DISSERTATION

# ON THE ROLE OF CIRCULATING ATP IN VASCULAR CONTROL AT REST AND DURING EXERCISE OF AGING HUMANS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BRETT SEAN KIRBY ENTITLED ON THE ROLE OF CIRCULATING ATP IN VASCULAR CONTROL AT REST AND DURING EXERCISE OF AGING HUMANS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

ON THE ROLE OF CIRCULATING ATP IN VASCULAR CONTROL AT REST AND DURING EXERCISE OF AGING HUMANS

The following investigation composes a series of experiments with the overall aim of determining the role for the circulating nucleotide adenosine triphosphate (ATP) in vascular control at rest and during exercise in humans of advanced age. We tested the general hypothesis that ATP has definite vasodilator and sympatholytic vasomotor properties in young adults during exercise, but that these actions are impaired in older adults; and that endogenously circulating levels of ATP are diminished during exercise in the aged population. Specifically, the experiments are outlined as such: 1) to determine whether exogenous ATP can modulate α-adrenergic vasoconstriction in the human forearm of young adults, 2) to determine whether vasodilator responsiveness to exogenous ATP is impaired in aging humans, and the contribution of adenosine to ATP-mediated vasodilation in aging humans, 3) to determine whether the ability of exogenous ATP to modulate α-adrenergic vasoconstriction in the human forearm of older adults is impaired similar to the typical response observed during exercise in aged humans, 4) to determine whether endogenous venous plasma [ATP] and ATP release is diminished during mild-to-moderate exercise in aging humans. Our collective findings indicate that alterations in the contribution of ATP to vascular tone in aging humans exist and may in

part be a potential mechanism by which aged adults have reductions in oxygen delivery to active skeletal muscle. In particular, circulating exogenous ATP has the ability to significantly blunt α-adrenergic vasoconstriction in young adults similar to that observed during exercise. In contrast to our hypothesis, the vasodilatory responsiveness and sympatholytic properties of exogenous ATP remain intact in aging humans. However, older adults demonstrate reduced venous plasma [ATP] and impaired ATP release during graded mild-to-moderate handgrip exercise which is associated with attenuations in skeletal muscle vasodilation and blood flow. Taken together, it is our belief that the typically observed impairments in skeletal muscle vasodilation and the inability to offset sympathetic vasoconstrictor tone during exercise is in part due to diminished endogenous levels of circulating ATP in aging humans. On the whole, ATP appears to be a significant regulator of vascular control in humans, and may act as a potential mechanism which in part explains the typically observed reductions in skeletal muscle blood flow and oxygen delivery to active tissue in aged humans thereby predisposing this population to an elevated risk for cardiovascular diseases, age-related declines in exercise capacity/tolerance, and an overall decline in quality of life in this population.

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# CHAPTER I – INTRODUCTION/ EXPERIMENTAL AIMS

Blood flow through a muscle vascular bed is primarily determined by means of the arteriovenous pressure difference across that bed ($\Delta P = P_a - P_v$) and its vascular conductance (VC), ($Q_a = \Delta P \times VC$, where $Q_a$ is arterial inflow). Therefore, adjustments in perfusion pressure or vascular tone could act together or independently as possible blood flow regulating mechanisms. In quiescent skeletal muscle under stable perfusion pressure gradients, blood flow is chiefly determined by the vascular tone of arterioles. Specifically, vascular conductance is directly proportional to the radius of the vessel raised to the fourth power and is inversely proportional to the length of the vessel and to the viscosity of the fluid through the vessel. On the other hand, the pressure gradient across a muscle bed is determined by the difference between arterial luminal pressure and a critical downstream 'back-pressure', which is commonly thought to be venous luminal pressure. Thus, blood flow could be augmented via changes in the perfusion pressure gradient, either by increases in arterial pressure or by reductions in venous pressure. Nonetheless, adjustments in either vascular tone or perfusion pressure can have profound independent effects on muscle blood flow.

During whole body exercise, both changes in perfusion pressure and vessel caliber occur, thus concomitantly assisting in the elevation of blood flow and oxygen delivery to match the metabolic demand of the active tissue without compromising blood pressure. Accordingly, the local control of vascular tone during exercise consists of a fine interplay

between sympathetic neural vasoconstriction and local metabolic vasodilation, and together dictates optimal blood flow and oxygen delivery to the active tissue. For over a century, experiments have been directed towards increasing our understanding of the factors that regulate vascular tone during exercise. Although it is clear that vasoconstriction occurs in both inactive and active tissue via elevations in sympathetic nervous system activity, the substances evoking vasodilation during exercise are somewhat more perplexing. To date, it has been difficult to identify any one single factor that is *obligatory* to observe exercise hyperemia in humans, although many vasodilators are recognized to contribute (adenosine, nitric oxide, vasodilating prostaglandins, $K^+$). Nonetheless, we have recently turned our attention the postulate that circulating nucleotides have an explicit role in the control of vascular tone at rest and during exercise.

Importantly, the study of ATP in vascular control has physiological relevance as it is understood that ATP is released extracellularly into the human circulation from a large host of cells. As such, *in vitro* evidence indicates that circulating ATP may result from deoxygenation and mechanical strain upon endothelial cells as well as erythrocytes and platelets, although other cells are recognized to have this ability as well. For many decades, nucleotides have been recognized to reside within the circulation of humans and to have robust vasodilator action when infused intra-arterially. More recently, evidence indicates that ATP has powerful vasodilatory action in that it can evoke elevations in blood flow similar to that observed during maximal exercise and results in the ability to significantly blunt (and in some instances abolish) sympathetically-mediated vasoconstriction in a dose-response fashion similar to that seen with graded intensity

exercise in young adults. In addition, although heavily touted as an endothelium-dependent vasodilator, ATP appears to evoke vasodilation independent of nitric oxide, vasodilating prostaglandins, as well as adenosine, and may chiefly cause vasodilation via spreading hyperpolarization (yet unexplored in humans). Interestingly, these vasomotor properties are specific to ATP and are typically not observed with downstream adenine nucleotide, AMP, or nucleoside, adenosine.

With respect to alterations observed with advancing age in humans, it is well recognized that aging serves as a primary independent risk factor for the development of cardiovascular disease. In particular, older adults demonstrate impaired vasodilation to endothelium-dependent stimuli as well as an attenuated increase in blood flow (and oxygen delivery) to various metabolic stimuli including exercise. Moreover, this is largely a result of impaired local vasodilation. As stated above, the endogenous circulating nucleotide ATP appears to maintain specific vasomotor properties in young adults, yet whether a deficit in either the responsiveness or the circulating content of this molecule results with advancing age is unknown.

Therefore, this body of work comprises four explicit hypotheses aimed to enhance our understanding of the contribution of circulating ATP to vascular control at rest and during exercise in humans. Considerable emphasis is placed upon how this control is altered with advancing age in humans.

**Overall hypothesis:** ATP has definite vasodilator and sympatholytic vasomotor properties in young adults during exercise, but that these actions are impaired in older adults; and that endogenously circulating levels of ATP are diminished during exercise in the aged population.

**Specific Aims**

**Experiment #1:** to determine whether exogenous ATP can modulate α-adrenergic vasoconstriction in the human forearm of young adults.

**Experiment #2:** to determine whether vasodilator responsiveness to exogenous ATP is impaired in aging humans, and the contribution of adenosine to ATP-mediated vasodilation in aging humans.

**Experiment #3:** to determine whether the ability of exogenous ATP to modulate α-adrenergic vasoconstriction in the human forearm of older adults is impaired similar to the typical response observed during exercise in aged humans.

**Experiment #4:** to determine whether endogenous venous plasma [ATP] and ATP release is diminished during mild-to-moderate exercise in aging humans.

To the best of our knowledge, the preceding collection of experiments provides novel and significant insight into the understanding of how circulating ATP assists in vascular control in aging humans. The collective findings from these studies indicate that

alterations in the contribution of ATP to vascular tone in aging humans exist and may in part be a potential mechanism by which aged adults have reductions in oxygen delivery to active skeletal muscle. Distinctively, exogenous ATP has the ability to blunt α-adrenergic vasoconstriction in young adults similar to that observed during exercise. In contrast to our hypothesis, the vasodilatory responsiveness and sympatholytic properties of exogenous ATP remain intact in aging humans. However, older adults demonstrate reduced venous plasma [ATP] and impaired ATP release during graded mild-to-moderate handgrip exercise which is associated with attenuations in skeletal muscle vasodilation and blood flow. Taken together, it is our belief that the typically observed impairments in skeletal muscle vasodilation and the inability to offset sympathetic vasoconstrictor tone during exercise is in part due to diminished endogenous levels of circulating ATP. On the whole, ATP appears to be a significant regulator of vascular control in humans, and may act as a potential mechanism which in part explains the typically observed reductions in skeletal muscle blood flow and oxygen delivery to active tissue in aged humans thereby predisposing this population to an elevated risk for cardiovascular diseases.

CHAPTER II – MANUSCRIPT I

# Graded Sympatholytic Effect of Exogenous ATP on Postjunctional α-adrenergic Vasoconstriction in the Human Forearm: implications for vascular control in contracting muscle

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**Abstract**

Recent evidence suggests that adenosine triphosphate (ATP) can inhibit vasoconstrictor responses to endogenous noradrenaline release via tyramine in the skeletal muscle circulation, similar to what is observed in contracting muscle. Whether this involves direct modulation of postjunctional α-adrenoceptor responsiveness, or is selective for $\alpha_1$- or $\alpha_2$-receptors, remains unclear. Therefore, in *Protocol 1*, we tested the hypothesis that exogenous ATP can blunt direct postjunctional α-adrenergic vasoconstriction in humans. We measured forearm blood flow (FBF; Doppler ultrasound) and calculated the vascular conductance (FVC) responses to local intra-arterial infusions of phenylephrine ($\alpha_1$-agonist) and dexmedetomidine ($\alpha_2$-agonist) during moderate rhythmic handgrip exercise (15% maximum voluntary contraction), during a control non-exercise vasodilator condition (adenosine), and during ATP infusion in 8 young adults. Forearm hyperaemia was matched across all conditions. Forearm vasoconstrictor responses to direct $\alpha_1$-receptor stimulation were blunted during exercise vs adenosine (ΔFVC = -11±3% vs -39±5%; $P < 0.05$), and were abolished during ATP infusion (-3±2%). Similarly, vasoconstrictor responses to $\alpha_2$-receptor stimulation were blunted during exercise vs adenosine (-13±4% vs -40±8%; $P < 0.05$), and were abolished during ATP infusion (-4±4%). In *Procotol 2* (n = 10), we tested the hypothesis that graded increases in ATP would reduce $\alpha_1$-mediated vasoconstriction in a dose-dependent manner compared with vasodilatation evoked via adenosine. Forearm vasoconstrictor responses during low dose adenosine (-38±3%) and ATP (-33±2%) were not significantly different from rest (-40±3%; $P > 0.05$). In contrast, vasoconstrictor responses during moderate (-22±6%) and high dose ATP (-8±5%) were significantly blunted compared with rest, whereas the

responses during adenosine became progressively greater (moderate = -48±4%, $P = 0.10$; high = -53±6%, $P < 0.05$). We conclude that exogenous ATP is capable of blunting direct postjunctional α-adrenergic vasoconstriction, that this involves both $\alpha_1$- and $\alpha_2$-receptor subtypes, and that this is graded with ATP concentrations. Collectively, these data are consistent with the conceptual framework regarding how muscle blood flow and vascular tone are regulated in contracting muscles of humans.

## Introduction

Blood flow and oxygen delivery increase in proportion to the oxygen demand of contracting skeletal muscle, a complex response involving competing influences of local vasodilator signals and sympathetic neural vasoconstriction particularly as the intensity of exercise and amount of muscle mass engaged increases (Saltin *et al.* 1998). Although it is clear that sympathetic restraint of active muscle blood flow is imperative for appropriate blood pressure regulation (Marshall *et al.* 1961; Rowell, 1997), it has also been repeatedly demonstrated that the vasoconstrictor responses in contracting muscle are significantly blunted compared with the responses under resting (quiescent) conditions (Remensnyder *et al.* 1962; Anderson & Faber, 1991; Thomas & Victor, 1998; Buckwalter *et al.* 2001; Tschakovsky ME *et al.* 2002; Dinenno & Joyner, 2003). A variety of substances/mechanisms have been proposed in this local modulation of sympathetic vasoconstriction including activation of ATP-sensitive potassium channels ($K_{ATP}$) (Thomas *et al.* 1997; Keller *et al.* 2004), adenosine (Nishigaki *et al.* 1991), nitric oxide (NO) (Thomas & Victor, 1998; Chavoshan *et al.* 2002), and vasodilating prostaglandins (PGs) (Faber *et al.* 1982). However, recent data indicate that independent inhibition of NO and PGs do not influence the ability of contractions to blunt sympathetic vasoconstriction (Dinenno & Joyner, 2003, 2004), and that combined inhibition of these substances only slightly augments the constrictor response to α-adrenoceptor stimulation (Dinenno & Joyner, 2004). Further, exogenous infusion of adenosine to mimic exercise hyperaemia does not blunt sympathetic vasoconstriction (Tschakovsky *et al.* 2002). Thus, identifying *the* sympatholytic factor associated with muscle contractions has proven difficult.

It has recently been proposed that circulating adenosine triphosphate (ATP) could be involved in matching muscle perfusion to oxygen demand during exercise (Ellsworth, 2000; Gonzalez-Alonso *et al.* 2002; Ellsworth, 2004). Although ATP can be produced in many cells, one source of ATP release during mismatches in oxygen delivery and demand appears to be the red blood cell, where ATP release is coupled with the level of deoxygenated hemoglobin (Ellsworth, 2000; Jagger *et al.* 2001; Gonzalez-Alonso *et al.* 2002; Ellsworth, 2004), and can also be augmented by hypercapnia, acidosis, and mechanical deformation (Bergfeld & Forrester, 1992; Ellsworth *et al.* 1995; Sprague *et al.* 1998). In addition to directly evoking vasodilatation via binding to $P_{2y}$-receptors on the endothelium (Burnstock & Kennedy, 1986; Rongen *et al.* 1994), work by Rosenmeier and colleagues (2004) indicate that ATP could assist in matching oxygen delivery with tissue demand by modulating local sympathetic vasoconstrictor tone. Indeed, data derived from this study suggest that circulating ATP can override sympathetic vasoconstriction in the leg circulation evoked via intra-arterial administration of tyramine (evokes endogenous noradrenaline (NA) release), similar to that observed in contracting muscle (Rosenmeier *et al.* 2004). This occurred despite similar increases in femoral venous NA concentrations, suggesting that this modulatory effect of ATP is at the level of postjunctional α-adrenoceptors. However, it must be emphasized that changes in venous NA concentrations do not always accurately reflect NA release from sympathetic nerve endings especially when there are marked changes in regional blood flow (Esler *et al.* 1990). Therefore, whether circulating ATP modulates direct postjunctional α-adrenoceptor responsiveness and whether this is selective for $\alpha_1$- or $\alpha_2$-adrenoceptors is unclear.

An additional question related to the role of circulating ATP in modulating sympathetic vasoconstriction is whether this is graded with the levels of circulating ATP. In this context, several studies utilizing isolated limb models have clearly demonstrated that the magnitude of sympatholysis during exercise is graded with exercise intensity, such that mild levels of muscle contraction (< 10% maximal effort) do not interfere with sympathetic vasoconstriction whereas progressive increases in exercise intensity above this level lead eventually to robust blunting of the vasoconstrictor response (Thomas *et al.* 1994; Buckwalter *et al.* 2001; Tschakovsky *et al.* 2002; Kirby *et al.* 2005). Thus, if circulating ATP does indeed play a role in muscle blood flow regulation during contractions, one would predict that low levels of ATP are not sympatholytic, whereas increasing circulating levels of ATP would lead to a progressive reduction in sympathetic vasoconstrictor responsiveness. This is an important hypothesis to test, as the only data regarding how ATP interacts directly with sympathetic vasoconstriction indicates that ATP completely overrides the vasoconstrictor response (Rosenmeier *et al.* 2004). As such, if ATP release from red blood cells occurred in all active muscle during high intensity large muscle mass exercise, and this completely abolishes sympathetic vasoconstriction as recently demonstrated (Rosenmeier *et al.* 2004), the vasodilatory capacity of exercising muscle would outstrip cardiac pumping capacity and arterial pressure would fall (Joyner & Thomas, 2003; Calbet *et al*. 2004). In healthy humans, this does not occur.

Therefore, in the present study we tested the hypothesis that exogenous ATP infusion blunts direct postjunctional α-adrenergic vasoconstriction in humans and determined whether this is selective for $\alpha_1$- or $\alpha_2$-adrenoceptors. Further, given that the

ability of muscle contractions to blunt a known sympathetic vasoconstrictor stimulus is graded with exercise intensity, we also tested the hypothesis that graded increases in arterial ATP concentrations would cause graded inhibition of sympathetic α-adrenergic vasoconstriction.

## Methods

### *Subjects*

With Institutional Review Board approval and after written informed consent, a total of 18 young healthy adults (12 men, 6 women; age = 22 ± 1 years; weight = 72.6 ± 3.0 kg; height = 176 ± 2 cm; body mass index = 23.1 ± 0.7 kg $m^{-2}$; means±S.E.M) participated in the present study. All were non-smokers, non-obese, normotensive, and not taking any medications. Studies were performed after a 4-hour fast with the subjects in the supine position. All studies were performed according to the Declaration of Helsinki.

### *Arterial Catheterization*

A 20-gauge, 7.6-cm catheter was placed in the brachial artery of the non-dominant arm under aseptic conditions after local anesthesia (2% lidocaine) for local administration of study drugs. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml $h^{-1}$ with heparinized saline (Dinenno & Joyner, 2003, 2004; Dinenno *et al.* 2005). The two side ports were used for infusions of vasoactive drugs.

***Forearm Blood Flow and Vascular Conductance***

A 4 MHz pulsed Doppler probe (Model 500V, Multigon Industries, Mt. Vernon, NY, USA) was used to measure brachial artery mean blood velocity (MBV) with the probe securely fixed to the skin over the brachial artery proximal to the catheter insertion site as previously described by our laboratory (Dinenno & Joyner, 2003, 2004; Dinenno *et al.* 2005). The probe insonation angle relative to the skin was 45 degrees. A linear 12 MHz echo Doppler ultrasound probe (GE Vingmed Ultrasound Vivid7, Horten, Norway) was placed in a holder securely fixed to the skin immediately proximal to the velocity probe to measure brachial artery diameter. Forearm blood flow was calculated as:

FBF = MBV ($cm \cdot s^{-1}$) * $\pi$ (brachial artery diameter/2)$^2$ * 60, where the FBF is in ml $min^{-1}$, the MBV is in cm $s^{-1}$, the brachial diameter is in cm, and 60 is used to convert from ml $s^{-1}$ to ml $min^{-1}$. Forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml $min^{-1}$ 100 $mmHg^{-1}$.

***Rhythmic Handgrip Exercise***

Maximum voluntary contraction (MVC) was determined for each subject as the average of at least three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3 percent of each other. For the exercise trials, weights corresponding to 15% MVC were 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 minute) using audio and visual signals to ensure the correct timing (Dinenno & Joyner, 2003, 2004). We chose this moderate workload because it significantly blunts, but does

not abolish, sympathetic vasoconstriction in contracting muscle (Tschakovsky *et al.* 2002; Dinenno & Joyner, 2003; Dinenno *et al.* 2005).

***Sympathetic α-Adrenergic Vasoconstrictor Drugs***

In male subjects, phenylephrine (a selective $\alpha_1$-agonist; Baxter, Irvine, CA) was infused at 0.0625 μg (dl forearm volume)$^{-1}$ min$^{-1}$ and dexmedetomidine (a selective $\alpha_2$-agonist; Hospira, Lake Forest, IL) was infused at 6.25 ng (dl forearm volume)$^{-1}$ min$^{-1}$. The doses of phenylephrine and dexmedetomidine were chosen based on our experience at rest (Dinenno *et al.* 2002; Smith *et al.* 2007) and during handgrip exercise (Dinenno & Joyner, 2003; Rosenmeier *et al.* 2003a; Dinenno & Joyner, 2004). Because young women typically have reduced vasoconstrictor responses to α-receptor stimulation (Kneale *et al.* 2000), the doses of phenylephrine and dexmedetomidine were doubled for the female participants. All vasoconstrictor drug infusions were adjusted for the hyperaemic conditions as previously described (see below) (Dinenno & Joyner, 2003, 2004).

