, ankyrin, and protein 4.1 in the cytoskeleton (Mohandas & Chasis, 1993; Mohandas &

Evans, 1994; Mohandas & Gallagher, 2009; Lux IV *et al.*, 2016). On average, six spectrin proteins, each of which forms a flexible spring-like structure through the intertwining of its α and β subunits, interact with actin to form the generally hexagonal "spoke and hub" framework of the cytoskeleton, which is further stabilized by the interaction of adducin and protein 4.1 with the spectrin-actin complex (Mohandas & Chasis, 1993; Mohandas & Evans, 1994; Lux IV *et al.*, 2016). The primary linkage between the cytoskeleton and the lipid bilayer appears to be facilitated by ankyrin, which simultaneously interacts with spectrin and the integral protein band 3 (Mohandas & Chasis, 1993; Mohandas & Evans, 1994; Mohandas & Gallagher, 2009; Lux IV *et al.*, 2016). However, other linkages between these two layers that have been shown to contribute to membrane deformability and stability include RhAG-ankyrin-spectrin, glycophorin C-protein 4.1-spectrin, and band 3-adducin-spectrin (Mohandas & Chasis, 1993; Mohandas & Evans, 1994; Anong *et al.*, 2009; Mohandas & Gallagher, 2009; Lux IV *et al.*, 2016).

Altering the associations between cytoskeletal proteins or between protein complexes at the anchor points of the cytoskeleton with the lipid bilayer can dramatically influence RBC deformability by affecting the ability of spectrin molecules to undergo a conformational rearrangement (Mohandas & Chasis, 1993; Mohandas & Evans, 1994). With regards to the present study, one action of Rho-kinase is the prevention of actin disassembly (Sumi *et al.*, 1999); thus, a potential mechanism by which Rho-kinase inhibition with Y-27632 increased RBC deformability is through an increase in actin disassembly and subsequent decrease in the number of associations between spectrin proteins in the cytoskeleton. In contrast, diamide decreases RBC deformability by increasing the associations between spectrin in the cytoskeleton through the formation of crosslinking disulfide bonds (Haest *et al.*, 1977, 1980; Maeda *et al.*, 1983; Fischer, 1988).

Red blood cell deformability and deoxygenation-induced ATP release

Acute, pharmacologically-induced increases or decreases in RBC deformability have been shown to produce corresponding increases or decreases in deoxygenation-induced ATP release (Sridharan *et al.*, 2010*b*; Thuet *et al.*, 2011), which strongly suggests that these two properties of RBCs are linked. However, the precise pathway for RBC ATP release in response to deoxygenation and the mechanism(s) by which RBC deformability alters this process remain to be fully elucidated. Deoxygenation-induced ATP release has been shown to be dependent on the activation of the heterotrimeric inhibitory G (G_i) protein, and although the exact nature of this activation has not been tested in RBCs, one proposal is that it is mechanically activated by the R- to T-state conformational change of hemoglobin (Jagger *et al.*, 2001; Olearczyk *et al.*, 2004*a*, 2004*b*; Ellsworth *et al.*, 2009; Sridharan *et al.*, 2010*b*; Thuet *et al.*, 2011) based on evidence that G_i proteins have mechanosensitive properties (Gudi *et al.*, 1998). Accordingly, this conformational change from oxygenated hemoglobin (oxyHb) to deoxygenated hemoglobin (deoxyHb) has been clearly linked to a number of RBC properties through the reversible association of deoxyHb with band 3 (the most abundant protein in the RBC membrane) (Chu *et al.*, 2008, 2016; Sega *et al.*, 2015). Specifically, as the percentage of deoxyHb and its subsequent association with band 3 increases, there is a corresponding increase in RBC metabolism and deformability through the displacement of a glycolytic enzyme complex (Campanella *et al.*, 2005, 2008; Chu & Low, 2006; Lewis *et al.*, 2009; Puchulu-Campanella *et al.*, 2013; Chu *et al.*, 2016) and ankyrin (Stefanovic *et al.*, 2013; Chu *et al.*, 2016) from band 3, as well as an increase in RBC ATP release (Chu *et al.*, 2016). Furthermore, increases in RBC intracellular ATP (as occurs during deoxygenation) produce fluctuations or “flickering” of the RBC membrane (Park *et al.*, 2010). Although it remains to be tested, fluctuations of the RBC membrane could activate mechanosensitive proteins like G_i proteins or Piezo1 channels (Cinar *et al.*, 2015) and facilitate the subsequent release of ATP.

The findings from the present study provide additional support for a link between RBC deformability and ATP release, and more importantly, provide the first experimental evidence that age-related decreases in RBC deformability (Fig. 2.1) are a primary mechanism of impaired deoxygenation-induced ATP release from RBCs of healthy older adult humans, as both increasing deformability of RBCs from older adults and decreasing deformability of RBCs from young adults (Fig. 2.1) abolished the difference in deoxygenation-induced ATP release between the age groups (Figs. 2.2 and 2.3). If the stimulus for RBC ATP release following hemoglobin deoxygenation is indeed mechanical in nature as the evidence described above suggests, then it is probable that the parallel effects of increasing or decreasing RBC deformability on deoxygenation-induced ATP release are due to respective increases or decreases in the activation of mechanosensitive pathways that facilitate RBC ATP release. Additionally, this study provides the first experimental evidence that the age-related impairment in deoxygenation-induced ATP release from RBCs of healthy older adults is not due to changes in RBC metabolism, as the absolute concentration of intracellular ATP was not different between the age groups under any conditions and the increase in glycolytic ATP synthesis during hypoxia was unaffected by donor age or pharmacological treatments (Fig. 2.4).

Mechanisms of decreased red blood cell deformability in older adults

Advancing donor age is associated with multiple deleterious changes in RBC properties, including increased fragility (Detraglia *et al.*, 1974; Bowdler *et al.*, 1981), morphological changes (Bowdler *et al.*, 1981), and decreases in membrane fluidity and deformability (Reid *et al.*, 1976; Hegner *et al.*, 1979; Gelmini *et al.*, 1987, 1989). However, these changes are not necessarily linked to RBC age *per se*, as older adults have increased RBC turnover and an overall higher proportion of chronologically younger RBCs relative to young adults (Glass *et al.*, 1985; Gershon & Gershon, 1988; Magnani *et al.*, 1988; Shperling & Danon, 1990; Pinkofsky, 1997). Indeed, comparing RBCs of similar chronological age from young and older adults reveals that

RBCs from aged individuals have an “older” phenotype based on enzyme activity (particularly those involved in protection against oxidative stress) and markers of cell damage and senescence (Glass & Gershon, 1984; Glass *et al.*, 1985; Jozwiak & Jasnowska, 1985; Gershon & Gershon, 1988).

Of these age-associated changes, the decline in antioxidant capacity (Glass & Gershon, 1984; Gershon & Gershon, 1988; Gil *et al.*, 2006; Rizvi & Maurya, 2007; Chaleckis *et al.*, 2016) is likely one of the most detrimental given that RBCs can generate substantial amounts of reactive oxygen/nitrogen species (Johnson *et al.*, 2005; Cimen, 2008; Rifkind & Nagababu, 2013; Kuhn *et al.*, 2017), which cause oxidative damage that has been clearly linked to decreased RBC deformability (Haest *et al.*, 1977; Wang *et al.*, 1999; Tsantes *et al.*, 2006; Rifkind & Nagababu, 2013; Mohanty *et al.*, 2014) and would only be exacerbated by the increased susceptibility of RBCs from older adults to oxidative damage (Glass & Gershon, 1984; Gershon & Gershon, 1988; Gil *et al.*, 2006; Rizvi & Maurya, 2007). Furthermore, oxidative stress may be detrimental to RBC anion transport, which is required for proper RBC ATP release in response to deoxygenation (Petty *et al.*, 1991; Bergfeld & Forrester, 1992). Despite this established decline in RBC antioxidant capacity with advancing age, studies testing the efficacy of administering exogenous antioxidants or inducing endogenous antioxidant production for improving age-associated decrements in RBC properties are limited (Nelson *et al.*, 2006; Cazzola *et al.*, 2012), and are nonexistent as it pertains to RBC ATP release. The majority of the work that has been performed with RBCs in this area has focused on demonstrating the ability of antioxidant administration to improve redox status (Pandey & Rizvi, 2010; Wojceiech *et al.*, 2010; Nakagawa *et al.*, 2011; Kumar *et al.*, 2015; Richie Jr. *et al.*, 2015) or protect against an oxidative challenge either acutely or under more chronic stress conditions (Brown *et al.*, 1997; Zou *et al.*, 2001; Amer *et al.*, 2006; Luqman & Rizvi, 2006; Vijayakumar & Nalini, 2006; Pandey & Rizvi, 2010; Wojceiech *et al.*, 2010; Soudani *et al.*, 2011; Salini *et al.*, 2016; Jagadish *et al.*, 2017), although some studies have found that antioxidant administration can actually

increase susceptibility to oxidative stress under certain conditions (Brown *et al.*, 1997; Zhang *et al.*, 2016).

Cholesterol is a major component of the RBC membrane and can influence multiple membrane material properties (Mohandas & Chasis, 1993; Mohandas & Evans, 1994; Mohandas & Gallagher, 2009). Relevant to the present study, decreasing the membrane cholesterol content in RBCs from healthy adults has been shown to increase RBC deformability and shear-induced ATP release, but interestingly, enriching cholesterol in the RBC membrane had no effect on RBC deformability or ATP release compared to control (Forsyth *et al.*, 2012). Although there was a slight negative correlation between total cholesterol and the mean percent change in extracellular ATP during hypoxia under control conditions in the present study (r^2 = 0.112; P = 0.049), there was no relationship between total cholesterol and RCTT (r^2 = 0.059; P = 0.178). These collective findings suggest that it is unlikely that the elevated total cholesterol in whole blood from older adults in the present study (Table 2.1) was a primary contributor to the age-related declines in RBC deformability and deoxygenation-induced ATP release.

Experimental considerations and limitations

In the present study, incubation of RBCs with 500 μM diamide significantly decreased intracellular ATP in normoxia and hypoxia compared to the saline control condition (Fig. 2.4). However, this effect of diamide was the same in RBCs from both age groups and it also did not affect the upregulation of glycolytic activity and increase in intracellular ATP in hypoxia (Fig. 2.4), which is required for deoxygenation-induced ATP release (Jagger *et al.*, 2001). In both age groups in present study, diamide did not alter RBC characteristics like hemoglobin concentration or oxygen saturation relative to saline in normoxia or hypoxia; furthermore, other functional properties of RBCs including survival, osmotic fragility, density distribution, and hemoglobin polymerization have been shown to be unaffected by treatment with diamide at similar concentrations (Kosower *et al.*, 1969; Schmid-Schönbein & Gaehtgens, 1981; Maeda *et*

al., 1983). Diamide-mediated decreases in RBC deformability and deoxygenation-induced ATP release have also been shown to be abolished by subsequent incubation of RBCs with Y-27632 (Thuet *et al.*, 2011). Thus, the decrease in deoxygenation-induced ATP release from RBCs of young adults following incubation with diamide in the present study is likely to be due primarily to the decrease in RBC deformability.

It has recently been suggested that RBC ATP release occurs primarily through hemolysis rather than a regulated export process (Sikora *et al.*, 2014; Grygorczyk & Orlov, 2017). However, while hemolysis can certainly contribute to extracellular ATP and is an important methodological challenge that must be controlled for in studies such as these (Keller *et al.*, 2017), the collective experimental evidence does not support the hypothesis that hemolysis is a primary mechanism for ATP release from human RBCs (Kirby *et al.*, 2015). Accordingly, there were no significant differences in hemolysis (hemoglobin absorbance at 405 nm) between age groups or drug treatments during normoxia or hypoxia in the present study, and no significant correlations between hemolysis and extracellular ATP in young or older adults during any of the experimental conditions (adjusted r^2 ranged from -0.042-0.056, $p = 0.118$-0.993). Thus, RBC ATP release in the present study was primarily due to a regulated export process that was dependent on the oxygenation state of hemoglobin and influenced by donor age and pharmacological manipulations of deformability.

Conclusions

ATP is a unique vasoactive molecule that can stimulate vasodilation and blunt sympathetically-mediated vasoconstriction to help facilitate appropriate regulation of blood flow to the skeletal muscle. RBCs are a primary source of circulating ATP, and the ability to release ATP in response to cell deformation and hemoglobin deoxygenation allows RBCs to act as both a ‘sensor’ of local elevations in tissue oxygen demand and an ‘effector’ to match oxygen supply through the release of ATP and subsequent vasodilation, which increases tissue blood flow and

oxygen delivery to the region to meet the metabolic need. Recent findings have demonstrated that deoxygenation-induced ATP release is impaired in RBCs from healthy older adults, but the underlying mechanisms of this impairment were unknown.

This series of studies demonstrates that age-related decreases in RBC deformability are a primary mechanism of impaired deoxygenation-induced ATP release, as improving deformability restored the ability of RBCs from older adults to release ATP to a level that was not different from RBCs of young adults, whereas decreasing deformability of RBCs from young adults attenuated the release of ATP such that it was identical to the typical impaired response in RBCs from older adults. It is unclear if this decrease in deformability with advancing age is the only mechanism of impaired ATP release in RBCs from healthy older adults, or if there are overlapping mechanisms with other conditions such as diabetes in which RBC ATP release is also impaired. The contribution of impaired RBC ATP release to the declines in vascular function and blood flow regulation in healthy older adults, and whether enhancing RBC ATP release *in vivo* can improve vascular function with age, also remains to be determined.

Table 2.1. Subject Characteristics

	Deformability		*Y-27632*		*Diamide*	
	Young	Older	Young	Older	Young	Older
Male:Female	6:3	4:5	7:6	6:8	8:5	4:6
Age (years)	24±1	64±2*	22±1	65±2*	23±1	65±3*
Body mass index (kg/m^2)	22.7±0.8	25.6±1.1	23.0±0.8	24.9±0.7*	23.2±0.8	25.1±0.9
Body fat (%)	24.3±2.2	34.2±3.0*	24.7±1.8	34.9±2.1*	25.6±2.4	34.0±2.9*
Total cholesterol (mg/dL)	158±12	193±9*	159±9	191±9*	161±9	195±11*
LDL cholesterol (mg/dL)	94±10	115±7	94±7	117±7*	97±7	118±10
HDL cholesterol (mg/dL)	49±3	58±6	50±3	54±5	50±3	60±6
LDL:HDL	2.0±0.2	2.2±0.3	1.9±0.2	2.5±0.3	2.0±0.2	2.1±0.3
Triglycerides (mg/dL)	80±8	103±19	75±12	100±11	77±10	87±9

*$P < 0.05$ vs. young (within condition)

Table 2.2. Isolated red blood cell gases

			pH	PO_2 (mmHg)	PCO_2 (mmHg)	tHb (g/dL)	FO_2Hb (%)	FHHb (%)
Normoxia	Young	Saline	7.327±0.010	112.1±1.9	35.4±1.0	7.1±0.2	95.1±0.2	3.4±0.1
		Y-27632	7.327±0.009	112.5±2.5	34.4±0.7	7.1±0.2	95.1±0.2	3.3±0.1
	Older	Saline	7.344±0.009	111.8±2.7	35.0±0.7	7.7±0.3	95.0±0.2	3.6±0.2
		Y-27632	7.360±0.006*†	114.0±2.7*	34.1±0.4	7.4±0.2	95.2±0.2	3.4±0.1*
Hypoxia	Young	Saline	7.340±0.010	19.3±1.2	36.5±1.1	7.1±0.2	24.1±2.8	72.1±2.6
		Y-27632	7.340±0.010	19.6±1.4	36.6±0.8	7.1±0.2	24.1±2.8	72.2±2.7
	Older	Saline	7.367±0.010	18.7±1.7	35.8±1.0	7.6±0.3	23.1±3.4	73.1±3.2
		Y-27632	7.375±0.008†	18.2±1.4	35.2±0.4	7.3±0.2	21.5±3.0	74.4±2.9
Normoxia	Young	Saline	7.324±0.007	109.6±1.1	34.8±0.6	6.7±0.1	94.6±0.2	3.6±0.1
		Diamide	7.319±0.007	110.0±0.8	35.1±0.6	6.7±0.1	94.6±0.2	3.5±0.1
	Older	Saline	7.314±0.008	111.8±1.0	33.9±0.9	6.5±0.2	94.5±0.1	3.7±0.2
		Diamide	7.306±0.009	111.9±1.2	34.5±0.6	6.5±0.2	94.5±0.3	3.5±0.2
Hypoxia	Young	Saline	7.348±0.007	16.8±0.8	34.6±0.6	6.5±0.1	17.4±1.2	78.1±1.2
		Diamide	7.347±0.007	17.5±1.0	35.8±0.7	6.6±0.2	19.6±2.1	76.1±1.9
	Older	Saline	7.332±0.010	18.1±1.1	35.0±0.5	6.4±0.2	17.9±2.0	77.7±1.9
		Diamide	7.339±0.016	21.1±1.7†	34.5±1.2	6.6±0.2	28.8±3.9	67.3±3.6

PO_2 = partial pressure of oxygen, PCO_2 = partial pressure of carbon dioxide, tHb = total hemoglobin, FO_2Hb = fraction of oxygenated hemoglobin, FHHb = fraction of deoxygenated hemoglobin *P < 0.05 vs. saline (within age); †P < 0.05 vs. young (within condition)

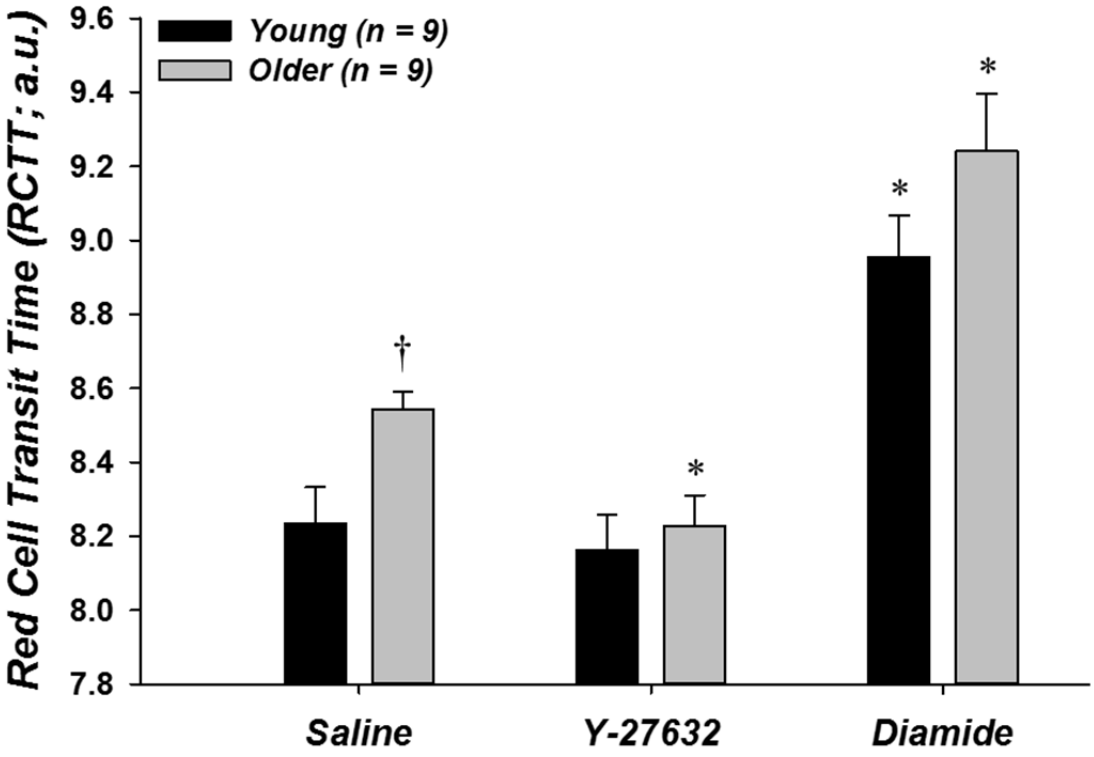

Figure 2.1. Effect of donor age, Y-27632, and diamide on red blood cell deformability
As a decrease in RCTT reflects an increase in RBC deformability, RBCs from older adults were less deformable than RBCs from young adults in control (saline) conditions. Incubation of RBCs with 1 μM Y-27632 restored deformability in older adults, whereas 500 μM diamide decreased RBC deformability similarly in young and older adults. *$P < 0.05$ vs. saline (within age); †$P < 0.05$ vs. young (within condition)

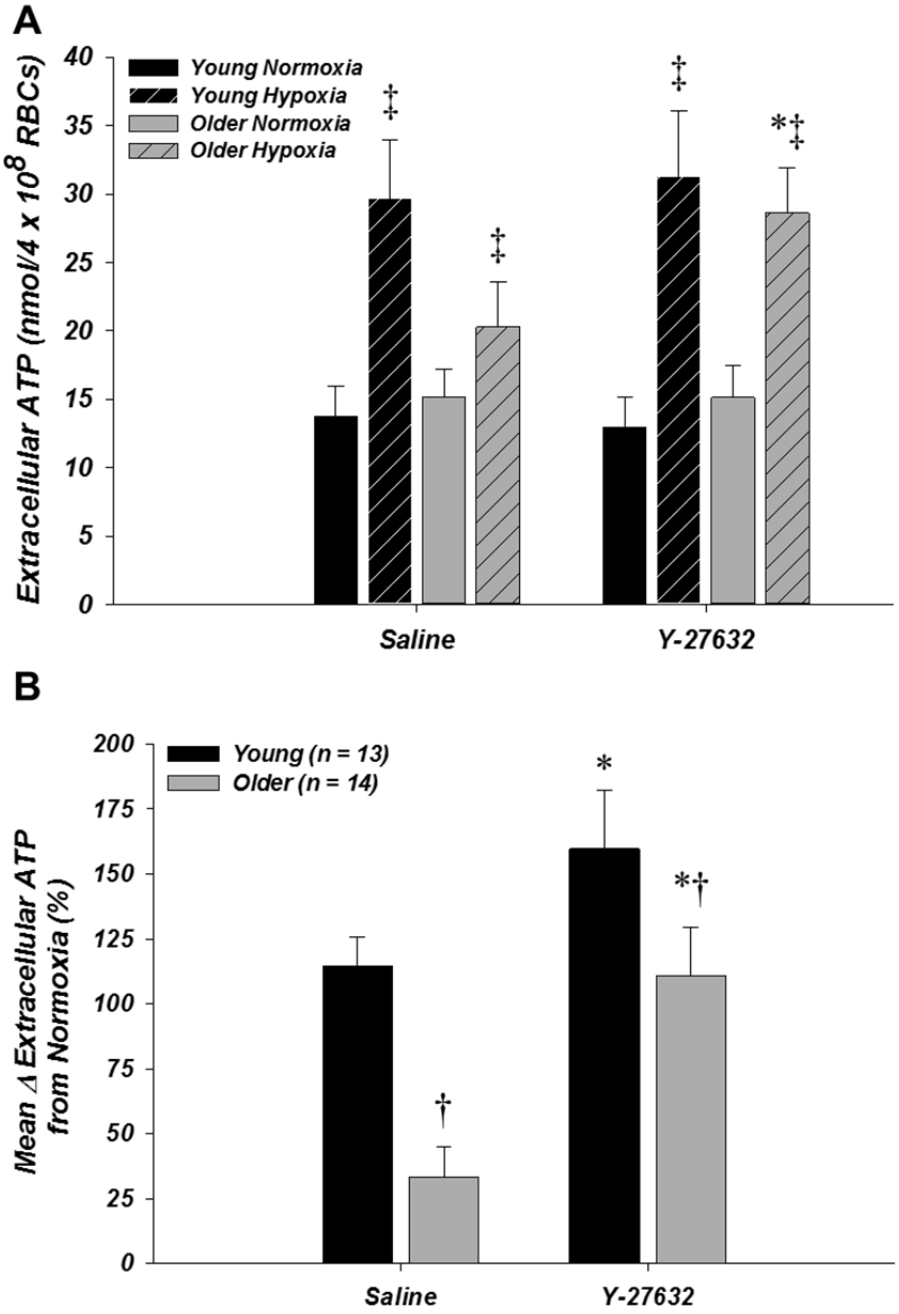

Figure 2.2. Effect of donor age and Y-27632 on red blood cell ATP release in normoxia and hypoxia

A: Y-27632 increased extracellular ATP during hypoxia in RBCs from older adults. In the hypoxia saline condition, extracellular ATP from RBCs of older adults trended towards being lower compared to young adults ($P = 0.15$). *B*: the mean percent increase in extracellular ATP from normoxia to hypoxia was impaired in RBCs from older adults in control (saline) conditions. Incubation with Y-27632 rescued this response in RBCs from older adults relative to the young saline control, but the age impairment remained within the Y-27632 condition due to an improvement in the young as well. *$P < 0.05$ vs. saline (within age); †$P < 0.05$ vs. young (within condition); ‡$P < 0.05$ vs. normoxia (within condition)

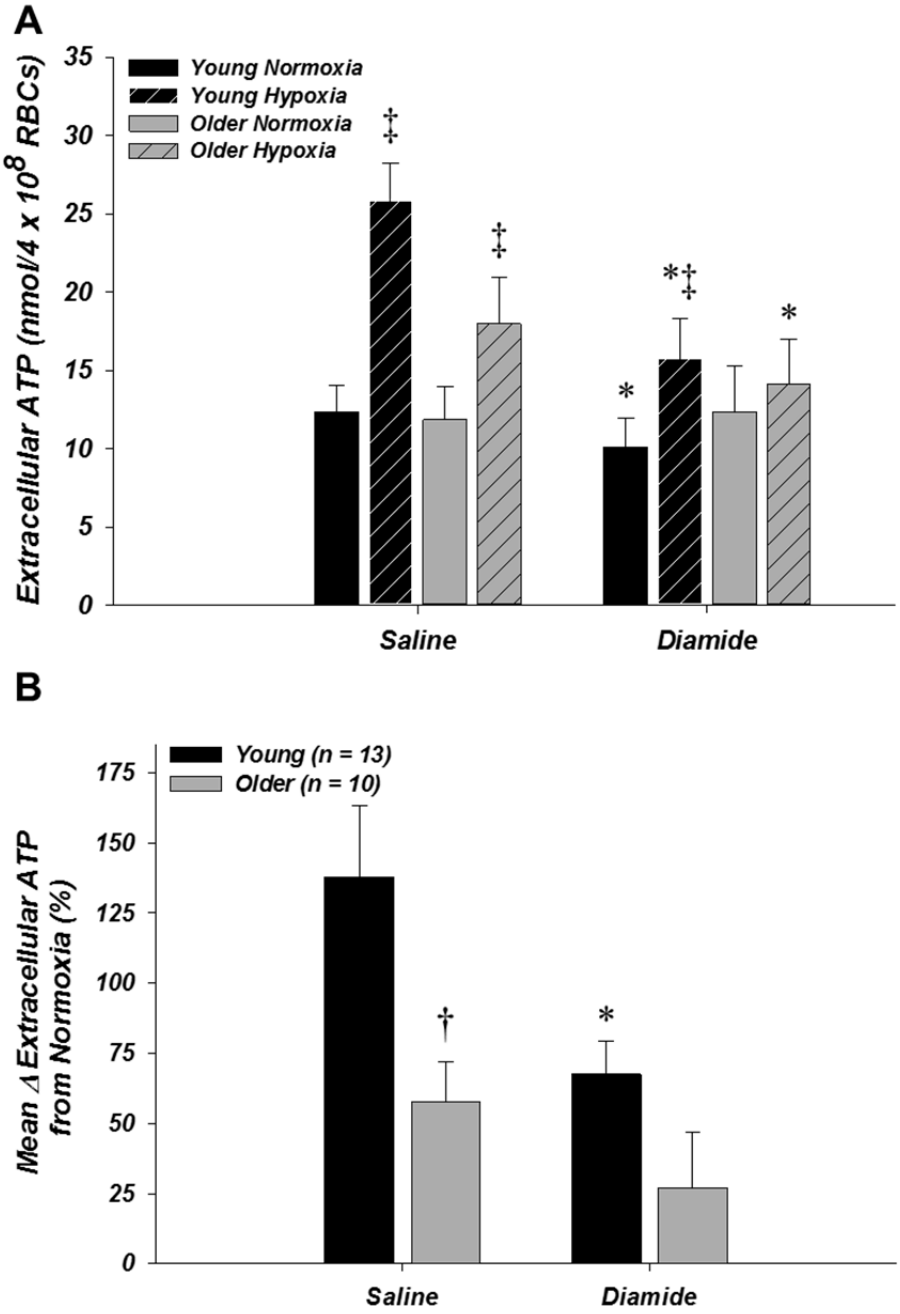

Figure 2.3. Effect of donor age and diamide on red blood cell ATP release in normoxia and hypoxia

A: diamide decreased extracellular ATP in both age groups during hypoxia and in the young during normoxia. In the hypoxia saline condition, extracellular ATP from RBCs of older adults trended towards being lower compared to young adults ($P = 0.097$). *B*: the mean percent increase in extracellular ATP from normoxia to hypoxia was impaired in RBCs from older adults in control (saline) conditions, but diamide significantly decreased ATP release in young adults such that it was no longer different from older adults. *$P < 0.05$ vs. saline (within age); †$P < 0.05$ vs. young (within condition); ‡$P < 0.05$ vs. normoxia (within condition)

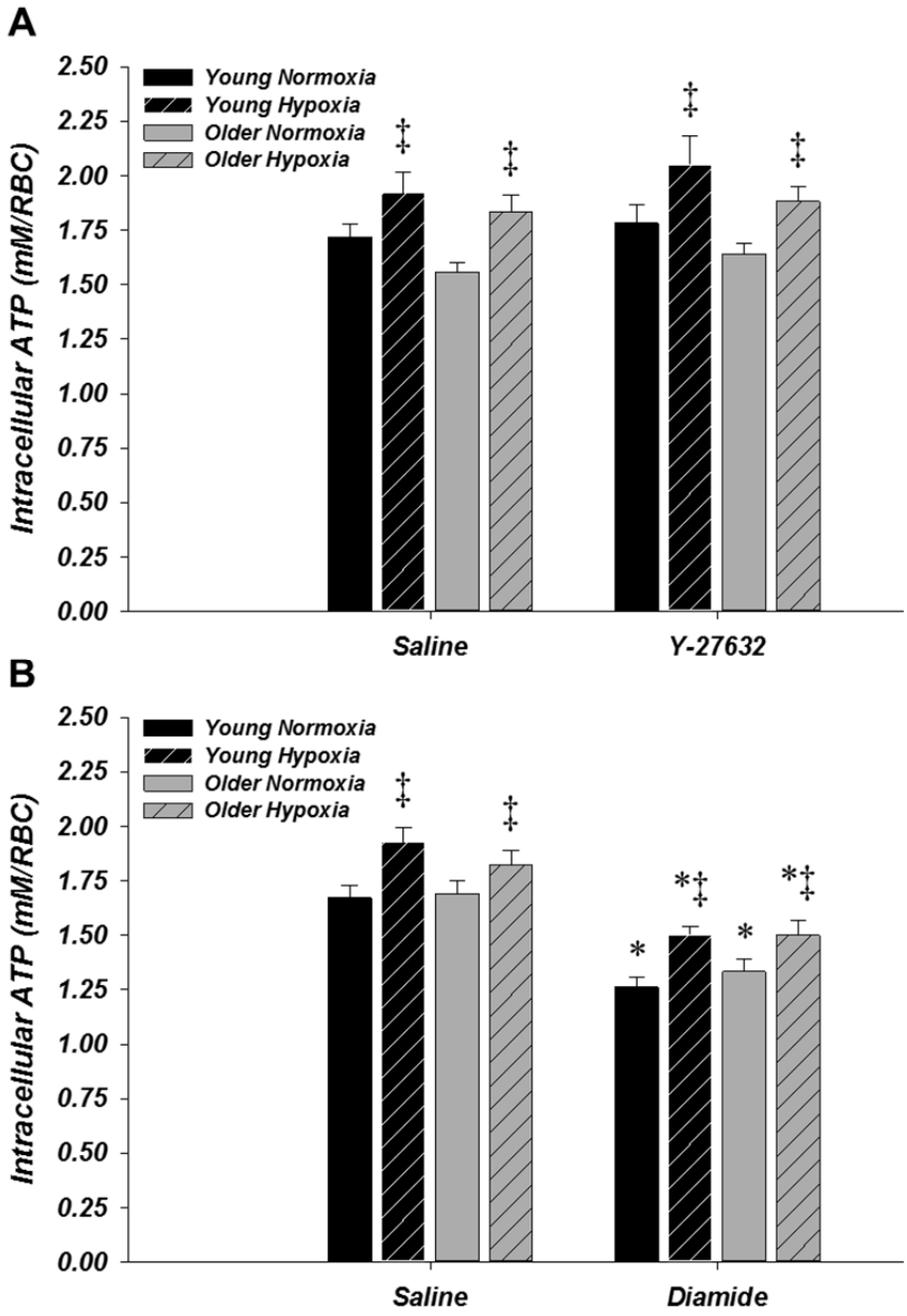

Figure 2.4. Effect of donor age, Y-27632, and diamide on red blood cell intracellular ATP in normoxia and hypoxia

A: RBC intracellular ATP increased in hypoxia and was unaffected by donor age or Y-27632; young (n = 9) and older (n = 10). *B*: intracellular ATP increased in hypoxia and was not different with donor age, but it was lower than saline conditions following incubation with diamide; young (n = 11) and older (n = 10). **P* < 0.05 vs. saline (within age); ‡*P* < 0.05 vs. normoxia (within condition)

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CHAPTER III – MANUSCRIPT II

Role of red blood cell cAMP in impaired deoxygenation-induced ATP release with age

Summary

Red blood cells (RBCs) release adenosine triphosphate (ATP) in direct proportion to the degree of hemoglobin deoxygenation, which binds to purinergic receptors on the endothelium and stimulates local and conducted vasodilation. Accordingly, RBCs act as both a 'sensor' for oxygen demand and an 'effector' for increasing oxygen delivery to facilitate the matching of tissue oxygen supply and demand. Deoxygenation-induced ATP release is impaired in RBCs from healthy older adults and age-associated reductions in RBC deformability contribute significantly to the impairment; however, it is unclear if other factors play a role as well. Type 2 diabetes is also associated with impaired RBC ATP release, and this appears to be at least partly due to alterations in cyclic AMP (cAMP) signaling given that treatment with cilostazol, to inhibit phosphodiesterase 3 (PDE3) hydrolysis of cAMP, improves deoxygenation-induced ATP release from RBCs of these patients. Thus, we hypothesized that treatment of RBCs with cilostazol would improve deoxygenation-induced ATP release from RBCs of healthy older adults, and sought to determine if RBC intracellular signaling related to cAMP is impaired with advancing age. Isolated RBC ATP release during normoxia (PO_2 ~114 mmHg) and hypoxia (PO_2 ~24 mmHg) was quantified in RBCs from young (26 ± 1 years; n = 10) and older (64 ± 2 years; n = 12) adults using the luciferin-luciferase technique following RBC incubation with dimethylformamide (DMF; vehicle control) or cilostazol (100 μM). With DMF, the relative change in ATP release from normoxia to hypoxia was significantly less in RBCs from older compared with young adults (~50% vs. ~120%; $P < 0.05$), and these responses were unaffected by cilostazol (~60% vs. ~140%, respectively; $P > 0.05$ vs. DMF). This finding suggests that altered cAMP signaling is not a mechanism of impaired deoxygenation-induced RBC ATP

release in healthy older adults. To confirm this, intracellular cAMP responses (n = 6 per age group) and ATP release (n = 4 per age group) in response to inhibitory G (G_i) protein stimulation by mastoparan 7 (Mas 7; 10 μM) were quantified in RBCs from young and older adults using a commercially available enzyme immunoassay and the luciferin-luciferase technique, respectively. With Mas 7, the relative increase in intracellular cAMP and ATP release from control and baseline conditions was not different between young and older adults (~55% and ~240%, respectively for both age groups; $P > 0.05$ for young vs. older). Collectively, these findings suggest that advancing age is not associated with alterations in RBC intracellular cAMP signaling or responsiveness to G_i stimulation, which may have implications for treating impaired vascular control in healthy older adults.

Introduction

Matching skeletal muscle blood flow and oxygen delivery with tissue metabolic demand is an essential physiological process, particularly during dynamic exercise when both skeletal muscle metabolic rate and blood flow can increase nearly 100-fold and exceed the pumping capacity of the heart when extrapolated to the whole-body level (Andersen & Saltin, 1985; Richardson *et al.*, 1993). This process requires the coordination and integration of multiple stimuli, including mechanical forces and vasoactive/metabolic substances resulting from muscle contraction, vasoconstrictor stimuli from the sympathetic nervous system, and vasodilator stimuli derived from the endothelium and circulating factors like red blood cells (RBCs). Changes to these stimuli that occur with primary (healthy) aging include chronic elevations in sympathetic nervous system activity and declines in the synthesis or general availability of important vasodilatory molecules like nitric oxide (NO) and adenosine triphosphate (ATP). Advancing age also results in impaired vascular responses and subsequent regulation of blood flow to the skeletal muscle during physiological stressors such as hypoxia and exercise, which contributes to the age-related reduction in aerobic exercise capacity (an independent predictor

of cardiovascular disease morbidity and mortality), functional independence, and overall quality of life.

Of the alterations in vasoactive stimuli that occur with advancing age, data from our laboratory indicate that augmented sympathetic vasoconstriction does not contribute to the reduction in peripheral vasodilation and skeletal muscle hyperemia during hypoxia or exercise in older adults (Richards *et al.*, 2014*a*, 2017). Therefore, the attenuation in local vasodilatory signaling with advancing age is likely to be a major contributor to this impairment. Of the local vasodilators that are affected by age, the blunted increases in circulating ATP during hypoxia and exercise (Kirby *et al.*, 2012) may be among the most important given the unique ability of ATP to stimulate both local and conducted vasodilation via binding to purinergic P_{2Y} receptors on the endothelium (Collins *et al.*, 1998; Winter & Dora, 2007; Dora, 2017), blunt adrenergic vasoconstriction (Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008; Hearon Jr. *et al.*, 2017), and limit adhesion and coagulation in the blood (Hrafnkelsdóttir *et al.*, 2001; Zhu *et al.*, 2011; Kirby *et al.*, 2014). Red blood cells (RBCs) are a primary source of circulating ATP and can contribute to the coupling of blood flow and oxygen delivery to tissue metabolic demand through the release of ATP in direct proportion to the degree of hemoglobin deoxygenation (Bergfeld & Forrester, 1992; Ellsworth *et al.*, 1995; Dietrich *et al.*, 2000; Ellsworth, 2000; Jagger *et al.*, 2001; González-Alonso *et al.*, 2002; Sprague *et al.*, 2009; Jensen, 2009; Ellsworth & Sprague, 2012; Kirby *et al.*, 2013), but this deoxygenation-induced ATP release is impaired in RBCs from healthy older adults (Kirby *et al.*, 2012).

The experiments described in Chapter II of this dissertation are the first to investigate the underlying mechanisms of the age-related impairment in RBC ATP release. While the results indicate that the decrease in deformability of RBCs from older adults is a significant contributor, this is the only mechanism of impaired RBC ATP release with healthy aging that has been studied and it is therefore unclear if other factors play a role as well. Type 2 diabetes is another condition that is associated with impaired RBC ATP release in response to cell deformation

(Subasinghe & Spence, 2008) and deoxygenation (Sprague *et al.*, 2010, 2011, Richards *et al.*, 2014*b*, 2015; Dergunov *et al.*, 2015), both of which have been shown to cause ATP release through the stimulation of heterotrimeric inhibitory G (G_i) proteins (Olearczyk *et al.*, 2004*a*, 2004*b*). The signaling cascade for RBC ATP release downstream of G_i stimulation has been demonstrated to involve the subsequent activation of adenylyl cyclase (AC) and increases in intracellular cyclic AMP (cAMP), the overall level of which is controlled by the balance between AC-mediated synthesis and hydrolysis by phosphodiesterase 3 (PDE3) (Sprague *et al.*, 2001, 2006, 2011; Conti & Beavo, 2007; Adderley & Sprague, 2010; Lomas & Zaccolo, 2014; Brescia & Zaccolo, 2016). Although recent findings dispute the importance of cAMP in this pathway (Keller *et al.*, 2017), RBCs from people with type 2 diabetes have also been shown to have impaired increases in intracellular cAMP and ATP release following direct G_i protein stimulation with mastoparan 7 (Mas 7) relative to RBCs from healthy controls (Sprague *et al.*, 2006, 2011).

Importantly, the impairments in intracellular cAMP and ATP release associated with type 2 diabetes or elevated insulin can be improved by incubation of RBCs with the PDE3 inhibitor cilostazol (Hanson *et al.*, 2010; Sprague *et al.*, 2011; Dergunov *et al.*, 2015), indicating that altered cAMP signaling is a significant underlying mechanism of impaired deoxygenation-induced ATP release from RBCs of individuals with type 2 diabetes. However, it is unclear if alterations in RBC intracellular cAMP signaling are a shared mechanism of impaired RBC ATP release between diabetes and healthy aging. Thus, the primary goal of the present study was to test the hypothesis that treatment of RBCs with the PDE3 inhibitor cilostazol would improve deoxygenation-induced ATP release from RBCs of healthy older adults, and to determine if cellular responses downstream of G_i activation (i.e., increased intracellular cAMP and ATP release) are impaired with advancing age.

Methods

Ethical approval and subjects

With Institutional Review Board approval and after written informed consent, a total of 14 young and 13 older healthy adults participated in the present investigation. Of those, 6 young and 6 older subjects participated in multiple experiments. All subjects were free from overt cardiovascular disease as assessed from a medical history, free of cardiovascular medications, non-smokers, non-obese, normotensive, and sedentary to moderately active. Young female subjects were studied during the early follicular phase of their menstrual cycle to minimize any potential cardiovascular effects of sex-specific hormones, whereas older female subjects were post-menopausal and not taking hormone replacement therapy. Additionally, older subjects were further evaluated for clinical evidence of cardiopulmonary disease with a physical examination and resting and exercise (Balke protocol) electrocardiograms. Body composition was determined by whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc., USA). Whole blood lipid panels were run using a Piccolo Xpress chemistry analyzer (Abaxis, USA). All studies were performed according to the *Declaration of Helsinki*.

Isolation of red blood cells

Blood was obtained by either catheterization of the brachial artery (if the subject was participating in another study in the laboratory) or venipuncture of the antecubital vein and collected into Vacutainer tubes containing sodium heparin (158 USP units) after a 4 hour fast and 12 hour abstention from caffeine, alcohol, and exercise. RBCs were isolated by centrifugation of the collected whole blood (500*g*, 4°C, 10 min) followed by removal of the plasma and buffy coat by aspiration. Packed RBCs were resuspended and washed three times in a cell wash buffer containing (in mM) 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0 Tris-base, 5.5 glucose, and 0.5% BSA, with pH adjusted to 7.4 at room temperature (Kirby *et al.*,

2012; Richards *et al.*, 2014*b*, 2015). All studies were performed immediately after blood collection and RBC isolation.

Red blood cell deoxygenation and measurement of extracellular ATP

As described previously by our laboratory (Kirby *et al.*, 2012), washed RBCs were diluted to 20% hematocrit with a bicarbonate-based buffer containing (in mM) 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 11.1 glucose, 23.8 $NaHCO_3$, and 0.5% BSA warmed to 37°C. This 20% hematocrit RBC suspension was placed in a rotating bulb tonometer (Eschweiler GmbH & Co. KG, Germany) and warmed to 37°C. RBCs were incubated in the tonometer bulbs with dimethylformamide (DMF; vehicle control; Sigma) or the PDE3 inhibitor cilostazol (100 μM; Sigma) for 30 min in normoxia (16% O_2, 6% CO_2, balanced nitrogen; P_{O_2} = 114.3 ± 0.7 mmHg and $F_{O_2}Hb$ = 95.0 ± 0.1% across both age groups and conditions) (Sprague *et al.*, 2011), after which RBCs were sampled from each tonometer bulb for measurement of extracellular and intracellular ATP in normoxia (details below). RBCs were then deoxygenated by exposure to hypoxia (2.25% O_2, 6% CO_2, balanced nitrogen; P_{O_2} = 24.1 ± 0.4 mmHg and $F_{O_2}Hb$ = 34.8 ± 1.2% across all age groups and conditions) for 15 min and RBC samples were taken for measurement of ATP as in normoxia. Normoxic and hypoxic gases were blended via gas blender (MCQ Gas Blender Series 100, Italy) and humidified before introduction into the tonometer bulbs. Blood gases were confirmed by blood gas analysis (Siemens Rapid Point 405 Series Automatic Blood Gas System, Los Angeles, CA) (Kirby *et al.*, 2012).

ATP was measured via the luciferin-luciferase technique as described previously (Sprague *et al.*, 2001, 2011, Richards *et al.*, 2012, 2014*b*, 2015; Kirby *et al.*, 2012), with light emission during the reaction detected by a luminometer (TD 20/20, Turner Designs). For extracellular ATP (i.e., ATP release) measurements, a 10 μL sample of the 20% hematocrit suspension was taken from each tonometer bulb and diluted 500-fold (0.04% hematocrit), from which a 200 μL sample was taken and injected into a cuvette containing 100 μL of firefly tail

extract (10 mg/mL DI water; Sigma) and 100 µL of D-luciferin (0.5 mg/mL DI water; Research Products International). Peak light output was measured at least in triplicate for each experimental condition and the mean was used for determination of ATP levels by comparison to a standard curve for ATP (Calbiochem) generated on the day of the experiment. Cell counts were obtained from each 0.04% RBC suspension and extracellular ATP was normalized to 4 x 10^8 cells. To confirm that ATP release was not due to hemolysis, the 0.04% RBC suspensions from which samples for ATP analysis and cell counting were taken were analyzed for free hemoglobin by measuring absorbance at 405 nm similar to previous reports, and samples with significant lysis were excluded (Sprague *et al.*, 1998, 2011, Kirby *et al.*, 2012, 2014, Richards *et al.*, 2013, 2014*b*, 2015).

Measurement of red blood cell total intracellular ATP

To confirm that the effects of donor age and cilostazol on RBC ATP release were not due to differences in total intracellular ATP or the increase in RBC glycolytic activity during hypoxia (Messana *et al.*, 1996; Campanella *et al.*, 2005; Lewis *et al.*, 2009), 50 µL samples of drug- and saline-treated RBCs (20% hematocrit) were taken from the tonometer bulbs in normoxia and hypoxia following measurement of extracellular ATP and lysed in DI water at room temperature (a 20-fold dilution). This lysate was diluted an additional 400-fold (8000-fold total) and ATP was measured using the same ATP assay used for determination of extracellular ATP (Sridharan *et al.*, 2010*b*, 2010*a*; Sprague *et al.*, 2011; Thuet *et al.*, 2011; Kirby *et al.*, 2012, 2014). Values were normalized to ATP concentration per RBC.

Measurement of red blood cell intracellular cAMP

As described previously (Olearczyk *et al.*, 2004*a*; Sprague *et al.*, 2005, 2006, 2011; Hanson *et al.*, 2010; Sridharan *et al.*, 2010*b*), washed RBCs were diluted to a 50% hematocrit in a cell wash buffer (as described for isolation of RBCs) and three 1 mL aliquots of this RBC

suspension were incubated at room temperature with either DMF for 45 min (vehicle and time control), the G_i activator mastoparan 7 (Mas 7; 10 μM; Sigma) for 15 min, or the PDE3 inhibitor cilostazol (100 μM) for 30 min followed by an additional 15 min co-incubation with Mas 7 (10 μM). The reaction was halted by the addition of 4 mL of ice cold acidified ethanol (1.3 μL of 11.6 M HCl in 15 mL of 200 proof ethanol), followed by vortexing and centrifugation (14,000*g*, 4°C, 10 min). The supernatant was removed and stored overnight at -20°C to precipitate the remaining proteins and centrifuged (3,700*g*, 4°C, 10 min) the next day. The final supernatant was removed, dried under vacuum centrifugation, and stored at -80°C until enough samples were collected to run the assay. The dried sample was reconstituted in an assay buffer and the concentration of cAMP (fmol) was determined using a commercially available enzyme immunoassay (GE Healthcare; non-acetylation protocol kit). RBCs from 6 young and 6 older subjects were used for this experiment. Treatment of RBCs and measurement of intracellular cAMP was performed in duplicate and averaged for each subject, and the mean was used to determine the relative (%) change in intracellular cAMP compared to the DMF vehicle control (Hanson *et al.*, 2010).

Red blood cell G_i activation (Mas 7) and measurement of extracellular ATP

Washed RBCs were diluted to a 10% hematocrit with a bicarbonate-based buffer (as described above for RBC deoxygenation), placed in a rotating bulb tonometer, and warmed to 37°C in normoxia (15% O_2, 6% CO_2, balanced nitrogen; P_{O_2} = 118.1 ± 1.1 mmHg and $F_{O_2}Hb$ = 93.7 ± 0.2% across both age groups and conditions). After a 15 min equilibration period, RBC samples were taken from each tonometer bulb for baseline measurement of extracellular ATP (details below), followed by incubation with saline (vehicle control) or 10 μM Mas 7 (Sprague *et al.*, 2005, 2006, Hanson *et al.*, 2009, 2010; Sridharan *et al.*, 2010*b*; Thuet *et al.*, 2011). Saline-treated RBCs were sampled for measurement of extracellular ATP at 15 min after the addition of saline, and RBCs incubated with Mas 7 were sampled for extracellular ATP at 5, 10, and 15

min after the addition of Mas 7 and the peak value was used for calculating the relative (%) change in extracellular ATP from baseline.

ATP was measured via the luciferin-luciferase technique as described previously (Sprague *et al.*, 2001, 2011, Richards *et al.*, 2012, 2014*b*, 2015; Kirby *et al.*, 2012), with light emission during the reaction detected by a luminometer (TD 20/20, Turner Designs). For extracellular ATP (i.e., ATP release) measurements, a 10 µL sample of the 10% hematocrit suspension was taken from each tonometer bulb and diluted 250-fold (0.04% hematocrit), from which a 200 µL sample was taken and injected into a cuvette containing 100 µL of firefly tail extract (10 mg/mL DI water; Sigma) and 100 µL of D-luciferin (0.5 mg/mL DI water; Research Products International). Peak light output was measured at least in triplicate for each experimental condition and the mean was used for determination of ATP levels by comparison to a standard curve for ATP (Calbiochem) generated on the day of the experiment. Cell counts were obtained from each 0.04% RBC suspension and extracellular ATP was normalized to 4 x 10^8 cells. To confirm that ATP release was not due to hemolysis, the 0.04% RBC suspensions from which samples for ATP analysis and cell counting were taken were analyzed for free hemoglobin by measuring absorbance at 405 nm similar to previous reports, and samples with significant lysis were excluded (Sprague *et al.*, 1998, 2011, Kirby *et al.*, 2012, 2014, Richards *et al.*, 2013, 2014*b*, 2015).

Statistics

All values are reported as mean ± SEM. Statistical analyses of absolute ATP values (intracellular and extracellular) were performed using R (R Core Team 2016, R Foundation for Statistical Computing, Vienna, Austria). Absolute ATP values were tested using a 3-way repeated measures ANOVA, with age as the between subjects factor (young vs. older) and drug/gas conditions as the within subject factors (DMF vs. cilostazol and normoxia vs. hypoxia, respectively). When an interaction or main effect was found, appropriate pairwise comparisons

were made. The relative increase in intracellular cAMP compared to zero was tested using a one-tailed t-test. For statistical analyses of blood gases, the relative (%) change in ATP release from normoxia to hypoxia and in response to incubation with Mas 7, and the relative (%) change in intracellular cAMP, SigmaPlot (Systat Software, San Jose, CA, USA) was used to perform a 2-way repeated measures ANOVA. In the event of a main effect of or interaction between age and drug condition, post hoc comparisons were made with Tukey's HSD test. Significance was set at $P < 0.05$.

Results

Subjects and blood gases

Subject characteristics are reported in Table 3.1. Compared to the young adults, older adults had either trending or significant elevations in body mass index (BMI), body fat percentage, and blood lipids, although all values were still within the normal healthy range. Blood gases for isolated RBCs are reported in Table 3.2. Most importantly, there were no significant differences in the fraction of oxygenated hemoglobin (FO_2Hb) between age groups or pharmacological treatments in normoxia or hypoxia.

Effect of donor age and cilostazol on deoxygenation-induced ATP release from red blood cells and red blood cell intracellular ATP

Extracellular ATP in normoxia was not different between age groups or drug condition (Fig. 3.1A). In the DMF vehicle control condition, extracellular ATP from RBCs of older adults in hypoxia tended to be lower compared to young adults (10.7 ± 1.4 nmol/4 x 10^8 RBCs vs. 15.0 ± 2.5 nmol/4 x 10^8 RBCs, respectively; $P = 0.196$) (Fig. 3.1A) and the mean percent increase in RBC ATP release from normoxia to hypoxia was significantly impaired in the older vs. young adults (46.7 ± 8.0% vs. 117.6 ± 13.3%, respectively; $P < 0.05$) (Fig. 3.1B). This age impairment in ATP release during hypoxia was unaffected by incubation of RBCs with cilostazol, as both the

trend for lower absolute levels of extracellular ATP from RBCs of older compared to young adults (10.7 ± 1.2 nmol/4 x 10^8 RBCs vs. 16.7 ± 3.4 nmol/4 x 10^8 RBCs, respectively; $P = 0.146$) (Fig. 3.1A) and the significantly blunted relative increase in RBC ATP release from normoxia to hypoxia in older vs. young (64.2 ± 11.4% vs. 141.0 ± 25.6%, respectively; $P < 0.05$) (Fig. 3.1B) persisted. RBC intracellular ATP was not different between age groups or drug condition ($P > 0.05$) and was significantly increased in hypoxia vs. normoxia (Fig. 3.2; $P < 0.05$).