Given that exercise increases forearm blood flow, adenosine was infused to elevate resting forearm blood flow to similar levels observed during exercise. We have previously demonstrated that exercise blunts the vasoconstrictor responses to direct $\alpha_1$- and $\alpha_2$-adrenoceptor stimulation, whereas these vasoconstrictor responses are maintained when blood flow is elevated with adenosine and hence it was used to create a "high flow" control state (Dinenno & Joyner, 2003; Rosenmeier *et al.* 2003a; Dinenno & Joyner, 2004). In an effort to normalise the concentration of each vasoconstricting drug in the blood perfusing the forearm, the infusions were adjusted on the basis of forearm blood

flow and forearm volume (measured via regional analysis of whole-body DEXA scans). Various concentrations of each compound were available to keep the absolute infusion rates less than 3 ml $min^{-1}$ in every trial.

## Experimental Protocols

### *General Experimental Protocol*

Figure 1 is an example of a time-line for the specific trials. In the supine position, subjects performed either a bout of handgrip exercise, or received intra-arterial adenosine (Sicor, Irvine, CA) or ATP (Sigma, USA); the total time for each trial was 8 minutes. After 2 minutes of baseline measurements, exercise or vasodilator infusion was initiated and steady-state FBF was reached within 3 minutes. Between 3 and 4 minutes of hyperaemia (minutes 5 and 6 of Figure 1) the dose of the $\alpha_1$- or $\alpha_2$- agonist (vasoconstrictor) was calculated on the basis of forearm volume and blood flow. The vasoconstrictor infusion began at the 6-minute mark and lasted for 2 minutes.

### *Protocol 1: Effects of Exogenous ATP on Postjunctional α-adrenergic Vasoconstrictor Responsiveness*

The purpose of this protocol was to determine whether exogenous ATP blunts direct postjunctional α-adrenergic responsiveness, and whether this is selective for $\alpha_1$- or $\alpha_2$-adrenoceptors. Therefore, in 8 subjects (6 men, 2 women), the vasoconstrictor responses to direct $\alpha_1$- and $\alpha_2$-adrenoceptor stimulation (via phenylephrine and dexmedetomidine, respectively) were assessed during control vasodilator infusion of adenosine, during moderate rhythmic handgrip exercise (15% MVC), and during infusion

of ATP. In total, there were 6 experimental trials for each subject. In this protocol, the goal was to match steady-state FBF during infusion of adenosine or ATP with that observed during exercise. To do so, adenosine (45 nmol $100ml^{-1}$ $min^{-1}$) and ATP (5 nmol $100ml^{-1}$ $min^{1}$) were initially infused and doses were increased to elevate FBF accordingly. The final average doses of adenosine and ATP were 73±8 and 11±2 nmol $100ml^{-1}$ $min^{-1}$, respectively. The order of the adenosine, exercise, and ATP trials were counterbalanced across subjects. Thus, for subjects that did not perform the exercise trial first, we had them perform 3-4 minutes of rhythmic handgrip exercise prior to any experimental trials with α-agonists to determine their individual steady-state FBF for this exercise intensity. Additionally, in one half of the subjects, vasoconstrictor responses to $\alpha_1$-adrenoceptor stimulation were determined under each hyperaemic condition, followed by the trials for $\alpha_2$-receptor stimulation. This order was reversed in the other 4 subjects, and all subjects rested for 15 minutes between each trial.

### *Protocol 2: Effects of Graded Infusions of ATP on Postjunctional α-adrenergic Vasoconstrictor Responsiveness*

The ability of muscle contractions to blunt a known sympathetic vasoconstrictor stimulus is graded with exercise intensity, such that greater inhibition of α-mediated vasoconstriction (greater sympatholysis) is observed with increasing workloads (Thomas *et al.* 1994; Buckwalter *et al.* 2001; Tschakovsky *et al.* 2002; Kirby *et al.* 2005). Therefore, the purpose of this protocol was to determine whether graded increases in exogenous ATP caused graded sympatholysis as has been observed during exercise. In 10 subjects (6 men, 4 women), we determined the vasoconstrictor responses to direct $\alpha_1$-

adrenoceptor stimulation via phenylephrine at rest (saline control), as well as during graded increases in ATP and adenosine. In total, there were 7 experimental trials for each subject and each trial was performed in a similar fashion as outlined in *Protocol 1*. For this protocol, the doses of ATP were calculated (based on resting forearm blood flow) to increase arterial concentrations by 500, 1000, and 2000 nmol $L^{-1}$ ("low", "moderate", and "high") provided no ATP degradation were to occur, and this was based on data obtained from the femoral vein during graded knee extensor exercise (Gonzalez-Alonso *et al.* 2002). Similar to *Protocol 1*, we infused adenosine as a control vasodilator at concentrations required to match the increase in FBF evoked via these doses of ATP. Thus, to do so, ATP trials were always performed prior to adenosine trials, but the order of ATP and adenosine doses (low, moderate, and high) were counterbalanced across subjects. All trials were separated by 15 minutes of rest.

***Data Acquisition and Analysis***

Data was collected and stored on computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Mean arterial pressure (MAP) was determined from the arterial pressure waveform. Baseline FBF, HR, and MAP represent an average of the last minute of the resting time period, the steady-state hyperaemic values represent an average of minutes 3-4 (minutes 5-6 of Figure 1; pre-vasoconstrictor) during exercise, adenosine, or ATP and the effects of the $\alpha$-agonists represent an average of the final 30-seconds of drug infusion (post-vasoconstrictor). The % reduction in FBF during vasoconstrictor administration was calculated as:

((FBF post constrictor - FBF pre constrictor)/(FBF pre constrictor)) x 100.

We also calculated % reduction in FVC as our standard index to compare vasoconstrictor responses to the α-agonists across conditions, as this appears to be the most appropriate way to compare vasoconstrictor responsiveness under conditions where there might be differences in vascular tone (Lautt, 1989; O'Leary, 1991; Thomas *et al.* 1994; Tschakovsky *et al.* 2002). In an effort to be comprehensive, we have also presented absolute values of forearm haemodynamics for all conditions in tabular form.

### *Statistics*

All values are reported as means ± S.E.M. Specific hypothesis testing within each of the exercise, adenosine, or ATP trials with the two different α-agonist infusions was performed using repeated measures ANOVA. Comparison of the hemodynamic values at specific time points between the exercise, adenosine, and ATP conditions were made with unpaired t-tests, and the values within each hyperaemic condition (exercise, adenosine, or ATP) with paired t-tests. Significance was set at $P<0.05$.

## Results

### *Protocol 1: Effects of Exogneous ATP on Postjunctional α-adrenergic Vasoconstrictor Responsiveness*

Forearm haemodynamics, HR, and MAP for *Protocol 1* are presented in Table 1. Intra-arterial infusion of both adenosine and ATP, as well as handgrip exercise, significantly increased FBF and FVC from baseline ($P < 0.05$). As desired by experimental design, steady-state (pre-vasoconstrictor) FBF responses to adenosine and

ATP infusion were effectively matched to that observed during 15% MVC handgrip exercise within both phenylephrine (Figure 2A) and dexmedetomidine conditions (Figure 2B; $P = 0.5 - 0.9$). Infusion of phenylephrine ($\alpha_1$-agonist) significantly reduced FBF from steady-state hyperaemia during adenosine and exercise ($P < 0.05$), whereas FBF was unchanged during ATP (*NS*; Figure 2A). Similarly, infusion of dexmedetomidine ($\alpha_2$-agonist) significantly reduced FBF from steady-state hyperaemia during adenosine and exercise ($P < 0.05$), whereas FBF was unchanged during ATP (*NS*; Figure 2B).

The forearm vasoconstrictor responses to direct $\alpha_1$-adrenoceptor stimulation were blunted during steady-state exercise vs adenosine ($\Delta$FVC = -11 ± 3% vs -39 ± 5%; $P < 0.05$), and were abolished during ATP infusion (-3 ± 2%; $P = 0.2$ vs zero; Figure 3A). Similarly, vasoconstrictor responses to $\alpha_2$-receptor stimulation were blunted during exercise vs adenosine (-13 ± 4% vs -40 ± 8%; $P < 0.05$), and were abolished during ATP infusion (-4 ± 4%; $P = 0.5$ vs zero; Figure 3B). MAP changed minimally within and between conditions (Table 1A and B), thus FBF responses were similar to FVC. Heart rate increased in response to exercise ($P < 0.05$), but otherwise was not significantly between or within trials and conditions (Table 1).

### *Protocol 2: Effects of Graded Infusions of ATP on Postjunctional α-adrenergic Vasoconstrictor Responsiveness*

Forearm haemodynamics, HR, and MAP for *Protocol 2* are presented in Table 2. Graded increases in exogenous ATP evoked a dose-dependent increase in FBF (Figure 4). Specifically, increasing the arterial concentration by 500, 1000, and 2000 nmol $L^{-1}$ increased FBF by ~2, 3, and 4-fold respectively (all $P < 0.05$ vs baseline). As desired by

experimental design, steady-state (pre-vasoconstrictor) FBF responses to adenosine infusion were effectively matched to that observed within each dose condition of ATP (Figure 4; $P = 0.9$ - 1.0). Infusion of phenylephrine ($\alpha_1$-agonist) significantly reduced FBF from steady-state hyperaemia during low and moderate dose adenosine and ATP (both $P < 0.05$; Figure 4A and B). However, although phenylephrine also reduced FBF during high dose adenosine ($P < 0.05$), it did not significantly affect FBF during high dose ATP ($P = 0.16$; Figure 4C).

The forearm vasoconstrictor responses to direct $\alpha_1$-adrenoceptor stimulation ($\Delta$FVC) under control resting conditions were -40 ± 3%. The vasoconstrictor responses during low dose adenosine (-38 ± 3%) and ATP (-33 ± 2%) were not significantly different from one another, and were similar to the responses observed at rest ($P = 0.3$ - 0.9). The vasoconstrictor responses during the moderate dose of adenosine tended to be greater versus resting conditions (-48 ± 4%; $P = 0.07$), and the responses during high dose adenosine were significantly greater than rest conditions (-53 ± 6%; $P < 0.05$), most likely due to the absolute amount of phenylephrine infused as part of the flow adjustment process. In contrast, the vasoconstrictor responses during moderate dose ATP (-22 ± 6%) were significantly blunted compared with rest, and the responses during high dose ATP (-8 ± 5%) were significantly blunted compared with rest and those observed during low dose ATP (both $P < 0.05$; Figure 5). Importantly, the vasoconstrictor responses during moderate and high dose ATP were significantly blunted compared with the responses during the conditions of matched forearm hyperaemia via adenosine (both $P < 0.05$). MAP changed minimally within and between conditions, thus FBF responses were

similar to FVC. Heart rate and MAP were not significantly different between trials or conditions (Table 2).

## Discussion

The primary findings from the present investigation are as follows. First, exogenous infusions of ATP required to match steady-state hyperaemia observed during moderate intensity dynamic handgrip exercise (15% MVC) abolishes postjunctional α-adrenoceptor mediated vasoconstriction in humans. Second, the ability of ATP to modulate α-mediated vasoconstriction under these conditions is not selective for $\alpha_1$-or $\alpha_2$-adrenoceptors, as both were similarly abolished by exogenous ATP. Third, increasing arterial ATP concentrations to mimic levels observed within the physiological range during dynamic exercise elevates resting forearm blood flow in a dose-dependent manner. Finally, low dose ATP infusion is not sympatholytic in the human forearm, however graded increases in ATP infusions progressively blunt postjunctional α-adrenergic vasoconstriction. Importantly, these data cannot be explained simply by the vasodilator properties *per se* of ATP, as forearm hyperaemia was matched via infusions of adenosine which did not blunt sympathetic α-adrenergic vasoconstriction. The physiological implications of these findings will now be discussed.

### *Exogenous ATP and Postjunctional α-adrenergic Vasoconstriction*

Data derived from experimental studies using a variety of approaches in both animals and humans have clearly demonstrated a unique ability of contracting muscle to blunt sympathetic vasoconstriction, a phenomenon believed to optimize blood flow and

oxygen delivery to active muscle when the sympathetic nervous system is engaged (Joyner & Thomas, 2003; VanTeeffelen & Segal, 2003). Several potential modulators of sympathetic vasoconstriction have been suggested to contribute to this phenomenon including adenosine, NO, and PGs, although elucidating a clear mechanism in humans has proved difficult (Dinenno& Joyner, 2003, 2004). Recently, however, Rosenmeier and colleagues (2004) demonstrated that exogenous ATP infusions abolished regional vasoconstriction in the skeletal muscle circulation evoked via intra-arterial tyramine, similar to what was observed during isolated knee extensor exercise at 25% of peak power output. These data are of significant interest in that infusions of other vasodilators such as adenosine and sodium nitroprusside (NO donor) to mimic exercise hyperaemia do not interfere with sympathetic vasoconstriction in humans (Tschakovsky *et al.* 2002; Dinenno & Joyner, 2003; Rosenmeier *et al.* 2003b). Further, emerging evidence that ATP released from red blood cells in proportion to deoxygenated hemoglobin might act to couple blood flow and oxygen delivery to demand during exercise make it an attractive candidate capable of directly causing vasodilatation and also limiting sympathetic vasoconstriction in active muscle. In this context, ATP appears to be a good candidate for explaining sympatholyis because it has a short half-life, is released when erythrocytes become deoxygenated which would occur in close proximity of active muscle fibers, and this would allow sympathetic vasoconstriction to occur in resistance vessels of less active fibers to maintain an appropriate match between oxygen demand and oxygen delivery at the microcirculatory level (Calbet *et al.* 2006; Lundby *et al.* 2008).

In the present study, we directly tested the hypothesis that exogenous ATP infusion blunts postjunctional α-adrenoceptor responsiveness similar to that observed

during exercise. In the study by Rosenmeier and colleagues (2004), intra-arterial administration of tyramine was used to evoke endogenous NA release and cause subsequent vasoconstriction. Although we (Dinenno & Joyner, 2003, 2004) and others (Ruble *et al.* 2002; Wilkins *et al.* 2006) have also used this approach, it must be emphasized that measuring changes in venous NA concentrations do not always accurately reflect NA release from sympathetic nerve endings especially when there are marked changes in regional blood flow (Esler *et al.* 1990). Therefore, we determined the forearm vasoconstrictor responses to direct $\alpha_1$- and $\alpha_2$-adrenoceptor simulation (via phenylephrine and dexmedetomidine, respectively) during moderate handgrip exercise (15% MVC), during control vasodilator infusion of adenosine, and during infusion of ATP required to match forearm hyperaemia during exercise (*Protocol 1*). As expected, there was marked vasoconstriction to both α-agonists during infusion of adenosine, whereas the responses were significantly blunted (but not abolished) during handgrip exercise. These data are consistent with our previous work in this area of investigation (Dinenno & Joyner, 2003, 2004). Somewhat similar to our observations during exercise, the vasoconstrictor responses to direct α-adrenoceptor stimulation were abolished during infusion of exogenous ATP. To the best of our knowledge, these data provide the first experimental evidence that exogenous ATP modulates direct postjunctional α-adrenoceptor vasoconstriction in humans.

With respect to the α-adrenoceptor subtypes, we observed no difference in the ability of muscle contractions to blunt $\alpha_1$- and $\alpha_2$-mediated vasoconstriction in the human forearm. Similarly, exogenous ATP abolished both $\alpha_1$- and $\alpha_2$-mediated vasoconstriction with no apparent selectivity for the α-receptor subtypes. These data are consistent with

previous studies indicating that both postjunctional $\alpha_1$- and $\alpha_2$-adrenoceptor responsiveness are significantly blunted during moderate forearm exercise in humans (Dinenno & Joyner, 2003; Rosenmeier *et al.* 2003a; Dinenno & Joyner, 2004). It should be noted that there are data in humans performing knee extensor exercise suggestive that $\alpha_2$-adrenoceptors are more sensitive to metabolic inhibition than $\alpha_1$-receptors (Wray *et al.* 2004), and this is conceptually similar with data derived from various experimental animal models (Anderson & Faber, 1991; Buckwalter *et al.* 2001). However, in this particular study in humans, there were marked pressor effects (and thus baroreflex activation) during infusion of the α-agonists that preclude clear interpretation of this data (Wray *et al.* 2004). Nevertheless, if $\alpha_2$-adrenoceptors are indeed more sensitive to metabolic inhibition in the leg circulation, it would be of interest to determine whether ATP has a greater modulatory effect on $\alpha_2$- versus $\alpha_1$-adrenoceptor mediated vasoconstriction in the leg vasculature.

***Exogenous ATP and Graded Modulation of Postjunctional α-adrenergic Vasoconstriction***

In *Protocol 1*, we demonstrated that exogenous ATP required to match steady-state hyperaemia observed during moderate dynamic handgrip exercise (15% MVC) completely abolished postjunctional $\alpha_1$- and $\alpha_2$-mediated vasoconstriction. From a physiological standpoint, however, if circulating ATP were to completely override sympathetic vasoconstriction in active muscle during large muscle mass exercise, excess vasodilatation would occur and arterial pressure would be compromised (Marshall *et al.* 1961; Rowell, 1997; Joyner & Thomas, 2003). Thus, in *Protocol 2*, we determined

whether graded ATP infusions evoked a dose-dependent modulation of α-adrenergic vasoconstriction, as has been demonstrated during graded levels of exercise (Thomas *et al.* 1994; Buckwalter *et al.* 2001; Tschakovsky *et al.* 2002; Kirby *et al.* 2005). Interestingly, we found that low dose ATP infusion (500 nmol $L^{-1}$) sufficient to increase resting forearm blood flow 2-fold did not blunt α-adrenergic vasoconstriction. This is strikingly similar to what is observed during very mild muscle contractions (5% MVC) that elevate forearm blood flow to a similar extent (Kirby *et al.* 2005). However, at moderate (1000 nmol $L^{-1}$) and higher (2000 nmol $L^{-1}$) doses of ATP selected to increase arterial concentrations similar to that observed in the femoral vein draining skeletal muscle during graded knee extensor exercise (Gonzalez-Alonso *et al.* 2002), α-adrenoceptor responsiveness was progressively blunted. In this context, the ability of graded ATP infusions to limit postjunctional α-adrenoceptor mediated vasoconstriction is quite similar to what is observed with graded exercise intensity, and provides further support for the hypothesis that circulating ATP could play a significant role in regulating muscle blood flow and vascular tone during exercise.

***Potential Mechanisms***

The mechanism(s) by which circulating ATP can modulate sympathetic α-adrenergic vasoconstriction in the skeletal muscle circulation are presently unknown. What is clear, however, is that this does not simply reflect the vasodilator properties of ATP *per se*, as infusions of other vasodilators to mimic exercise hyperaemia do not blunt sympathetic vasoconstriction (Tschakovsky *et al.* 2002; Rosenmeier *et al.* 2003b). Indeed, in the present study (*Protocol 2*), adenosine was not capable of inhibiting α-

adrenergic vasoconstriction. Thus, we speculate that cellular mechanisms by which ATP-induced $P_{2y}$ receptor activation evokes smooth muscle cell relaxation are involved. In this context, recent studies indicate that ATP-mediated vasodilatation in humans is independent of NO and PGs (van Ginneken *et al.* 2004), which is consistent with recent findings that independent inhibition of NO (Dinenno & Joyner, 2003) and PGs (Hansen *et al.* 2000; Dinenno & Joyner, 2004) do not significantly impact on functional sympatholysis in humans. Further, when we performed combined NO and PG inhibition, sympathetic vasoconstriction in contracting muscle was only slightly augmented compared with control conditions, suggesting other modulatory factors were involved (Dinenno & Joyner, 2004). Taken together, it seems plausible to speculate that ATP evokes smooth muscle cell hyperpolarization, and this in turn blunts sympathetic α-adrenergic vasoconstriction. Future studies will be needed to determine the exact mechanism underlying the sympatholytic effect of circulating ATP.