Effect of donor age and cilostazol on the red blood cell intracellular cAMP response to Mas 7

Unstimulated (DMF vehicle control) intracellular cAMP concentration was not significantly different between young and older adults (233.2 ± 41.0 fmol vs. 191.4 ± 38.0 fmol, respectively; $P = 0.47$). Incubation of RBCs with the G_i activator Mas 7 (10 μM) significantly increased the mean relative (%) change in intracellular cAMP from DMF vehicle control in RBCs from both young and older adults (52.3 ± 15.2% and 60.8 ± 22.8%, respectively; $P < 0.05$ vs. zero) (Fig. 3.3). This relative increase in intracellular cAMP to 10 μM Mas 7 remained greater than zero in RBCs from both young and older adults after pretreatment with the PDE3 inhibitor cilostazol (100 μM) (36.5 ± 18.9% and 34.7 ± 14.9%; $P < 0.05$) (Fig. 3.3), and was not significantly different between age groups or from Mas 7 alone (Fig. 3.3; $P > 0.05$).

Effect of donor age on red blood cell ATP release in response to Mas 7

Baseline extracellular ATP prior to the addition of saline or Mas 7 was not different in young (10.8 ± 5.6 nmol/4 x 10^8 RBCs and 12.5 ± 6.1 nmol/4 x 10^8 RBCs, respectively; $P > 0.05$) or older (7.5 ± 1.2 nmol/4 x 10^8 RBCs and 6.4 ± 1.9 nmol/4 x 10^8 RBCs, respectively; $P > 0.05$) adults. ATP release from RBCs of both young and older adults did not increase significantly following incubation with saline for 15 min (20.0 ± 18.5% and 1.6 ± 17.9%, respectively; $P > 0.05$ vs. zero) (Fig. 3.4). Peak ATP release from measurements taken at 5, 10, and 15 min after the addition of Mas 7 (10 μM) was significantly greater than saline in RBCs from both young and

older adults (231.8 ± 91.4% and 259.8 ± 43.8%, respectively; $P < 0.05$) (Fig. 3.4) and was not different between age groups ($P > 0.05$).

Discussion

The primary novel findings from the present investigation are as follows. First, treatment of RBCs from older adults with the PDE3 inhibitor cilostazol does not improve the age-related impairment in deoxygenation-induced ATP release. Second, the increase in RBC intracellular cAMP in response to the G_i activator Mas 7 is not impaired in healthy older adults. Finally, RBC ATP release in response to the G_i activator Mas 7 also remains intact in healthy older adults. These collective findings provide the first evidence that advancing age is not associated with alterations in RBC intracellular cAMP signaling or responsiveness to G_i stimulation, and therefore, that this is not a mechanism of impaired deoxygenation-induced ATP release from RBCs of healthy older adults.

Impaired red blood cell ATP release in healthy older vs. young adults

In support of our previous findings (Kirby *et al.*, 2012; Chapter II of this dissertation), the results of the present study demonstrate that ATP release from RBCs of healthy older adults in response to deoxygenation is impaired relative to RBCs from healthy young adults (Fig. 3.1). More importantly, this study provides the first experimental evidence that this age-related impairment in deoxygenation-induced RBC ATP release is not the result of a broad decline in RBC function with advancing donor age, as multiple cellular functions remained intact when comparing RBCs from healthy young and older adults. First, RBCs from older adults retained the ability to increase glycolysis in hypoxia (Fig. 3.2) as a result of deoxygenated hemoglobin (deoxyHb) reversibly associating with band 3 and displacing a complex of glycolytic enzymes (Campanella *et al.*, 2005, 2008; Chu & Low, 2006; Lewis *et al.*, 2009; Puchulu-Campanella *et al.*, 2013; Chu *et al.*, 2016), which has been shown to be required for deoxygenation-induced

ATP release despite the presence of a large intracellular pool of ATP (Jagger *et al.*, 2001; Chu *et al.*, 2016). Second, RBCs from healthy older adults had preserved increases in intracellular cAMP following direct G_i stimulation with 10 μM Mas 7 compared to RBCs from healthy young adults (Fig. 3.3). An increase in intracellular cAMP has been proposed to be a crucial component of the signaling cascade for RBC ATP release (Sprague *et al.*, 2001; Ellsworth & Sprague, 2012), therefore the finding that this response is intact in RBCs from healthy older adults suggests that it is not a contributing factor to impaired deoxygenation-induced ATP release with age. Finally, RBC ATP release in response to 10 μM Mas 7 was also the same between young and older adults (Fig. 3.4), which provides additional evidence in support of the conclusion that primary aging does not affect RBC intracellular cAMP signaling or responsiveness to G_i stimulation, and that the mechanisms of impaired deoxygenation-induced ATP release from RBCs of healthy older adults must involve other factors such as the age-associated decline in RBC deformability.

Impaired red blood cell ATP release with primary aging vs. diabetes: distinct mechanisms

In contrast to RBCs from healthy older adults, evidence from the literature demonstrates that RBCs from individuals with type 2 diabetes have blunted increases in intracellular cAMP and ATP release following the same G_i stimulus used in the present study (10 μM Mas 7) compared to RBCs from healthy subjects (Sprague *et al.*, 2006, 2011) in addition to impaired deoxygenation-induced ATP release (Sprague *et al.*, 2010, 2011, Richards *et al.*, 2014*b*, 2015; Dergunov *et al.*, 2015). Furthermore, all three of these impaired responses associated with type 2 diabetes can be improved by treatment with the PDE3 inhibitor cilostazol (Hanson *et al.*, 2010; Sprague *et al.*, 2011; Dergunov *et al.*, 2015), whereas the same concentration of cilostazol (100 μM) had no effect on deoxygenation-induced ATP release from RBCs of older adults in the present study (Fig. 3.1). In comparison to the literature, the findings from the present study provide the first experimental evidence that impaired deoxygenation-induced RBC ATP release

with primary aging and type 2 diabetes is at least partly due to distinct mechanisms, with altered cAMP signaling not contributing in healthy older adults whereas it does in RBCs from individuals with type 2 diabetes.

ATP release from RBCs has been studied more extensively with diabetes than primary aging, and therefore an examination of the literature can provide some additional mechanistic insight into the factors unique to diabetes that underlie the impaired responses to deoxygenation and direct G_i activation. First, although the mechanisms are unclear, RBCs from people with type 2 diabetes have reduced expression of the G_i isoform G_{i2} (Sprague *et al.*, 2006), whereas the expression of other components in the signaling cascade that are related to regulation intracellular cAMP concentrations, including AC and PDE3, are unaffected (Sprague *et al.*, 2006, 2011). Second, hyperinsulinemia associated with type 2 diabetes could increase cAMP hydrolysis given that insulin can activate PDE3 (Degerman *et al.*, 1997; Conti & Beavo, 2007). Accordingly, insulin has been shown to blunt intracellular cAMP responses and ATP release following G_i stimulation with Mas 7 as well as deoxygenation-induced ATP release when it is co-incubated with RBCs from healthy humans (Hanson *et al.*, 2010; Richards *et al.*, 2013). Third, during the process of insulin production in pancreatic β-cells, the enzymatic cleavage of proinsulin produces mature insulin and connecting peptide (C-peptide), which are both released into the circulation and equilibrate at a C-peptide to insulin ratio of 1:1 or greater due to the longer half-life of C-peptide compared to insulin (30 min vs. 3-5 min, respectively) (Polonsky *et al.*, 1986; Duckworth *et al.*, 1998; Steiner, 2004). Similar to insulin, C-peptide can also blunt deoxygenation-induced ATP release when co-incubated with RBCs from healthy humans (Richards *et al.*, 2013). However, incubating insulin and C-peptide together at concentrations and ratios that reflect normal physiological levels no longer impairs deoxygenation-induced ATP release from RBCs of healthy humans (Richards *et al.*, 2013). More importantly, this co-incubation of C-peptide and insulin reverses the impairment in deoxygenation-induced ATP release from RBCs of people with type 2 diabetes by a mechanism that is proposed to involve

balanced activation of PDE3 (Richards *et al.*, 2014*b*, 2015), although both of these beneficial effects are lost when supraphysiological concentrations or ratios of C-peptide and insulin are used (Richards *et al.*, 2013, 2014*b*). Thus, overproduction of insulin in the earlier stages of type 2 diabetes or administration of exogenous insulin in the treatment of diabetes without co-administration of C-peptide could impair RBC ATP release by altering the normal physiological balance of these two compounds. Collectively, these findings suggest that the distinct contribution of altered cAMP signaling to impaired deoxygenation-induced ATP release from RBCs of people with type 2 diabetes compared to healthy older adults results from a combination of reduced cAMP synthesis due to lower G_i expression and an increase in PDE3-mediated hydrolysis of cAMP caused by hyperinsulinemia or altered C-peptide to insulin ratios.

Impaired red blood cell ATP release with primary aging vs. diabetes: common mechanisms

While the experimental evidence from the present and previous studies strongly suggests that there are distinct mechanisms between primary aging and diabetes that contribute to impaired deoxygenation-induced RBC ATP release, there are also overlapping changes to RBC properties with age and diabetes. As discussed in Chapter II of this dissertation, aging is associated with a decline in RBC antioxidant capacity and increased susceptibility to oxidative damage of RBC membrane proteins (Glass & Gershon, 1984; Gershon & Gershon, 1988; Gil *et al.*, 2006; Rizvi & Maurya, 2007; Chaleckis *et al.*, 2016), which can result in decreased RBC deformability (Haest *et al.*, 1977; Wang *et al.*, 1999; Tsantes *et al.*, 2006; Rifkind & Nagababu, 2013; Mohanty *et al.*, 2014). Similarly, RBCs from both type 1 and type 2 diabetics have increased oxidative stress (Schwartz *et al.*, 1991; Subasinghe & Spence, 2008; Maellaro *et al.*, 2013), reduced deformability due to oxidative damage of membrane proteins and increased tubulin content in the membrane (the latter unique to diabetes) (McMillan *et al.*, 1978; McMillan & Gion, 1981; Ernst & Matrai, 1986; Schwartz *et al.*, 1991; Linderkamp *et al.*, 1999; Caimi & Lo Presti, 2004; Hach *et al.*, 2008; Nigra *et al.*, 2016), and alterations in RBC shape (Piagnerelli *et*

al., 2007) relative to RBCs from healthy controls, all of which may be caused at least in part by elevated glucose concentrations. Accordingly, impaired deformability may depend on disease severity or the quantification methodology used as changes with diabetes are not always observed (Schwartz *et al.*, 1991; Richards *et al.*, 2014*b*). Based on the results of the experiments described in Chapter II of this dissertation, changes in RBC deformability associated with diabetes would almost certainly contribute to the impairment in deoxygenation-induced ATP release, but this has not been directly tested using pharmacological manipulation of deformability in RBCs from people with diabetes. However, the impaired responses to G_i activation in RBCs from diabetic patients are likely unrelated to decreased RBC deformability with diabetes, as neither improving deformability with Y-27632 or simvastatin nor decreasing deformability with diamide alters the response of healthy RBCs to Mas 7 (Sridharan *et al.*, 2010*b*; Thuet *et al.*, 2011; Clapp *et al.*, 2013); furthermore, the responses to G_i stimulation with Mas 7 in the present study were not impaired with age (Figs. 3.3 and 3.4) despite age-related reductions in RBC deformability (Ch. 2, Fig. 2.1; Reid *et al.*, 1976; Hegner *et al.*, 1979; Gelmini *et al.*, 1987, 1989).

Experimental considerations and limitations

The primary limitation of the present study is that cilostazol did not significantly increase the intracellular cAMP response to Mas 7 in RBCs from young or older adults (Fig. 3.3). This raises an important question about the efficacy of cilostazol-mediated inhibition of PDE3. The concentration of cilostazol used in the present study (100 μM) has been shown to increase the intracellular cAMP response to Mas 7 in RBCs from healthy humans and those with type 2 diabetes, although the improvement appears to be greater in the RBCs from type 2 diabetics compared to healthy humans (~45% increase vs. ~15% increase, respectively) (Sprague *et al.*, 2011). Given that the cAMP response to Mas 7 alone was not impaired in RBCs from healthy humans in that previous study and that it was also not different between RBCs from healthy

young and older adults in the present study (Fig. 3.3), one possible explanation is that the efficacy of cilostazol is limited when the intracellular response to G_i stimulation is not impaired and thus an effect on intracellular cAMP was not detected in the present study. Accordingly, the concentration of cilostazol used in the present study has also been shown to reverse the inhibitory effects of insulin on the intracellular cAMP and ATP release responses to Mas 7 in RBCs from healthy humans (Hanson *et al.*, 2010). Unfortunately, intracellular cAMP responses in that study were not determined with co-incubation of just Mas 7 and cilostazol, thus the efficacy of cilostazol under normal conditions (i.e., not influenced by the inhibitory effects of insulin) cannot be confirmed. However, these findings from the present and previous studies in combination with the finding that G_i-mediated increases in ATP release were also unaffected by age (Fig. 3.4) suggest that cilostazol's lack of effect on deoxygenation-induced ATP release from RBCs of healthy older adults is due to this signaling pathway not being involved in the age-related impairment rather than a lack of efficacy.

An alternative explanation for why cilostazol did not have an effect on ATP release in the present study is that cAMP may not actually be involved in the signaling cascade for RBC ATP release, as suggested by Keller *et al.* (2017) based on their recent experimental findings. This proposal is based on the specific findings that incubation of RBCs with the active cAMP analog 8Br-cAMP did not induce any ATP release independent of significant increases in RBC lysis (Fig. 5) and that treatment of RBCs with various compounds that increased intracellular cAMP had no effect of ATP release (Fig. 6) (Keller *et al.*, 2017). These findings are in direct contrast to work performed by Sprague *et al.* (2001) demonstrating that incubation of RBCs with the active cAMP analog Sp-cAMP stimulates ATP release, whereas pretreatment with Rp-cAMP, an inactive cAMP analog and inhibitor of protein kinase A, blocks deformation-induced RBC ATP release. The aforementioned studies demonstrating the efficacy of cilostazol for improving intracellular cAMP responses and ATP release from RBCs of people with diabetes or RBCs co-incubated with insulin also supports a role for cAMP in the signaling cascade for ATP release

(Hanson *et al.*, 2010; Sprague *et al.*, 2011; Dergunov *et al.*, 2015). The reasons for this discrepancy are unclear, but this is an issue that warrants further investigation in order to define more clearly the intracellular factors that regulate the release of ATP from RBCs in response to physiological stimuli such as deformation and deoxygenation, while taking special care to control and account for RBC lysis.

Accordingly, in the present study there were no significant differences in hemolysis (hemoglobin absorbance at 405 nm) between age groups during normoxia or hypoxia, and no significant correlations between hemolysis and extracellular ATP in both the DMF and cilostazol conditions (r^2 = 0.051 and -0.008, respectively; $P > 0.05$). There was also no correlation between total cholesterol and the mean percent change in extracellular ATP during hypoxia under control conditions (r^2 = 0.06; P = 0.147). Thus, RBC ATP release in the present study was primarily due to a regulated export process that was dependent on the oxygenation state of hemoglobin and influenced by donor age.

Conclusions

This series of studies demonstrates that primary aging is not associated with changes in cAMP signaling within RBCs, as both intracellular cAMP responses and ATP release following direct G_i activation remained intact in RBCs from older adults compared to young, while treatment with the PDE3 inhibitor cilostazol did not improve the age-related impairment in deoxygenation-induced ATP release from RBCs of healthy older adults. These novel findings provide the first evidence of distinct mechanisms underlying the impairment in RBC ATP release with age vs. diabetes, and suggest that treatments which aim to improve RBC-mediated vascular control in diabetes may not be applicable to aging *per se* given the collection of studies which indicate that improving intracellular cAMP responses can restore deoxygenation-induced ATP release from RBCs of people with type 2 diabetes. With this in mind, the collective findings from this dissertation thus far suggest that reductions in RBC deformability associated with

advancing donor age are the primary mechanism of impaired deoxygenation-induced ATP release in healthy older adults and should be the principal therapeutic target for improving this age-related decrement in RBC function. Whether these findings in isolated RBCs are translatable to the *in vivo* environment in humans and whether potential increases in circulating ATP following restored responsiveness of RBCs to physiological stimuli are associated with improved vascular control of skeletal muscle blood flow and oxygen delivery in response to stressors such as hypoxia or exercise remains to be determined.

Table 3.1. Subject Characteristics

	ATP release in hypoxia with cilostazol		*Intracellular cAMP*		*Mas 7-induced ATP release*	
	Young	Older	Young	Older	Young	Older
Male:Female	5:5	7:5	3:3	3:3	2:2	2:2
Age (years)	26±1	64±2*	27±1	65±3*	23±2	67±2*
Body mass index (kg/m^2)	22.3±0.7	25.4±0.8*	22.2±0.8	25.3±0.8*	22.6±1.3	23.8±0.4
Body fat (%)	23.2±2.6	32.3±1.9*	24.3±2.2	32.1±3.0	25.0±2.0	28.3±4.3
Total cholesterol (mg/dL)	158±8	200±11*	165±13	207±19*	152±10	222±24*
LDL cholesterol (mg/dL)	79±7	118±8*	82±12	126±11*	83±10	131±18
HDL cholesterol (mg/dL)	63±3	61±4	66±4	60±8	52±2	71±6*
LDL:HDL	1.3±0.1	2.0±0.2*	1.3±0.2	2.2±0.2*	1.6±0.2	1.8±0.1
Triglycerides (mg/dL)	79±8	107±10*	89±11	102±11	84±5	99±14

*P < 0.05 vs. young (within condition)

Table 3.2. Isolated red blood cell gases

			pH	PO_2 (mmHg)	PCO_2 (mmHg)	tHb (g/dL)	FO_2Hb (%)	FHHb (%)
Normoxia	Young	DMF	7.330±0.010	113.1±1.2	34.9±0.6	6.6±0.1	94.9±0.2	3.8±0.1
		Cilostazol	7.325±0.012	114.1±1.7	34.7±0.7	6.5±0.2	94.9±0.2	3.7±0.1
	Older	DMF	7.362±0.013	115.7±1.3	35.4±0.8	6.8±0.1	95.1±0.1	3.3±0.2
		Cilostazol	7.350±0.013*	114.0±1.1	37.0±0.6	6.8±0.2	95.2±0.1	3.5±0.1*
Hypoxia	Young	DMF	7.352±0.010	23.5±1.1	34.8±0.5	6.9±0.2	33.3±3.2	63.2±3.0
		Cilostazol	7.339±0.009	24.5±1.2	36.4±0.8*	6.8±0.2	35.7±3.2	60.8±2.9
	Older	DMF	7.375±0.012	24.3±0.6	36.2±0.5	7.0±0.1	34.8±1.7	61.9±1.6
		Cilostazol	7.361±0.013	24.1±0.6	36.0±0.7	7.0±0.2	35.5±1.8	61.1±1.7
Normoxia	Young	Saline	7.439±0.024	120.6	35.1±1.7	3.2±0.1	94.3±0.3	3.7±0.4
		Mas 7	7.433±0.018	114.5±1.8	36.9±0.7	3.2±0.2	94.2±0.3	3.8±0.2
	Older	Saline	7.399±0.006	120.0±0.8	35.4±0.9	2.9±0.1	92.6±0.0†	4.4±0.5
		Mas 7	7.396±0.009	119.6±1.3	36.7±1.0	3.0±0.1	93.2±0.4	4.0±0.3

PO_2 = partial pressure of oxygen, PCO_2 = partial pressure of carbon dioxide, tHb = total hemoglobin, FO_2Hb = fraction of oxygenated hemoglobin, FHHb = fraction of deoxygenated hemoglobin *$P < 0.05$ vs. vehicle control (within age); †$P < 0.05$ vs. young (within condition)

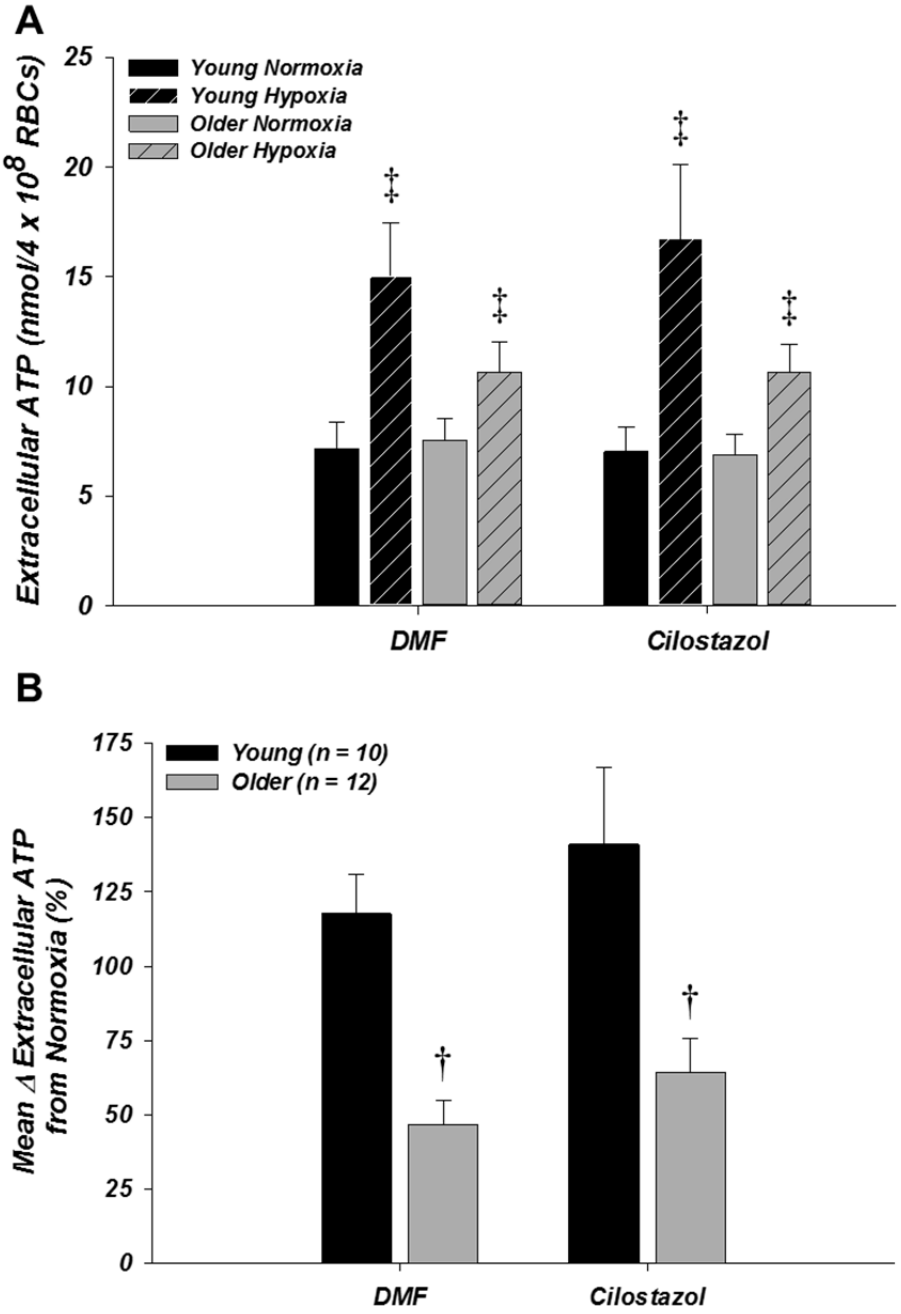

Figure 3.1. Effect of donor age and cilostazol on red blood cell ATP release in normoxia and hypoxia.

A: cilostazol had no effect on extracellular ATP in young or older adults in normoxia or hypoxia. During hypoxia, extracellular ATP from RBCs of older adults trended towards being lower compared to young adults in both the dimethylformamide (DMF; vehicle control) and cilostazol conditions (P = 0.196 and 0.146, respectively). *B*: the mean percent increase in extracellular ATP from normoxia to hypoxia was impaired in RBCs from older adults in both the DMF and cilostazol conditions. †P < 0.05 vs. young (within condition); ‡P < 0.05 vs. normoxia (within condition)

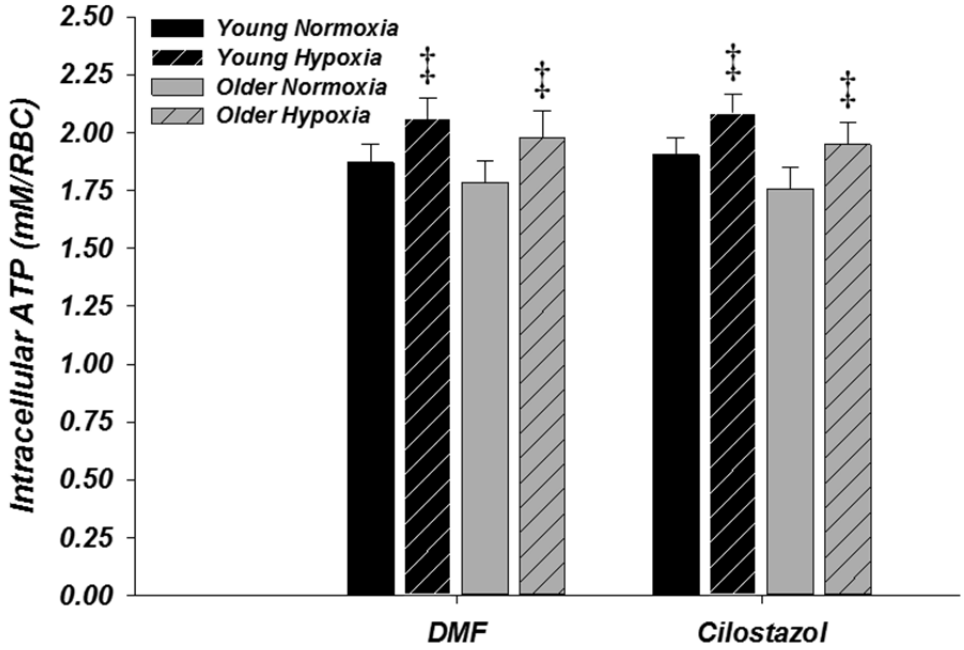

Figure 3.2. Effect of donor age and cilostazol on red blood cell intracellular ATP in normoxia and hypoxia

RBC intracellular ATP increased in hypoxia and was unaffected by donor age or cilostazol; young (n = 10) and older (n = 12). ‡$P < 0.05$ vs. normoxia (within condition)

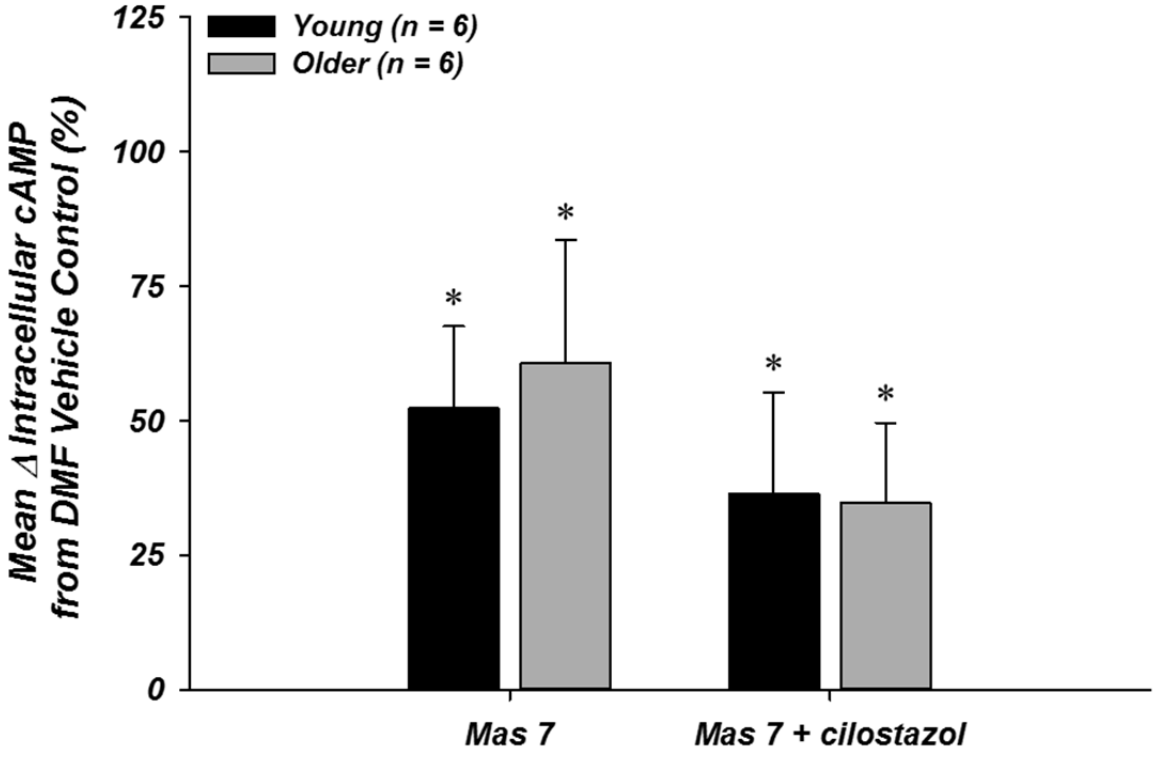

Figure 3.3. Effect of donor age and cilostazol on the red blood cell intracellular cAMP response to Mas 7

The G_i activator Mas 7 increased intracellular cAMP similarly in RBCs from young and older adults, and this response was unaffected by pretreatment of RBCs with cilostazol.

*P < 0.05 vs. zero

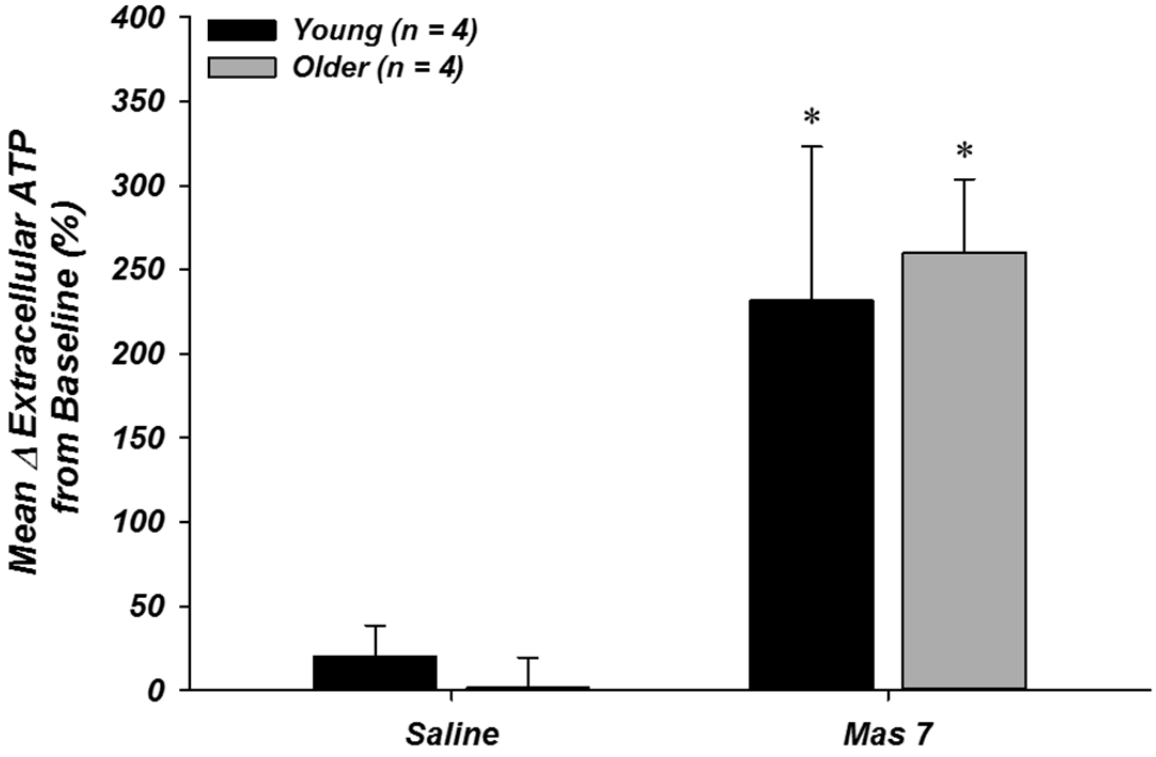

Figure 3.4. Effect of donor age on red blood cell ATP release in response to incubation with Mas 7

Extracellular ATP was unaffected by incubation with saline (vehicle and time control), whereas the G_i activator Mas 7 increased extracellular ATP similarly in RBCs from young and older adults. *P < 0.05 vs. saline (within age)

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CHAPTER IV – MANUSCRIPT III

Effect of Rho-kinase inhibition on hemodynamic responses and circulating ATP during hypoxia and exercise in healthy older adults

Summary

Circulating adenosine triphosphate (ATP) is a potent vasodilator believed to assist in the matching of tissue oxygen delivery to metabolic demand. Older adults have impaired skeletal muscle hemodynamic responses to hypoxia and exercise and blunted increases in circulating ATP during these stimuli, which may be due to reduced deoxygenation-induced red blood cell (RBC) ATP release. Based on previous findings that Rho-kinase inhibition improves RBC deformability and ATP release from isolated RBCs of older adults, the goal of the present study was to test the hypothesis that *in vivo* Rho-kinase inhibition via fasudil would improve hemodynamic responses and circulating ATP during hypoxia and exercise in older adults. Healthy young (Y; 25 ± 1 years; n = 11) and older (O; 66 ± 1 years; n = 12) adults participated in a double-blind, randomized, placebo-controlled, crossover design study on 2 days (≥ 5 days between visits). A deep venous catheter in the forearm was used to administer saline (100 mL/60 min; placebo control) or fasudil (60 mg/60 min) and to sample blood for plasma [ATP]. Forearm vascular conductance (FVC; mean arterial pressure from finometry and forearm blood flow (FBF) from Doppler ultrasound) was calculated at rest, during 5 min of isocapnic hypoxia (80% Sp_{O_2}), and during graded intensity rhythmic handgrip exercise at 5%, 15%, and 25% of maximum voluntary contraction (MVC; 4 min per workload). All age- and drug-related effects are similar when data are presented as FVC or FBF, so only FVC is reported here. Venous plasma concentration of ATP ($[ATP_V]$) was measured at rest and at the end of each condition and RBCs were isolated to measure ATP release in response to normoxic (P_{O_2} ~123 mmHg) and hypoxic (P_{O_2} ~25 mmHg) stimuli, both measured using the luciferin-luciferase technique. With saline, ΔFVC during hypoxia was ~60% lower in O vs Y and the greatest age impairment

during exercise occurred with ΔFVC from rest to 25% MVC (220.2 ± 19.4 vs 339.5 ± 25.5 mL/min/100mmHg; $P < 0.05$). There was also no increase in $[ATP]_V$ or ATP effluent (FBF x $[ATP]_V$; an index of the total circulating rate of ATP to account for the impact of changes in FBF on $[ATP]_V$ measurements) from normoxia to hypoxia in O vs Y ($P > 0.05$), and ΔATP effluent from rest to 25% MVC was also lower in O vs Y (22.5 ± 4.3 vs 44.4 ± 10.3 nmol/min; $P < 0.05$). The % Δ in isolated RBC ATP release from normoxia to hypoxia was impaired by ~75% in washed and unwashed RBCs from O vs Y as well ($P < 0.05$). In O, fasudil restored ΔFVC during hypoxia and 25% MVC compared to saline (7.8 ± 1.4 vs 2.7 ± 1.0 mL/min/100mmHg and 276.5 ± 17.3 vs 220.2 ± 19.4 mL/min/100mmHg, respectively; $P < 0.05$), abolishing the impairment vs Y. Similarly, fasudil tended to improve the increase in $[ATP]_V$ during hypoxia ($P = 0.10$ vs. normoxia and $P = 0.12$ vs. zero) and significantly improved ΔATP effluent during hypoxia and 25% MVC vs. saline in O (0.96 ± 0.38 vs. 0.24 ± 0.14 nmol/min and 36.8 ± 7.6 vs. 22.5 ± 4.3 nmol/min; $P < 0.05$). Fasudil also tended to improve isolated RBC ATP release from unwashed cells in O vs. saline (53.0 ± 13.5 vs. 15.0 ± 14.6%, respectively; $P = 0.08$). Finally, the % Δ in brachial artery diameter during exercise, a nitric oxide- (NO) dependent response, was impaired in O vs. Y at 15% (1.0 ± 0.4 vs 5.0 ± 0.9%) and 25% MVC (3.9 ± 0.7 vs 9.3 ± 1.3%) and was improved with fasudil in O at 15% (3.4 ± 0.8%) and 25% MVC (7.6 ± 1.0%) ($P < 0.05$). These data suggest that *in vivo* Rho-kinase inhibition improves hemodynamic responses to hypoxia and exercise in O at least partly via improved ATP release and NO bioavailability.

Introduction

Cardiovascular diseases (CVD) remain the leading cause of death worldwide and the majority of CVD-related mortality is associated with arterial dysfunction (Benjamin *et al.*, 2017). Advancing age is the primary risk factor for CVD, and it is estimated that over 90% of all deaths associated with CVD are observed in adults over 60 years old (Benjamin *et al.*, 2017). Furthermore, healthy (primary) aging is associated with a decline in functional capacity that

leads to reductions in exercise tolerance, functional independence, and overall quality of life (WHO, 1993). All of these age-associated changes, as well as vascular pathologies like atherosclerosis and ischemic disease, involve impairments in vascular control and the subsequent regulation of tissue blood flow and oxygen delivery.

The multifaceted nature of local blood flow regulation requires an integrated and coordinated balance between vasodilatory factors, which can arise from the vascular endothelium, circulating elements in the blood, tissue metabolites, and mechanical forces, and vasoconstricting signals from the sympathetic nervous system, vasculature, and surrounding tissues (Clifford & Hellsten, 2004; Segal, 2005; Harold Laughlin *et al.*, 2012; Hellsten *et al.*, 2012; Mortensen & Saltin, 2014). Primary aging is associated with reductions in skeletal muscle blood flow during physiological stimuli like exercise (for review, see Proctor & Parker, 2006; Wray & Richardson, 2015; Hearon Jr. & Dinenno, 2016) and hypoxia (Casey *et al.*, 2011; Richards *et al.*, 2017), as well as increases in sympathetic nervous system activity (reviewed by Dinenno & Joyner, 2006) and declines in the production or bioavailability of vasodilatory molecules. Of these alterations in vasoactive stimuli, attenuated local vasodilatory signaling is likely to be the major contributor to the age-related impairment in blood flow regulation, as data from our laboratory indicate that augmented sympathetic vasoconstriction does not contribute to the reduction in peripheral vasodilation and skeletal muscle hyperemia during hypoxia or exercise in older adults (Richards *et al.*, 2014, 2017).

Among the vasodilatory signals that are affected by aging, the blunted increases in circulating adenosine triphosphate (ATP) during hypoxia and exercise (Kirby *et al.*, 2012) may be one of the most significant impairments given the unique ability of circulating ATP to both stimulate local and conducted vasodilation via binding to purinergic P_{2Y} receptors on the endothelium (Collins *et al.*, 1998; Winter & Dora, 2007; Dora, 2017) while also blunting adrenergic vasoconstriction (Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008; Hearon Jr. *et al.*, 2017), as well as its anti-adhesive and anti-coagulative properties (Hrafnkelsdóttir *et al.*, 2001; Zhu *et*

al., 2011; Kirby *et al.*, 2014). Importantly, it has been demonstrated that the vasodilatory responsiveness to exogenous ATP is preserved in the forearm of older adults (Kirby *et al.*, 2010), and although some work indicates that this differs in other vascular beds (e.g., the leg) in a manner that is influenced by physical activity status (Mortensen *et al.*, 2012), the collective evidence suggests that potential age-related impairments in the contribution of ATP to vascular control and regulation of skeletal muscle blood flow must be related to the source of ATP.

Red blood cells (RBCs) release ATP in response to cell deformation and in direct proportion to the degree of hemoglobin deoxygenation, and can therefore contribute to the coupling of blood flow and oxygen delivery to tissue metabolic demand (Bergfeld & Forrester, 1992; Ellsworth *et al.*, 1995; Dietrich *et al.*, 2000; Ellsworth, 2000; Jagger *et al.*, 2001; González-Alonso *et al.*, 2002; Sprague *et al.*, 2009; Jensen, 2009; Ellsworth & Sprague, 2012). Furthermore, increases in circulating ATP during exercise are dependent on intact skeletal muscle perfusion (i.e., intravascular sources) (Kirby *et al.*, 2013) and deoxygenation-induced ATP release from isolated RBCs is impaired with primary aging (Kirby et al., 2012). The experimental findings from Chapters II and III of this dissertation indicate that reduced RBC deformability in older adults is the primary underlying mechanism of impaired deoxygenation-induced ATP release and that improving deformability via treatment of isolated RBCs from healthy older adults with a Rho-kinase inhibitor can restore their ability to release ATP in response to deoxygenation. However, there have been no attempts to improve RBC deformability and ATP release *in vivo*, and it is unknown if successfully doing so would improve the hemodynamic responses to hypoxia and exercise in healthy older adults. Thus, the primary goal of the present study was to test the hypothesis that systemic administration of the Rho-kinase inhibitor fasudil would improve the hemodynamic responses to hypoxia and exercise in healthy older adults and that this would be accompanied by improvements in circulating ATP and deoxygenation-induced ATP release from isolated RBCs.

Methods

Ethical approval and subjects

The study conformed to the standards set by the *Declaration of Helsinki*, except for registration in a database. With approval from the Institutional Review Board at Colorado State University, a total of 11 young and 12 older healthy adults participated in the present investigation after providing their informed, written consent. All subjects were free from overt cardiovascular disease as assessed from a medical history, free of cardiovascular medications, non-smokers, non-obese (body mass index < 30 kg/m^2), normotensive (resting blood pressure < 140/90), and sedentary to moderately active. Young female subjects were studied during the early follicular phase of their menstrual cycle to minimize any potential cardiovascular effects of sex-specific hormones, whereas older female subjects were post-menopausal and not taking hormone replacement therapy. Additionally, older subjects were further evaluated for clinical evidence of cardiopulmonary disease with a physical examination and resting and exercise (Balke protocol) electrocardiograms. Body composition was determined by whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc., USA). Whole blood lipid panels were run using a Piccolo Xpress chemistry analyzer (Abaxis, USA). Studies were performed in the Human Cardiovascular Physiology Laboratory at Colorado State University (altitude: ~1500 m) after a 4 hour fast, 24 hour abstention from alcohol/substance use, and a 12 hour abstention from caffeine and exercise, with subjects in the supine position with the experimental arm abducted to 90° and slightly elevated above heart level upon a tilt-adjustable table.

Experimental design and general experimental protocol

The overall experimental design and timeline for each experimental visit is depicted in Fig. 4.1. Using a double-blind, placebo-controlled, crossover design, subjects were randomized to receive an infusion of either saline (placebo control) or fasudil for their first experimental visit.

Subjects then received the opposite treatment for their second experimental visit, with at least five days and no more than two months between the first and second visit. Fasudil and hydroxyfasudil are metabolized quickly (half-life of ~45 min and ~280 min, respectively with a 60 mg/60 min infusion of fasudil; Shibuya *et al.*, 2005) and thus typically administered two to three times per day in clinical practice (Shibuya *et al.*, 1992, 2005, Suzuki *et al.*, 2007, 2008; Zhao *et al.*, 2011; Satoh *et al.*, 2014; Jiang *et al.*, 2015). Therefore, at least five days between visits was deemed to be sufficient for washout of any potential effects of fasudil administration.

All experimental measures were performed in the same order for each visit within a subject, with the order of hypoxia and graded-intensity rhythmic handgrip exercise trials randomized and counterbalanced between subjects. Arterial stiffness measures (augmentation index and carotid-femoral pulse wave velocity) were performed before and after placement of the venous catheter and 60 min treatment administration. For both the hypoxia and exercise trials, resting hemodynamics were measured for 2-3 min until a steady-state was observed, after which the physiological stimulus was initiated. The hypoxia trial consisted of 3 min of steady-state hypoxia at an oxygen saturation of ~80% as assessed via pulse oximetry on the earlobe (Sp_{O_2}; plus ~2 min for the normoxia to hypoxia transition). The exercise trial consisted of 4 min at each workload to ensure that steady-state hemodynamics were achieved. Timing of blood sampling is indicated by arrows; most importantly, blood samples for plasma [ATP] were taken under steady-state conditions at rest, the end of hypoxia, and the end of each exercise workload.

Venous catheterization

As described previously by our lab (Crecelius *et al.*, 2011, 2013, Kirby *et al.*, 2012, 2013), an 18- or 20-gauge (depending on inspection of vein size) 5.1 cm catheter was inserted in retrograde fashion into an antecubital vein of the experimental arm for treatment administration and deep venous blood samples. The catheter was connected to a three-way

stopcock, with one connection to an intravenous solution set for treatment administration followed by continuous flushing with saline at a rate of approximately 2 mL/min for the duration of the study to keep it patent and the other connection to a 10- or 3-mL syringe for blood sampling.

Intravenous fasudil and placebo (saline) administration

Fasudil monohydrochloride (fasudil; LC Laboratories, Woburn, MA, USA) was prepared in saline (10 mg/1 mL sodium chloride 0.9% PF injection; Pencol Compounding Pharmacy, Denver, CO, USA) and passed all measures for purity by HPLC, sterility, endotoxins, and fungal presence (Analytical Research Laboratories, Oklahoma City, OK, USA) prior to use. 60 mg of fasudil (6 mL vial) was added to a 100 mL saline bag immediately prior to administration, covered to protect it from exposure to light, and infused intravenously over 60 min (Shibuya *et al.*, 2005). This single dose of fasudil was well tolerated by both young and older adults, and no adverse events were observed or reported in either age group. For the placebo control trial, saline administration was performed identically to fasudil administration, with a covered 100 mL saline bag infused intravenously over 60 min. Fasudil mixed in saline was indistinguishable from saline alone, thus all investigators remained blinded during treatment administration.

Forearm blood flow and vascular conductance

A 12 MHz linear-array ultrasound probe (Vivid 7, General Electric, Milwaukee, WI, USA) was used to determine brachial artery mean blood velocity (MBV) and diameter proximal to the catheter insertion site as described previously (Crecelius *et al.*, 2011; Kirby *et al.*, 2012; Hearon Jr. *et al.*, 2017; Richards *et al.*, 2017). Foam tape was used to mark the outline of the probe for consistent placement and measurement over the course of the experiments. For blood velocity measurements, the probe insonation angle was maintained at < 60 degrees and the frequency used was 5 MHz. The Doppler shift frequency spectrum was analyzed via a Multigon 500M

TCD (Multigon Industries, Mt. Vernon, NY, USA) spectral analyzer from which MBV 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 (at least in triplicate) during steady-state conditions (Crecelius *et al.*, 2011; Kirby *et al.*, 2012; Richards *et al.*, 2017). Forearm blood flow (FBF) was calculated as: FBF = MBV × π × (brachial artery diameter/2)^2 × 60, where FBF is expressed as mL/min, MBV as cm/s, brachial diameter as cm, and 60 was used to convert from mL/s to mL/min. Forearm vascular conductance (FVC) was calculated as (FBF/MAP) × 100 and expressed as mL/min/100 mmHg (Kirby *et al.*, 2012; Richards *et al.*, 2014, 2017; Hearon Jr. *et al.*, 2017). All studies were performed in a semi-darkened, cool (20-22°C), temperature-controlled environment with a fan directed toward the forearm to minimize the contribution of skin blood flow to forearm hemodynamics.

Systemic isocapnic hypoxia

The systemic isocapnic hypoxia trial was performed using a self-regulating partial rebreathe system developed by Banzett et al. (2000) and more recently described by our laboratory (Markwald *et al.*, 2011; Crecelius *et al.*, 2011; Kirby *et al.*, 2012; Richards *et al.*, 2017). This system allows for constant alveolar fresh air ventilation independent of changes in breathing frequency or tidal volume (Banzett *et al.*, 2000; Dinenno *et al.*, 2003; Wilkins *et al.*, 2008). Using this system, we were able to clamp end-tidal CO_2 levels despite the hypoxia-induced increases in ventilation. The level of oxygen was manipulated by mixing nitrogen with medical air via an anesthesia gas blender. Specifically, inspired oxygen was titrated to achieve an Sp_{O_2} of ~80%. Subjects breathed through a scuba mouthpiece with a nose-clip to prevent nasal breathing. An anesthesia monitor (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA) was used to determine heart rate (HR; 3-lead ECG) and expired CO_2 sampled at the mouthpiece. Ventilation was measured via a turbine pneumotachograph (model 17125 UVM, Vacu-Med, Ventura, CA, USA).

Graded-intensity rhythmic handgrip exercise

Maximum voluntary contraction (MVC) was determined for the experimental arm as the average of three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3% of each other. Rhythmic handgrip exercise during the trials was performed with weights corresponding to 5%, 15%, and 25% MVC attached to a pulley system and lifted 4-5 cm over the pulley at a duty cycle of 1 s contraction-2 s relaxation (20 contractions per min) using both visual and auditory feedback to ensure the correct timing (Dinenno & Joyner, 2003, 2004; Kirby *et al.*, 2012; Richards *et al.*, 2014). Handgrip exercise was performed for four min at each workload, for a total of 12 min.

Blood sampling and measurement of [fasudil], [hydroxyfasudil], plasma [ATP], plasma [Hb], and blood gases

Timing of deep venous blood samples is indicated by arrows in Fig. 4.1. Based on preliminary pharmacokinetics experiments performed in our laboratory (data not shown), a blood sample for peak plasma concentrations of fasudil and hydroxyfasudil was taken ~15 min after the treatment infusion ended and an additional sample was taken at the end of the study immediately prior to catheter removal to confirm that concentrations of each compound remained at a level that can effectively inhibit Rho-kinase, which has been shown to range from 0.08-1.9 μM for fasudil (Davies *et al.*, 2000; Wickman *et al.*, 2003; Shibuya *et al.*, 2005; Rikitake *et al.*, 2005; Jacobs *et al.*, 2006; Satoh *et al.*, 2012) and from 0.04-1.8 μM for hydroxyfasudil (Shimokawa *et al.*, 1999; Shimokawa, 2002; Shibuya *et al.*, 2005; Rikitake *et al.*, 2005; Jacobs *et al.*, 2006; Satoh *et al.*, 2012).

The plasma concentrations of fasudil and hydroxyfasudil were measured by triple quadrupole UPLC-MS/MS. Stock solutions of standards fasudil (1 mg/mL), hydroxyfasudil (0.5 mg/mL) and ranitidine (1 mg/mL) were prepared in methanol. A 9-point calibration curve was prepared with fasudil and hydroxyfasudil using ranitidine as an internal standard in methanol,

and in a pooled blank plasma created by mixing aliquots of plasma from test subjects following saline injection. Analytical samples and matrix calibration solutions were prepared by mixing a 230 µL aliquot of plasma, 400 µL methanol and 70 µL of a 5 µg/mL solution of ranitidine in an Eppendorf tube, they were vortexed for 30s, and centrifuged at 11,000 rpm for 5 min. 150 µL of the supernatant was transferred to an autosampler vial. QC samples and blanks were run every 8 injections and all injections were introduced in duplicate. A Waters H-class Acquity UPLC systems in-line with a Waters triple quadrupole mass spectrometer (TQD) equipped with an electrospray ionization (ESI) source was used for separation and detection of the target analytes. A Waters Acquity BEH UPLC column (50 x 2.1 mm 1.7 µm particle size) was used with gradient separation. Mobile phase A was water with 0.1% formic acid and 2 mM ammonium acetate, mobile phase B was methanol. The gradient started at 50% B, held for 0.1 min, ramped to 80% B over 2.5 min, held at 80% B for 0.5 min, returned to 50% B over 0.1 min, and equilibrated at 50% B for 0.9 min for total UPLC run time of 4 min. Source conditions on the TQD were as follows: capillary voltage 2.4 kV, cone voltage 40 V, source temperature 150°C, desolvation temperature 200°C, desolvation gas flow 550 L/hr, cone gas flow 1 L/hr, extractor 3 V and RF lens 2.5 V. Transitions used for quantitation (quant) as confirmatory qualifiers (qual) along with dwell times, cone voltage and collision voltages were as follows: fasudil parent m/z 292.1, daughter m/z 69.1, dwell 0.008 s, cone 40 V, collision 24 V (qual); fasudil parent m/z 292.1, daughter 99.2, dwell 0.008 s, cone 40 V, collision 18 V (quant); hydroxyfasudil parent 308.2, daughter 69.0, dwell 0.008 s, cone 54 V collision 24 V (qual); hydroxyfasudil parent 308.2, daughter 99.2, dwell 0.008 s, cone 50 V, collision 26 V (quant); ranitidine parent 315.2, daughter 97.0, dwell 0.005 s, cone 34 V, collision 52 V (qual); ranitidine parent 315.2, daughter 175.9, dwell 0.005 s, cone 34 V, collision 19 V (quant). Data were processed using the response ratio for target analytes fasudil and hydroxyfasudil to the internal standard ranitidine (Chen *et al.*, 2010).

Blood samples for plasma [ATP] and blood gases were taken immediately after the treatment infusion (ATP standard curve sample) and at the end of rest, hypoxia, and each exercise intensity. Our method for blood sampling, preparation, and measurement of plasma [ATP] (Kirby *et al.*, 2012, 2013; Crecelius *et al.*, 2013) generally follows the procedures established by Gorman and colleagues (Gorman *et al.*, 2003, 2007) and was performed as previously described in detail (Kirby *et al.*, 2012). Briefly, ~3-5 mL of venous blood was drawn directly into a pre-heparinized 10 mL syringe, from which 2 mL was gently and at once expelled into a tube containing 2.7 mL of an ATP-stabilizing solution to equal a blood:diluent ratio of 1.35 (Gorman *et al.*, 2003, 2007, Kirby *et al.*, 2012, 2013; Crecelius *et al.*, 2013). This ATP-stabilizing solution is used to inhibit the degradation of ATP via nucleotidases and additional ATP release post-sampling. The blood-diluent mixture was immediately centrifuged at 4000 rpm (~1200*g*) for 3 min at 22°C, and 100 µL of the supernatant was taken for measurement of plasma [ATP] via luciferin-luciferase assay. An ATP standard curve was created on each visit prior to hypoxia and exercise trials using plasma from each subject studied as the medium. All plasma ATP measures were performed at least in triplicate.