***Experimental Considerations***

In *Protocol 2* of the present investigation, we chose to only use phenylephrine ($\alpha_1$- agonist) to test whether the ability of circulating ATP to blunt postjunctional α-adrenoceptor vasoconstriction was graded with ATP concentrations, as opposed to using both $\alpha_1$- and $\alpha_2$-adrenoceptor agonists. However, in *Protocol 1*, our data indicated that exogenous infusion of ATP at concentrations to match forearm hyperaemia observed during moderate intensity handgrip exercise abolished both $\alpha_1$- and $\alpha_2$-mediated vasoconstriction, with no apparent difference between the receptor subtypes. Additionally, we were concerned about giving dexmedetomidine ($\alpha_2$-agonist) over 7

experimental trials, as this would significantly increase the risk of $\alpha_2$-adrenoceptor effects on the central nervous system (e.g., hypotension, sedation). This is important not only from a subject safety standpoint, but also if there were central $\alpha_2$ effects, this would inhibit basal sympathetic outflow (Lang *et al.* 1997) and cloud interpretation of the data for the remaining experimental trials .

An additional consideration for *Protocol 2* relates to the dose adjustment of phenylephrine we employed based on changes in forearm blood flow, which was performed to reduce any potential "dilution effect" of the $\alpha_1$-agonist during the various doses of adenosine and ATP. Our findings indicate that the vasoconstrictor responses during the moderate dose of adenosine tended to be greater, and that the vasoconstrictor responses during the high dose of adenosine were greater compared with control (saline) conditions. Although seemingly counterintuitive, this is consistent with what has been demonstrated when adjustments in the dose of tyramine were performed for similar reasons during hyperaemic conditions associated with adenosine infusions (Tschakovsky *et al.* 2002). Thus, although we were aware this might occur, we needed to be sure that any apparent sympatholytic effect of exogenous ATP was not due to a dilution effect, as this has never been determined before during ATP infusions. Regardless, our data clearly indicate a graded sympatholytic effect of exogenous ATP whether compared with the vasoconstriction observed during control conditions, or conditions of matched hyperaemia via adenosine.

***Experimental Limitations***

One limitation of the present study relates to the use of moderate intensity exercise with a small muscle mass, and thus moderate hyperaemic conditions, to test our hypotheses. However, our experimental model allows for moderate intensity dynamic muscle contractions to be performed without increasing sympathetic outflow, and allows for well-controlled vasoactive drug infusions that do not alter arterial blood pressure and thus do not evoke baroreflex-mediated alterations in sympathetic outflow. This is important in that changes in sympathetic nervous system activity would cloud interpretation of our vasoconstrictor responses during the α-agonist infusions. Nevertheless, it is important to recognize that the potential interaction between ATP and sympathetic vasoconstriction during larger muscle mass, higher intensity exercise could be more complex than observed in our studies as indicated by recent data demonstrating that some degree of local vasoconstriction is necessary for the precise matching of oxygen delivery to oxygen demand under such conditions (Calbet *et al.* 2006; Lundby *et al.* 2008).

Another limitation of the present study relates to the lack of measurement of circulating arterial or venous plasma ATP concentrations during the experimental trials. In *Protocol 1*, our goal was to match forearm blood flow to that observed during match steady-state exercise hyperaemia, thus we were not concerned with how much exogenous ATP was required to achieve this. However, in *Protocol 2*, our doses were chosen to mimic average increases in ATP concentrations observed in the venous circulation during graded knee extensor exercise in humans (Gonzalez-Alonso *et al.* 2002). We used data from the venous circulation (versus arterial) as ATP release from red blood cells would

occur at the level of the microcirculation and thus venous concentrations would provide a better estimate of this. Using this approach, we were able to demonstrate a 2-4 fold increase in forearm blood flow across this range of exogenous ATP and further, that increasing ATP concentrations within this predicted range caused graded sympatholysis. However, it still remains unclear what exact concentrations of ATP at the level of the resistance vessel network are ultimately observed during exercise, and thus are sympatholytic under these conditions. A final limitation of the present investigation relates to the inability to inhibit $P_{2y}$-receptors in humans due to the lack of available pharmacological agent. In this context, to definitively determine if ATP is mechanistically-linked with the ability of muscle contractions to blunt sympathetic vasoconstriction, similar studies will need to be performed before and after $P_{2y}$-receptor blockade and demonstrate that muscle contractions are incapable of modulating sympathetic vasoconstriction under conditions of $P_{2y}$-receptor inhibition.

***Conclusions***

The results from the present investigation demonstrate that exogenous ATP infusions required to match the hyperaemic responses observed during moderate handgrip exercise abolish postjunctional $\alpha_1$- and $\alpha_2$-adrenoceptor responsiveness in the human forearm. Importantly, graded increases in arterial concentrations of ATP within the physiological range that evoke moderate limb hyperaemia causes graded inhibition of α-mediated vasoconstriction, such that low levels are not sympatholytic whereas progressive reductions in α-adrenoceptor mediated vasoconstriction are observed with increasing ATP concentrations. Collectively, these data are consistent with the

conceptual framework regarding how muscle blood flow and vascular tone are regulated in contracting muscles of humans.

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| Table 1A. *Protocol 1* Forearm and Systemic Haemodynamics: Phenylephrine Trials | | | | | |
|---|---|---|---|---|---|
| Time | Condition | Forearm Blood Flow (ml $min^{-1}$) | Mean Arterial Pressure (mmHg) | Forearm Vascular Conductance (ml $min^{-1}$ 100 $mmHg^{-1}$) | Heart Rate (beats $min^{-1}$) |
| Baseline | Adenosine | 30 ± 4 | 92 ± 3 | 32 ± 4 | 56 ± 3 |
| | Exercise | 26 ± 4 | 94 ± 3 | 28 ± 4 | 55 ± 4 |
| | ATP | 29 ± 5 | 92 ± 2 | 31 ± 5 | 54 ± 4 |
| Pre-Phenylephrine | Adenosine | 143 ± 16* | 93 ± 3 | 156 ± 19* | 57 ± 3 |
| | Exercise | 154 ± 13* | 96 ± 3 | 160 ± 12* | 60 ± 4* |
| | ATP | 138 ± 19* | 94 ± 2 | 149 ± 21* | 56 ± 3 |
| Phenylephrine | Adenosine | 89 ± 9*† | 98 ± 3* | 92 ± 10*† | 56 ± 3 |
| | Exercise | 136 ± 12*†‡ | 96 ± 3 | 141 ± 10*†‡ | 60 ± 3* |
| | ATP | 133 ± 19*‡ | 93 ± 1 | 143 ± 21*‡ | 54 ± 3 |

| Table 1B. *Protocol 1* Forearm and Systemic Haemodynamics: Dexmedetomidine Trials | | | | | |
|---|---|---|---|---|---|
| Time | Condition | Forearm Blood Flow (ml $min^{-1}$) | Mean Arterial Pressure (mmHg) | Forearm Vascular Conductance (ml $min^{-1}$ 100 $mmHg^{-1}$) | Heart Rate (beats $min^{-1}$) |
| Baseline | Adenosine | 27 ± 4 | 95 ± 2 | 28 ± 4 | 53 ± 3 |
| | Exercise | 30 ± 6 | 92 ± 3 | 33 ± 7 | 54 ± 4 |
| | ATP | 30 ± 6 | 97 ± 2 | 31 ± 6 | 54 ± 3 |
| Pre-Dexmedetomidine | Adenosine | 141 ± 25* | 98 ± 3 | 144 ± 25* | 54 ± 4 |
| | Exercise | 150 ± 16* | 94 ± 2 | 158 ± 15* | 57 ± 4* |
| | ATP | 153 ± 20* | 95 ± 2 | 162 ± 21* | 54 ± 3 |
| Dexmedetomidine | Adenosine | 85 ± 15*† | 101 ± 3 | 84 ± 15*† | 55 ± 3 |
| | Exercise | 132 ± 12*†‡ | 95 ± 2 | 136 ± 12*†‡ | 57 ± 4* |
| | ATP | 152 ± 23*‡ | 96 ± 2 | 159 ± 24*‡ | 53 ± 3 |

***** $P < 0.05$ vs baseline within condition; † $P < 0.05$ vs steady-state (Pre-vasoconstrictor; Phenylephrine/Dexmedetomidine) within condition; ‡ $P < 0.05$ vs adenosine during α-agonist infusion. Forearm vascular conductance was calculated as (forearm blood flow/mean arterial pressure) x 100.

| Table 2. *Protocol 2* Forearm and Systemic Haemodynamics during Graded Infusions of ATP and Adenosine | | | | | |
|---|---|---|---|---|---|
| Time | Condition/ Dose | Forearm Blood Flow (ml $min^{-1}$) | Mean Arterial Pressure (mmHg) | Forearm Vascular Conductance (ml $min^{-1}$ 100 $mmHg^{-1}$) | Heart Rate (beats $min^{-1}$) |
| Baseline | Adenosine 1 | 21 ± 4 | 86 ± 3 | 25 ± 5 | 58 ± 5 |
| | ATP 1 | 23 ± 3 | 86 ± 3 | 27 ± 4 | 59 ± 5 |
| | Adenosine 2 | 23 ± 3 | 86 ± 3 | 27 ± 4 | 58 ± 5 |
| | ATP 2 | 22 ± 3 | 86 ± 3 | 26 ± 4 | 59 ± 4 |
| | Adenosine 3 | 23 ± 3 | 86 ± 3 | 28 ± 4 | 60 ± 5 |
| | ATP 3 | 24 ± 3 | 86 ± 3 | 28 ± 4 | 59 ± 5 |
| Pre-Phenylephrine | Adenosine 1 | 51 ± 8* | 86 ± 3 | 61 ± 9* | 60 ± 5 |
| | ATP 1 | 50 ± 9* | 84 ± 3 | 61 ± 12* | 58 ± 5 |
| | Adenosine 2 | 66 ± 9* | 87 ± 2 | 77 ± 11* | 59 ± 5 |
| | ATP 2 | 66 ± 9* | 86 ± 3 | 78 ± 12* | 59 ± 5 |
| | Adenosine 3 | 90 ± 12* | 87 ± 3 | 105 ± 14* | 60 ± 5 |
| | ATP 3 | 88 ± 13* | 84 ± 3 | 106 ± 17* | 59 ± 5 |
| Phenylephrine | Adenosine 1 | 33 ± 6*† | 88 ± 4 | 38 ± 7*† | 60 ± 5 |
| | ATP 1 | 35 ± 7*† | 87 ± 3 | 41 ± 9*† | 59 ± 5 |
| | Adenosine 2 | 36 ± 6*† | 90 ± 3* | 41 ± 8*† | 59 ± 5 |
| | ATP 2 | 53 ± 10*† | 88 ± 4 | 62 ± 12*† | 58 ± 5 |
| | Adenosine 3 | 43 ± 8*† | 90 ± 4* | 50 ± 10*† | 60 ± 5 |
| | ATP 3 | 79 ± 11*‡ | 86 ± 3 | 94 ± 14*‡ | 59 ± 5 |

***** $P < 0.05$ vs baseline within condition; † $P < 0.05$ vs steady-state (Pre-vasoconstrictor; Phenylephrine) within condition; ‡ $P < 0.05$ vs adenosine during phenylephrine ($\alpha_1$-agonist) infusion. 1 = Low dose condition; 2 = Moderate dose condition; 3 = High dose condition (see text for details). Forearm vascular conductance was calculated as (forearm blood flow/mean arterial pressure) x 100.

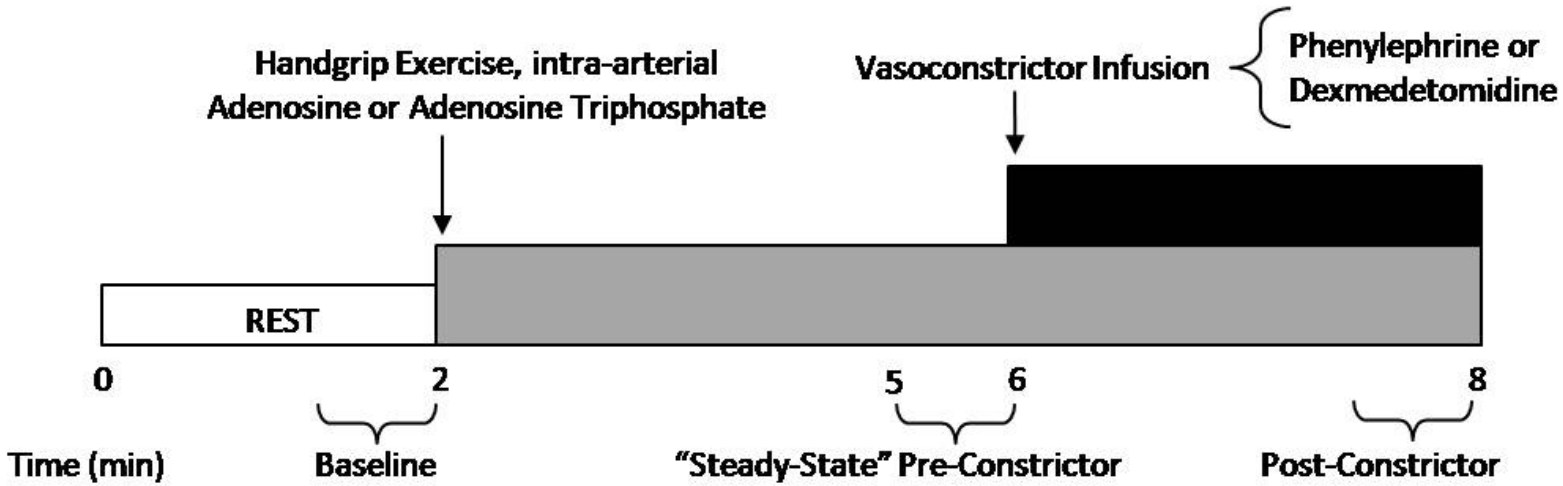
**Figure 1: General Experimental Trial.** Each trial consisted of a 2-minute baseline period. After this time period, subjects either began rhythmic handgrip exercise or received intra-arterial adenosine or adenosine triphosphate (ATP) to elevate resting forearm blood flow to levels observed during exercise. During minutes 5-6 (pre-constrictor), the doses of the $\alpha_1$- or $\alpha_2$-adrenoceptor agonists (phenylephrine or dexmedetomidine, respectively) were calculated on the basis of steady-state hyperaemic forearm blood flow and forearm volume. Subsequently, the α-agonist was infused for 2 minutes until minute 8. An average of forearm blood flow and mean arterial blood pressure during the final 30 seconds of α-agonist infusion was used to calculate the vasoconstrictor effect during all hyperaemic conditions.

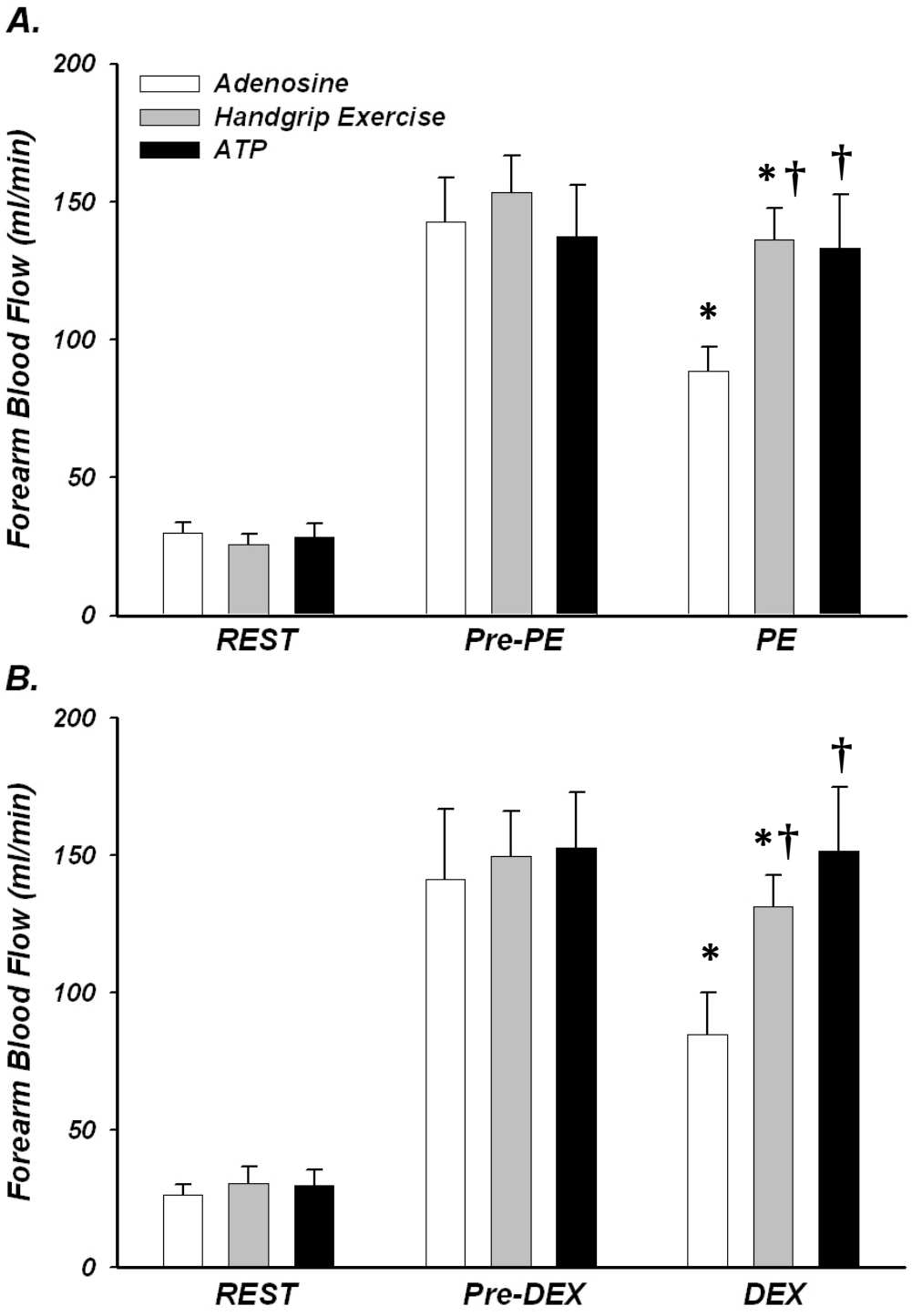
**Figure 2: Forearm blood flow at rest, during each hyperaemic condition, and during infusion of α-agonists.** Steady-state hyperaemia was similar during rhythmic handgrip exercise, adenosine, and ATP infusions for trials involving the $\alpha_1$-agonist phenylephrine (A; Pre-PE) and the $\alpha_2$-agonist dexmedetomidine (B; Pre-Dex). Forearm blood flow was reduced significantly with both α-agonists during adenosine and exercise, but the response was attenuated during exercise. In contrast, α-agonist infusion did not significantly reduce forearm blood flow during ATP. ***** $P < 0.05$ vs steady state (Pre-vasoconstrictor; PE/Dex) within condition; † $P < 0.05$ vs adenosine during α-agonist infusion.

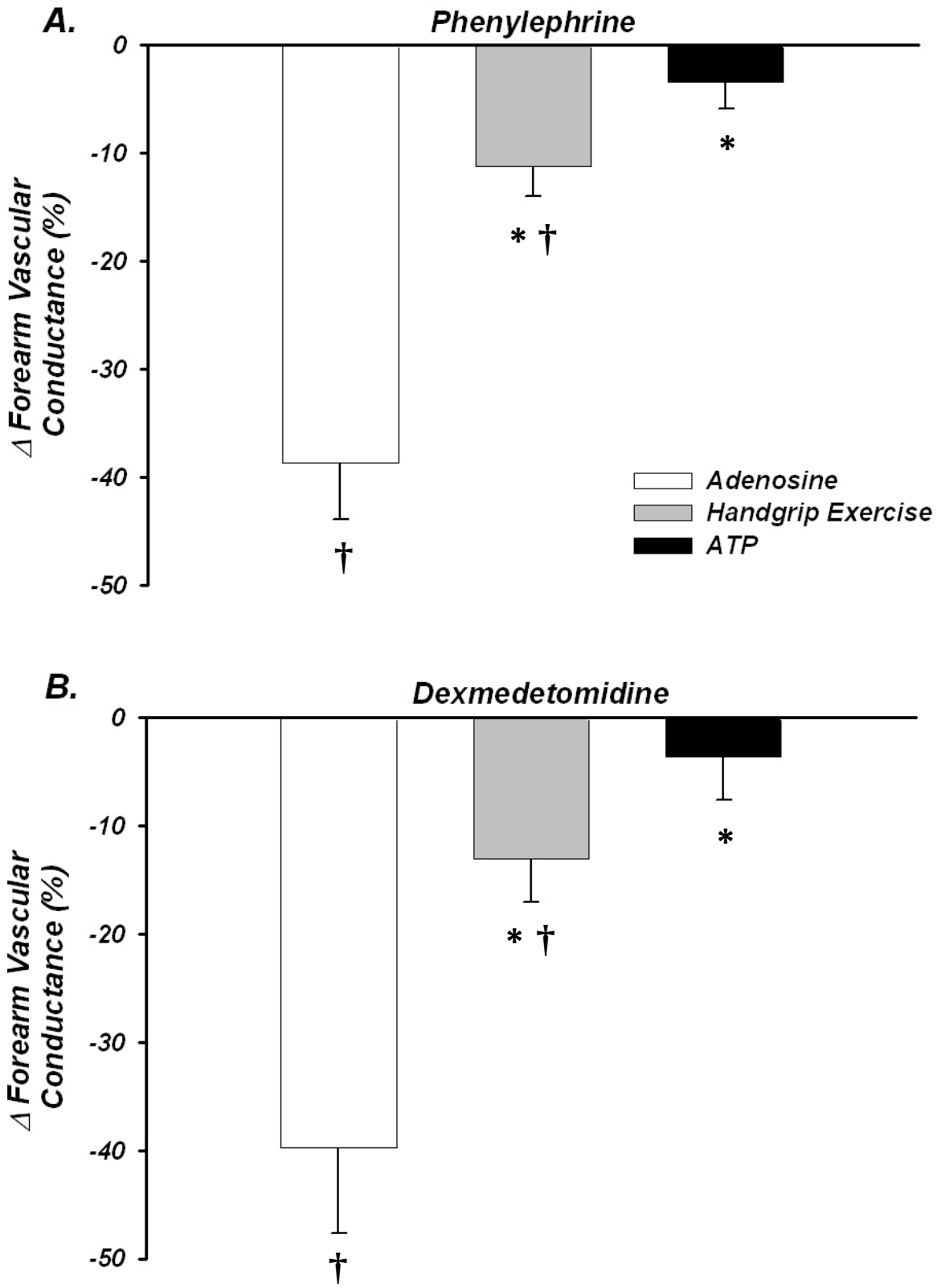
**Figure 3: Forearm vasoconstrictor responses to $\alpha_1$- and $\alpha_2$-adrenoceptor stimulation.** Percentage reductions in forearm vascular conductance to phenylephrine ($\alpha_1$-agonist) (A) were significantly blunted during exercise and abolished during ATP compared with adenosine infusions. Similar data was obtained in response to $\alpha_2$-adrenoceptor stimulation via dexmedetomidine (B). ***** $P < 0.05$ vs adenosine; † $P < 0.05$ vs zero.

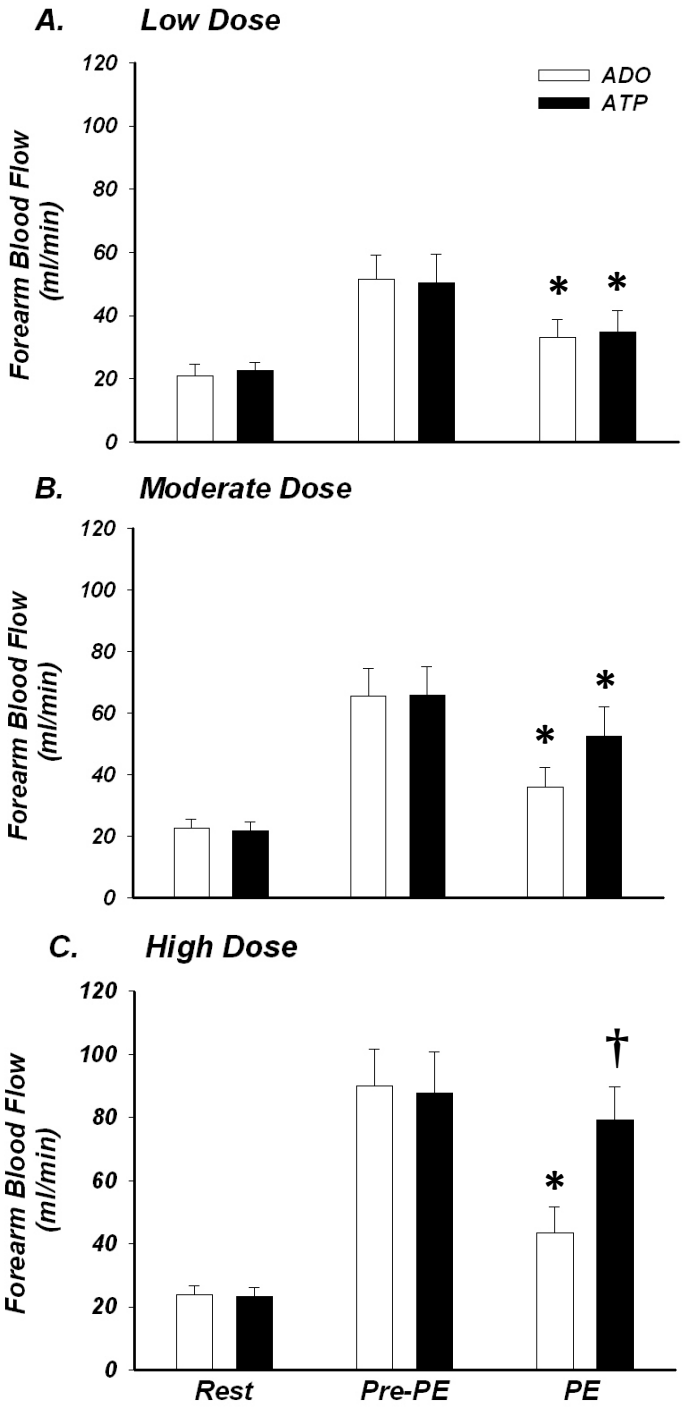
**Figure 4: Forearm blood flow at rest, during the adenosine and ATP hyperaemic conditions, and during infusion of the $\alpha_1$-agonist.** Forearm blood flow was significantly elevated in a dose-dependent manner with exogenous ATP ($P < 0.05$), and forearm hyperaemia was effectively matched via infusion of adenosine. $\alpha_1$-adrenoceptor stimulation with phenylephrine (PE) significantly reduced forearm blood flow during all doses (low, moderate, high) of adenosine. In contrast, phenylephrine significantly reduced forearm blood flow during low and moderate dose ATP, whereas this was not significant during high dose ATP. ***** $P < 0.05$ vs steady state (Pre-PE) within condition; † $P < 0.05$ vs adenosine during phenylephrine.

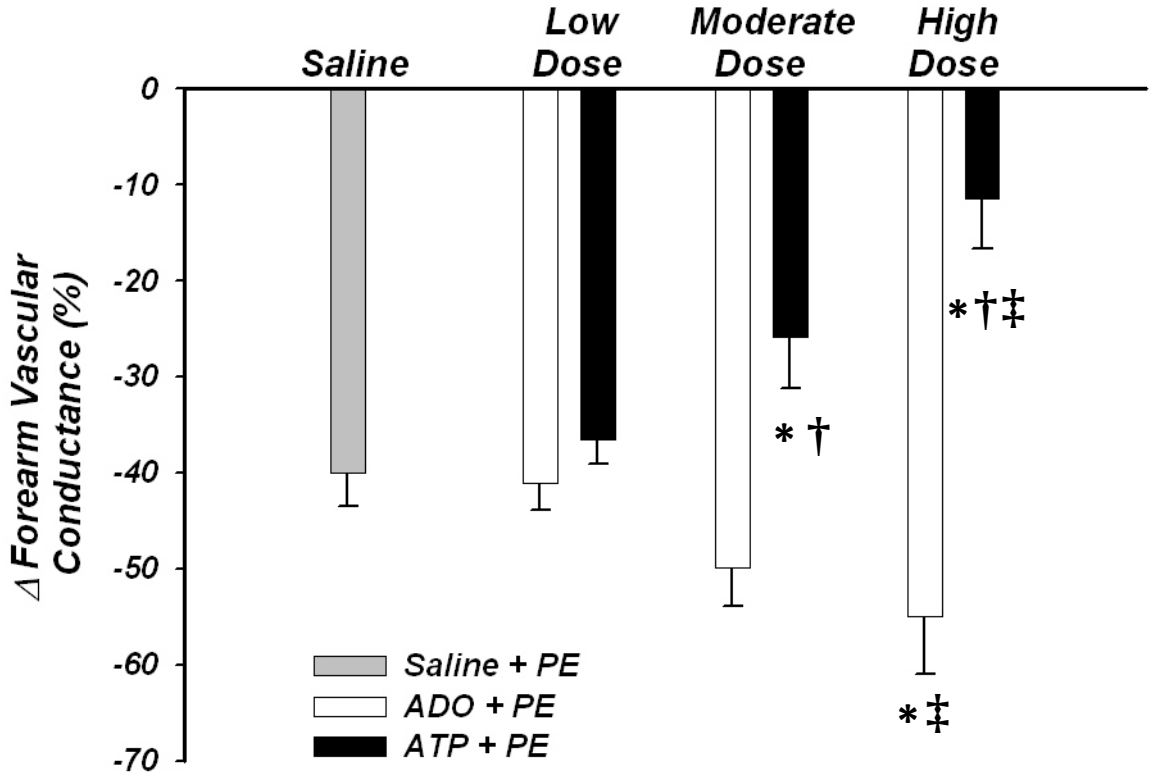
**Figure 5: Forearm vasoconstrictor responses to $\alpha_1$-adrenoceptor stimulation.** Percentage reduction in forearm vascular conductance in response to $\alpha_1$-adrenoceptor stimulation (via phenylephrine; PE) during low dose infusion of adenosine and ATP were not significantly different than during saline (control) conditions. $\alpha_1$-mediated vasoconstriction was greater during high dose infusion of adenosine compared with control, whereas the vasoconstrictor responses were blunted during moderate and high dose ATP. ***** $P < 0.05$ vs saline (control); † $P < 0.05$ vs adenosine within dose condition; ‡ $P < 0.05$ vs low dose within drug condition.


Handgrip Exercise, intra-arterial
Adenosine or Adenosine Triphosphate
Vasoconstrictor Infusion
Phenylephrine or
Dexmedetomidine
REST
0
2
5
6
8
Time (min)
Baseline
"Steady-State" Pre-Constrictor
Post-Constrictor


A.
Adenosine
Handgrip Exercise
ATP
Forearm Blood Flow (ml/min)
200
150
100
50
0
*
*†
†
REST
Pre-PE
PE
B.
Forearm Blood Flow (ml/min)
200
150
100
50
0
*
*†
†
REST
Pre-DEX
DEX


A.
Phenylephrine
0
-10
-20
-30
-40
-50
Δ Forearm Vascular Conductance (%)
*
* †
†
Adenosine
Handgrip Exercise
ATP
B.
Dexmedetomidine
0
-10
-20
-30
-40
-50
Δ Forearm Vascular Conductance (%)
*
* †
†


A.
Low Dose
ADO
ATP
Forearm Blood Flow (ml/min)
120
100
80
60
40
20
0
*
*
B.
Moderate Dose
Forearm Blood Flow (ml/min)
120
100
80
60
40
20
0
*
*
C.
High Dose
Forearm Blood Flow (ml/min)
120
100
80
60
40
20
0
*
†
Rest
Pre-PE
PE


Saline
Low Dose
Moderate Dose
High Dose
0
-10
-20
-30
-40
-50
-60
-70
Δ Forearm Vascular Conductance (%)
Saline + PE
ADO + PE
ATP + PE
* †
* †‡
* ‡

CHAPTER III – MANUSCRIPT II

# Vasodilatory Responsiveness to Adenosine Triphosphate in Ageing Humans

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**Non-technical Summary**

The innermost lining of a blood vessel (endothelium) is important for increasing vessel diameter and facilitating increases in blood flow and oxygen delivery to muscle. This process is typically impaired in older adults. We demonstrate that increases in blood flow to the naturally circulating vessel relaxant, ATP, are intact in older adults who have an impaired innermost lining of muscle blood vessels. Our results show that an unhealthy endothelium does not always translate to an inability to relax blood vessels and aids in the overall understanding of how blood vessels control oxygen delivery to muscle as humans get older.

**Abstract**

Endothelium-dependent vasodilatation is reduced with advancing age in humans, as evidenced by blunted vasodilator responsiveness to acetylcholine (ACH). Circulating adenosine triphosphate (ATP) has been implicated in the control of skeletal muscle vascular tone during mismatches in oxygen delivery and demand (e.g., exercise) via binding to purinergic receptors ($P_{2y}$) on the endothelium evoking subsequent vasodilatation, and ageing is typically associated with reductions in muscle blood flow under such conditions. Therefore, we tested the hypothesis that ATP-mediated vasodilatation is impaired with age in healthy humans. We measured forearm blood flow (venous occlusion plethysmography) and calculated vascular conductance (FVC) responses to local intra-arterial infusions of ACH, ATP, and sodium nitroprusside (SNP) before and during AA infusion in 13 young and 13 older adults. The peak increase in FVC to ACH was significantly impaired in older compared with young adults (262±71% vs 618±97%; $P<0.05$), and this difference was abolished during AA infusion (510±82% vs 556±71%; *NS*). In contrast, peak FVC responses were not different between older and young adults to either ATP (675±105% vs 734±126%) or SNP (116±111% vs 1138±148%) and AA infusion did not alter these responses in either age group (both *NS*). In another group of 6 young and 6 older adults, we determined whether vasodilator responses to adenosine and ATP were influenced by $P_1$-receptor blockade via aminophylline. The peak FVC responses to adenosine were not different in young (350±65%) versus older adults (360±80%), and aminophylline blunted these responses by ~50% in both groups. The peak FVC responses to ATP were again not different in young and older adults, and aminophylline did not impact the vasodilatation in either

group. Thus, in contrast to the observed impairments in ACH responses, the vasodilatory response to exogenous ATP is not reduced with age in healthy humans. Further, our data also indicate that adenosine mediated vasodilatation is not reduced with age, and that ATP-mediated vasodilatation is independent of $P_1$-receptor stimulation in both young and older adults.

**Abbreviation List**

AA, ascorbic acid; ACH, acetylcholine; ADO, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; APH, aminophylline; ATP, adenosine triphosphate; DEXA, dual-energy X-ray absorptiometry; ECG, electrocardiogram; FAV, forearm volume; FBF, forearm blood flow; FVC, forearm vascular conductance; HDL, high-density lipoprotein; HR, heart rate; LDL, low-density lipoprotein; MAP, mean arterial pressure; MBV, mean blood velocity; MVC, maximal voluntary contraction; NO, nitric oxide; NOS, nitric oxide synthase; P-receptor, purinergic-receptor; SNP, sodium nitroprusside.

## Introduction

Over the past decade, evidence has begun to point towards the circulating endogenous purine nucleotide ATP as having an increasingly important role in vascular homeostasis. As such, circulating ATP is thought to have a role in curtailing platelet aggregation (Soslau *et al.*, 1995; Hrafnkelsdottir *et al.*, 2001) and has been implicated in the control of skeletal muscle vascular tone during exercise via binding to purinergic receptors ($P_{2Y}$) on the endothelium evoking both considerable vasodilatation and attenuating sympathetic vasoconstriction (Gonzalez-Alonso *et al.*, 2002; Kirby *et al.*, 2008). In addition, ATP appears to induce vasodilatation primarily via endothelium-dependent mechanisms and results in spreading vasodilation which has been proposed as a significant contributor to the full expression of the exercise hyperemic response (Winter & Dora, 2007). Of note is the fact that other putative endothelium-dependent vasodilators such as nitric oxide, prostaglandins, and adenosine do not clearly blunt sympathetic vasoconstriction nor evoke spreading dilation, highlighting the unique and important properties of ATP (Hoepfl *et al.*, 2002; Winter & Dora, 2007; Kirby *et al.*, 2008). Interestingly, the majority of data in young adult humans also suggests that these putative vasodilators are not the primary downstream signals by which direct intra-arterial ATP administration evokes hyperemia (Rongen *et al.*, 1994; van Ginneken *et al.*, 2004).

In addition to the aforementioned properties, it is our belief that the contribution of circulating ATP to vascular tone in humans is of particular significance from a genuine physiological standpoint. Considerable evidence has mounted demonstrating that the erythrocyte can act as an oxygen ‘sensor’ in addition to its traditional role of an oxygen

'carrier', whereby ATP is released from the red cell in direct proportion to the degree of hemoglobin deoxygenation (Jagger *et al.*, 2001). Additionally, ATP is documented to be released from endothelial cells into circulation in response to shear stress (a known endothelium-dependent stimulus) (Bodin & Burnstock, 1995). Accordingly, circulating endogenous concentrations of ATP increase in young adults during the physiological stressors of hypoxia and exercise (Forrester & Lind, 1969; Gonzalez-Alonso *et al.*, 2002). Collectively, given the vasomotor properties of ATP and that endogenous [ATP] increase in circulation during natural physiological stressors, the responsiveness to this proposed endothelium-dependent vasodilator in humans may offer substantial insight that is unobtainable with the use of other vasodilators whose control of the skeletal muscle vasculature during physiological stressors could be questioned.

With respect to human ageing, advancing age is widely recognized as the primary risk factor associated with increased cardiovascular disease risk, and older adults typically display characteristics of endothelial cell injury (Celermajer *et al.*, 1994; Lloyd-Jones *et al.*, 2009). A normal, healthy endothelium functions to minimize the pro-inflammatory environment that promotes the initiation and progression of atherosclerotic vascular disease. Further, the endothelial cell layer of the vasculature is significant in regulating vasomotor tone, thus facilitating in the balance of blood flow and oxygen delivery to the metabolic needs of the tissue (Wu & Thiagarajan, 1996; Vanhoutte, 1997). Accordingly, older adults have a blunted responsiveness to endothelium-dependent vasodilator stimuli and this endothelial dysfunction contributes to negative alterations in vascular control (Taddei *et al.*, 2000; Taddei *et al.*, 2001; Kirby *et al.*, 2009).

During physiological metabolic stress such as exercise, older adults typically have an attenuated hyperaemic response to exercise which may be mediated via reductions in endothelium-dependent vasodilatation (Poole *et al.*, 2003; Proctor & Parker, 2006). As such, we recently demonstrated that acute improvements in endothelial function are associated with significant increases in muscle blood flow during dynamic exercise in ageing humans (Kirby *et al.*, 2009). Importantly, the regulation of blood flow during such a stress is a complex response that involves a fine balance between metabolic vasodilation and sympathetic vasoconstriction. In addition to blunted endothelium-dependent dilation, ageing is also associated with an impaired ability to blunt sympathetic vasoconstriction within the active muscle which may limit oxygen delivery to the tissue when oxygen requirements are elevated (Koch *et al.*, 2003; Dinenno *et al.*, 2005). Taken together, the control of vascular tone and thus oxygen delivery to skeletal muscle at rest and during exercise is impaired in older compared to young adults and is related to decrements in overall endothelial health and function.

To date, the understanding of vasodilator responsiveness to intra-arterial ATP administration in aging humans is not fully understood. Therefore, we tested the hypothesis that ATP-mediated vasodilatation is impaired in older compared with young healthy humans. Additionally, circulating ectonucleotidases (which hydrolyze and breakdown ATP toward adenosine) have been suggested to be elevated in disease states that are increased with age and who demonstrate endothelial dysfunction (Duarte *et al.*, 2007; Schetinger *et al.*, 2007; Lunkes *et al.*, 2008). Given the possibility that ATP could be rapidly degraded to its downstream by-products via interactions with circulating ectonucleotidases and that adenosine-mediated vasodilatation could possibly mask any

real impairment to ATP infusion in older adults, in a subgroup of subjects we determined ATP-mediated vasodilatation during $P_1$-receptor blockade via aminophylline. Again, this question is of particular importance given that ATP possesses unique vasoactive properties (i.e. maximal vasodilator capacity, sympatholysis, and propagated vasodilatation) exhibited by ATP but not other common vasodilators including adenosine.