To account for the potential influence of RBC hemolysis on measures of plasma [ATP], which can increase significantly with only small amounts of hemolysis, a 1 mL sample of plasma supernatant from the blood-diluent mixture was taken immediately following ATP measurements and analyzed for plasma hemoglobin (Hb) via spectrophotometry (SpectraMax, Molecular Devices, Sunnyvale, CA, USA) at wavelengths of 415, 380, and 450 nm as described previously by our laboratory (Kirby *et al.*, 2012, 2013; Crecelius *et al.*, 2013). The percentage hemolysis was then calculated as ((100 – hematocrit) × plasma [Hb]/total [Hb]) × 100. Any sample that was more than two standard deviations from the mean percentage hemolysis was excluded from the analysis and regarded as a technical error.

Blood gas samples (~2 mL) were immediately (< 1 min) analyzed with a clinical blood gas analyzer (Rapid Point 400 Series Automatic Blood Gas System, Siemens Healthcare

Diagnostics, Deerfield, IL, USA) for partial pressures of oxygen and carbon dioxide (PO_2 and PCO_2), pH, fraction of oxygenated hemoglobin (FO_2Hb), oxygen content, and Hb.

Isolation of red blood cells

Blood was collected from the antecubital vein catheter into two Vacutainer tubes containing sodium heparin (158 USP units). It was unclear if the potential effects of *in vivo* Rho-kinase inhibition would be diminished by cell washing during the normal RBC isolation process as described previously by our lab (Kirby *et al.*, 2012) given that both fasudil and hydroxyfasudil inhibit Rho-kinase via competitive inhibition at the ATP binding site (Jacobs *et al.*, 2006), therefore RBCs were isolated from one tube of blood with washing and from the second tube without washing. For both tubes, whole blood was initially centrifuged (500*g*, 4°C, 10 min) followed by removal of the plasma and buffy coat by aspiration. For unwashed RBCs, excess blood was aspirated in order to remove as much of the buffy coat as possible. For washed RBCs, packed cells were resuspended in cell wash buffer (CWB) containing (in mM) 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 21.0 Tris-base, 5.5 glucose, and 0.5% BSA, with pH adjusted to 7.4 at room temperature. Centrifugation, buffy coat aspiration, and resuspension in CWB were repeated two additional times for a total of three wash cycles. All RBC ATP measures were performed immediately after RBC isolation was completed.

Red blood cell deoxygenation and measurement of extracellular ATP

The paired washed and unwashed RBCs were diluted to 20% hematocrit with a bicarbonate-based buffer containing (in mM) 4.7 KCl, 2.0 $CaCl_2$, 1.2 $MgSO_4$, 140.5 NaCl, 11.1 glucose, 23.8 $NaHCO_3$, and 0.5% BSA warmed to 37°C. These 20% hematocrit RBC suspensions were placed in a rotating bulb tonometer (Eschweiler GmbH & Co. KG, Germany) and incubated for 30 min in normoxia (16% O_2, 6% CO_2, balanced nitrogen; PO_2 ~123 mmHg and FO_2Hb ~95% across all age groups and conditions) at 37°C. Samples of washed and

unwashed RBCs were removed from each tonometer bulb for measurement of extracellular and intracellular ATP in normoxia (details below). RBCs were then deoxygenated by exposure to hypoxia (1.5% O_2, 6% CO_2, balanced nitrogen; PO_2 ~25 mmHg and FO_2Hb ~34% across all age groups and conditions) for 15 min and RBC samples were taken for measurement of ATP as in normoxia. Normoxic and hypoxic gases were blended via gas blender (MCQ Gas Blender Series 100, Italy) and humidified before introduction into the tonometer bulbs. Blood gases were confirmed by blood gas analysis (Siemens Rapid Point 405 Series Automatic Blood Gas System, Los Angeles, CA) (Kirby *et al.*, 2012).

ATP was measured via luciferin-luciferase technique as described previously (Sprague *et al.*, 2001; Sridharan *et al.*, 2010*b*, 2010*a*; Thuet *et al.*, 2011; Kirby *et al.*, 2012; Richards *et al.*, 2013), with light emission during the reaction detected by a luminometer (TD 20/20, Turner Designs). For extracellular ATP (i.e., ATP release) measurements, a 10 µL sample of the 20% hematocrit suspension was taken from each tonometer bulb and diluted 500-fold (0.04% hematocrit), from which a 200 µL sample was taken and injected into a cuvette containing 100 µL of firefly tail extract (10 mg/mL DI water; Sigma) and 100 µL of D-luciferin (0.5 mg/mL DI water; Research Products International). Peak light output was measured at least in triplicate for each experimental condition and the mean was used for determination of ATP levels by comparison to a standard curve for ATP (Calbiochem) generated on the day of the experiment. Cell counts were obtained from each 0.04% RBC suspension and extracellular ATP was normalized to 4×10^8 cells. To confirm that ATP release was not due to hemolysis, the 0.04% RBC suspensions from which samples for ATP analysis and cell counting were taken were analyzed for free hemoglobin by measuring absorbance at 405 nm similar to previous reports (Sprague *et al.*, 1998, 2011; Sridharan *et al.*, 2010*a*; Thuet *et al.*, 2011; Kirby *et al.*, 2012, 2014; Richards *et al.*, 2013) as well as at 570 nm and subtracting out the background at 700 nm as recently suggested by Keller and colleagues (Keller *et al.*, 2017), and samples with significant lysis were excluded.

Measurement of total red blood cell intracellular ATP

To determine if donor age or *in vivo* fasudil administration affected total intracellular ATP or the increase in RBC glycolytic activity during hypoxia (Messana *et al.*, 1996; Campanella *et al.*, 2005; Lewis *et al.*, 2009), 50 µL samples of drug- and saline-treated RBCs (20% hematocrit) were taken from the tonometer bulbs in normoxia and hypoxia and lysed in DI water at room temperature (a 20-fold dilution). This lysate was diluted an additional 400-fold (8000-fold total) and ATP was measured using the same ATP assay used for determination of extracellular ATP (Sridharan *et al.*, 2010*b*, 2010*a*; Sprague *et al.*, 2011; Thuet *et al.*, 2011; Kirby *et al.*, 2012, 2014). Values were normalized to ATP concentration per RBC.

Central artery stiffness: carotid-femoral pulse wave velocity and central augmentation index

Carotid-femoral pulse wave velocity (cfPWV) and central augmentation index (AIx) were determined noninvasively using the SphygmoCor XCEL (AtCor Medical) as described previously (Butlin *et al.*, 2013; Hwang *et al.*, 2014; Butlin & Qasem, 2016; Shoji *et al.*, 2017; Suleman *et al.*, 2017; Nakagomi *et al.*, 2018). For determination of cfPWV, the device automatically calculates the ratio of the time delay between femoral pulse waves acquired using a cuff-based approach and carotid pulse waves acquired by applanation tonometry to the corrected distance between pulse measuring sites. Distance correction was performed by factoring in measurements from the suprasternal notch to the carotid site and the proximal edge of the thigh cuff (placed midway between hip and the knee) to the femoral artery at the inguinal ligament, both made using a nonstretchable tape measure, and a measurement from the suprasternal notch to the proximal edge of the thigh cuff that was made with tree calipers to avoid overestimation of the distance between these two points. For AIx measures, the SphygmoCor XCEL utilizes a validated cuff-based approach to derive AIx automatically from measurements over the brachial artery that are processed and transformed by the device's software using a proprietary generalized transfer function. In the present experiment, the

brachial cuff was placed midway between the shoulder and elbow of the arm contralateral to the catheter.

Data acquisition and analysis

All *in vivo* data were collected and stored on a computer at 250 Hz and were analyzed offline with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Resting MAP was determined non-invasively over the brachial artery (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA). Beat-by-beat MAP was measured at the heart level by finger photoplethysmography (Finometer, FMS, Netherlands) on the middle finger of the control hand during hypoxia and rhythmic handgrip exercise trials (Kirby *et al.*, 2012). FBF, HR, MAP, and oxygen saturations (pulse oximetry) represent an average of the last 30 seconds of the appropriate time period. Minute ventilation and end-tidal CO_2 in the hypoxia trial were determined from an average of the data over the last minute of each time period in order to ensure an adequate number of sampling points.

Venous oxygen content (CvO_2) determined from deep venous blood samples taken at the end of rest, hypoxia, and each exercise intensity was combined with estimates of arterial oxygen content (CaO_2) in normoxia (203.7 mL O_2 / L blood) and hypoxia (165.4 mL O_2 / L blood) based on data collected previously by our laboratory via brachial artery catheter in both young and older adults (Richards *et al.*, 2014, 2017; no difference with age) in order to quantify oxygen delivery, extraction, and consumption. Arteriovenous oxygen difference was calculated as $CaO_2 - CvO_2$. Oxygen delivery was calculated as ($CaO_2 \times FBF \times 0.001$) and expressed in mL/min. Oxygen extraction, reported as a percent, was calculated as $((CaO_2 - CvO_2)/CaO_2 \times 100)$. Oxygen consumption across the forearm ($\dot{V}_{O2}$) was calculated as $((CaO_2 - CvO_2) \times FBF \times 0.001)$ and expressed in mL/min.

To account for changes in FBF and its impact on [ATP] concentration measurements and to quantify the rate of total ATP draining the active muscle, ATP effluent was calculated as

FBF × [ATP] × 0.001, as quantified previously by our laboratory (Kirby *et al.*, 2012; Crecelius *et al.*, 2013) and similar to other methods of data quantification when blood flow is altered (González-Alonso *et al.*, 2002; Giannarelli *et al.*, 2009).

Statistics

All values are reported as mean ± SEM. All analyses were performed using R (R Core Team 2016, R Foundation for Statistical Computing, Vienna, Austria) with the lme4, lmerTest, pbkrtest, and lsmeans packages. Age (young or older), drug (saline or fasudil), condition (rest or exercise intensity or hypoxia), and age×drug×condition for three-way repeated measures or age×drug for two-way repeated measures ANOVA were treated as fixed effects. In order to account for the crossover design, subject and subject×drug were in included in the model as random effects for the three-way repeated measures analyses and subject was included as a random effect for the two-way repeated measures analyses. When an interaction or main effect was found, appropriate pairwise comparisons were made and a Tukey test was included when necessary. Comparisons of variables relative to zero were tested using a one-tailed t-test, and differences in subject characteristics were tested using a two-tailed t-test. Comparisons between saline and fasudil were performed within age group and comparisons between young and old were performed within condition. Significance was set at $P < 0.05$.

Results

Subject characteristics and plasma [fasudil] and [hydroxyfasudil]

Subject characteristics are reported in Table 4.1. The mean age difference between the young and older adults was 41 years. Compared to the young adults, older adults had higher body fat percentage, total cholesterol, and LDL cholesterol ($P < 0.05$); however, all values were still within the normal healthy range. In a subset of subjects (n = 14; 7 young and 7 older adults), blood samples were taken approximately 15 min after the treatment infusion stopped

(peak) and at the end of the study just before the venous catheter was removed (end) for measurement of plasma concentrations of fasudil and hydroxyfasudil. The average [fasudil] at peak and end was 1.003 ± 0.321 μM and 0.071 ± 0.010 μM, respectively, and the average [hydroxyfasudil] at peak and end was 2.208 ± 0.190 μM and 1.013 ± 0.133 μM, respectively. Importantly, there were no differences between young and older adults and fasudil was well tolerated in all subjects with no adverse events.

Effects of age and fasudil on ventilatory, hemodynamic, and plasma ATP responses during systemic isocapnic hypoxia

Hemodynamic and ventilatory responses during normoxia and systemic isocapnic hypoxia are reported in Table 4.2. SpO_2, minute ventilation, and end-tidal CO_2 were not significantly different between age groups in the saline condition, whereas in the fasudil condition there were some age-related differences in SpO_2 and minute ventilation as well as an effect of fasudil on minute ventilation; however, ~80% SpO_2 was achieved in all conditions. MAP was significantly higher in older vs. young adults during normoxia and hypoxia with saline and this was decreased with fasudil ($P < 0.05$) (Table 4.2 and Fig. 4.2E). There were no differences in baseline (normoxia) FBF or FVC between age groups or treatment conditions (Table 4.2, Figs. 4.2A and 4.2B). The increase in FBF and FVC from normoxia to hypoxia was impaired in older vs. young adults in the saline control condition (2.2 ± 0.6 vs. 6.3 ± 1.2 mL/min and 2.7 ± 1.0 vs. 7.1 ± 1.4 mL/min/100 mmHg, respectively; $P < 0.05$), and this was completely reversed for both FBF and FVC following fasudil administration such that there was no longer a difference between older and young adults (7.2 ± 1.6 vs. 6.2 ± 1.2 mL/min and 7.8 ± 1.4 vs. 6.2 ± 1.1 mL/min/100 mmHg, respectively; $P > 0.05$) (Figs. 4.2C and 4.2D).

Resting venous plasma [ATP] and ATP effluent in normoxia were not different between young and older adults in saline (79.9 ± 16.1 vs. 101.2 ± 16.6 nmol/L and 1.6 ± 0.4 vs. 2.2 ± 0.4 nmol/min, respectively; $P > 0.05$) or fasudil (81.3 ± 16.8 vs. 66.9 ± 12.8 nmol/L and 1.5 ± 0.3 vs.

1.5 ± 0.3 nmol/min, respectively; $P > 0.05$) conditions, and within each age group these values were not different between saline and fasudil ($P > 0.05$) (Figs. 4.3A and 4.3B). In older adults, $[ATP]_V$ and ATP effluent did not increase during hypoxia compared to normoxia with saline (105.0 ± 20.4 vs. 101.2 ± 16.6 nmol/L and 2.4 ± 0.4 vs. 2.2 ± 0.4 nmol/min, respectively; $P > 0.05$) (Figs. 4.3A and 4.3B), whereas this increase during hypoxia vs. normoxia was improved with fasudil for $[ATP]_V$ (82.8 ± 23.5 vs. 66.9 ± 12.8 nmol/L, respectively; $P = 0.10$) and ATP effluent (2.5 ± 0.7 vs. 1.5 ± 0.3 nmol/min, respectively; $P < 0.05$) (Figs. 4.3A and 4.3B). Similarly, $\Delta[ATP]_V$ from normoxia to hypoxia in older adults was not significantly different from zero with saline, but tended to be with fasudil ($P = 0.12$) (Fig. 4.3C), and ΔATP effluent during hypoxia was impaired in older vs. young adults with saline (0.24 ± 0.14 vs. 1.08 ± 0.21 nmol/min, respectively; $P < 0.05$) and restored with fasudil (0.96 ± 0.38 nmol/min; $P < 0.05$ vs. saline) (Fig. 4.3D).

Effects of age and fasudil on hemodynamic, plasma ATP, and brachial artery diameter responses during graded-intensity rhythmic handgrip exercise

Hemodynamic responses at baseline and during graded-intensity rhythmic handgrip exercise are reported in Table 4.3. MAP was significantly higher in older vs. young adults at all time points with saline ($P < 0.05$) and this difference was improved with fasudil (Table 4.3 and Fig. 4.4E; $P < 0.05$). Importantly, fasudil did not alter the increase in MAP from rest at each exercise intensity in either age group relative to saline (Fig. 4.4F; $P > 0.05$).

There were no effects of age or fasudil on resting FBF or FVC (Table 4.3, Figs. 4.4A and 4.4B; $P > 0.05$). With saline, the increase in FBF and FVC from rest to exercise was impaired in older vs. young adults at 25% MVC (248.0 ± 20.1 vs. 327.8 ± 31.1 mL/min and 220.2 ± 19.4 vs. 339.5 ± 25.5 mL/min/100 mmHg, respectively; $P < 0.05$) and also at 15% MVC for FVC (134.3 ± 13.3 vs. 194.0 ± 17.4 mL/min/100 mmHg, respectively; $P < 0.05$) (Figs. 4.4C and 4.4D). With fasudil, the increase in FBF and FVC from rest to exercise was blunted in young adults at 25%

MVC compared to saline (290.1 ± 26.7 vs. 327.8 ± 31.1 mL/min and 286.8 ± 21.9 vs. 339.5 ± 25.5 mL/min/100 mmHg, respectively; $P < 0.05$) (Figs. 4.4C and 4.4D). In contrast, fasudil significantly improved the change in FBF and FVC at 25% MVC in older adults compared to saline (289.5 ± 22.3 vs. 248.0 ± 20.1 mL/min and 276.5 ± 17.3 vs. 220.2 ± 19.4 mL/min/100 mmHg, respectively; $P < 0.05$) (Figs. 4.4C and 4.4D).

Venous plasma [ATP] and ATP effluent at rest were not different between young and older adults in saline (75.7 ± 12.9 vs. 53.5 ± 6.0 nmol/L and 1.6 ± 0.4 vs. 1.2 ± 0.2 nmol/min, respectively; $P > 0.05$) or fasudil (74.3 ± 11.2 vs. 81.0 ± 15.6 nmol/L and 1.8 ± 0.4 vs. 2.1 ± 0.5 nmol/min, respectively; $P > 0.05$) conditions, and within each age group these values were not different between saline and fasudil ($P > 0.05$) (Figs. 4.5A and 4.5B). During 5% MVC exercise in the saline condition, older adults had significantly lower plasma $[ATP]_V$ compared to young adults (61.5 ± 9.8 vs. 111.8 ± 15.9, respectively; $P < 0.05$) and there was a trend for lower ATP effluent in older vs. young adults as well (4.6 ± 1.1 vs. 8.0 ± 1.3, respectively; $P = 0.07$), both of which significantly improved with fasudil (132.9 ± 22.3 nmol/L and 9.5 ± 2.0 nmol/min, respectively; $P < 0.05$ vs. saline) (Figs. 4.5A and 4.5B). Fasudil also tended to increase plasma $[ATP]_V$ at 15% and 25% MVC in older adults compared to saline (127.4 ± 28.1 vs. 88.5 ± 10.8 nmol/L, $P = 0.09$ and 123.6 ± 23.6 vs. 83.8 ± 12.1 nmol/L, $P = 0.08$, respectively) (Fig. 4.5A). Similarly, ATP effluent was significantly impaired in older vs. young adults with saline at 25% MVC (23.8 ± 4.4 vs. 46.3 ± 10.5 nmol/min; $P < 0.05$) and this response was improved with fasudil in older adults (38.9 ± 7.8; $P < 0.05$ vs. saline) (Fig. 4.5B). In the saline condition, $\Delta[ATP]_V$ from rest to exercise was only impaired with age at 5% MVC and this was significantly improved with fasudil (Fig. 4.5C). The ΔATP effluent from rest to exercise was also significantly impaired in older vs. young adults with saline at 5% and 25% MVC (3.31 ± 0.85 vs. 6.36 ± 1.04 nmol/min and 22.54 ± 4.27 vs. 44.43 ± 10.28 nmol/min, respectively; $P < 0.05$), and this impairment was improved with fasudil in older adults at 5% MVC (7.10 ± 1.70 nmol/min) and 25% MVC (36.83 ± 7.56 nmol/min) compared to saline ($P < 0.05$) (Fig. 4.5D).

Absolute brachial artery diameter was not different between age groups at any time point, although fasudil did increase brachial artery diameter relative to saline at rest and 25% MVC in young adults and 15% and 25% MVC in older adults ($P < 0.05$) (Fig. 4.6A). The relative (%) change in brachial artery diameter from rest to exercise, measured during the last ~30 sec at a given workload, was significantly impaired in older vs. young adults with saline at 15% MVC (1.0 ± 0.4 vs. 5.0 ± 0.9%, respectively) and 25% MVC (3.9 ± 0.7 vs. 9.3 ± 1.3%, respectively) ($P < 0.05$) (Fig. 4.6B). Fasudil significantly improved this increase in brachial artery diameter at 15% and 25% MVC in older adults (3.4 ± 0.8% and 7.6 ± 1.0%, respectively; $P < 0.05$ vs. saline) such that there was no longer an age-related impairment (Fig. 4.6B).

Effects of age and fasudil on oxygen delivery, extraction, and consumption during systemic isocapnic hypoxia and graded-intensity rhythmic handgrip exercise

In the hypoxia trial, there was a trend for forearm $\dot{V}_{O2}$ to decrease during hypoxia relative to normoxia in older adults with saline (2.0 ± 0.3 vs. 2.4 ± 0.2 mL/min, respectively; $P = 0.07$) (Fig. 4.7E). Fasudil significantly increased oxygen delivery and forearm $\dot{V}_{O2}$ during hypoxia in older adults compared to saline (4.8 ± 0.5 vs. 3.8 ± 0.4 mL/min and 2.5 ± 0.4 vs. 2.0 ± 0.3 mL/min, respectively; $P < 0.05$) (Figs. 4.7A and 4.7E). In the exercise trial, oxygen delivery and forearm $\dot{V}_{O2}$ were both impaired in older vs. young adults at 25% MVC with saline (55.0 ± 4.4 vs. 73.2 ± 8.8 mL/min and 36.3 ± 3.0 vs. 43.8 ± 4.1 mL/min, respectively; $P < 0.05$) (Figs. 4.7B and 4.7F). These age-related impairments at 25% MVC were reversed with fasudil due to improvements in both oxygen delivery (64.2 ± 4.8 mL/min) and forearm $\dot{V}_{O2}$ (40.6 ± 3.4 mL/min) in the older adults relative to saline ($P < 0.05$) (Figs. 4.7B and 4.7F).

Effects of age and fasudil on isolated red blood cell extracellular and intracellular ATP

Blood gases for isolated RBCs are shown in Table 4.4. The only differences between age groups or drug condition in the fraction of oxygenated hemoglobin ($F_{O_2}Hb$), which provides

an index of the stimulus for ATP release given the linear relationship between hemoglobin oxygenation state and extracellular ATP, was a higher value during hypoxia in washed RBCs from older vs. young adults with fasudil (35.2 ± 1.6 vs. 26.7 ± 3.6%, respectively; $P < 0.05$) (Table 4.4). With washed RBCs, the increase in extracellular ATP from normoxia to hypoxia was significantly impaired in older vs. young adults with saline (52.5 ± 16.7 vs. 165.4 ± 53.0%, respectively; $P < 0.05$) and fasudil (17.4 ± 13.2 vs. 113.7 ± 25.4%, respectively; $P < 0.05$) (Fig. 4.8C). With unwashed RBCs, the increase in extracellular ATP from normoxia to hypoxia was also significantly impaired in older vs. young adults with saline (15.0 ± 14.6 vs. 92.7 ± 15.8%, respectively; $P < 0.05$) and fasudil (53.0 ± 13.5 vs. 105.4 ± 16.3%, respectively; $P < 0.05$), although there was a trend for fasudil to improve this change in extracellular ATP in the older adults relative to saline (53.0 ± 13.5 vs. 15.0 ± 14.6%, respectively; $P = 0.08$) (Fig. 4.8D). Similarly, extracellular ATP from unwashed RBCs of older adults was not elevated in hypoxia compared to normoxia with saline (14.1 ± 2.6 vs. 12.3 ± 1.8 nmol/4x10^8 RBCs, respectively; $P > 0.05$) whereas it was with fasudil (16.5 ± 2.7 vs. 11.6 ± 2.0 nmol/4x10^8 RBCs, respectively; $P < 0.05$) (Fig. 4.8B). Finally, intracellular ATP increased in hypoxia ($P < 0.05$) and there were no differences between age groups or drug conditions (Figs. 4.8E and 4.8F).

Effects of age and fasudil on central artery stiffness

Carotid-femoral pulse wave velocity (cfPWV) was significantly higher in older vs. young adults and was not significantly changed by fasudil administration in either age group (Fig. 4.9A). Similarly, central augmentation index (AIx) was also significantly elevated in older relative to young adults and was unaffected by fasudil administration (Fig. 4.9B).

Discussion

This is the first study to investigate the use of fasudil in healthy older adult humans as a means to improve the age-related impairments in circulating ATP responses to systemic

hypoxia and exercise, and the control of vascular tone and peripheral blood flow during these physiological stimuli in this population. The primary novel findings are as follows. First, fasudil completely reversed the impairments in local vasodilatory and blood flow responses to systemic hypoxia in older adults, accompanied by a general trend for improvements in ATP release based on measures of venous plasma [ATP] and the rate of ATP effluent from skeletal muscle during the hypoxic stimulus. Second, fasudil significantly improved the age-related impairment in vasodilation and blood flow during high-intensity (25% MVC) rhythmic handgrip exercise, and this was also accompanied by improvements in circulating ATP in older adults based on trends for increased venous plasma [ATP] and significantly improved ATP effluent. Third, the improvements in the hemodynamic responses to systemic hypoxia and exercise with fasudil resulted in increased oxygen delivery to the skeletal muscle and improved forearm $\dot{V}_{O2}$ during these physiological stimuli in older adults. Fourth, fasudil improved the age-related impairment in flow-mediated dilation of the brachial artery during progressive, graded-intensity rhythmic handgrip exercise such that there was no longer a difference between young and older adults. Fifth, fasudil significantly lowered mean arterial blood pressure in healthy, normotensive older adults at rest, during systemic hypoxia, and during exercise such that age-related elevations in control conditions were abolished. Sixth, *in vivo* fasudil administration tended to improve the impairment in deoxygenation-induced ATP release from unwashed RBCs of older adults. Finally, fasudil had no effect on the age-related increases in central artery stiffness in older adults. These collective findings provide the first experimental evidence that systemic fasudil administration improves ATP release *in vivo* and the regulation of vascular tone and skeletal muscle blood flow during the physiological stimuli of systemic hypoxia and exercise in healthy older adults, which may have therapeutic implications for reducing cardiovascular disease risk and increasing exercise tolerance and functional independence in aging populations.

Possible mechanisms of fasudil-mediated improvements in vascular function in older adults: ATP release

The design of the present study does not allow the underlying mechanisms of the fasudil-mediated improvements in vasodilatory and blood flow responses to systemic hypoxia and graded-intensity rhythmic handgrip exercise in older adults to be identified conclusively; however, some insight can be gained by evaluating the effects of fasudil on other outcomes of interest. The experimental basis for the present study was the novel finding from experiments performed in Chapter II of this dissertation that age-related declines in RBC deformability are a primary mechanism of impaired deoxygenation-induced ATP release from RBCs of healthy older adults, and more specifically, that improvements in deformability of RBCs from older adults following Rho-kinase inhibition restored the ability of these cells to release ATP in response to hemoglobin deoxygenation relative to RBCs from young adults. Although methodological concerns prevented experimental confirmation of the effects of systemic fasudil administration on RBC deformability (discussed in more detail in the 'Experimental considerations and limitations' section below), the general improvements in circulating ATP measures during systemic hypoxia and rhythmic handgrip exercise in older adults with fasudil strongly suggest that fasudil improved RBC deformability and ATP release *in vivo* given our previous findings that increases in circulating ATP during exercise are dependent on skeletal muscle perfusion and thus an intravascular cell source like RBCs (Kirby *et al.*, 2013), as well as the aforementioned effects of Rho-kinase inhibition on RBC deformability and ATP release from Chapter II. Accordingly, there was also a trend for improved deoxygenation-induced ATP release from isolated RBCs of older adults following *in vivo* administration of fasudil, but only in the unwashed RBCs ($P = 0.08$). This discrepancy between the effect of fasudil on washed and unwashed RBCs may be due to methodological limitations as well (discussed below). Considering the ability of ATP to stimulate both local and conducted vasodilation (Winter & Dora, 2007; Dora, 2017), the preserved vasodilatory responsiveness to exogenous ATP in the

forearm of older adults (Kirby *et al.*, 2010), and the associations between plasma [ATP] and FBF in young and older adults (Kirby *et al.*, 2012), the increase in circulating ATP following fasudil administration in the present study likely contributed to the enhanced vascular responses during hypoxia and exercise.