## Methods

### *Subjects*

With Institutional Review Board approval and after written informed consent, a total of 19 young and 19 older healthy adult men and women participated in the present study. All subjects were normotensive and free from overt cardiovascular disease as assessed from casual blood pressure measurements and a medical history. Older subjects were further evaluated for clinical evidence of cardiopulmonary disease with a physical examination and resting and maximal exercise electrocardiograms. All subjects were sedentary to moderately active, non-smokers, not taking any medications including antioxidants, and studies were performed after a minimum of a 4-hour fast. Subjects provided written, informed consent after all potential risks and procedures were explained. This study was approved by the Human Research Committee of Colorado State University and was performed according to the Declaration of Helsinki.

### *Arterial Catheterization*

A 20-gauge, 7.6-cm catheter was placed in the brachial artery of the non-dominant arm under aseptic conditions after local anesthesia (2% lidocaine) for local administration of study drugs. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml $h^{-1}$ with heparinized saline. The two side ports were used for infusions of vasoactive drugs (Kirby *et al.*, 2008; Kirby *et al.*, 2009).

### *Blood Samples*

Measures of total cholesterol, low- and high-density lipoproteins (LDL and HDL), and triglycerides were performed via conventional methods by the clinical laboratory of the Poudre Valley Hospital (Fort Collins, CO, USA). Oxidized-LDL was measured via standard ELISA (Mercodia, Inc., Uppsala, Sweden) as a marker of circulating oxidative stress via the General Clinical Research Center of the Milton S. Hershey Medical Center (Hershey, PA, USA).

### *Body Composition and Forearm Volume*

Body composition was determined by dual-energy X-ray absorptiometry (DEXA; Hologic, Inc; Bedford, MA, USA). Total forearm volume was calculated from regional analysis of the experimental forearm (from the proximal to distal radioulnar joint) from whole-body DEXA scans with QDR series software for normalization of individual drug doses. Body mass index was calculated as bodyweight (kg) divided by height (meters) squared.

### *Forearm Blood Flow and Vascular Conductance*

Forearm blood flow (FBF) was measured via venous occlusion plethysmography using mercury-in-salistic strain gauges (Greenfield *et al.*, 1963; Dinenno *et al.*, 2002). A paediatric blood pressure cuff was placed around the wrist of the experimental arm and inflated to suprasystolic pressure (~200 mmHg) to arrest the hand circulation. Additionally, a venous occlusion cuff was placed around the upper portion of the experimental arm and cycled between rapid inflation at ~50 mmHg (7 seconds) and deflation (8 seconds) yielding one blood flow measurement every 15 seconds. FBF was expressed as millilitres per 100 millilitres of tissue per minute (ml (100 ml)$^{-1}$ min$^{-1}$). As an index of forearm vasodilatation and to account for individual differences in baseline vascular tone, forearm vascular conductance (FVC) was calculated as (FBF/MAP) x 100 expressed as ml min$^{-1}$ (100mmHg)$^{-1}$. In an effort to minimize the contribution of cutaneous blood flow to FBF measurements, a fan was directed at the experimental arm throughout the experimental protocol.

### *Vasoactive Drug Administration*

As a standard test of endothelium-dependent vasodilatation, the muscarinic receptor agonist acetylcholine (ACH; Miochol-E, Novartis Inc.) was infused via brachial artery catheter at 1, 4, 8, and, 16 μg 100 ml$^{-1}$ forearm volume min$^{-1}$ for 4 minutes each. Additionally, endothelium-independent vasodilatation was assessed via intra-arterial infusion of sodium nitroprusside (SNP; Nitropress, Hospira Inc.) at 0.25, 0.5, 1, and 2 μg 100 ml forearm volume$^{-1}$ min$^{-1}$ for 4 minutes each (Taddei *et al.*, 2001; DeSouza *et al.*,

2002). To test our primary hypothesis, the $P_{2Y}$ receptor agonist adenosine triphosphate (ATP; Sigma A7699) was infused at 1.25, 2.5, 5, and 10 μg 100 ml forearm volume$^{-1}$ min$^{-1}$ for 4 minutes each (Rongen *et al.*, 1994; Kirby *et al.*, 2008). ATP was confirmed sterile and free of endotoxin with a standard microbiology report (JCB-Analytical Research Labs) (Kirby *et al.*, 2008). As a method of acutely improving endothelium-dependent vasodilatation, the potent antioxidant ascorbic acid (Vitamin C; American Regent Inc.) was infused at 8 mg 100 ml forearm volume$^{-1}$ min$^{-1}$ for 10 minutes as a loading dose (see *Experimental Protocol* below), and at 40% of this loading dose for maintenance infusion throughout the remainder of the experiment (Taddei *et al.*, 2001; Kirby *et al.*, 2009).

In a second group of subjects, adenosine (Sicor, Irvine, CA) was infused at 3.125, 6.25, and 12.5 μg 100 ml forearm volume$^{-1}$ min$^{-1}$ for 4 minutes as described by Martin and colleagues (Martin *et al.*, 2006). In an effort to produce vasodilatation of a similar magnitude as adenosine, the doses of ATP were estimated to be 1.25, 2.5, and 5 μg 100 ml forearm volume$^{-1}$ min$^{-1}$ for 4 minutes based on data from the first group of subjects. The $P_1$-receptor blocker, aminophylline (American Reagent, Shirley, NY), was infused for 20 minutes at 100 μg 100 ml forearm volume$^{-1}$ min$^{-1}$ prior to experimental trials and continued through the remainder of the study (Leuenberger *et al.*, 1999; Martin *et al.*, 2006). Pilot data in our laboratory indicated this dose of aminophylline markedly decreases adenosine-mediated vasodilation without affecting basal forearm vascular tone.

***Experimental Protocol***

The primary purpose of the main experimental protocol was to determine whether ATP-mediated vasodilatation is impaired in older compared with young adults. A secondary purpose of this protocol was to determine whether ATP-mediated vasodilatation would be improved in older adults by acute antioxidant administration of AA similar to that observed previously to ACH (Taddei *et al.*, 2001; Kirby *et al.*, 2009). Thirteen young (eight men; five women) and thirteen older (eight men; five women) were studied in the supine position with the experimental (non-dominant) arm abducted 90º laterally at heart level. The most distal portion of the arm was slightly elevated in order to facilitate venous return during blood flow measurements with venous occlusion plethysmography. After a minimum of 30 minutes following catheterization and experimental set-up, the first of three vasoactive drugs (ACH, SNP, or ATP) was infused for 4 minutes at each dose totaling 16 minutes of infusion time. Four minutes of quiet rest preceded all vasodilator trials where baseline measurements were performed and saline was infused. Twenty minutes of quiet rest was allotted between all trials and the order of vasodilator drug infusion was randomized and counterbalanced across subjects. After completion of these initial trials, ascorbic acid was infused at the loading dose for 10 minutes, then reduced to 40% of this dose for the remainder of the study. The infusions of ACH, SNP, and ACH were then repeated in the same order as before AA for each subject.

In a second experimental protocol in an additional 6 young (4 men; 2 women) and 6 older (3 men; 3 women) subjects, we determined whether adenosine-mediated vasodilatation was impaired with age and whether any age-associated differences in

ATP-mediated vasodilatation could be attributed to greater breakdown to adenosine and subsequent $P_1$-receptor stimulation. Therefore, in randomized and counterbalanced fashion, adenosine and ATP were infused before and after local $P_1$-receptor inhibition via aminophylline. The timeline for this protocol was similar to that described above for the primary experimental protocol.

***Data Acquisition and Analysis***

Data was collected and stored on computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). FBF was determined from the derivative of the forearm plethysmogram. Mean arterial pressure (MAP) was determined from the arterial pressure waveform and heart rate (HR) was determined via standard 3-lead ECG. Baseline FBF, FVC, HR, and MAP represent an average of the last minute of the resting time period prior to all pharmacological vasodilatory tests. In addition, all haemodynamic responses from drug infusions represent an average of the last minute of data from that specific dose of infusion. The % change in FBF and FVC during drug infusions was calculated as:

((FBF drug - FBF baseline)/(FBF baseline)) x 100.

Changes in FVC were calculated in a similar fashion.

***Statistics***

All values are reported as means ± S.E.M. Comparison of subject characteristics and the haemodynamic values at specific time points between groups were made with unpaired t-tests, and the within group values for each hyperaemic condition with paired t-

tests. Specific hypothesis testing within trials was performed to assess mean group differences between young and older adults using two-way repeated measures analysis of variance. *Post-hoc* analysis was performed using the Tukey's test when significance was observed. Significance was set at $P<0.05$.

## Results

### *Subject Characteristics*

The mean age difference in age between young and older subjects was ~45 years (Table 1). There were no significant differences between young and older adults in forearm volume, HDL-cholesterol, or triglycerides. Older adults had a greater BMI, body fat percentage, total cholesterol, and LDL-cholesterol (all $P<0.05$), although these values were within normal levels. Baseline FBF, FVC, and HR for all trials were not different between young and older adults. Although normotensive, MAP was elevated in older compared with young adults ($P<0.05$).

### *Vasodilator Drug Administration: Effect of Age*

The vasodilatory responses expressed as the percentage increase in FVC from baseline during all doses of ACH, ATP, and SNP are shown in Figure 1A-C. As expected, older adults demonstrated an impaired vasodilatory response to ACH compared to that observed in young adults (Figure 1A; $P<0.05$). The vasodilatory response to the endothelium-independent vasodilator, SNP, was not affected by age (Figure 1C). In contrast to our hypothesis, older adults had a preserved vasodilator response to ATP infusion (Figure 1B). FBF responses followed a similar pattern to that of FVC for all

conditions (Table 2). Within both age groups, MAP was unchanged during ACH and ATP, however at the highest 2 doses of SNP, MAP was significantly lower than baseline ($P$<0.05; Table 2). Similarly, HR was not different between or within ACH and ATP infusion, yet the 2 highest doses of SNP resulted in elevated HR compared to baseline for both age groups ($P$<0.05; Table 2).

***Vasodilator Drug Administration: Effect of Ascorbic Acid***

Acute infusion of AA restored endothelium-dependent vasodilatation to ACH in older adults but had no effect on the dilatory response in young adults (Figure 1A). In contrast, concurrent AA administration during ATP and SNP infusion did not impact the vasodilatory responses in either young or older adults (Figure 1B-C). FBF responses followed a similar pattern to that of FVC for all conditions (Table 2). Neither MAP nor HR was significantly affected by administration of AA within any drug condition ($P$=NS; Table 2).

***Effect of $P_1$-receptor Blockade on Adenosine- and ATP-mediated Vasodilatation***

The vasodilatory responses expressed as the percentage increase in FVC from baseline during all doses of adenosine and ATP are shown in Figure 2A & 2B. Adenosine-mediated vasodilatation was not different between young and older adults at any dose (Figure 2A). Similar to subjects from the primary experiment, vasodilatory responsiveness to ATP infusion was again not different between young and older adults (Figure 2B). $P_1$-receptor blockade via aminophylline blunted peak adenosine-mediated vasodilatation in both young (~47%) and older (~44%) adults (both $P$<0.05 vs zero),

however had little to no impact on ATP-mediated vasodilatation (~10% vs ~10%; both NS vs zero). Importantly, the effect of aminophylline during either adenosine or ATP infusion was not different between age groups (adenosine: $P$=0.8; ATP: $P$=0.9, Figures 2A-B). FBF responses followed a similar pattern to that of FVC in both age groups and within all drug conditions (Table 3). Neither MAP nor HR was significantly affected during ATP or adenosine infusions before or during aminophylline administration ($P$=NS; Table 3).

### *Plasma Markers of Oxidative Stress*

At baseline, plasma oxidized-LDL was greater in the older compared with young subjects (53±3 vs 39±3 U $L^{-1}$; $P$<0.05). Infusion of ascorbic acid did not affect these plasma levels in either young (38±3 U $L^{-1}$) or older adults (54±3 U $L^{-1}$), which most likely reflects that the ascorbic acid was administered locally via brachial artery catheter and was dose-adjusted to forearm volume. Importantly, the improvements in acetylcholine-mediated vasodilatation (see above) in older subjects is consistent with prior studies and provides evidence that ascorbic acid was effective at the level of the forearm vasculature (Taddei *et al.*, 2001; Kirby *et al.*, 2009).

## Discussion

Circulating ATP has been implicated in the control of skeletal muscle vascular tone during mismatches in oxygen delivery and demand (e.g., exercise) via binding to purinergic receptors on the endothelium, and ageing is typically associated with reductions in muscle blood flow under such conditions. Further, diminished endothelial

function is observed in aged humans and has been closely related to oxidative stress (Taddei *et al.*, 2001; Forstermann & Munzel, 2006; Ashfaq *et al.*, 2008). The key finding of the present investigation is that vasodilator responsiveness to exogenous ATP in ageing humans is preserved despite the presence of local endothelial dysfunction as evidenced by impaired vasodilatation to acetylcholine infusion. Further, the presence of the antioxidant ascorbic acid reversed the observed impairments to acetylcholine, but had no impact on the vasodilatory response to exogenous ATP. Importantly, the vasodilatory response to exogenous ATP was unaffected under conditions of $P_1$-receptor blockade (with aminophylline), negating the possibility that adenosine-mediated vasodilatation is, in part, masking a genuine diminished vasodilatory response to exogenous ATP. Collectively, our findings demonstrate that older adults with endothelial dysfunction respond with a similar vasodilatory response to ATP as that observed in young adults.

***Ageing and Endothelium-dependent Vasodilatation***

In older adults, endothelium-dependent vasodilatation in response to ACH infusion is attenuated compared to young adults, and this impaired vasodilatory response is classically referred to as "endothelial dysfunction" (Yasue *et al.*, 1990). Despite the preponderance of evidence supporting blunted ACH-mediated vasodilatation in older adults, data suggests that not all endothelium-dependent agonists demonstrate reduced vasodilator responsiveness in aged humans (DeSouza *et al.*, 2002). Recently, ATP has become more recognized as a significant endogenous circulating modulator of vascular tone that affects oxygen delivery to meet the metabolic demand of active tissue (Ellsworth, 2004; Gonzalez-Alonso, 2008). Further, isolated vessels studies clearly

indicate that vasodilatation to ATP is largely dependent on the presence of a healthy endothelium (Busse *et al.*, 1988; Winter & Dora, 2007). Therefore, the primary purpose of the present experiment was to test the hypothesis that the vasodilatory response to exogenous infusion of ATP is impaired in ageing humans. As expected, healthy older adults demonstrated obvious blunted endothelium-dependent vasodilatation in response to the muscarinic agonist, ACH, and unaltered vasodilatation to the endothelium-independent dilator, SNP, compared with young adults. However, in contrast to our hypothesis, no age-associated decrement in ATP-mediated vasodilatation (in the same subjects that demonstrated blunted ACH responses) was observed.

To the best of our knowledge, only one other group has attempted to determine ATP-mediated vasodilatory responsiveness in healthy ageing humans (Imaizumi *et al.*, 1990). Similar to the present study, ATP-mediated vasodilatation was not attenuated in older subjects (age 57 ± 1 yrs). However, the findings from this previous study were difficult to interpret given that vasodilatation to ACH was not impaired with age (i.e. endothelial dysfunction was not present). Although there is limited direct information in healthy aging humans, there appears to be a fairly robust vasodilator response to intra-coronary artery infusion of ATP in coronary artery diseased patients, a population which typically exhibits endothelial dysfunction (Takase *et al.*, 1998). Interestingly, ACH infusion in this population often results in vasoconstriction or profoundly weak vasodilatation further supporting different vasodilator responses between the endothelium-dependent agonists ACH and ATP (Vita *et al.*, 1990; Yasue *et al.*, 1990). Nonetheless, the present findings clearly indicate that vasodilatory responsiveness to

ATP is not impaired in older adults demonstrating typical 'endothelial dysfunction' as evidenced by substantially blunted responses to ACH.

Classically, age-associated endothelial dysfunction is characterized by a functional loss of bioavailable NO (Herrera *et al.*, 2009). Our laboratory and others have demonstrated that acute intra-arterial administration of ascorbic acid can abolish age-associated impairments in endothelium-dependent vasodilatation to ACH (Taddei *et al.*, 2001; Kirby *et al.*, 2009). As such, we anticipated vasodilatation to ATP would be impaired in older adults and that we would acutely restore endothelial health with ascorbic acid similar to ACH. In contrast to our hypothesis, ATP-mediated vasodilatation was not impaired, thus predictably AA had no effect on ATP vasodilator responsiveness. The majority of studies examining the mechanism by which AA can modulate blood flow indicate an ability of AA to scavenge superoxide or stabilize tetrahydrobiopterin; both which would preserve the bioavailability of NO (Forstermann & Munzel, 2006). Consequently, the contribution of NO to ACH-mediated vasodilatation in aged humans has been shown to be greatly enhanced following AA administration (Taddei *et al.*, 1998; Taddei *et al.*, 2001). In relation to vascular control during metabolic stress where circulating ATP has been proposed as particularly important, data indicate that AA may be restoring blood flow in older adults via NOS-mediated pathways (Crecelius *et al.*, 2009; Kirby *et al.*, 2009). Nonetheless, in the present study we did not observe a reduction in vasodilatation to exogenous ATP and this was unaffected by AA administration, indirectly supporting previous studies indicating a minor contribution of NO to ATP-mediated vasodilatation in humans (Rongen *et al.*, 1994; Shiramoto *et al.*, 1997; Hrafnkelsdottir *et al.*, 2001; van Ginneken *et al.*, 2004).

Although *in vitro* preparations suggest a significant contribution of NO to ATP-induced vasodilatation (Burnstock, 1990), human data is less convincing. Rongen and colleagues were the first to demonstrate that while NOS blockade can reduce vasodilatation to ACH infusion, it does not decrease the vasodilatory response to ATP (Rongen *et al.*, 1994). Following this, multiple other studies including unpublished pilot studies from our lab indicate little role for nitric oxide in mediating the vasodilation by ATP (Rongen *et al.*, 1994; Shiramoto *et al.*, 1997; Hrafnkelsdottir *et al.*, 2001; van Ginneken *et al.*, 2004; Mortensen *et al.*, 2009). In actuality, clarifying the potential downstream vasodilating factors appears to be quite complex even in young people alone as investigators have been unable to firmly declare the mechanisms by which ATP results in smooth muscle relaxation (van Ginneken *et al.*, 2004). Most recently, one study in the human lower limb suggested that combined blockade of NOS and cycloxygenase may blunt some of the ATP-mediated vasodilatation in the human leg, however this study could be interpreted differently when calculating % change in vasodilation and is somewhat clouded by systemic baroreflex activation (Mortensen *et al.*, 2009). On the other hand, it's possible the different dose of ATP in these studies could explain the contrasted findings (Stanford *et al.*, 2001). Additionally, keeping in mind that ATP has dual vasomotor properties in that it not only results in potent vasodilatation but can also blunt vasoconstrictor action; current understanding lends a small role for NO or PG's in the ability to override sympathetic vasoconstriction (Dinenno & Joyner, 2003, 2004). Collectively, evidence seems to suggest that downstream signaling of ATP may be more closely related to hyperpolarizing factors rather than NO and PGs when initiating vasodilatation and attenuating sympathetic vasoconstriction.

### *Ageing and Adenosine-mediated Vasodilatation*

The finding that vasodilatation to ATP was not different between groups was unexpected given the endothelium-dependence of ATP and current knowledge of ACH responses in this population. In this context, we questioned whether ectonucleotidases that assist in the breakdown of ATP towards adenosine are elevated in older individuals thereby allowing adenosine-mediated vasodilatation to mask any true impairment to ATP. Accordingly, evidence indicates a variety of diseased populations that demonstrate endothelial dysfunction exhibit increases in ATP-degrading ectonucleotidases presumably as a means to offset inflammatory mediated platelet aggregation by ADP (Schetinger *et al.*, 2007; Lunkes *et al.*, 2008). In particular, ATP hydrolysis is closely related to ox-LDL, in which aging humans typically have elevated levels of ox-LDL compared to younger adults and was observed in the present study (Duarte *et al.*, 2007). Therefore, in a subgroup of subjects we determined adenosine-mediated vasodilatation in older adults before and after $P_1$-receptor blockade via aminophylline. We observed that vasodilatation to adenosine infusion was not different in older adults and that the contribution of adenosine-mediated vasodilatation during ATP infusion was not significant in both young and older adults. Our findings in aging humans are similar to other observations in young adults using adenosine receptor blocking agents (Rongen *et al.*, 1994; Mortensen *et al.*, 2009). Although ectonucleotidase activity in older adults under these conditions is not directly known, our observations clearly indicate that adenosine-mediated vasodilatation does not appear to mask a true decrement in vasodilatory responsiveness to ATP administration in ageing humans.

***Experimental Considerations***

A few experimental considerations should be acknowledged for the present study. First, the ideal method to determine the contribution of ATP to basal vascular tone in aging humans would be to use a $P_{2Y}$ receptor antagonist, however the pharmacological agents to do this safely in humans is not yet available. Next, other investigators have demonstrated impairments to ACH-mediated vasodilatation yet no attenuated vasodilator responsiveness to different endothelium-dependent agonists in ageing individuals (DeSouza *et al.*, 2002). Why this is the case is not clearly known but most likely indicates that downstream signaling from muscarinic receptor binding is the most prominent age-associated impairment. Nonetheless, we clearly demonstrate that the vasodilatory response to ATP infusion was not blunted in the aged group and that this is considerably different from what is observed during ACH infusion.

Because vasodilatation to ATP was not different between age groups and evidence suggests that this population may have increased ectonucleotidase activity (Duarte *et al.*, 2007), we aimed to test in a subgroup of adults whether vasodilatation to adenosine was masking a true vasodilatory responsiveness to ATP in older adults. Therefore, we infused a $P_1$-receptor antagonist during both adenosine and ATP infusions and observed ~50% reduction to adenosine-mediated vasodilatation in both age groups but a non-significant reduction in vasodilatation during ATP infusions. Despite our attempts to uncover extraneous vasodilatation via the ATP-breakdown product adenosine, ADP as well as AMP also results in vasodilatation (Rosenmeier *et al.*, 2008). Whether the balance of nucleotide-mediated vasodilatation is shifted in older adults is difficult to address since ATP, ADP, and AMP all have the potential to bind to $P_2$ receptors on the

endothelium and specific pharmacological antagonists are currently unavailable for human use (Ralevic & Burnstock, 1998). Hence, we were unable to determine the contribution of ADP and AMP to exogenous ATP infusion. Regardless, our data are the first clear evidence that adenosine-mediated forearm vasodilatation is not impaired in older adults and that vasodilatation via adenosine binding to $P_1$ receptors during ATP infusion is minimal in both young and older adults.

***Physiological Perspectives***

Exogenous ATP administration can modulate vascular tone in a multifaceted manner as a result of evoking both profound dilatation as well as having the ability to blunt sympathetic vasoconstriction (Rosenmeier *et al.*, 2004; Gonzalez-Alonso, 2008; Kirby *et al.*, 2008). Thus, from a physiological standpoint, the extent to which ATP is present and acts in circulation is of considerable interest. For many years nucleotides have been identified in circulation (Forrester, 1969), however only more recently have investigators begun to determine the stimuli that increase [ATP] in blood. In view of this, plasma [ATP] elevate during exercise (Gonzalez-Alonso *et al.*, 2002), and specific red blood cell release of ATP is enhanced under conditions of hypoxia, hypercapnia, acidosis, and mechanical deformation (Sprague *et al.*, 1996; Ellsworth *et al.*, 2009). Despite this understanding in young adults, whether an age-associated impairment in circulating [ATP] exists at rest or during various stimuli is uncertain. Corroborating this perspective is the fact that older adults typically demonstrate reduced exercise and hypoxic vasodilatation compared to younger individuals in conditions where ATP would be presumed to be high in concentration and resulting in active vasodilatation (Kravec *et*

*al.*, 1972; Proctor & Parker, 2006; Kirby *et al.*, 2009). Building upon the present observations that vasodilatory responsiveness to exogenous ATP infusion appears intact in ageing humans, future work should be aimed at determining whether circulating ATP is hampered with age and how this may contribute to altered haemodynamic regulation.

### *Conclusions*

The findings from the present investigation indicate that vasodilatory responsiveness to exogenous ATP is not impaired in ageing humans despite the presence of endothelial dysfunction as evidenced by attenuated ACH-mediated vasodilatation. Further, adenosine-mediated vasodilatation is intact in older adults and the degradation of ATP to adenosine does not appear to 'mask' a genuine impairment in endothelium-dependent vasodilatation to ATP. Given the physiological relevance of circulating ATP to vascular tone, it is plausible that advancing age has little impact on receptor sensitivity but results in diminished endogenous release of ATP in circulation. Taken together, the collective observations further emphasize the need to understand downstream vasodilator signaling as it relates to endothelial dysfunction and the control of vascular tone *in vivo*.

## Author Contributions

B.S.K contributed to the experimental design, data acquisition, data analysis, data interpretation, and drafting of the manuscript. A.R.C contributed to data acquisition and interpretation, and critical review of the manuscript. W.F.V provided clinical support, invasive methodology, and contributed to data acquisition and interpretation, as well as critical review of the manuscript. F.A.D. contributed to the conception and experimental design, data acquisition and interpretation, and critical review of the manuscript. All authors approved the final version of the manuscript.

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## Table 1: Subject Characteristics and Baseline Haemodynamics

| Variable | Young | Older |
|---|---|---|
| Male:Female | 8:5 | 8:5 |
| Age (years) | 21 ± 1 | 66 ± 3* |
| Body mass index (kg $m^{-2}$) | 22.0 ± 0.5 | 24.4 ± 0.9* |
| Body fat (%) | 17.4 ± 2.1 | 28.1 ± 2.2* |
| Forearm volume (ml) | 931 ± 77 | 876 ± 59 |
| Total cholesterol (mmol $l^{-1}$) | 3.4 ± 0.2 | 4.4 ± 0.2* |
| LDL cholesterol (mmol $l^{-1}$) | 2.0 ± 0.1 | 2.9 ± 0.2* |
| HDL cholesterol (mmol $l^{-1}$) | 1.2 ± 0.1 | 1.1 ± 0.1 |
| Triglycerides (mmol $l^{-1}$) | 0.7 ± 0.1 | 0.9 ± 0.1 |
| Mean arterial pressure (mmHg) | 85 ± 2 | 97 ± 2* |
| Heart rate (beats $min^{-1}$) | 56 ± 2 | 57 ± 1 |
| Forearm blood flow (ml dl $FAV^{-1}$ $min^{-1}$) | 2.2 ± 0.3 | 2.0 ± 0.2 |
| Forearm vascular conductance (ml dl FAV $min^{-1}$ 100 mmHg $^{-1}$) | 2.7 ± 0.4 | 2.0 ± 0.2 |

Data presented as mean ± SEM. LDL = low density lipoprotein; HDL = high density lipoprotein; FAV = Forearm volume. *$P < 0.05$ *vs* younger.

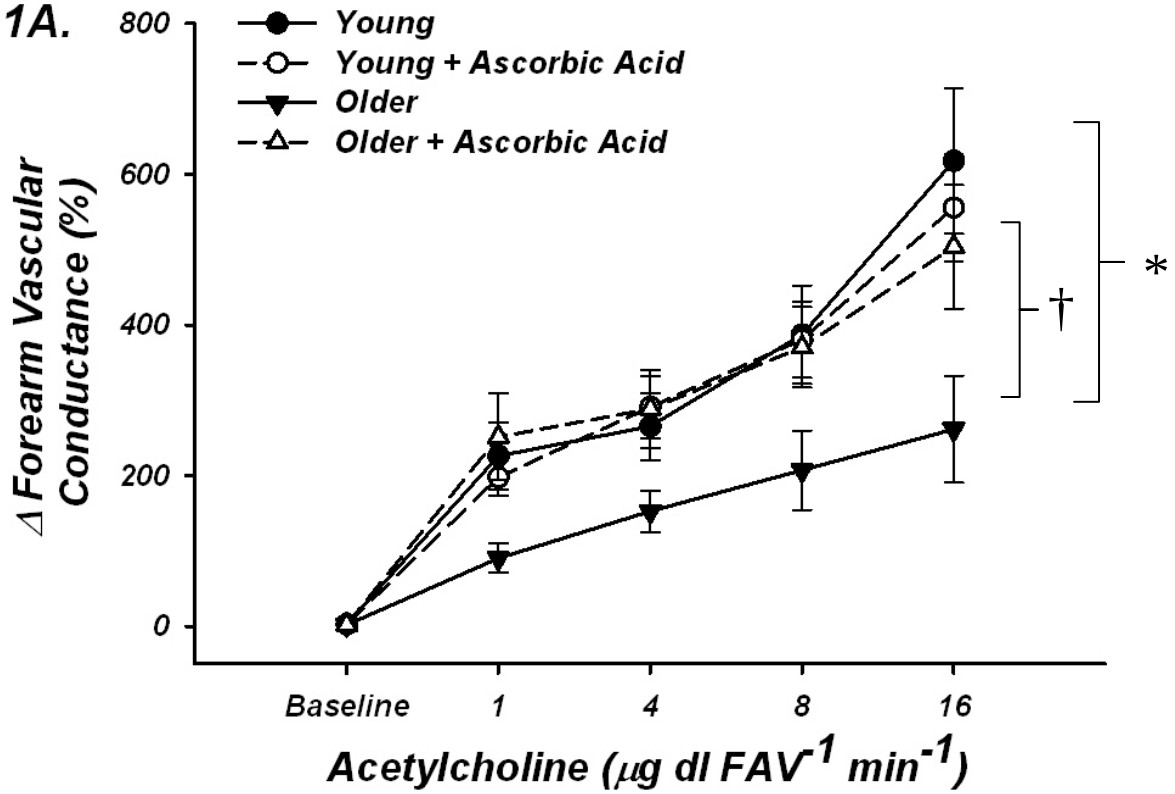
| *Table 2* | | *BASELINE* | | | *DOSE 1* | | | *DOSE 2* | | | *DOSE 3* | | | *DOSE 4* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **FBF** | **MAP** | **HR** | **FBF** | **MAP** | **HR** | **FBF** | **MAP** | **HR** | **FBF** | **MAP** | **HR** | **FBF** | **MAP** | **HR** |
| ***ACH*** | **Young** | 2.1 ± 0.3 | 86 ± 2 | 55 ± 1 | 6.7 ± 1.0 | 86 ± 2 | 57 ± 3 | 7.4 ± 1.0 | 86 ± 2 | 55 ± 2 | 9.6 ± 1.0 | 87 ± 2 | 55 ± 2 | 14.4 ± 1.9 | 86 ± 2 | 55 ± 2 |
| | **Young + AA** | 2.1 ± 0.2 | 89 ± 2 | 54 ± 2 | 6.3 ± 0.7 | 89 ± 2 | 54 ± 1 | 8.2 ± 1.0 | 89 ± 2 | 55 ± 2 | 10.1 ± 1.1 | 89 ± 2 | 54 ± 2 | 13.6 ± 1.5 | 89 ± 2 | 57 ± 2 |
| | **Older** | 1.9 ± 0.1 | 98 ± 2 | 56 ± 1 | 3.5 ± 0.4*† | 99 ± 2 | 57 ± 1 | 4.6 ± 0.3*† | 100 ± 2 | 58 ± 2 | 5.3 ± 0.6*† | 99 ± 2 | 58 ± 2 | 6.3 ± 0.9*† | 100 ± 2 | 58 ± 2 |
| | **Older + AA** | 1.9 ± 0.1 | 101 ± 2 | 56 ± 2 | 6.8 ± 1.1 | 102 ± 3 | 55 ± 2 | 7.7 ± 1.1 | 103 ± 2 | 57 ± 2 | 9.4 ± 1.2 | 103 ± 2 | 57 ± 2 | 12.2 ± 1.9 | 103 ± 2 | 57 ± 1 |
| ***ATP*** | **Young** | 2.1 ± 0.2 | 86 ± 2 | 53 ± 3 | 8.2 ± 1.0 | 87 ± 2 | 55 ± 2 | 11.6 ± 1.5 | 88 ± 2 | 56 ± 2 | 14.2 ± 1.7 | 87 ± 2 | 56 ± 2 | 15.7 ± 2.1 | 87 ± 2 | 55 ± 2 |
| | **Young + AA** | 2.0 ± 0.2 | 90 ± 2 | 54 ± 2 | 7.5 ± 1.0 | 91 ± 2 | 54 ± 2 | 10.1 ± 1.2 | 91 ± 2 | 55 ± 2 | 12.5 ± 1.9 | 90 ± 2 | 56 ± 1 | 15.1 ± 2.5 | 91 ± 2 | 57 ± 2 |
| | **Older** | 2.0 ± 0.2 | 98 ± 2 | 56 ± 1 | 7.9 ± 0.9 | 99 ± 2 | 57 ± 2 | 9.8 ± 1.0 | 98 ± 2 | 58 ± 2 | 11.6 ± 1.6 | 98 ± 2 | 58 ± 2 | 13.9 ± 1.7 | 98 ± 2 | 58 ± 2 |
| | **Older + AA** | 1.9 ± 0.1 | 100 ± 3 | 56 ± 2 | 8.1 ± 0.1 | 101 ± 3 | 56 ± 1 | 10.7 ± 1.4 | 102 ± 2 | 57 ± 2 | 12.9 ± 1.3 | 101 ± 2 | 58 ± 2 | 16.4 ± 1.7 | 102 ± 3 | 58 ± 2 |
| ***SNP*** | **Young** | 2.1 ± 0.3 | 86 ± 2 | 55 ± 2 | 8.2 ± 1.2 | 87 ± 2 | 56 ± 2 | 11.7 ± 1.4 | 85 ± 2 | 56 ± 2 | 17.7 ± 1.9 | 83 ± 2 | 61 ± 2 | 21.3 ± 2.1 | 82 ± 2 | 62 ± 2 |
| | **Young + AA** | 2.1 ± 0.2 | 89 ± 2 | 55 ± 2 | 8.4 ± 1.0 | 92 ± 2 | 56 ± 2 | 11.8 ± 1.3 | 90 ± 2 | 55 ± 2 | 17.9 ± 1.8 | 87 ± 2 | 57 ± 2 | 21.5 ± 2.0 | 85 ± 2 | 60 ± 2 |
| | **Older** | 1.8 ± 0.1 | 98 ± 3 | 57 ± 1 | 7.8 ± 0.5 | 99 ± 3 | 57 ± 1 | 11.1 ± 1.2 | 96 ± 3 | 58 ± 1 | 15.6 ± 1.2 | 94 ± 2 | 59 ± 2 | 19.4 ± 1.8 | 89 ± 2 | 61 ± 2 |
| | **Older + AA** | 2.0 ± 0.2 | 101 ± 3 | 56 ± 2 | 8.8 ± 1.1 | 103 ± 3 | 56 ± 2 | 12.0 ± 1.6 | 101 ± 2 | 57 ± 2 | 17.0 ± 1.6 | 96 ± 2 | 58 ± 2 | 20.6 ±1.9 | 93 ± 2 | 61 ± 2 |

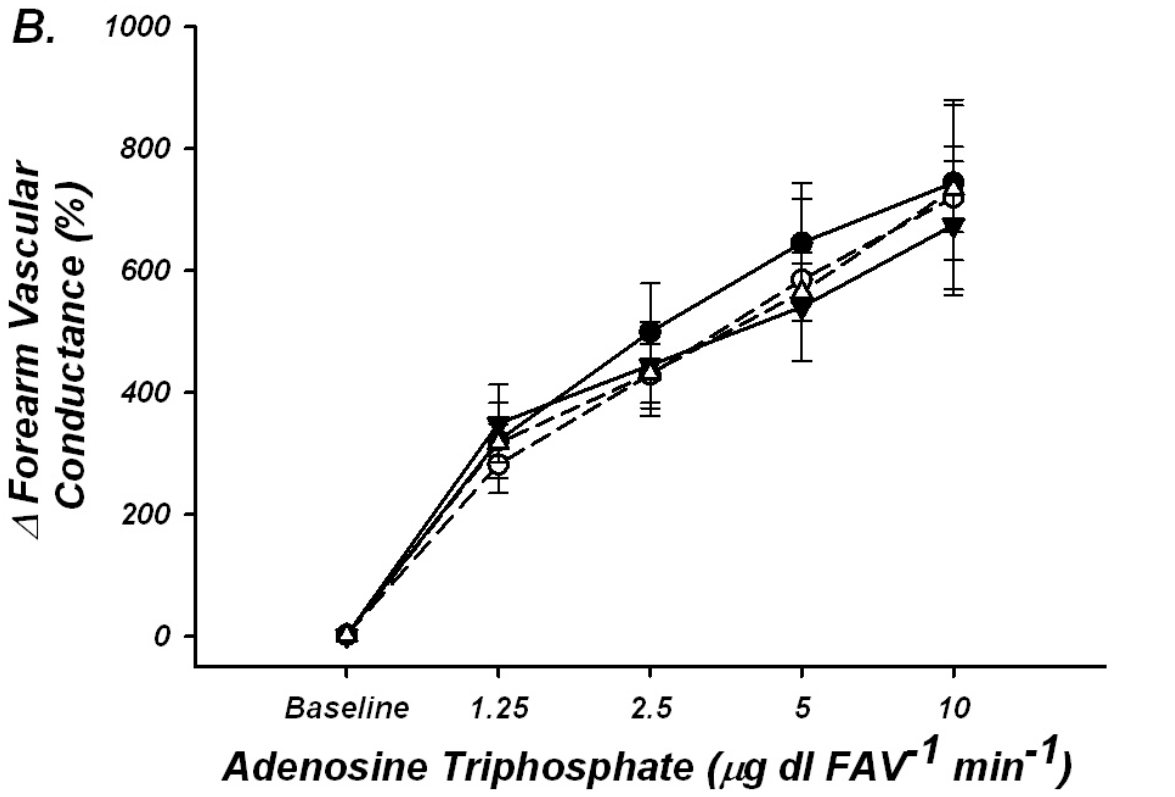
FBF = forearm blood flow (ml (100 ml)$^{-1}$ min$^{-1}$); MAP = mean arterial pressure (mmHg); HR = heart rate (beats min$^{-1}$); * $P < 0.05$ *vs* young; † $P < 0.05$ *vs* with AA; note: all absolute MAP values for older adults are significantly greater than young.

| *Table 3* | | *BASELINE* | | | *DOSE 1* | | | *DOSE 2* | | | *DOSE 3* | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **FBF** | **MAP** | **HR** | **FBF** | **MAP** | **HR** | **FBF** | **MAP** | **HR** | **FBF** | **MAP** | **HR** |
| ***ADO*** | **Young** | 1.7 ± 0.3 | 91 ± 3 | 60 ± 5 | 4.5 ± 0.9 | 94 ± 4 | 58 ± 4 | 6.2 ± 0.9 | 95 ± 4 | 59 ± 4 | 7.0 ± 0.8 | 95 ± 5 | 58 ± 5 |
| | **Young + APH** | 2.0 ± 0.5 | 93 ± 4 | 57 ± 5 | 4.1 ± 0.9 | 98 ± 5 | 58 ± 4 | 4.8 ± 1.0 | 97 ± 5 | 57 ± 4 | 5.6 ± 1.2 | 98 ± 6 | 58 ± 4 |
| | **Older** | 1.2 ± 0.2 | 98 ± 5 | 57 ± 2 | 3.6 ± 0.8 | 99 ± 5 | 58 ± 2 | 4.8 ± 0.9 | 99 ± 5 | 57 ± 2 | 5.6 ± 1.1 | 100 ± 4 | 58 ± 2 |
| | **Older + APH** | 1.2 ± 0.3 | 102 ± 6 | 56 ± 2 | 2.9 ± 1.1 | 102 ± 6 | 56 ± 2 | 3.5 ± 1.4 † | 101 ± 6 | 56 ± 2 | 3.9 ± 1.5 | 102 ± 7 | 58 ± 2 |
| ***ATP*** | **Young** | 1.8 ± 0.3 | 92 ± 5 | 59 ± 5 | 5.8 ± 1.2 | 93 ± 4 | 59 ± 5 | 7.5 ± 1.5 | 93 ± 5 | 58 ± 5 | 9.1 ± 1.7 | 93 ± 5 | 59 ± 5 |
| | **Young + APH** | 2.0 ± 0.3 | 92 ± 5 | 58 ± 5 | 6.2 ± 1.0 | 98 ± 5 | 57 ± 4 | 7.3 ± 1.1 | 96 ± 5 | 59 ± 4 | 8.6 ± 1.2 | 97 ± 5 | 58 ± 4 |
| | **Older** | 1.3 ± 0.2 | 98 ± 3 | 58 ± 2 | 4.7 ± 0.9 | 98 ± 4 | 58 ± 1 | 5.8 ± 1.4 | 102 ± 5 | 57 ± 2 | 7.4 ± 1.1 | 99 ± 4 | 57 ± 2 |
| | **Older + APH** | 1.3 ± 0.2 | 98 ± 5 | 56 ± 1 | 4.6 ± 1.0 | 102 ± 5 | 56 ± 2 | 5.4 ± 1.1 | 102 ± 5 | 56 ± 2 | 6.5 ± 1.0 | 101 ± 6 | 57 ± 2 |

APH = aminophylline; ADO = adenosine; FBF = forearm blood flow (ml (100 ml)$^{-1}$ min$^{-1}$); MAP = mean arterial pressure (mmHg); HR = heart rate (beats min$^{-1}$)

Figure 1A.