Possible mechanisms of fasudil-mediated improvements in vascular function in older adults: nitric oxide

Rho-kinase has a variety of molecular targets, one of which is the nitric oxide-producing enzyme endothelial nitric oxide synthase (eNOS) (Satoh *et al.*, 2014; Shimokawa *et al.*, 2016). Specifically, Rho-kinase inhibits eNOS-mediated synthesis of nitric oxide (NO) via phosphorylation of the enzyme at threonine 495 (Sugimoto *et al.*, 2007). Accordingly, some of the vascular effects of fasudil and hydroxyfasudil have been shown to be mediated in large part by improved NO bioavailability as a result of decreasing the inhibition of eNOS by Rho-kinase (Büssemaker *et al.*, 2007; Satoh *et al.*, 2014). Pertinent to the present study, the increase in brachial artery diameter during progressive, graded-intensity handgrip exercise has been shown to be largely dependent on NO in healthy young adults and impaired in older adults as a result of age-related declines in NO-mediated vascular function (Wray *et al.*, 2011; Trinity *et al.*, 2013). In the present study, the increase in brachial artery diameter during graded-intensity rhythmic handgrip exercise was also significantly impaired in older adults with saline and this impairment was reversed with fasudil administration such that there was no longer an age-related difference (Fig. 4.6). Taken together, these findings suggest that at least some of the fasudil-mediated improvements in vascular function in older adults were due to enhanced NO bioavailability.

Possible mechanisms of fasudil-mediated improvements in vascular function in older adults: literature-based evidence of additional pathways

The evidence for increases in ATP and NO bioavailability as possible mechanisms of the improvements in vascular function during hypoxia and exercise following fasudil administration in older adults is based on both experimental findings in the present study and results of previous investigations by various groups. However, given the diverse molecular targets of Rho-kinase *in vivo* and the systemic administration of fasudil in the present study, there are multiple additional pathways that could have contributed to the fasudil-mediated improvements in vascular function in older adults that can only be addressed at this point by turning to the scientific literature. An additional vasodilatory pathway that may have been affected by fasudil administration is the K_V7 subfamily of voltage-gated K^+ (K_V) channels, which contribute to both cyclic GMP- (cGMP) and cyclic AMP- (cAMP) dependent vasodilation and have been shown be selectively activated by fasudil (specifically $K_V7.4$ and $K_V7.4/K_V7.5$) (Stott & Greenwood, 2015; Zhang *et al.*, 2016). In contrast, the Rho-kinase inhibitors fasudil and Y-27632 have both been shown to blunt the vascular response to multiple vasoconstrictors. Specifically, fasudil can limit α-adrenergic vasoconstriction induced by norepinephrine and abolish endothelin-mediated vasoconstriction (Büssemaker *et al.*, 2007) and Y-27632 has been shown to cause a dose-dependent decrease in α_1-adrenergic vasoconstriction stimulated by phenylephrine (Löhn *et al.*, 2005). If some degree of inhibition of α-adrenergic vasoconstriction occurred in the present study, it is unlikely that it contributed to the improved vascular responses in older adults given our previous observations that local adrenoceptor blockade does not improve the age-related impairments in vasodilation during hypoxia (80% Sp_{O_2}) or graded-intensity rhythmic handgrip exercise (5%, 15%, and 25% MVC). However, the effect of fasudil on endothelin-mediated constriction (Büssemaker *et al.*, 2007) may have contributed in the present study given that an age-related increase in vasoconstriction mediated by endothelin A (ET_A) receptors has been

identified in the leg of healthy older adults both at rest and during exercise (Barrett-O'Keefe *et al.*, 2015).

Experimental considerations and limitations

The primary experimental limitation of the present study is that fasudil could not be delivered specifically to RBCs and therefore systemic drug administration was required in order to inhibit Rho-kinase in the circulating pool of RBCs. Given that Rho-kinase is distributed widely throughout the body and has a diverse range of cellular targets, follow-up studies will be needed to gain more definitive mechanistic insight into the fasudil-mediated improvements in vascular control in healthy older adults. Such studies could utilize systemic fasudil administration in combination with local brachial artery infusion of pharmacological antagonists of the pathways proposed to be altered by Rho-kinase inhibition, such as barium chloride for blocking ATP-mediated dilation via inwardly rectifying potassium (K_{IR}) channels (Crecelius *et al.*, 2012), L-NMMA for blocking eNOS-derived NO, and BQ-123 for blocking the ET_A receptor (Barrett-O'Keefe *et al.*, 2015), with subsequent analysis of how these antagonists alter the effects of fasudil providing insight into which pathways were involved.

Another limitation of systemic drug administration is that fasudil and its active metabolite hydroxyfasudil can have off-target effects from Rho-kinase, including myosin light chain kinase, protein kinase A, and protein kinase C. However, the inhibitor constant (K_i) of fasudil is much more specific for Rho-kinase, ranging from 0.33-1.9 μM (Davies *et al.*, 2000; Wickman *et al.*, 2003; Shibuya *et al.*, 2005; Rikitake *et al.*, 2005; Jacobs *et al.*, 2006; Satoh *et al.*, 2012) compared to 55 μM for myosin light chain kinase (Satoh *et al.*, 2012), ~10 μM on average for protein kinase A (Davies *et al.*, 2000; Rikitake *et al.*, 2005; Jacobs *et al.*, 2006; Satoh *et al.*, 2012), and ranging from 3.3 μM to over 100 μM for protein kinase C (Rikitake *et al.*, 2005; Satoh *et al.*, 2012). Importantly, hydroxyfasudil is an even more potent and specific inhibitor of Rho-kinase than fasudil, with a K_i ranging from 0.039-1.8 μM (Shimokawa *et al.*, 1999; Shimokawa,

2002; Shibuya *et al.*, 2005; Rikitake *et al.*, 2005; Jacobs *et al.*, 2006; Satoh *et al.*, 2012) compared to 140 μM for myosin light chain kinase (Satoh *et al.*, 2012), 2.2-37 μM for protein kinase A (Rikitake *et al.*, 2005; Jacobs *et al.*, 2006; Satoh *et al.*, 2012), and 18-100 μM for protein kinase C (Shimokawa *et al.*, 1999; Rikitake *et al.*, 2005; Satoh *et al.*, 2012), and it has a significantly longer half-life in circulation compared to fasudil (over four hours vs. less than one) (Shibuya *et al.*, 2005). Given that plasma [fasudil] and [hydroxyfasudil] were within the K_i range specific for Rho-kinase, it is unlikely that any effects of fasudil in the present study were due to off-target effects of these compounds.

Finally, we were unable to confirm that systemic fasudil administration altered RBC deformability due to methodological considerations associated with RBC isolation that could affect deformability and ATP release measures, as well as methodological limitations associated with measuring deformability via blood filtrometry. Beginning with RBC isolation, the primary methodological consideration is that it is unclear how removal of RBCs from the *in vivo* environment in which fasudil and hydroxyfasudil are circulating alters the effects of these compounds and influences the *ex vivo* measures of RBC function (i.e., deformability and ATP release) given that both act via competitive inhibition at the ATP binding site of Rho-kinase as opposed to inducing a more permanent or long-lasting change (Jacobs *et al.*, 2006). As opposed to the *ex vivo* studies described in Chapters II and III where pharmacological agents are incubated with isolated cells followed by the initiation of physiological measures within 5-30 min, at least 90 min pass between removal of RBCs from the *in vivo* environment in which fasudil and hydroxyfasudil are circulating and the initial ATP release measures in normoxia for washed RBCs as a result of the time associated with multiple cell washes, which may also alter the drug effects independent from time. Although there is less of a time delay with the unwashed RBCs, at least 50 min still pass between removal of RBCs and the initial ATP release measures in normoxia. These differences between washed and unwashed RBCs may explain why there was a trend for fasudil to increase deoxygenation-induced ATP release in unwashed

RBCs from older adults compared to saline (P = 0.08) whereas there was no effect of fasudil in washed RBCs (Figs. 4.8C and 4.8D).

Regarding the methodological limitations associated with blood filtrometry, the measure is highly influenced by the presence of non-RBCs given that it is dependent on cells deforming to pass through filter pores 5 μm in diameter. RBCs (~8 μm in diameter) are generally able to accomplish this, but other monocytes (ranging from 7-30μm in diameter), which inevitably persist without cell washing despite the removal of plasma and the visible buffy coat, cannot. To confirm this, we performed pilot tests of RBC deformability using washed and unwashed RBCs (n = 3 young adults) and found that red blood cell transit time (RCTT), where lower values indicate greater deformability, was significantly elevated and more than twice as variable in unwashed RBCs compared to washed (data not shown). Thus, given these considerations associated with cell washing and the limitations associated with measuring RBC deformability in unwashed cells, we were unable to confirm an effect of *in vivo* fasudil administration on RBC deformability. However, based on the experiments performed in Chapter II of this dissertation, it is likely that the improvements in circulating ATP *in vivo* and the trend for increased ATP release from unwashed RBCs in the present study resulted from fasudil-mediated improvements in RBC deformability and ATP release *in vivo*.

Conclusions

This investigation provides the first experimental evidence that age-related impairments in the peripheral vasodilatory and hyperemic responses to systemic hypoxia and graded-intensity rhythmic handgrip exercise can be significantly improved by Rho-kinase inhibition, and that this is accompanied by improvements in circulating ATP during these physiological stimuli. These findings also provide the necessary foundation for performing future investigations to determine the underlying mechanisms by which fasudil improves these hemodynamic responses in healthy older adults. Collectively, the work performed in this dissertation indicates

that RBCs may be a promising therapeutic target for improving vascular control of blood flow and oxygen delivery in older adults through the enhanced release of ATP, and for ultimately ameliorating the age-related increases in cardiovascular disease risk and declines in exercise tolerance and functional independence associated with impaired vascular function with advancing age.

Table 4.1. Subject Characteristics

	Young	Older
Male:Female	6:5	5:7
Age (years)	25 ± 1	66 ± 1*
Body mass index (kg/m^2)	23.4 ± 0.6	24.7 ± 0.9
Body fat (%)	24.1 ± 2.2	33.6 ± 2.6*
Forearm volume (ml)	897.2 ± 78.4	967.6 ± 95.1
Forearm fat-free mass (g)	749.4 ± 89.2	701.3 ± 84.0
Maximum voluntary contraction (kg)	34 ± 3	28 ± 2
5% workload (kg)	1.7 ± 0.2	1.4 ± 0.1
15% workload (kg)	5.1 ± 0.5	4.2 ± 0.4
25% workload (kg)	8.5 ± 0.8	7.0 ± 0.6
Total cholesterol (mg/dl)	151 ± 10	185 ± 12*
LDL cholesterol (mg/dl)	82 ± 7	107 ± 10*
HDL cholesterol (mg/dl)	53 ± 4	61 ± 3
LDL:HDL	1.6 ± 0.1	1.8 ± 0.2
Triglycerides (mg/dl)	77 ± 6	86 ± 8

* $P < 0.05$ vs. young (within condition)

Table 4.2. Hemodynamic and ventilatory responses during systemic isocapnic hypoxia

	Young		Older	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Saline				
MAP (mmHg)	88±2	89±2	100±3†	99±4†
HR (beats/min)	57±2	78±4	58±2	68±2†
FBF (ml/min)	18.6±1.6	24.9±2.7	20.7±2.1	22.8±2.2
FVC (ml/min/100mmHg)	21.1±1.9	28.2±3.1	20.7±2.0	23.4±2.3
Sp_{O_2} (%)	98.6±0.3	79.1±1.0	97.5±0.4	79.8±0.6
Minute vent (L/min; BTPS)	8.5±0.7	16.6±1.1	7.7±0.6	14.4±1.0
End-tidal CO_2 (mmHg)	40.7±0.8	39.9±0.9	38.8±1.1	37.6±0.8
Fasudil				
MAP (mmHg)	87±3	90±4	93±3*	92±3*
HR (beats/min)	59±3	79±3	56±2	66±3†
FBF (ml/min)	18.7±2.4	25.0±3.2	21.6±2.9	28.8±3.1*
FVC (ml/min/100mmHg)	21.1±2.2	27.3±2.7	23.1±2.8	30.9±2.6*
Sp_{O_2} (%)	98.7±0.4	77.5±1.0	97.1±0.6†	79.6±0.8†
Minute vent (L/min; BTPS)	8.9±0.8	19.0±1.5*	8.4±0.8	12.7±0.6†
End-tidal CO_2 (mmHg)	39.6±1.1	38.8±0.9	37.6±1.6	37.2±1.1

* $P < 0.05$ vs. saline; † $P < 0.05$ vs. young (within condition)

Table 4.3. Hemodynamic responses during graded-intensity rhythmic handgrip exercise

	Young				Older			
	Rest	5% MVC	15% MVC	25% MVC	Rest	5% MVC	15% MVC	25% MVC
Saline								
MAP (mmHg)	87±3	92±4	91±4	95±5	100±3†	103±5†	107±5†	113±5†
HR (beats/min)	56±3	59±3	61±2	66±3	58±2	60±2	61±2	63±2
FBF (ml/min)	19.2±1.5	70.5±7.3	198.9±19.9	347.1±31.6	21.8±1.9	71.3±8.5	166.5±15.9	269.7±21.5†
FVC (ml/min/100mmHg)	22.1±1.8	76.2±6.2	216.2±17.7	361.7±26.3	21.8±1.8	69.1±7.4	156.0±14.6†	242.0±20.7†
Fasudil								
MAP (mmHg)	87±3	90±3	93±3	99±4	93.1±3.4*	96±4*	98±4*	103±4*
HR (beats/min)	55±3	63±2	64±3	71±5	54±2	57±2	59±2	61±2†
FBF (ml/min)	23.4±2.9	76.4±8.7	181.1±19.1	313.5±27.9*	25.5±2.8	69.2±6.5	169.3±16.1	315.0±23.4*
FVC (ml/min/100mmHg)	26.6±2.8	83.9±8.0	193.3±18.3	313.4±23.5*	27.2±2.4	71.7±5.6	171.6±14.7	303.7±18.4*

* $P < 0.05$ vs. saline; † $P < 0.05$ vs. young (within condition)

Table 4.4. Isolated red blood cell gases

			pH	PO_2 (mmHg)	PCO_2 (mmHg)	tHb (g/dL)	FO_2Hb (%)	FHHb (%)
Washed RBCs								
Normoxia	Young	Saline	7.33±0.02	119.8±2.6	34.3±0.8	6.6±0.2	95.1±0.2	3.1±0.1
		Fasudil	7.33±0.01	122.5±4.2	34.6±1.8	6.8±0.3	95.5±0.2	3.3±0.1
	Older	Saline	7.32±0.01	123.4±2.0	36.6±0.8	6.6±0.1	94.9±0.2	3.5±0.1
		Fasudil	7.32±0.01	125.0±1.4	37.1±0.7†	6.9±0.2*	95.2±0.1	3.1±0.1
Hypoxia	Young	Saline	7.35±0.01	23.4±1.5	36.2±0.7	6.6±0.2	30.9±3.0	65.6±2.8
		Fasudil	7.37±0.01	21.4±1.6	36.2±0.8	6.9±0.3	26.7±3.6	69.7±3.4
	Older	Saline	7.32±0.01	26.3±1.3	39.0±0.7	6.6±0.2	34.3±2.4	62.0±2.3
		Fasudil	7.33±0.01	26.8±0.8	39.6±0.6†	7.1±0.2*	35.2±1.6†	61.3±1.6†
Unwashed RBCs								
Normoxia	Young	Saline	7.42±0.02	121.5±2.6	33.9±0.7	6.3±0.1	95.5±0.2	2.9±0.1
		Fasudil	7.44±0.02	123.0±4.5	33.8±2.8	6.3±0.2	95.8±0.2	3.0±0.1
	Older	Saline	7.39±0.02	123.7±2.4	38.1±1.3	6.7±0.1†	95.5±0.1	3.0±0.1
		Fasudil	7.41±0.01	126.4±1.6	36.2±0.6	6.7±0.1†	95.7±0.1	2.8±0.1
Hypoxia	Young	Saline	7.44±0.01	22.6±1.1	35.4±0.8	6.3±0.2	34.1±3.1	62.2±3.0
		Fasudil	7.46±0.01	21.3±1.3	34.8±0.9	6.3±0.2	32.1±3.9	64.5±3.6
	Older	Saline	7.41±0.01	25.5±0.7	39.2±0.8	6.8±0.2†	37.9±1.5	58.4±1.5
		Fasudil	7.41±0.01	24.8±0.9	40.1±0.8†	6.7±0.1	36.0±2.8	60.5±2.8

PO_2 = partial pressure of oxygen, PCO_2 = partial pressure of carbon dioxide, tHb = total hemoglobin, FO_2Hb = fraction of oxygenated hemoglobin, FHHb = fraction of deoxygenated hemoglobin. * $P < 0.05$ vs. saline; † $P < 0.05$ vs. young

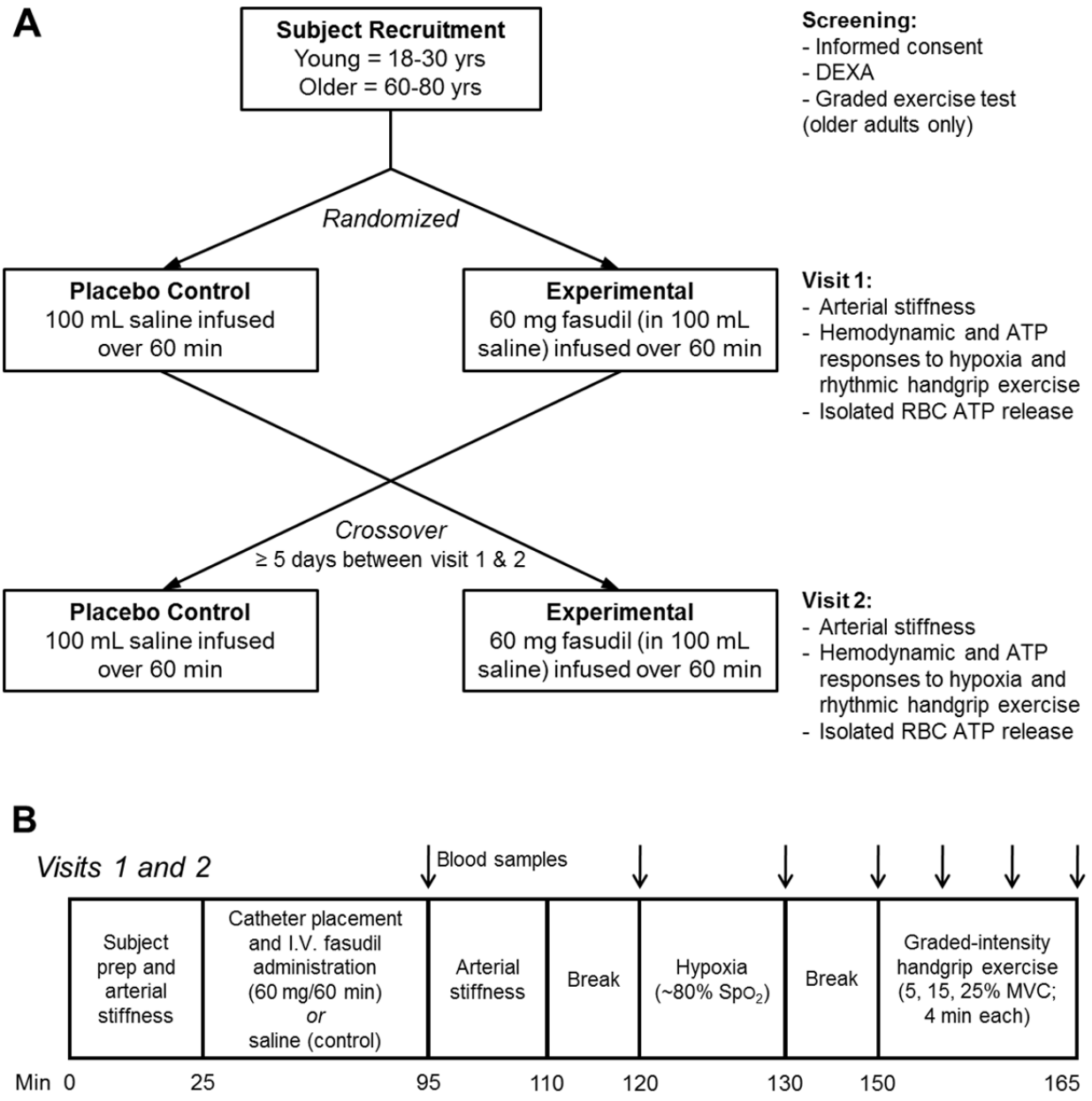

Figure 4.1. Overall experimental design and experimental visit timeline
A: double-blind, placebo-controlled, randomized, crossover experimental design; after successful screening, subjects were randomized in a double-blind manner to receive an infusion of either saline (placebo control) or fasudil for their first experimental visit. Subjects then received the opposite treatment for their second experimental visit, with at least five days between visit 1 and visit 2. *B*: experimental visit timeline; arterial stiffness measures were performed before and after venous catheter placement and treatment infusion followed by either hypoxia or graded-intensity rhythmic handgrip exercise trials, the order of which was randomized and counterbalanced between subjects, but kept the same for both visits within a subject. Timing of blood sampling is indicated by arrows, with samples for plasma [ATP] taken under steady-state conditions at rest, during hypoxia, and the end of each exercise workload.

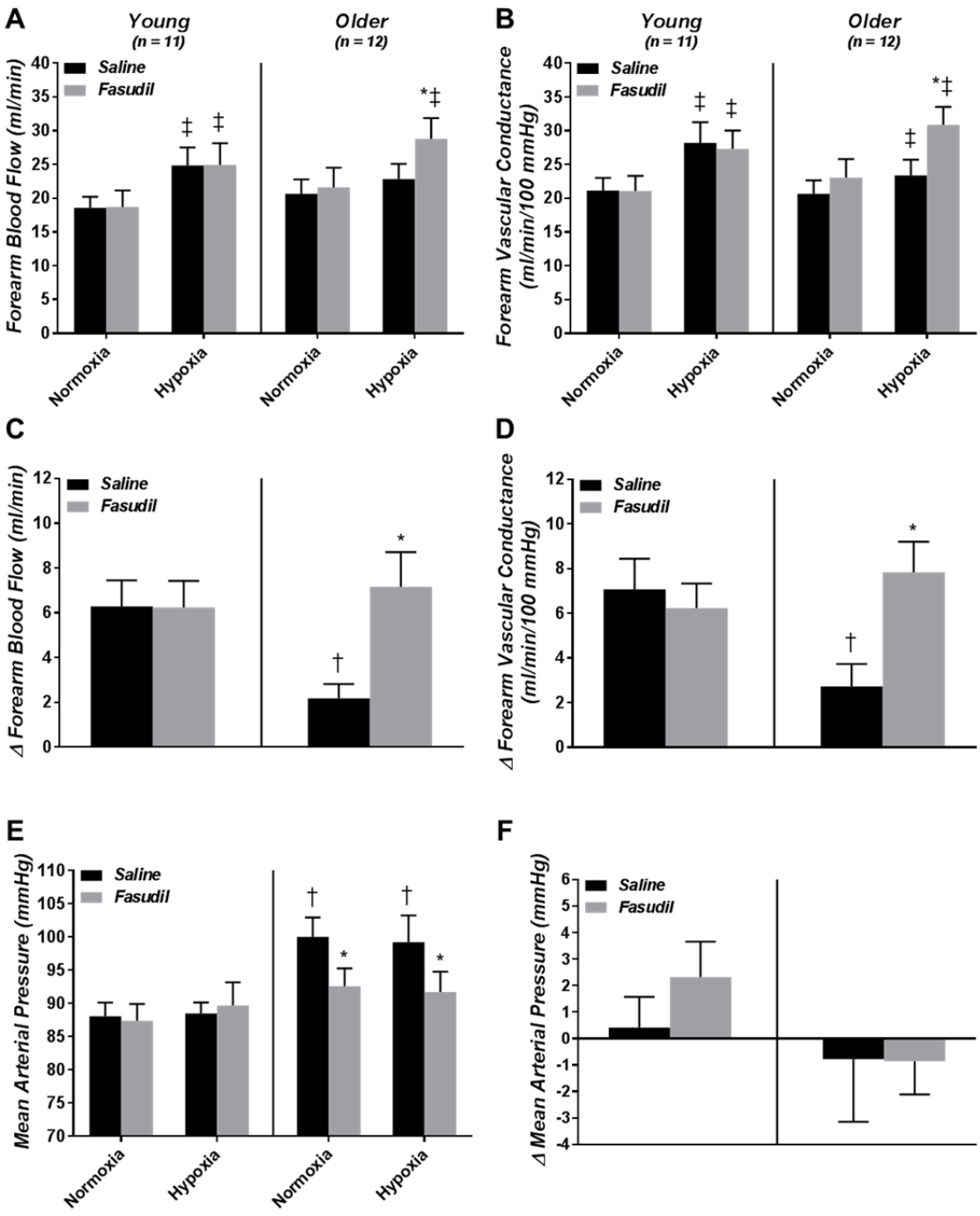

Figure 4.2. Effects of age and fasudil on hemodynamic responses during systemic hypoxia

A,B: there were no significant differences in absolute forearm blood flow (FBF) and vascular conductance (FVC) between young and older adults, but both increased significantly during hypoxia with fasudil in older adults. *C,D*: ΔFBF and ΔFVC from normoxia to hypoxia were impaired with age in the saline condition and were restored with fasudil. *E*: mean arterial pressure (MAP) was elevated in older adults with saline and was reduced with fasudil. *F*: ΔMAP from normoxia to hypoxia was unaffected by age or fasudil. * $P < 0.05$ vs. saline; † $P < 0.05$ vs. young; ‡ $P < 0.05$ vs. normoxia

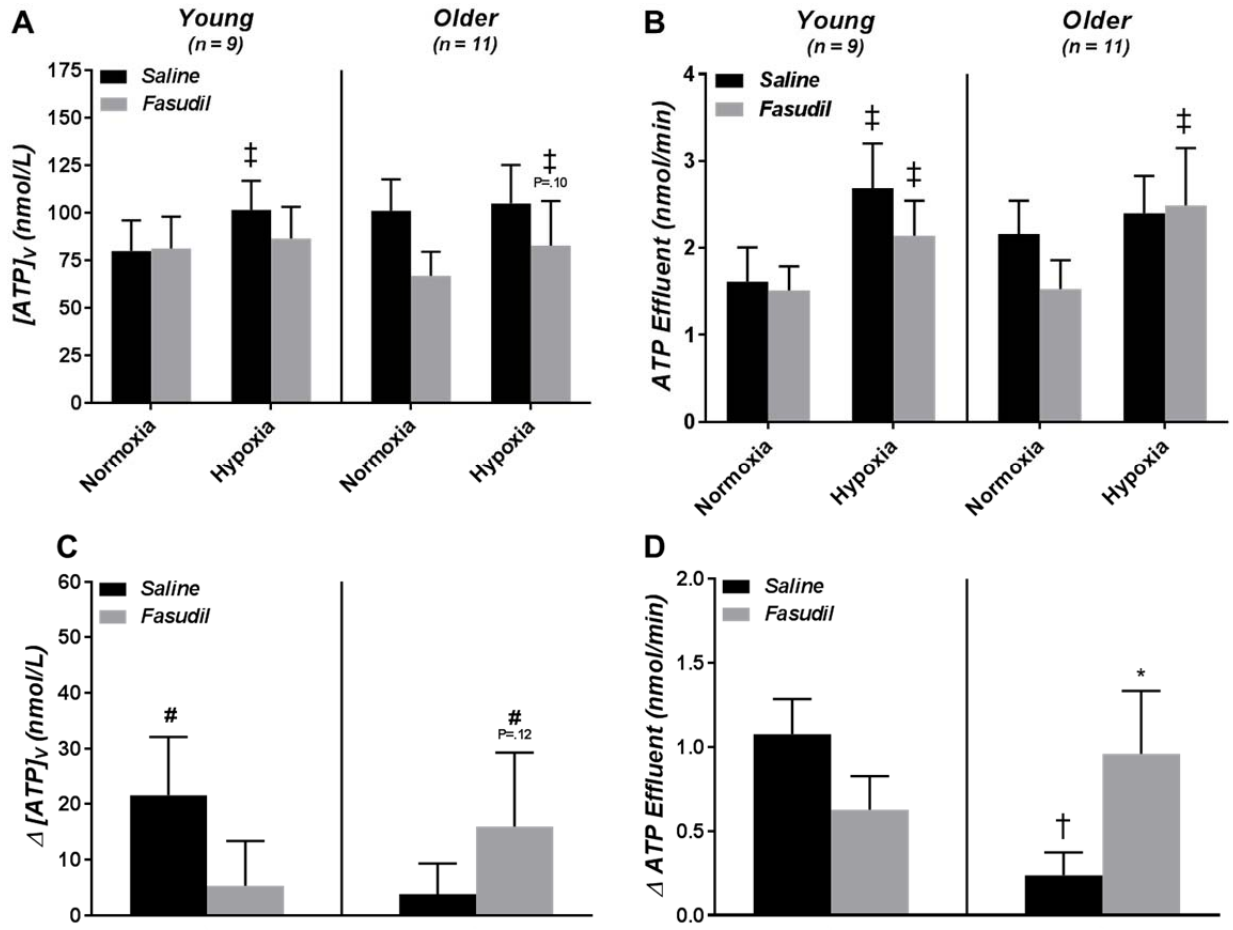

Figure 4.3. Effects of age and fasudil on ATP release during systemic hypoxia
A,C: in young adults, plasma $[ATP]_V$ increased during hypoxia from normoxia with saline and this $\Delta[ATP]_V$ was significantly greater than zero with saline, but not fasudil. In older adults, fasudil tended to improve $[ATP]_V$ during hypoxia compared to normoxia and $\Delta[ATP]_V$ from normoxia to hypoxia relative to zero. *B,D*: ATP effluent increased significantly from normoxia to hypoxia in young adults. In older adults, fasudil improved ATP effluent during hypoxia compared to normoxia and reversed the age-related impairment in Δ ATP effluent. * $P < 0.05$ vs. saline; † $P < 0.05$ vs. young; ‡ $P < 0.05$ vs. normoxia; # $P < 0.05$ vs. zero ($\Delta[ATP]_V$ only)

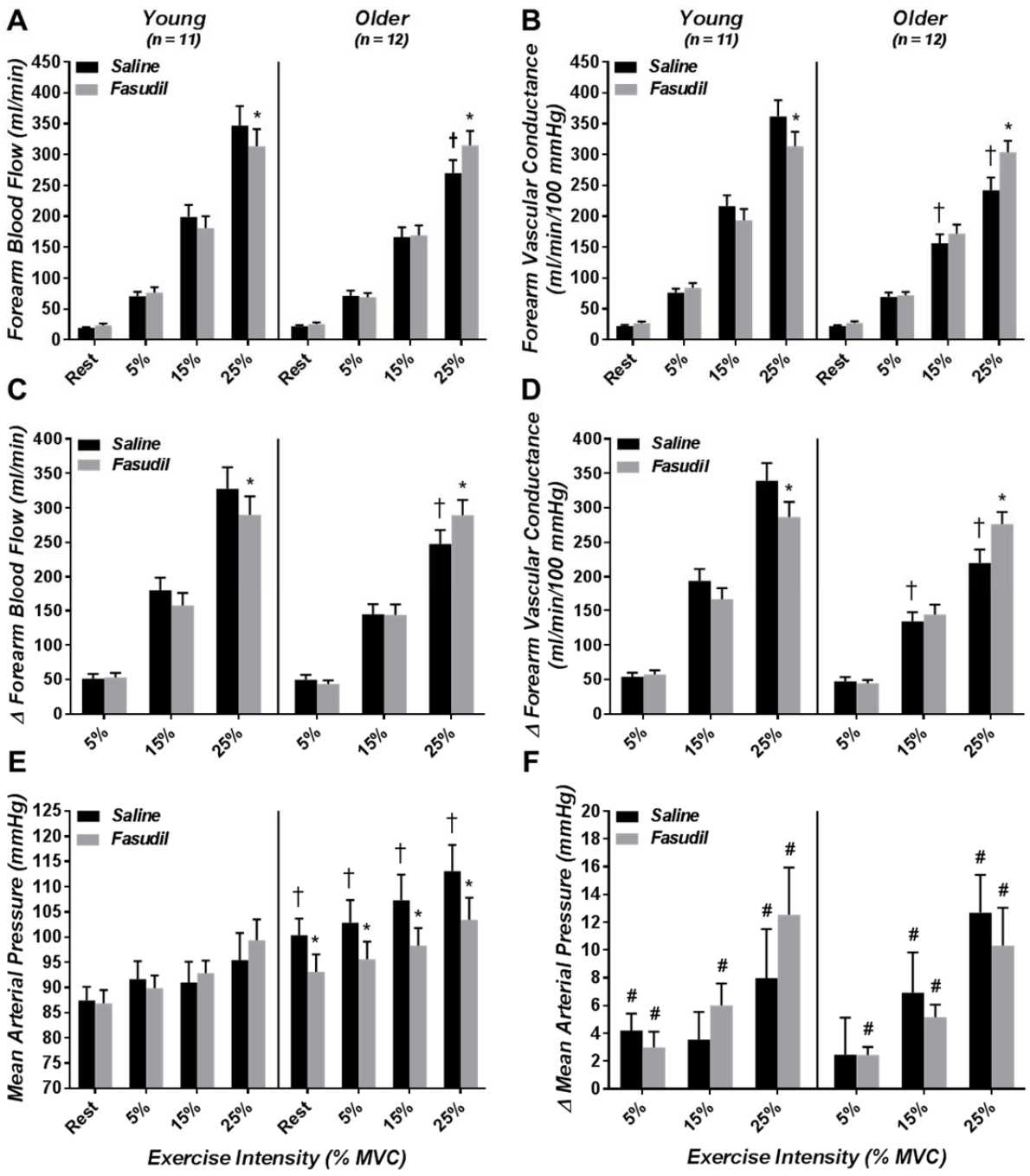

Figure 4.4. Effects of age and fasudil on hemodynamic responses during rhythmic handgrip exercise

A,B: with saline, forearm blood flow (FBF) was impaired during 25% maximum voluntary contraction (MVC) and forearm vascular conductance (FVC) was impaired during 15% and 25% MVC in older adults. Fasudil decreased FBF and FVC at 25% MVC in young adults and increased FBF and FVC at 25% in older adults. *C,D*: effects of age and fasudil on ΔFBF and ΔFVC from rest to exercise were the same as for absolute values in Panels A and B. *E*: mean arterial pressure (MAP) was elevated in older adults with saline and was reduced with fasudil at rest and during exercise. *F*: Δ MAP from rest to exercise was unaffected by age or fasudil.
* $P < 0.05$ vs. saline; † $P < 0.05$ vs. young; # $P < 0.05$ vs. zero (Δ MAP only)

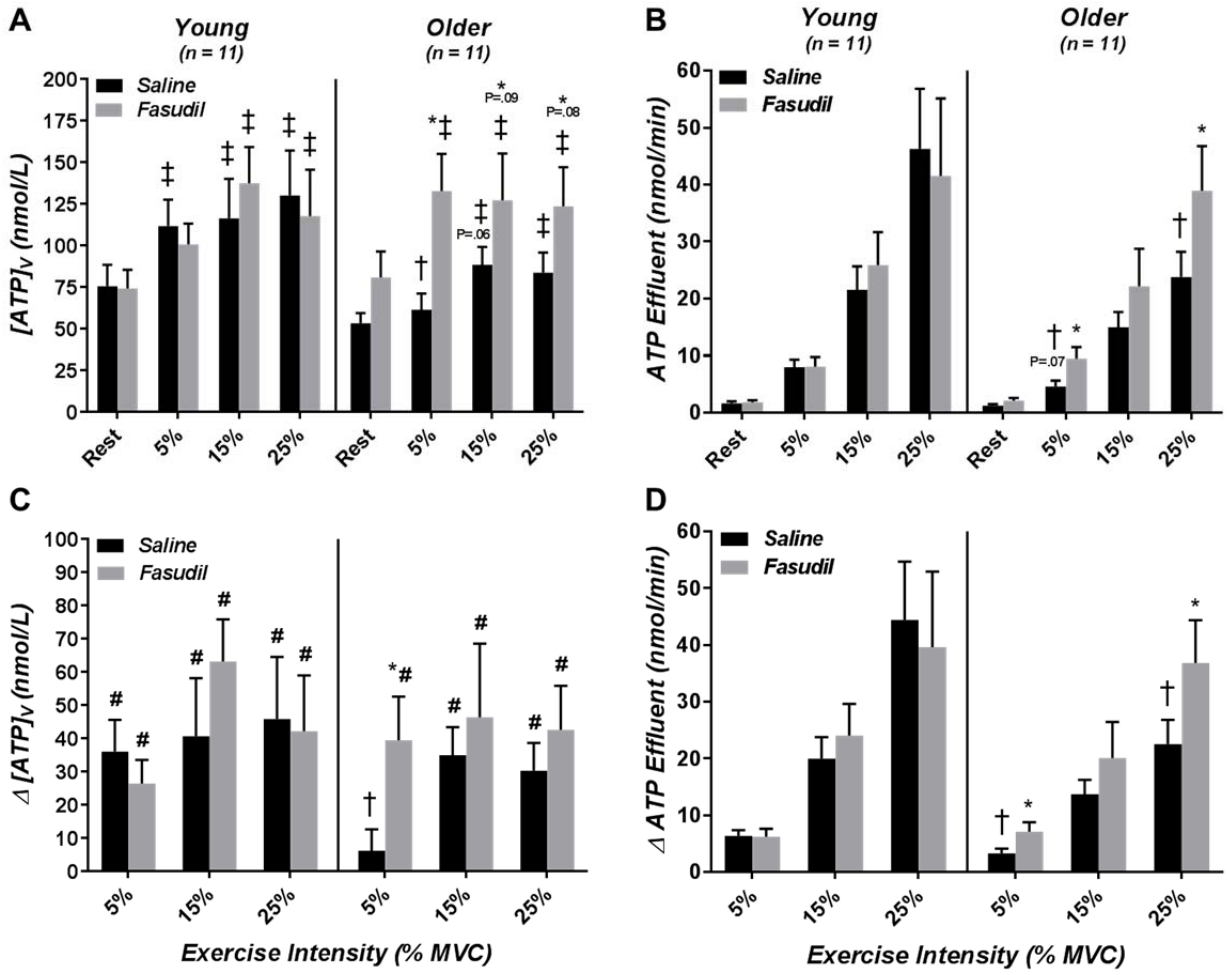

Figure 4.5. Effects of age and fasudil on ATP release during rhythmic handgrip exercise
A,C: with saline, plasma $[ATP]_V$ increased at all exercise intensities from rest in young adults and at 15% and 25% maximum voluntary contraction (MVC) in older adults, but was lower at 5% MVC in older vs. young adults. Fasudil increased absolute and $\Delta[ATP]_V$ at 5% MVC and tended to increase $[ATP]_V$ at 15% and 25% MVC in older adults. *B,D*: absolute and Δ ATP effluent were lower in older vs. young adults at 5% and 25% MVC with saline and were improved with fasudil at both exercise intensities **P* < 0.05 vs. saline; † *P* < 0.05 vs. young; ‡ *P* < 0.05 vs. rest ($[ATP]_V$ only); # *P* < 0.05 vs. zero ($\Delta[ATP]_V$ only)

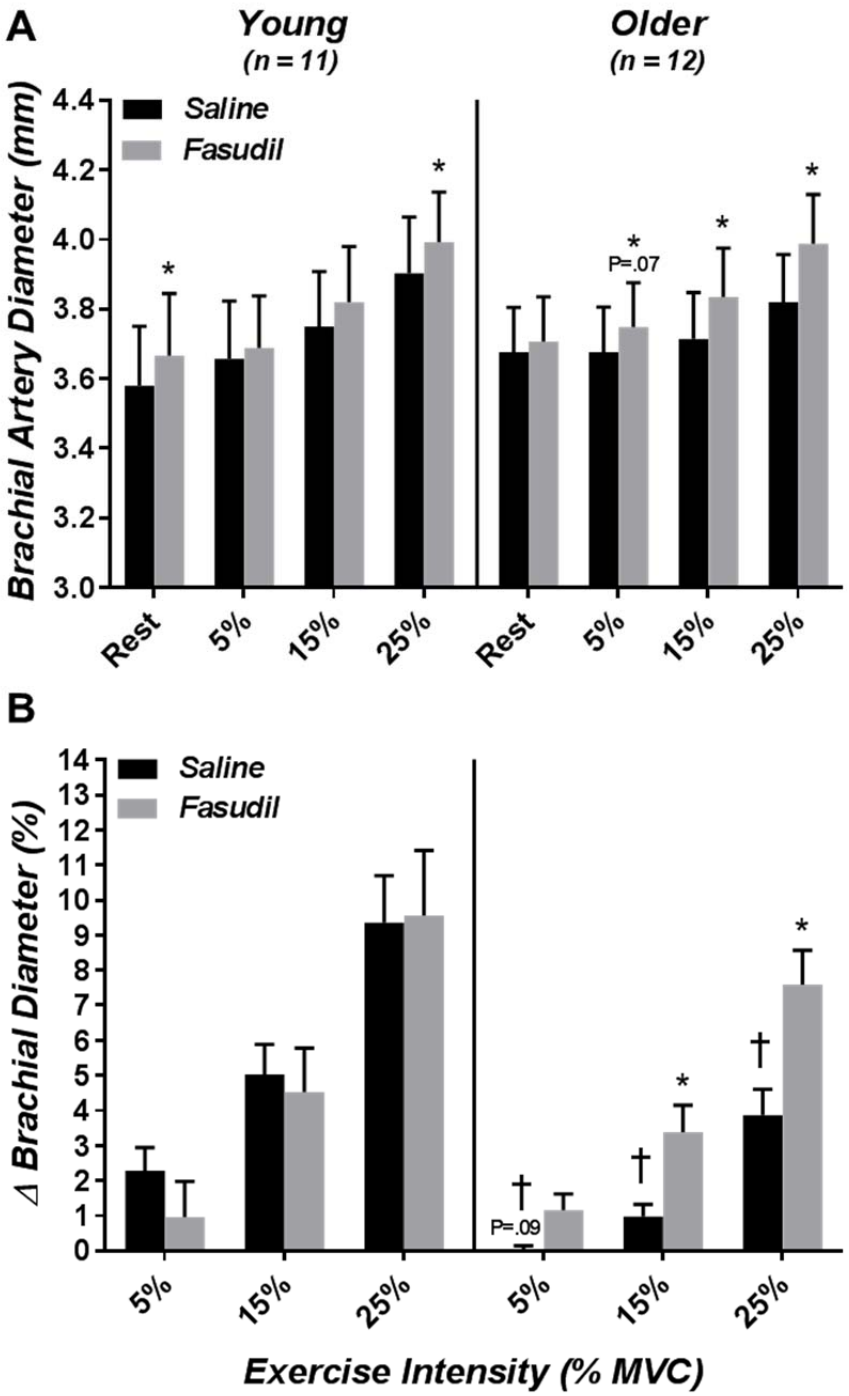

Figure 4.6. Effects of age and fasudil on brachial artery flow-mediated dilation during rhythmic handgrip exercise

A: aging did not affect absolute brachial artery diameter at rest or during exercise. Fasudil increased brachial diameter at rest and 25% maximum voluntary contraction (MVC) in young adults and at 15% and 25% MVC in older adults. *B*: the relative (%) change in brachial artery diameter from rest to exercise was impaired in older adults with saline and significantly improved with fasudil at 15% and 25% MVC. * $P < 0.05$ vs. saline; † $P < 0.05$ vs. young

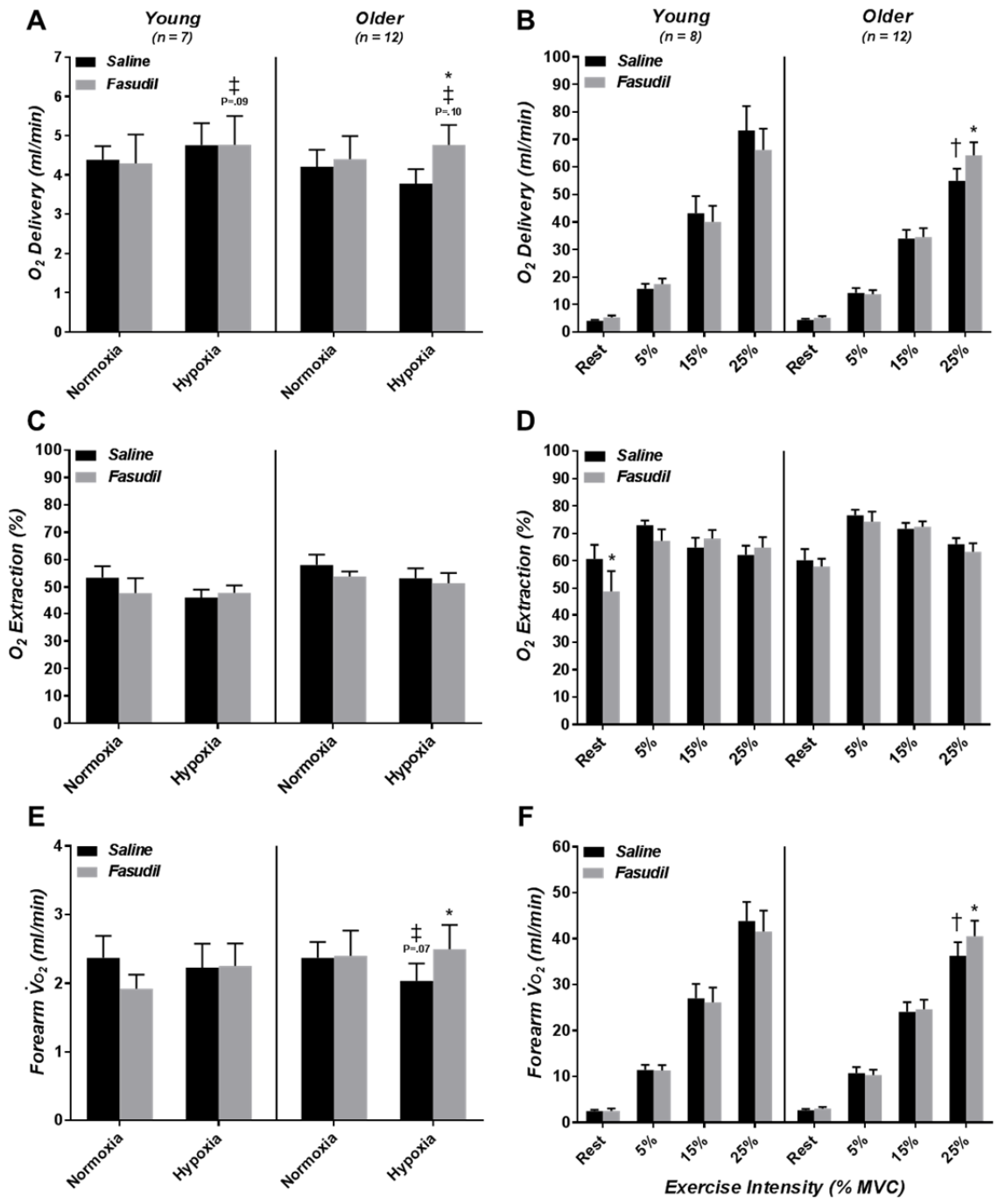

Figure 4.7. Effects of age and fasudil on forearm oxygen delivery, extraction, and consumption during hypoxia (A,C,E) and rhythmic handgrip exercise (B,D,F)

A,C,E: there were no differences in O_2 delivery (*A*), extraction (*C*), or consumption (*E*) between young and older adults. $\dot{V}_{O2}$ tended to decrease during hypoxia in older adults with saline (*E*) and both O_2 delivery and $\dot{V}_{O2}$ were improved with fasudil (*A*,*E*). *B,D,F:* O_2 delivery (*B*) and $\dot{V}_{O2}$ (*F*) were both impaired in older vs. young adults at 25% maximum voluntary contraction (MVC) with saline and this was reversed with fasudil. * $P < 0.05$ vs. saline; † $P < 0.05$ vs. young; ‡ $P < 0.05$ vs. normoxia

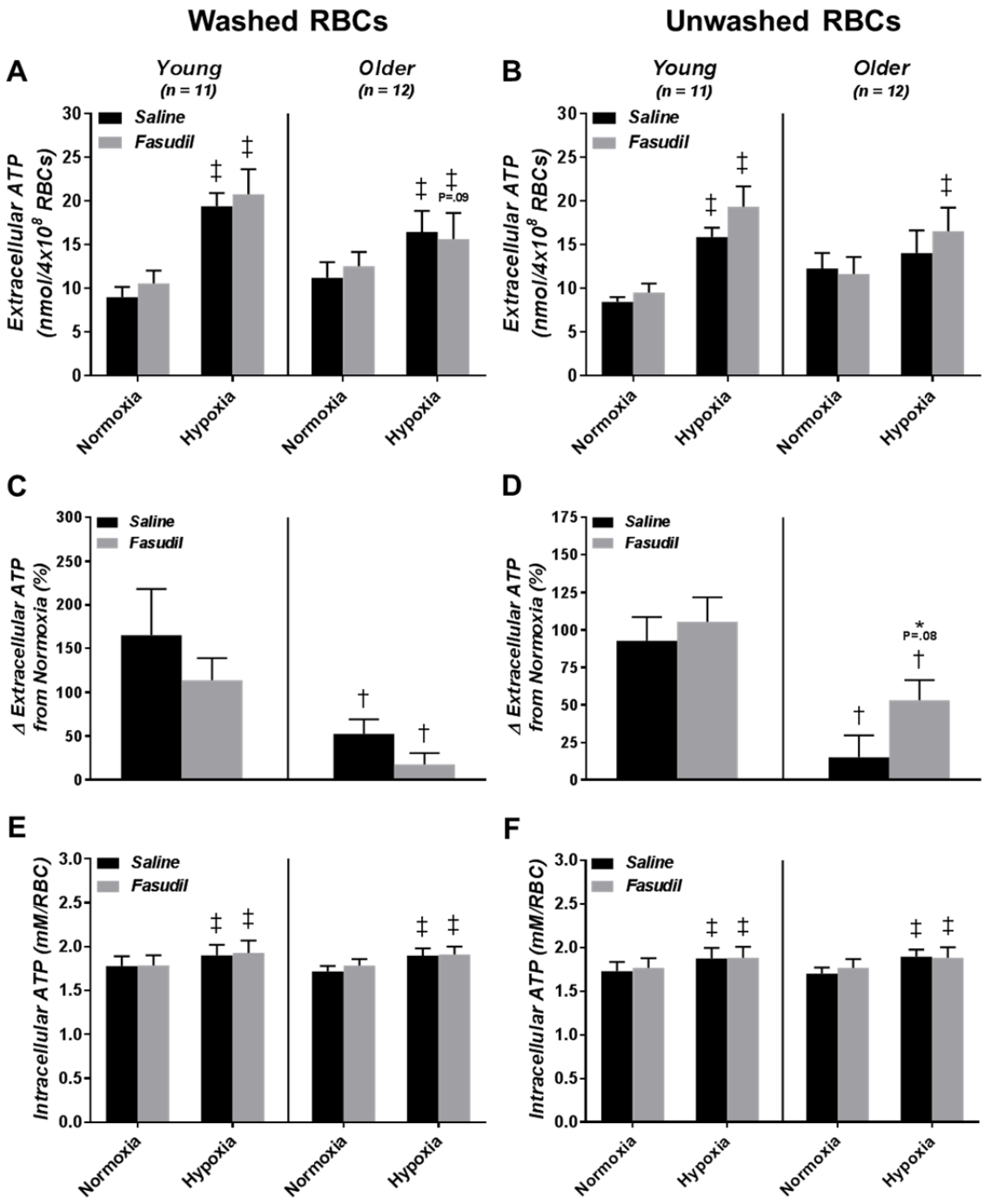

Figure 4.8. Effects of age and fasudil on deoxygenation-induced ATP release and intracellular ATP from washed (A,C,E) and unwashed (B,D,F) red blood cells (RBCs)
A,B: in young adults, extracellular ATP significantly increased from normoxia to hypoxia. In older adults, extracellular ATP significantly increased during hypoxia with saline for washed RBCs (*A*) and with fasudil for unwashed RBCs (*B*). *C,D*: the relative (%) change in extracellular ATP from normoxia to hypoxia was significantly impaired with age in all conditions, but fasudil tended to improve ATP release compared to saline in the unwashed RBCs (*D*). *E,F*: intracellular ATP increased from normoxia to hypoxia in all conditions and there were no differences between young and older adults. * $P < 0.05$ vs. saline; † $P < 0.05$ vs. young; ‡ $P < 0.05$ vs. normoxia