Young
Young + Ascorbic Acid
Older
Older + Ascorbic Acid
800
600
400
200
0
Δ Forearm Vascular Conductance (%)
†
*
Baseline
1
4
8
16
Acetylcholine (μg dl FAV-1 min-1)


B.
1000
800
600
400
200
0
Δ Forearm Vascular Conductance (%)
Baseline
1.25
2.5
5
10
Adenosine Triphosphate (μg dl FAV-1 min-1)

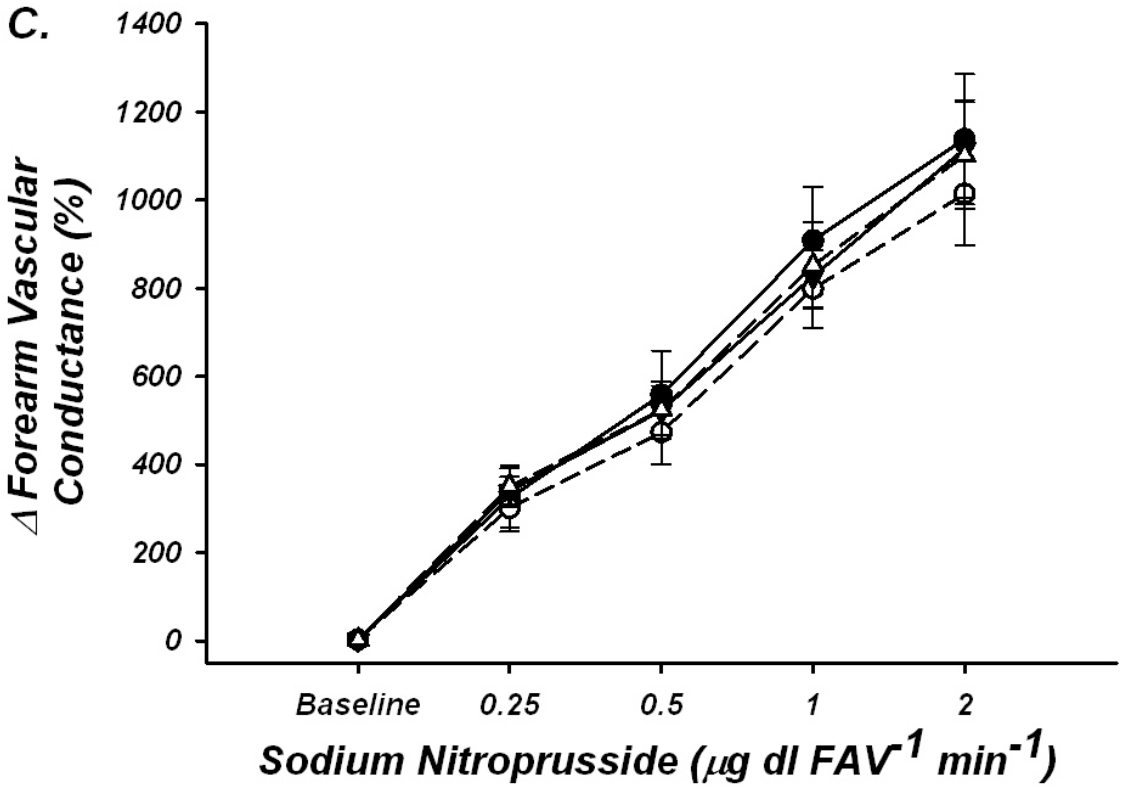



**Figure 1: Forearm Vasodilatory Responses to Acetylcholine, Adenosine Triphosphate, and Sodium Nitroprusside Infusion.** Vasodilatation was significantly impaired in older adults during muscarinic receptor agonist infusion (A; ACH) and this was abolished during simultaneous AA administration. There was no significant age-related impairment in vasodilatation to the purinergic receptor agonist ATP (B) or to the NO donor SNP (C), and these responses were unaffected by AA. ***** $P$ <0.05 vs young within condition; † $P$ <0.05 vs without AA in older adults.

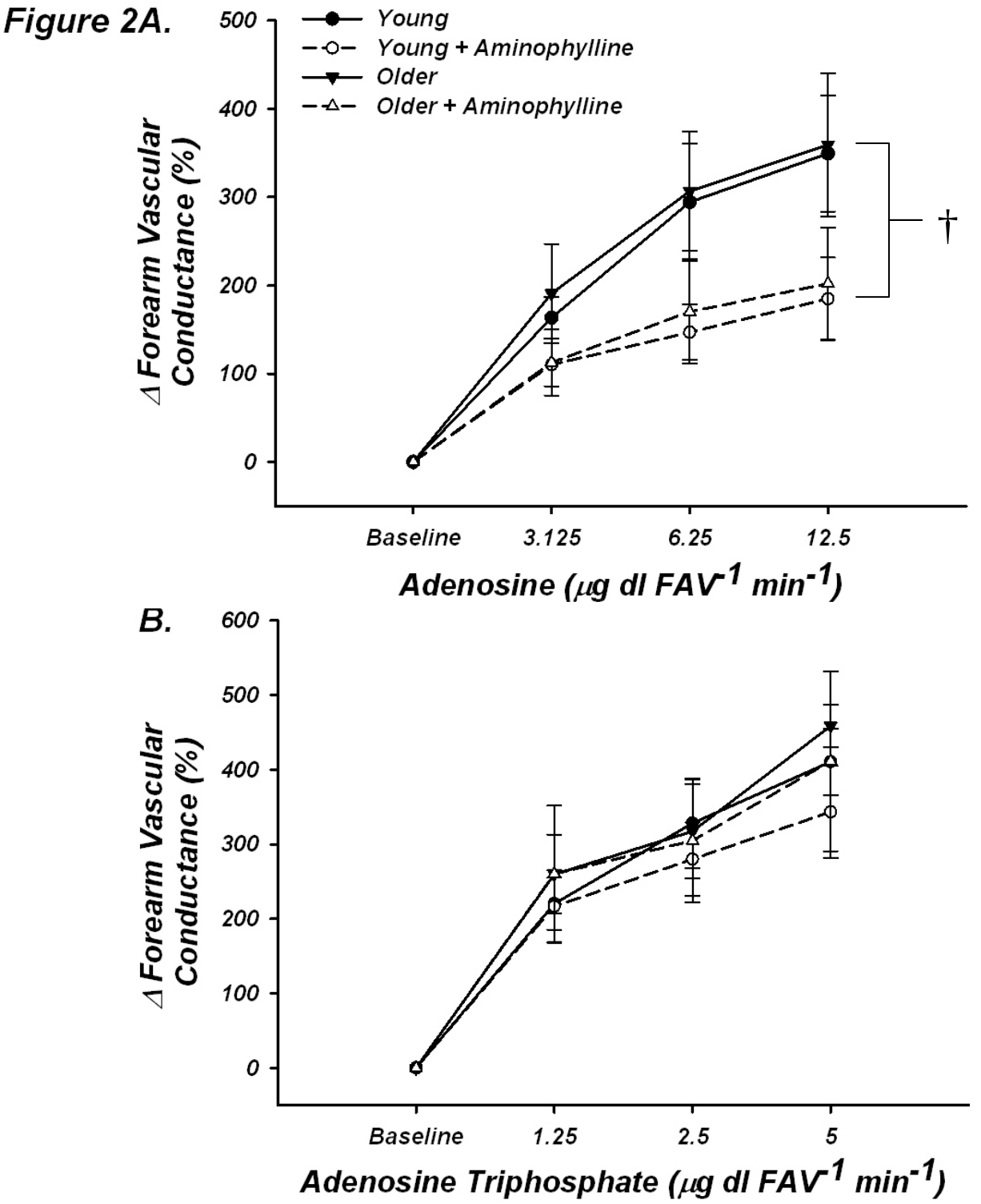



**Figure 2: Forearm Vasodilatory Responses to Adenosine Triphosphate and Adenosine Infusion.** Vasodilatation to $P_1$-receptor agonist adenosine infusion was not significantly impaired in older adults (A). Aminophylline significantly reduced vasodilatation to adenosine at all drug doses in both young and older adults. No significant age-related impairment in vasodilatation to the purinergic receptor agonist ATP was observed and this vasodilatation was unaffected during aminophylline infusion regardless of age (B). † $P$ <0.05 vs without aminophylline.

CHAPTER IV – Manuscript III

# Modulation of Postjunctional α-adrenergic Vasoconstriction during Exercise and Exogenous ATP Infusions in Aging Humans

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**Non-technical Summary**

Muscle contractions activate the sympathetic nervous system to assist in blood pressure regulation, but also increase blood flow to muscle to support delivery oxygen to the active tissue. A balance between these factors must exist, yet in older adults, this process of offsetting the sympathetic control of the vasculature is impaired and may be limited blood flow to muscle during exercise. We recently showed that a circulating factor called ATP can limit the ability of the sympathetic nervous system to evoke vasoconstriction in young adults, however whether this occurs ineffectively and explains the impairment in exercise muscle in older adults is unknown. We demonstrate that older adults have limited ability to increase blood flow during exercise, however this does not result from decreases in ATP to effectively offset sympathetic vasoconstriction.

**Abstract**

The ability to modulate α-adrenergic vasoconstriction in contracting muscle is impaired with age. In young adults, adenosine triphosphate (ATP) has been shown to inhibit vasoconstrictor responsiveness similar to exercise. We tested the hypothesis that modulation of postjunctional α-adrenergic vasoconstriction to exogenous ATP is impaired in aging humans. We measured forearm blood flow (FBF; Doppler ultrasound) and calculated vascular conductance (FVC) to intra-arterial infusions of phenylephrine ($\alpha_1$-agonist) and dexmedetomidine ($\alpha_2$-agonist) during rhythmic handgrip exercise (15% MVC), a control non-exercise vasodilator condition (adenosine), and ATP infusion in 7 young (22±1) and 7 older (64±3 yrs) adults. Forearm hyperemia was matched across all conditions. During adenosine, forearm vasoconstrictor responses to direct $\alpha_1$-stimulation were lower in older adults (ΔFVC= -25±3 vs -41±5%); the responses to $\alpha_2$-stimulation were not different (-35±6% vs -44±8%). The ability to blunt $\alpha_1$- and $\alpha_2$-vasoconstriction was impaired in older adults during exercise ($\alpha_1$ = 32±13 vs 74±8%; $\alpha_2$ = 19±8 vs 60±10%). In contrast, ATP reduced the vasoconstrictor responses by ~85-90% in both age groups. We conclude that the sympatholytic effect of exogenous ATP is not reduced with age, and thus blunted ATP release during exercise may contribute to the impaired exercise sympatholysis in older adults.

**Abbreviation List**

ADO, adenosine; ADP, adenosine diphosphate; AMP, adenosine monophosphate; ATP, adenosine triphosphate; DEX, dexmedetomidine; DEXA, dual-energy X-ray absorptiometry; ECG, electrocardiogram; FAV, forearm volume; FBF, forearm blood flow; FVC, forearm vascular conductance; HR, heart rate; MAP, mean arterial pressure; MBV, mean blood velocity; MVC, maximal voluntary contraction; PE, phenylephrine; P-receptor, purinergic-receptor.

## Introduction

Vascular regulation within contracting muscle exhibits a fine interplay between local vasodilator and sympathetic neural vasoconstrictor signals, and ultimately dictates blood flow and oxygen delivery to the active tissue. Moreover, the competition between these factors appears to increase with the intensity of exercise and the amount of muscle mass engaged (Rowell, 1993; Saltin *et al.*, 1998; Saltin, 2007). Although some debate has existed in the past, collective evidence indicates that vasoconstrictor responses in contracting muscle are blunted compared to resting (quiescent) muscle presumably to minimize a "blood flow error", however some degree of sympathetic restraint must occur to counteract against large declines in blood pressure (Remensnyder *et al.*, 1962; Rowell, 1997; Buckwalter & Clifford, 2001). Indeed, we and others have shown in young adult humans that muscle contractions have the ability to blunt sympathetically mediated vasoconstriction which is graded with the level of exercise intensity is coupled with the metabolic demand of contracting skeletal muscle (Buckwalter *et al.*, 2001; Tschakovsky *et al.*, 2002; Kirby *et al.*, 2005)

In an effort to gain insight into this control of vascular tone, investigations have aimed at determining the factor(s) which may consist of such dual vasomotor properties distinctively observed within contracting skeletal muscle. While many substances result in vasodilation, few have demonstrated the additional capacity to blunt sympathetic vasoconstriction; including but not limited to adenosine, vasodilating prostaglandins, nitric oxide, and beta-adrenergic receptor stimulation via isoproterenol (Tschakovsky *et al.*, 2002; Dinenno & Joyner, 2004) Nevertheless, we and others have recently demonstrated the ability of exogenous ATP to blunt $\alpha$-adrenergic vasoconstriction

(Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008). Further, graded increases in arterial concentrations of ATP that evoke moderate limb hyperemia result in graded inhibition of direct postjunctional alpha stimulation, such that low levels are not sympatholytic where as progressive reductions in α-adrenergic receptor mediated vasoconstriction are observed with increasing [ATP] (Kirby *et al.*, 2008). This is strikingly similar to the intensity dependent sympatholytic nature of muscle contractions. Importantly, these unique responses appear specific to ATP and are not mediated by its degradation by-products (ADP, AMP, or adenosine) (Rosenmeier *et al.*, 2008).

With respect to human aging, which poses an elevated cardiovascular disease risk, literature suggests a classic "upregulation-desensitization" response whereby older adults have clear elevations in muscle sympathetic nervous system activity yet have diminished α-adrenergic receptor responsiveness (Seals & Esler, 2000; Seals & Dinenno, 2004; Dinenno & Joyner, 2006). Although sympathetic vasoconstrictor responses differ slightly between upper and lower limbs at rest (Dinenno *et al.*, 2002; Smith *et al.*, 2007) general consensus point towards enhanced vasoconstriction and blunted vasodilation to imposed stimuli during muscle contractions in older adults compared to young (Seals & Dinenno, 2004; Dinenno & Joyner, 2006; Versari *et al.*, 2009). Therefore, it was recently hypothesized that aging is associated with an impaired modulation of α-adrenergic vasoconstriction in the vascular beds of contracting muscle (i.e. impaired functional sympatholysis). Accordingly, Dinenno and colleagues demonstrated that this in fact was the case and postulated that this impairment may underscore reductions in blood flow and oxygen delivery in active muscle, thereby potentially limiting oxygen uptake and exercise capacity (Dinenno *et al.*, 2005).

Despite information in young healthy humans, to date, a coherent understanding on the interactions of the vasodilator ATP and α-adrenergic vasoconstrictor stimuli in aging humans in unclear. Therefore, we tested the hypothesis that modulation of postjunctional α-adrenergic vasoconstriction to exogenous ATP is impaired in aging humans. To do so, we utilized the exact study design as previously described in young subjects (Kirby *et al.*, 2008) by measuring forearm hemodynamics (Doppler ultrasound) to intra-arterial infusions of phenylephrine ($\alpha_1$-agonist) and dexmedetomidine ($\alpha_2$-agonist) during rhythmic handgrip exercise (15% MVC), a control non-exercise vasodilator condition (adenosine), and ATP infusion in discrete groups of young and older adults. Our findings indicate that the sympatholytic effect of exogenous ATP is not reduced with age, and thus we speculate that circulating endogenous concentrations of ATP are reduced during exercise in older adults and may contribute to the typically observed impaired exercise sympatholysis in this population.

## Methods

### *Subjects*

With Institutional Review Board approval and after written informed consent, a total of 7 older healthy adults (5 men, 2 women; age = 64 ± 2 years; weight = 80 ± 5 kg; height = 180 ± 3 cm; body mass index = 25 ± 1 kg $m^{-2}$; means±S.E.M) participated in the present study. All were non-smokers, non-obese, normotensive, and not taking any medications. Studies were performed after a 4-hour fast with the subjects in the supine position. All studies were performed according to the Declaration of Helsinki.

### *Arterial Catheterization*

A 20-gauge, 7.6-cm catheter was placed in the brachial artery of the non-dominant arm under aseptic conditions after local anesthesia (2% lidocaine) for local administration of study drugs. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml $h^{-1}$ with heparinized saline (Kirby *et al.*, 2008). The two side ports were used for infusions of vasoactive drugs.

### *Forearm Blood Flow and Vascular Conductance*

A 4 MHz pulsed Doppler probe (Model 500V, Multigon Industries, Mt. Vernon, NY, USA) was used to measure brachial artery mean blood velocity (MBV) with the probe securely fixed to the skin over the brachial artery proximal to the catheter insertion site as previously described by our laboratory (Kirby *et al.*, 2007; Kirby *et al.*, 2008). The probe insonation angle relative to the skin was 45 degrees. A linear 12 MHz echo Doppler ultrasound probe (GE Vingmed Ultrasound Vivid7, Horten, Norway) was placed in a holder securely fixed to the skin immediately proximal to the velocity probe to measure brachial artery diameter. Forearm blood flow was calculated as:

FBF = MBV ($cm \cdot s^{-1}$) * $\pi$ (brachial artery diameter/2)$^2$ * 60, where the FBF is in ml $min^{-1}$, the MBV is in cm $s^{-1}$, the brachial diameter is in cm, and 60 is used to convert from ml $s^{-1}$ to ml $min^{-1}$. Forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml $min^{-1}$ 100 $mmHg^{-1}$.

***Rhythmic Handgrip Exercise***

Maximum voluntary contraction (MVC) was determined for each subject as the average of at least three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3 percent of each other. For the exercise trials, weights corresponding to 15% MVC were 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 minute) using audio and visual signals to ensure the correct timing (Kirby *et al.*, 2008). We chose this moderate workload because it significantly blunts, but does not abolish, sympathetic vasoconstriction in contracting muscle (Kirby *et al.*, 2005; Kirby *et al.*, 2008).

***Sympathetic α-Adrenergic Vasoconstrictor Drugs***

In male subjects, phenylephrine (a selective $\alpha_1$-agonist; Baxter, Irvine, CA) was infused at 0.0625 µg (dl forearm volume)$^{-1}$ min$^{-1}$ and dexmedetomidine (a selective $\alpha_2$-agonist; Hospira, Lake Forest, IL) was infused at 6.25 ng (dl forearm volume)$^{-1}$ min$^{-1}$. The doses of phenylephrine and dexmedetomidine were chosen based on our experience at rest and during handgrip exercise. All vasoconstrictor drug infusions were adjusted for the hyperaemic conditions as previously described (see below) (Kirby *et al.*, 2008).

Given that exercise increases forearm blood flow, adenosine was infused to elevate resting forearm blood flow to similar levels observed during exercise. We have previously demonstrated that exercise blunts the vasoconstrictor responses to direct $\alpha_1$- and $\alpha_2$-adrenoceptor stimulation, whereas these vasoconstrictor responses are maintained when blood flow is elevated with adenosine and hence it was used to create a “high flow”

control state (Tschakovsky *et al.*, 2002; Kirby *et al.*, 2008). In an effort to normalise the concentration of each vasoconstricting drug in the blood perfusing the forearm, the infusions were adjusted on the basis of forearm blood flow and forearm volume (measured via regional analysis of whole-body DEXA scans). Various concentrations of each compound were available to keep the absolute infusion rates less than 3 ml $min^{-1}$ in every trial.