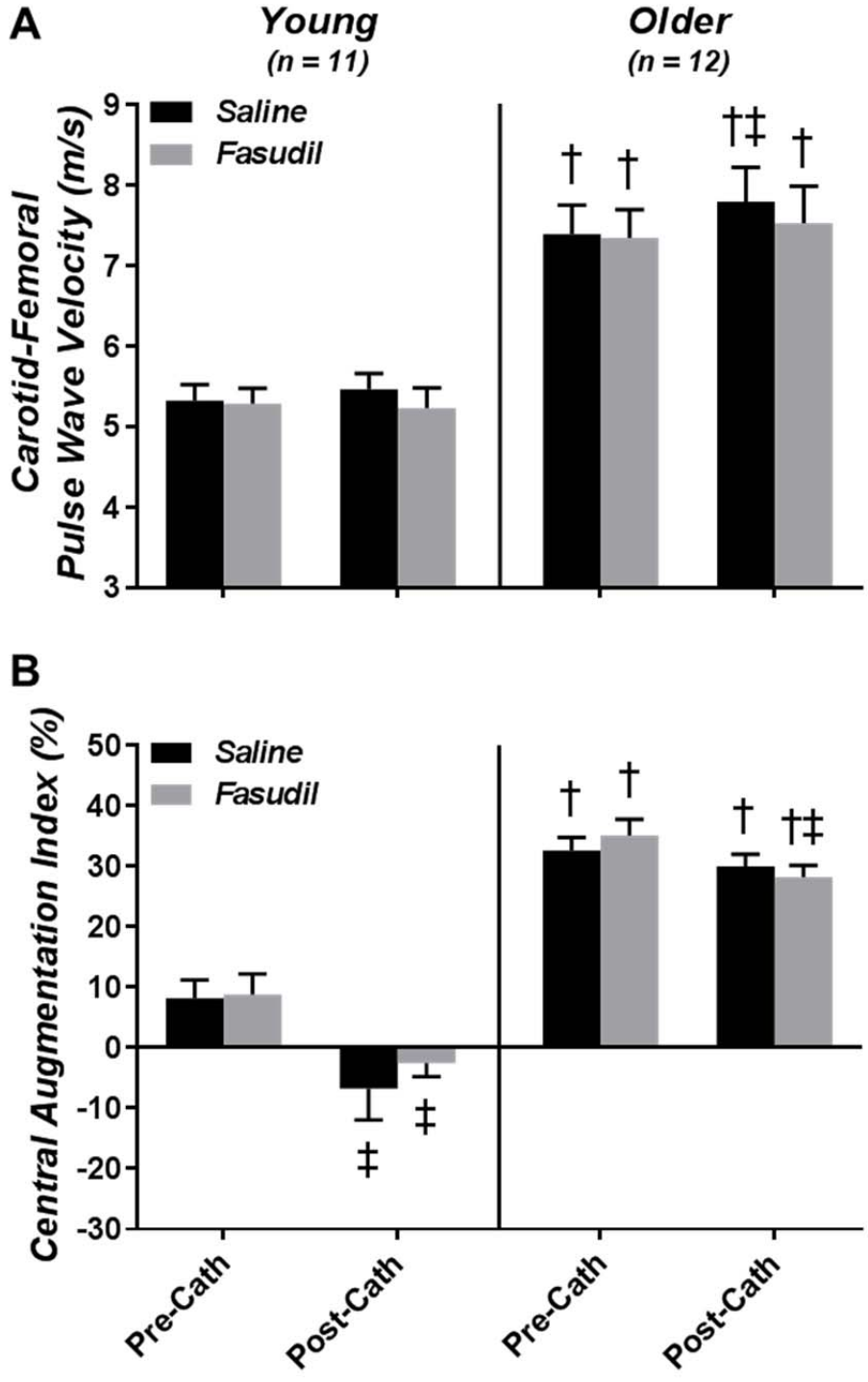

Figure 4.9. Effects of age and fasudil on measures of central artery stiffness
A: carotid-femoral pulse wave velocity (cfPWV) was elevated in older compared to young adults and there was no effect of fasudil. *B*: central augmentation index (AIx) was elevated in older compared to young adults and was unaffected by fasudil administration. † $P < 0.05$ vs. young; ‡ $P < 0.05$ vs. pre-catheter

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CHAPTER V – LIMITATIONS AND PERSPECTIVES

General Experimental Limitations

The first two sets of investigations provided novel mechanistic insight into the effects of primary aging on deoxygenation-induced ATP release from isolated red blood cells (RBCs). However, the major limitation of this dissertation is that the experimental approach utilized does not allow for the mechanisms of fasudil-mediated improvements in the hemodynamic responses to hypoxia and exercise in older adults to be determined; thus, we cannot establish a conclusive link between the first investigation, which demonstrated the ability of Rho-kinase inhibition to rescue deoxygenation-induced ATP release from RBCs of healthy older adults, and the third investigation in which systemic Rho-kinase inhibition *in vivo* significantly improved the hemodynamic responses to hypoxia and exercise in older adults. In order to address this limitation, the underlying mechanisms of the hemodynamic improvements following fasudil administration in older adults could be tested in follow up studies utilizing experimental approaches with which our laboratory has extensive expertise. Specifically, coupling fasudil administration with the use of local infusions of pharmacological antagonists into the brachial artery to inhibit the vasodilatory pathways thought to be improved by fasudil administration (e.g., ATP and nitric oxide) would provide a means to 'pharmacodissect' the contributing factors based on how blockade of these pathways affects the response to fasudil. Although this approach has limitations of its own, namely that there are currently no specific antagonists of purinergic P2 receptors available for use in humans, it is possible to block pathways that are downstream of ATP binding to P2 receptors such as activation of inwardly rectifying potassium channels, which we have demonstrated is the primary pathway for ATP-mediated dilation in humans.

Related to this primary limitation is the fact that systemic administration of fasudil was required in this study in order to expose the circulating RBC pool to the drug. Given that it is

currently not possible in humans to target the delivery of fasudil specifically to RBCs and that Rho-kinase is prevalent throughout the body, systemic drug administration may have been associated with inhibition of Rho-kinase in other tissues for which it would be difficult to control. However, our use of the forearm model is advantageous in this respect given that (i) the small muscle mass of the forearm is minimally impacted by systemic cardiovascular changes that can occur with systemic drug administration; (ii) handgrip exercise limits the stimulation of systemic cardiovascular reflexes that occur with whole body exercise and, when engaged, can confound the interpretation of peripheral vascular responses; and (iii) it allows for the broadest range of pharmacological agents to be utilized *in vivo* with the aforementioned 'pharmacodissection' approach that would otherwise not be possible due to risks and potential confounding effects that accompany the need to infuse higher drug doses when studying a larger muscle mass.

In addition to the potential confounding effects of inhibiting Rho-kinase in a variety of cell types, fasudil and hydroxyfasudil, the active metabolite of fasudil, can have off-target effects beyond Rho-kinase that include myosin light chain kinase and multiple protein kinases. However, the plasma concentrations that were achieved for both compounds in the third investigation were well below the average concentrations needed to inhibit targets other than Rho-kinase. Furthermore, hydroxyfasudil is a much more selective inhibitor of Rho-kinase than fasudil and it has a significantly longer elimination half-life (over four hours compared to less than one hour for fasudil). Thus, it is unlikely that the effects of fasudil on hemodynamics and circulating ATP in the third investigation were due to effects that were independent from Rho-kinase inhibition.

Perspectives

The regulation of blood flow to the tissues requires the complex integration and coordination of many different stimuli in order to appropriately match oxygen supply with the metabolic demand of the tissues. Over decades of research, it has become increasingly evident

that RBCs are more than just simple carriers of oxygen. Instead, through the regulated release of ATP in response to a variety of stimuli, including hemoglobin deoxygenation and cell deformation, RBCs are ideally positioned to both detect local changes in tissue metabolic demand and initiate a vasodilatory response to increase blood flow and participate in this crucial matching of oxygen supply and demand. While the effects of primary aging on vascular function have been widely studied, it was only identified recently that aging is also associated with impaired RBC ATP release in response to hemoglobin deoxygenation. However, the mechanisms of this impairment and its contribution to age-related declines in vascular function have remained completely unknown.

The data in this dissertation provide the first experimental evidence of the underlying mechanisms of impaired deoxygenation-induced ATP release from RBCs of healthy older adults, identifying declines in RBC membrane deformability as a primary mechanism whereas the cellular responses to G_i activation (e.g., increases in intracellular cAMP and ATP release) remain intact. Furthermore, the translation of these findings from isolated RBCs represents the first attempt to improve RBC ATP release *in vivo* in healthy older adults, and these studies are therefore the first to demonstrate that systemic Rho-kinase inhibition can improve hemodynamic and circulating ATP responses to hypoxia and exercise in older adults. Accordingly, RBC ATP release represents a novel therapeutic target for improving vascular function in aging populations, which may provide significant benefits for exercise tolerance, cardiovascular disease risk, functional independence, and overall quality of life.

APPENDIX A – HUMAN SUBJECTS APPROVAL

Colorado State University
Knowledge to Go Places

Research Integrity & Compliance Review Office
Office of the Vice President for Research
321 General Services Building - Campus Delivery 2011 eprotocol
TEL: (970) 491-1553
FAX: (970) 491-2293

NOTICE OF APPROVAL FOR HUMAN RESEARCH

DATE:	April 12, 2016	
TO:	Dinenno, Frank, Health & Exercise Science	
	Braun, Barry, Health & Exercise Science, Richards, Jennifer, Health & Exercise Science, Racine, Matt, Health & Exercise Science	
FROM:	Swiss, Evelyn, CSU IRB 1	
PROTOCOL TITLE:	Red Blood Cell ATP Release and Vascular Function in Humans	
FUNDING SOURCE:	Funding - Fellowships	
PROTOCOL NUMBER:	16-6361	
APPROVAL PERIOD:	Approval Date: March 23, 2016	Expiration Date: January 21, 2017

The CSU Institutional Review Board (IRB) for the protection of human subjects has reviewed the protocol entitled: Red Blood Cell ATP Release and Vascular Function in Humans. The project has been approved for the procedures and subjects described in the protocol. This protocol must be reviewed for renewal on a yearly basis for as long as the research remains active. Should the protocol not be renewed before expiration, all activities must cease until the protocol has been re-reviewed.

Important Reminder: If you will consent your participants with a signed consent document, it is your responsibility to use the consent form that has been finalized and uploaded into the consent section of eProtocol by the IRB coordinators. Failure to use the finalized consent form available to you in eProtocol is a reportable protocol violation.

If approval did not accompany a proposal when it was submitted to a sponsor, it is the PI's responsibility to provide the sponsor with the approval notice.

This approval is issued under Colorado State University's Federal Wide Assurance 00000647 with the Office for Human Research Protections (OHRP). If you have any questions regarding your obligations under CSU's Assurance, please do not hesitate to contact us.

Please direct any questions about the IRB's actions on this project to:

IRB Office - (970) 491-1553; RICRO_IRB@mail.Colostate.edu
Evelyn Swiss, Senior IRB Coordinator - (970) 491-1381; Evelyn.Swiss@Colostate.edu
Tammy Felton-Noyle, Assistant IRB Coordinator - (970) 491-1655; Tammy.Felton-Noyle@Colostate.edu

Evelyn Swiss

Swiss, Evelyn

Conditional approval has been granted for this research protocol. No enrollment may occur until FDA IND approvals have been submitted to the IRB at this time. Please submit your IND submission packet and any responses from the FDA regarding your INDs including the submission date of your applications as an amendment to this protocol.

Approval Period:	March 23, 2016 through January 21, 2017
Review Type:	FULLBOARD
IRB Number:	00010468
Funding:	National Institute of Health
Investigational Drugs:	Fasudil, Cilostazol (Pletal)

APPENDIX B – CONSENT FORM

Consent to Participate in a Research Study
Colorado State University

TITLE OF STUDY: *Red Blood Cell ATP Release and Vascular Function in Humans*

PRINCIPAL INVESTIGATOR: *Frank A. Dinenno, Ph.D. Associate Professor, Health and Exercise Science; frank.dinenno@colostate.edu; 970-491-3203*

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH? *You are a man or woman between the ages of 18-30 or 60-80 years, you are not pregnant, currently not taking any other medications, are sedentary to moderately active, and are otherwise healthy. Our research is looking at the effect of aging and exercise on regional blood flow control and how your blood vessels work.*

WHO IS DOING THE STUDY? *This research is being performed by Frank Dinenno, Ph.D of the Health and Exercise Science Department, and also by Gary Luckasen, M.D., of the Medical Center of the Rockies (University of Colorado Health). Trained graduate students, undergraduate students, research assistants, or research associates are assisting with the research. These studies are paid for by the National Heart Lung and Blood Institute, a part of the US Government (National Institutes of Health).*

WHAT IS THE PURPOSE OF THIS STUDY? *The way in which blood flow (and oxygen delivery) and blood vessels are regulated by local factors and nerves during exercise and during changes in the composition of air you breathe is being studied. Importantly, cardiovascular regulation under these conditions might change in older people, it might be different between men and women, and it might be affected by regular physical exercise. The purpose of the research is to understand differences in how blood vessels work in various groups of adults and what role the red blood cell plays in controlling vascular function.*

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST? *This whole research project will take place over a period of approximately five years. However, your part of this study will be:*

_____*1) one or two visits over a several day period* _____ ***(your initials)***

This project is a randomized, double-blind, placebo-controlled, crossover study, which means that you will receive an active drug on one visit and a placebo (a substance that does not have an effect) on your other visit (in a random order), but neither you nor any lab member collecting or analyzing data will know what treatment you are receiving on each visit.

WHAT WILL I BE ASKED TO DO? *This consent form applies to a large research project. You are only being asked to participate in one part of the total project. Depending on the part of the research project that you are involved in, you will be asked to participate in some of the following procedures. A member of the research team will fully explain each checked procedure that applies to your participation and specifically how long each session (total time) in the laboratory will be.*

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FOR ALL STUDY PARTICIPANTS, DURING THE SCREENING VISIT YOU WILL COMPLETE THE FOLLOWING:

_____ ***Health and Physical Activity Questionnaire.*** *You will be asked to answer some questions about your health and exercise habits to determine if you can participate in the study. (~20 minutes)*

_____ ***(your initials)***

____ ***Body Composition.*** *The fat, muscle, and bone in your body will be measured using an x-ray device (dual-energy x-ray absorptiometry) that will scan you from head to toe while you lie quietly on a special table for approximately 20 minutes. The amount of x-ray radiation you will receive is extremely low. (~20 minutes)*

_____ ***(your initials)***

_____ ***Maximum Voluntary Contraction.*** *This will consist of 3-4 trials where you will squeeze your muscles (either forearm, calf, or thigh) and generate as much force as you can. You will be asked to generate as much force over the course of ~3 seconds and hold this force another 5 seconds. After a 2-3 minute rest period, you will be asked to do this again. This is typically used to determine how heavy of exercise you perform so everybody is exercising at similar percentages of their maximum. (~ 10 minutes)*

_____ ***(your initials)***

_____ ***Graded Exercise Test.*** *If you are in the* ***60-80*** *yr-old age group, you will be asked to perform a maximal exercise test on a treadmill under the supervision of a physician. This test will occur in the Human Performance Clinical/Research Laboratory in the Department of Health and Exercise Science on the CSU campus. Sticky electrodes will be placed on your chest, and you will walk briskly or jog while the steepness of the treadmill is increased. Your blood pressure and heart beat will be closely monitored during and immediately after the test. (~1 hour)*

_____ ***(your initials)***

FOR ALL STUDY PARTICIPANTS, DURING THE STUDY DAY VISIT YOU MAY COMPLETE THE FOLLOWING:

_____ ***Heart Rate and Blood Pressure.*** *Heart rate will be measured by placing three sticky electrodes on your chest and reading the electrocardiogram (ECG) signal. Blood pressure will be measured with an automated machine that requires the placement of a cuff around your upper arm (bicep), or a small cuff on your finger. (continuous monitoring throughout study)*

_____ ***(your initials)***

____ ***Venous Catheterization.*** *Your skin will be cleaned and a catheter (plastic needle) will then be inserted on the front side of your elbow and secured to the skin.*

_____ ***(your initials)***

____ ***Blood Sample.*** *Less than 100 mL (approximately 60 mL) of your blood will be drawn from a vein on the front of your elbow or artery in a standard fashion using a sterilized hypodermic needle. (~15 minutes)*

_____ ***(your initials)***

____ ***Doppler Ultrasound.*** *The blood flow in your arm, leg, neck, or brain will be measured using an ultrasound machine which produces sound waves to measure your blood vessel size and the speed of your blood. This also provides information about how elastic or stiff your blood vessels are. (2-3 hours)*

_____ ***(your initials)***

_____ ***Forearm Exercise.*** *You will lay flat on a bed and squeeze your hand and forearm muscles using a handgrip device while your hand and arm are comfortably secured. The intensity of the exercise will range from very easy to moderately difficult, and you will be asked to perform this exercise for ~10 minutes several different times throughout the study with plenty of rest in between exercise trials. (1 – 2 hours)*

_____ ***(your initials)***

____ ***Lower Body Negative Pressure.*** *You will be placed in a sealed wooden chamber while you are lying flat on a bed. The chamber is sealed at your waist. Using a standard vacuum that is attached to the chamber, suction will be applied to mimic what happens when you go from laying to standing up. This will occur several times throughout the study for about 5 minutes at a time. (~10 min)*

_____ ***(your initials)***

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FOR ALL STUDY PARTICIPANTS (YOUNG AND OLDER) DURING THE STUDY DAY VISIT YOU MAY COMPLETE THE FOLLOWING:

____ ***Breathing a low Oxygen or high Carbon Dioxide Gas Mixture.*** *The purpose of this test is to mimic what happens when you go up to altitude or if you were to stop breathing. You will be asked to place your mouth around a scuba mouthpiece while wearing a nose clip to prevent breathing through your nose. The amount of oxygen or carbon dioxide you are breathing will be changed carefully with a specially designed system, and you will breathe this for a maximum of 20 minutes at a time. You will be asked to do this several times throughout the study, with plenty of time in-between each trial. The amount of oxygen that is in your blood will be measured with a light sensor on your fingertip or earlobe. (1-1.5 hours)*

_____ ***(your initials)***

____ ***Drug Administration (~ 2 - 4 hours).*** *The administration of one of the following drugs might occur during the study by way of the venous catheter or oral ingestion.*

Vasodilators – temporarily relax the blood vessels (minutes)
___ Cilostazol (200 mg; Oral Ingestion)
___ Fasudil (60 mg/ 60 min; Intra-venous infusion)

No major effects
___ Probenecid (2000 mg; Oral Ingestion; Young adults only)

**The drug doses for cilostazol and probenecid are above what is clinically recommended, but have been safely used in humans previously and the research team will be closely monitoring the subject for any adverse reactions. Fasudil is not used clinically in the United States, but has been safely administered to humans (in both healthy people and those with medical issues such as pulmonary hypertension and cerebral vasospasm) at this dose in research/clinical studies in the United States and abroad, and has been very well tolerated in all instances.*

_____ ***(your initials)***

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?
If you are not 18-30 or 60-80 years of age, are pregnant, are a regular smoker, or have any diseases (gout, kidney stones, cardiovascular disease) that would affect our measurements or significantly increase the risks associated with this study, we will not be able to include you in the research.

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WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?
(The procedures that apply to your proposed participation are checked)

_____ ***Health and Physical Activity Questionnaire*** *– there are no known risks associated with answering health questions. All information is kept strictly confidential.*

_____ ***(your initials)***

_____ ***Graded Exercise Test*** *– there is a risk of fatigue (temporary muscle tiredness), muscle strain, heart beat abnormalities (arrhythmias), a 0.01% chance of death (in people who have heart problems), a 0.02% risk of cardiac arrhythmias that would require you to go to a hospital (in people who have heart problems), and a risk of an increase or decrease in blood pressure.*

_____ ***(your initials)***

_____ ***Body composition (DEXA) scan*** *– The risks associated with the DEXA are very low. The maximum radiation dose you will receive in this study is less than 1/1000th of the federal and state occupational whole body dose limit allowed to radiation workers (5,000 mrem). Put another way, the maximum dose from any scan we utilize with this DEXA ranges from 1.2 mrem (Whole body scan) to 12.2 mrem (for several of the regional scans, such as lumbar, femur, and forearm scans). The average annual background radiation you already receive is at least 620 mrem/year. The more radiation you receive over the course of your life, the more the risk increases of developing a fatal cancer or inducing changes in genes. The radiation in this scan is not expected to significantly increase these risks, but the exact increase in such risks is not known. There are no discomforts associated with this procedure.*

_____ ***(your initials)***

_____ ***Muscle contractions (Exercise)*** *– There is a slight risk of muscle strain and muscle soreness resulting from brief strong muscle contractions. Soreness should not last more than two days or affect your normal function.*

_____ ***(your initials)***

_____ ***Breathing a low oxygen or high carbon dioxide content gas mixture*** *- The risks associated with this include light-headedness, headache and fainting. However, we will be monitoring all of your vital signals and will stop the procedure if this occurs. Symptoms will end momentarily after breathing normal room air.*

_____ ***(your initials)***

_____ ***Venous Catheterization*** *- The risk of allergic reaction to lidocaine is extremely low. There is a risk of bruising, slight risk of infection, local soreness, and fainting.*

_____ ***(your initials)***

_____ ***Blood sample*** *– The risks associated with blood drawing include bruising, slight risk of infection, soreness, and fainting. These are minor risks that usually do not last more than one day if they occur.*

_____ ***(your initials)***

_____ ***Lower Body Negative Pressure*** *- There is a small risk of feeling nauseous or fainting. These symptoms will be relieved when the vacuum is turned off.*

_____ ***(your initials)***

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_____ ***Drug/Supplement Administration*** *- The risks associated with drug administration include:*

Fasudil: *vasodilation, decrease in blood pressure, increase in heart rate, headache. These risks are extremely low (< 1% of people have a decrease in blood pressure). Given this, fasudil may be administered without a physician being physically present in the building. However, if you would prefer to receive fasudil only when a physician is present, please let us know.*

Cilostazol: *(Participants regularly taking proton pump inhibitors (omeprazole/Prilosec) should not participate and all participants should avoid taking these medications for one week prior to and following participation in this study. Individuals with III-IV congestive heart failure or active pathologic bleeding should be excluded).*
More Common: headache; diarrhea; abnormal stools; dizziness; palpitation; infection; back pain; abdominal pain; tachycardia (faster heart rate at rest); dyspepsia (indigestion or heartburn); flatulence; peripheral edema (swelling); myalgia (muscle pain); pharyngitis (swollen throat); rhinitis (inflammation of the nose); increased cough; nausea.
Less common (requiring immediate attention): abnormal bleeding; bloody or black tarry stools; bruises or red spots on the skin; fainting; severe nausea; severe or continuing indigestion; stiff neck; stomach pain (severe cramping or burning); swelling of the tongue; vomiting of blood or materials that look like coffee grounds.

Probenecid: *(Renal side effects have been noted with this drug. Renal function will be checked during screening.)*
More Common: headache; joint pain (redness or swelling); loss of appetite; nausea or vomiting (mild); anemia.
Less Common (requiring immediate attention): Bloody urine; Difficult or painful urination; Lower back or side pain (severe or sharp); skin rash, hives, or itching; dizziness; flushing or redness of the face (occurring with other allergic reaction); frequent urge to urinate; sore gums; change in skin color of the face; unusual bleeding or bruising; unusual tiredness or weakness.

***Note**: these drug reactions are listed for use of these medications daily over an extended duration of time; study participants will only be exposed to the drug one time.*

Substances, symptoms, or conditions to avoid administering these drugs with:

Fasudil: none.

Cilostazol: Grapefruit juice, individuals currently taking antibiotics, selective serotonin reuptake inhibitors (SSRI) anti-depressants, and patients with congestive heart failure.

Probenecid: kidney stones, gout, and individuals currently taking aspirin, acetaminophen, naproxen, antibiotics, and theophylline.

It is not possible to identify all potential risks in research procedures, but the researcher(s) have taken reasonable safeguards to minimize any known and potential, but unknown, risks.

_____ ***(your initials)***

WILL I BENEFIT FROM TAKING PART IN THIS STUDY? *There are no direct benefits to you for participating in this study beyond receiving information on your body composition and cardiovascular risk factors.*

DO I HAVE TO TAKE PART IN THE STUDY? *Your participation in this research is voluntary. If you decide to participate in the study, you may withdraw your consent and stop participating at any time without penalty or loss of benefits to which you are otherwise entitled.*

WHAT WILL IT COST ME TO PARTICIPATE? *There is no cost to you for participating except that associated with your transportation to our facilities.*

WHO WILL SEE THE INFORMATION THAT I GIVE? *We will keep private all research records that identify you, to the extent allowed by law. Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be identified in these written materials. We may publish the results of this study; however, we will keep your name and other identifying information private.*

We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key. You should know, however, that there are some circumstances in which we may have to show your information to other people: The Institutional Review Board (IRB); Department of Health and Human Services, the Food and Drug Administration, as the regulatory bodies for this research.

To help us protect your privacy, National Institutes of Health have extended added protections called a "Certificate of Confidentiality." With this Certificate, we cannot be forced by a court order or subpoena to disclose information that could identify you in any civil, criminal, administrative, legislative or other proceeding.

There are circumstances where the Certificate does not protect against disclosure of your personally identifiable information:

- *When the US government is inspecting or evaluating federally-funded studies*
- *When information must be disclosed to meet FDA requirements*
- *If you give someone written permission to receive research information or you voluntarily disclose your study information*
- *If the researcher reports that you threatened to harm yourself or others*
- *In cases of child abuse reported by the researcher*
- *If the investigator reports cases of contagious disease (such as HIV) to the state*

A description of this clinical trial will be available on http://www.ClinicalTrials.gov, as required by U.S. Law. This Web site will not include information that can identify you. At most, the Web site will include a summary of the results. You can search this Web site at any time.

CAN MY TAKING PART IN THE STUDY END EARLY? *Your participation in the study could end in the rare event of muscle strain, if you become pregnant, or if you miss an excessive number of appointments.*

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WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? *For experiments that involve the blood sample and arterial or venous catheterization, you will be paid $15/hour.*
Your identity/record of receiving compensation (NOT your data) may be made available to CSU officials for financial audits.

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? *We will arrange to get you medical care if you have an injury that is caused by this research. However, you or your insurance company will have to pay for that care. The Colorado Governmental Immunity Act determines and may limit Colorado State University's legal responsibility if an injury happens because of this study. Claims against the University must be filed with Colorado State University within 180 days of the injury.*

WHAT IF I HAVE QUESTIONS? *Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the principal investigator, Frank Dinenno, Ph.D., at (970)491-3203, or via email at frank.dinenno@colostate.edu. If you would like to ask a medical doctor about your participation in the study, you may contact one of the physicians listed below at the corresponding phone number. If you have any questions about your rights as a volunteer in this research, contact the CSU IRB at: RICRO_IRB@mail.colostate.edu; 970-491-1553. We will give you a copy of this consent form to take with you.*

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 8 pages.

_____ _____
Signature of person agreeing to take part in the study Date

_____
Printed name of person agreeing to take part in the study

_____ _____
Name of person providing information to participant Date

_____
Signature of Research Staff

** **List of Contact Numbers in Case of Medical Emergency**

Gary Luckasen, M.D.	Work: 970-221-1000 (24 hours a day)
Frank A. Dinenno, Ph.D.	Work: 970-491-3203 Home: 970-682-2552

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