## Experimental Protocols

### *General Experimental Protocol*

Figure 1 is an example of a time-line for the specific trials. In the supine position, subjects performed either a bout of handgrip exercise, or received intra-arterial adenosine (Sicor, Irvine, CA) or ATP (Sigma, USA); the total time for each trial was 8 minutes. After 2 minutes of baseline measurements, exercise or vasodilator infusion was initiated and steady-state FBF was reached within 3 minutes. Between 3 and 4 minutes of hyperaemia (minutes 5 and 6 of Figure 1) the dose of the $\alpha_1$- or $\alpha_2$- agonist (vasoconstrictor) was calculated on the basis of forearm volume and blood flow. The vasoconstrictor infusion began at the 6-minute mark and lasted for 2 minutes.

### *Effects of Exogenous ATP on Postjunctional α-adrenergic Vasoconstrictor Responsiveness*

The purpose of this protocol was to determine whether exogenous ATP blunts direct postjunctional α-adrenergic responsiveness in aging humans, and whether this is selective for $\alpha_1$- or $\alpha_2$-adrenoceptors. Therefore, in 7 subjects (5 men, 2 women), the

vasoconstrictor responses to direct $\alpha_1$- and $\alpha_2$-adrenoceptor stimulation (via phenylephrine and dexmedetomidine, respectively) were assessed during control vasodilator infusion of adenosine, during moderate rhythmic handgrip exercise (15% MVC), and during infusion of ATP. In total, there were 6 experimental trials for each subject. In this protocol, the goal was to match steady-state FBF during infusion of adenosine or ATP with that observed during exercise. To do so, adenosine (45 nmol $100ml^{-1}$ $min^{-1}$) and ATP (5 nmol $100ml^{-1}$ $min^{1}$) were initially infused and doses were increased to elevate FBF accordingly. The final average doses of adenosine and ATP were 75±21 and 10±2 nmol $100ml^{-1}$ $min^{-1}$, respectively. The order of the adenosine, exercise, and ATP trials were varied across subjects. Thus, for subjects that did not perform the exercise trial first, we had them perform 3-4 minutes of rhythmic handgrip exercise prior to any experimental trials with α-agonists to determine their individual steady-state FBF for this exercise intensity. Additionally, in 4 of the subjects, vasoconstrictor responses to $\alpha_1$-adrenoceptor stimulation were determined under each hyperaemic condition, followed by the trials for $\alpha_2$-receptor stimulation. This order was reversed in the other 3 subjects, and all subjects rested for 15 minutes between each trial.

***Previously Published Results from Young Adults***

This experimental protocol has been previously published in young adults, therefore any reference to young adults refers to this published experiment (Kirby *et al.*, 2008). The protocol was exactly replicated in older adults for the present study as in young adults, and thus enhances the findings when determining the impact of age on the ability of exogenous ATP to blunt direct postjunctional α-adrenergic vasoconstriction.

The exact protocol and equipment were emulated and used for data collection and analysis. Because vasoconstrictor responsiveness is not equal between age groups at rest in the forearm for a given receptor subtype (Dinenno *et al.*, 2002; Dinenno *et al.*, 2005) we calculated the magnitude of sympatholysis as a means of determining the ability of exercise or ATP to blunt alpha receptor stimulation (Dinenno *et al.*, 2005). The percent magnitude of sympatholysis was calculated as [(% $\Delta$ $FVC_{constriction}$ Adenosine – % $\Delta$ $FVC_{constriction}$ Exercise)/ % $\Delta$ $FVC_{constriction}$ Adenosine] *100.

***Data Acquisition and Analysis***

Data was collected and stored on computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Mean arterial pressure (MAP) was determined from the arterial pressure waveform. Baseline FBF, HR, and MAP represent an average of the last minute of the resting time period, the steady-state hyperaemic values represent an average of minutes 3-4 (minutes 5-6 of Figure 1; pre-vasoconstrictor) during exercise, adenosine, or ATP and the effects of the $\alpha$-agonists represent an average of the final 30-seconds of drug infusion (post-vasoconstrictor). The % reduction in FBF during vasoconstrictor administration was calculated as:

((FBF post constrictor - FBF pre constrictor)/(FBF pre constrictor)) x 100.

We also calculated % reduction in FVC as our standard index to compare vasoconstrictor responses to the $\alpha$-agonists across conditions, as this appears to be the most appropriate way to compare vasoconstrictor responsiveness under conditions where there might be differences in vascular tone (Lautt, 1989; O'Leary, 1991; Thomas *et al.*, 1994). In an

effort to be comprehensive, we have also presented absolute values of forearm haemodynamics for all conditions in tabular form.

### *Statistics*

All values are reported as means ± S.E.M. Specific hypothesis testing within each of the exercise, adenosine, or ATP trials with the two different α-agonist infusions was performed using repeated measures ANOVA. Comparison of the hemodynamic values at specific time points between the exercise, adenosine, and ATP conditions were made with unpaired t-tests, and the values within each hyperaemic condition (exercise, adenosine, or ATP) with paired t-tests. Significance was set at $P<0.05$.

## Results

### *Effects of Exogneous ATP on Postjunctional α-adrenergic Vasoconstrictor Responsiveness in Older adults*

Forearm haemodynamics, HR, and MAP for are presented in Tables 2A & B. Intra-arterial infusion of both adenosine and ATP, as well as handgrip exercise, significantly increased FBF and FVC from baseline ($P<0.05$). As desired by experimental design, steady-state (pre-vasoconstrictor) FBF responses to adenosine and ATP infusion were effectively matched to that observed during 15% MVC handgrip exercise within both phenylephrine (Figure 2A) and dexmedetomidine conditions (Figure 2B; $P = 0.4 - 0.8$). Infusion of phenylephrine ($\alpha_1$-agonist) significantly reduced FBF from steady-state hyperaemia during adenosine and exercise ($P < 0.05$), whereas FBF was unchanged during ATP (*NS*; Figure 2A). Similarly, infusion of dexmedetomidine

($\alpha_2$-agonist) significantly reduced FBF from steady-state hyperaemia during adenosine and exercise ($P < 0.05$), whereas FBF was unchanged during ATP (*NS*; Figure 2B).

The forearm vasoconstrictor responses to direct $\alpha_1$-adrenoceptor stimulation were blunted during steady-state exercise vs adenosine ($\Delta$FVC = -15 ± 2% vs -25 ± 3%; $P < 0.05$), and were abolished during ATP infusion (-1 ± 4%; $P = 0.8$ vs zero; Figure 3A). Similarly, vasoconstrictor responses to $\alpha_2$-receptor stimulation were blunted during exercise vs adenosine (-26 ± 4% vs -35 ± 8%; $P < 0.05$), and were abolished during ATP infusion (-5 ± 5%; $P = 0.3$ vs zero; Figure 4A). MAP changed minimally within and between conditions (Tables 2A and B), thus FBF responses were similar to FVC. Heart rate increased in response to exercise during phenylephrine ($P < 0.05$), but otherwise was not different between or within trials and conditions (Tables 2A & B).

***Magnitude of Sympatholysis: Impact of Advancing Age***

A comparison between young and older adults of the vasoconstrictor responsiveness during handgrip exercise, and intra-arterial infusions of adenosine and ATP are presented in figures 3 & 4. The 'magnitude of sympatholysis' or the ability to blunt α-adrenergic vasoconstriction during exercise was significantly blunted in older adults compared to young adults for each receptor subtype ($\alpha_1$ = 32% vs 74%; $\alpha_2$ = 19% vs 60%; respectively, Figure 3B & 4B). In contrast, exogenous ATP appeared to blunt vasoconstriction to both α-receptor subtype independent of age ($\alpha_1$ = 94% vs 88%; $\alpha_2$ = 88% vs 84%; respectively, Figure 3C & 4C).

**Discussion**

The primary findings from the present investigation are as follows. Exogenous ATP required to match forearm hyperemia during moderate handgrip exercise abolishes direct postjunctional α-adrenoceptor mediated vasoconstriction in older adults, and this involves both $\alpha_1$- and $\alpha_2$-receptor subtypes. The present data corroborate previous findings that at rest in the human forearm, $\alpha_1$-adrenoceptor responsiveness is blunted in older adults compared to young while $\alpha_2$-adrenoceptor sensitivity is relatively intact. As expected, aged humans have enhanced vasoconstriction to direct postjunctional α-adrenergic receptor stimulation during modest intensity forearm exercise, thus demonstrating a reduced magnitude of sympatholysis with exercise. However in contrast to our hypothesis, the ability of exogenous ATP to blunt postjunctional α-adrenergic vasoconstriction is similar in young and older adults.

The rationale for the present study stems from the understanding that a competition exists between local vasodilator and neural sympathetic vasoconstrictor signals during exercise as a means to regulate blood flow and oxygen delivery to active skeletal muscle without compromising blood pressure (Rowell, 1997; Buckwalter & Clifford, 2001). As such, it is well recognized that vasodilatation and sympathetic activation simultaneously occur with the degree of exercise intensity and muscle mass recruited (Rowell, 1997; Saltin *et al.*, 1998). Although once heavily debated, accumulating evidence appears to now indicate that exercise has the ability to blunt sympathetic vasoconstriction within the active muscle, presumably as a means of defending against a 'blood flow error' (Tschakovsky *et al.*, 2002; Dinenno & Joyner, 2006). This phenomenon has been termed 'functional sympatholysis' (Remensnyder *et*

*al.*, 1962). Over the last decade, investigators have placed a substantial amount of effort into finding the factor(s) responsible for blunting sympathetic vasoconstriction within active muscle, and appear to have collectively indicated only a minimal role for the putative vasodilator action of adenosine, nitric oxide, prostaglandins, and beta-adrenergic stimulation (Tschakovsky *et al.*, 2002; Dinenno & Joyner, 2004). Further, it does not appear that any physical mechanical effect of muscle contraction explains this phenomenon (Kirby *et al.*, 2005). However, more recently, we and others have demonstrated that exogenous ATP has the ability to significantly blunt sympathetically-mediated vasoconstriction in young adults (Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008). In addition, given that both circulating ATP levels as well as the ability to blunt sympathetic vasoconstriction increase in an exercise intensity dependent manner (Buckwalter *et al.*, 2001; Gonzalez-Alonso *et al.*, 2002), we had questioned whether this response was graded with the dose of ATP infusion, such that low doses of ATP are not sympatholytic yet higher doses of ATP produce progressive blunting of constriction (Kirby *et al.*, 2008). This in fact does occur.

In relation to aging humans, it is well recognized that control of the vasculature is altered with advancing age whereby elevations in sympathetic vasoconstrictor activity are elevated and vasodilator action is diminished both at rest and during exercise (Dinenno & Joyner, 2006; Kirby *et al.*, 2009b). With specific relevance to the present study, Dinenno and colleagues demonstrated that aged men have impaired modulation of sympathetic α-adrenergic vasoconstriction in contracting muscle (Dinenno *et al.*, 2005) supporting previous findings observed in older women (Fadel *et al.*, 2004) and the leg of older men (Koch *et al.*, 2003). Accordingly, in the present study we questioned and hypothesized

that the sympatholytic properties of exogenous ATP are impaired in aging humans when challenged with direct postjunctional α-adrenergic receptor stimulation. In contrast, we observed that the ability of exogenous ATP to blunt sympathetic vasoconstriction is intact and is similar between young and older adults. Despite these responses with exogenous ATP, older adults still exhibited an impaired ability to modulate direct α-adrenergic constriction during exercise. Collectively, we therefore speculate that the impairment in exercise sympatholysis presumably occurs prior to vasodilator signaling via ATP and may be a result of attenuated circulating ATP release during exercise in aging humans.

It has been previously demonstrated that vasoconstrictor responsiveness to α-adrenergic receptor stimulation in the human forearm at rest is altered in aging humans such that $\alpha_1$ but not $\alpha_2$ receptor sensitivity is reduced (Dinenno *et al.*, 2002). Our findings support such a concept and can be seen in Figure 3A & 4A. Regardless, it is clear that despite reduced vasoconstriction to $\alpha_1$ stimulation during adenosine, vasoconstriction was significantly blunted during exercise and abolished during ATP in older adults. This discrepant observation between exercise and exogenous ATP administration are novel and highlight the unique vasomotor properties of ATP in the human circulation.

As a means of concisely determining the ability of either exercise or exogenous ATP to blunt α-adrenergic constriction relative to a control vasodilator (i.e. adenosine), we calculated a % magnitude of sympatholysis. In the present study, we clearly demonstrate and support earlier findings that the magnitude of sympatholysis via exercise is significantly attenuated in older compared to young adults which are independent of α-receptor subtype (Dinenno *et al.*, 2005). However in contrast to our hypothesis, our data

show that the magnitude of sympatholysis via exogenous ATP was similar between young and older adults (~90%). Although these findings may be somewhat surprising considering an impaired ability of exercise to blunt sympathetic constriction in aging humans, we have recently demonstrated that aging humans have intact vasodilator responsiveness to graded doses of exogenous ATP despite clear endothelial dysfunction as evidenced by substantial reductions in vasodilation during acetylcholine infusion (Kirby *et al.*, 2009a). Moreover, when comparing the average dose of ATP used in the present study (5.6±1 μg/dl FAV/min) in light with our previous findings in young adults (6.2±1 μg/dl FAV/min) (Kirby *et al.*, 2008), no significant difference between young and older adults is observed. Collectively, these data imply that the dual vasoactive signaling properties of ATP are maintained with advancing age in humans.

***Potential Mechanisms***

It is important to note that to date, "the" factor(s) directly involved in functional sympatholysis have yet to be firmly clarified. The most promising data supports that ATP and UTP (both of which bind to purinergic $2_Y$ receptors along the endothelium) are of the few agents currently known to have sympatholytic properties (Rosenmeier *et al.*, 2008); however whether these substances are obligatory to observe functional sympatholysis during muscle contractions cannot currently be directly assessed as no receptor antagonist is available for human use. Further, the specific downstream vasodilator mechanisms of ATP also appears to be fraught with unexpected complexity even in young adults, as all investigations specifically in the human forearm have demonstrated little to no role for the primary endothelium dependent vasodilators of nitric

oxide and vasodilating prostaglandins (Rongen *et al.*, 1994; van Ginneken *et al.*, 2004). In addition, it does not appear that degradation of ATP to adenosine underlies ATP-mediated dilation in young nor older adults and fits with the current theme that adenosine is not sympatholytic in nature (Tschakovsky *et al.*, 2002; Kirby *et al.*, 2009a; Mortensen *et al.*, 2009). Studies also show that neither ADP nor AMP blunt sympathetic vasoconstriction (Rosenmeier *et al.*, 2008). Interestingly, ATP and UTP but not adenosine evoke endothelium-dependent spreading vasodilation/hyperpolarization that is independent of nitric oxide yet inhibited with $K_{Ca}$ channel blockade (Winter & Dora, 2007; Dora, 2010). When integrating these findings, it is important to note that functional sympatholysis and spreading dilation (both of which can result from ATP) have been implicated as crucial in optimizing oxygen delivery and blood flow to active tissue (Saltin *et al.*, 1998; Dora, 2010). Whether spreading vasodilation is a fundamental component underscoring the phenomenon of functional sympatholysis has yet to be determined.

Another possibility explaining the present observations relates to the endogenous release of ATP in human circulation. Accordingly, evidence indicates that ATP is released in young adults in an exercise intensity-dependent manner and is closely related to changes in the hemoglobin oxygenation, acidosis, as well as mechanical deformation of erythrocytes all which occur during muscle contractions (Gonzalez-Alonso *et al.*, 2002). We have most recently observed that venous plasma [ATP] and ATP release are significantly diminished in older compared to young adults during mild to moderate intensity forearm exercise. As such, we speculate that although both the vasodilatory and sympatholytic properties of ATP are intact in older adults, the impaired ability of

exercising muscle to modulate sympathetic vasoconstriction with advancing age may be a result of reduced endogenous levels of circulating ATP during such a stimulus.

***Experimental Considerations***

It should be considered that we were unable to determine the role of endogenous ATP in modulating direct α-adrenergic stimulation during exercise as no pharmacological antagonist for the purinergic $2_Y$ receptor is yet available for human use. In addition, we did not measure circulating ATP levels in either group, however this was not the main purpose of the study and the population characteristics within the present study are similar to our previous report involving ATP measures. Nonetheless, it is clear that exogenous ATP has the ability to blunt sympathetic vasoconstriction in aging humans despite an observed impairment of contracting muscle to modulate α-adrenoceptor stimulation.

Additionally, it is plausible that although vasoconstriction was blunted at this dose of exogenous ATP, perhaps a reduced ability to blunt α-adrenergic stimulation could be observed at lower ATP doses. We have previously demonstrated that low ATP doses do not evoke sympatholysis yet progressively increasing the ATP dose results in a progressive ability to offset the constrictor response (Kirby *et al.*, 2008). Nevertheless, in the present study our dose of ATP was chosen to match the hyperaemic response observed during 15% MVC, and at this exercise intensity older adults demonstrate a clearly blunted magnitude of sympatholysis.

### *Conclusions*

Contracting muscle of aged humans has an impaired ability to modulate sympathetic vasoconstriction, and this is not specific to the α-adrenergic receptor subtype. In contrast, the vasodilatory and sympatholytic properties of exogenous ATP are intact in older adults, likely indicating that perhaps blunted endogenous ATP release during exercise may contribute to the impaired exercise sympatholysis in older adults. Collectively, the present findings emphasize the unique vasomotor properties of circulating ATP and draw attention to the fact that impaired vasodilation exists in aging humans which may result in reduced blood flow and oxygen delivery during exercise.

**Author Contributions**

B.S.K contributed to the experimental design, data acquisition, data analysis, data interpretation, and drafting of the manuscript. A.R.C contributed to data acquisition and interpretation, and critical review of the manuscript. W.F.V provided clinical support, invasive methodology, and contributed to data acquisition and interpretation, as well as critical review of the manuscript. F.A.D. contributed to the conception and experimental design, data acquisition and interpretation, and critical review of the manuscript. All authors approved the final version of the manuscript.

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**TABLE 1: Subject characteristics**

| Variable | Young | Older |
|---|---|---|
| Male, Female | 5, 2 | 5, 2 |
| Age (years) | 22 ± 1 | 64 ± 2* |
| BMI ($kg \cdot m^{-2}$) | 23 ± 1 | 25 ± 1 |
| Body fat (%) | 17 ± 2 | 19 ± 2 |
| Forearm volume (ml) | 946 ± 25 | 1104 ± 96 |
| MVC (kg) | 43 ± 2 | 42 ± 3 |
| Total cholesterol ($mmol\ l^{-1}$) | NA | 4.7 ± 0.2 |
| LDL cholesterol ($mmol\ l^{-1}$) | NA | 4.2 ± 0.2 |
| HDL cholesterol ($mmol\ l^{-1}$) | NA | 1.0 ± 0.1 |
| Triglycerides ($mmol\ l^{-1}$) | NA | 1.0 ± 0.1 |

MVC = Maximum voluntary contraction, LDL = Low density lipoprotein, HDL = High density lipoprotein. * $P<0.05$ vs. young adults. Note: Young data are adapted from Kirby et al. (Kirby *et al.*, 2008)

| Table 2A. Forearm and Systemic Haemodynamics in Older Adults: Phenylephrine Trials | | | | | |
|---|---|---|---|---|---|
| Time | Condition | Forearm Blood Flow (ml $min^{-1}$) | Mean Arterial Pressure (mmHg) | Forearm Vascular Conductance (ml $min^{-1}$ 100 $mmHg^{-1}$) | Heart Rate (beats $min^{-1}$) |
| Baseline | Adenosine | 26 ± 4 | 102 ± 4 | 25 ± 4 | 58 ± 3 |
| | Exercise | 28 ± 5 | 105 ± 4 | 27 ± 5 | 59 ± 4 |
| | ATP | 27 ± 5 | 103 ± 3 | 26 ± 4 | 58 ± 3 |
| Pre-Phenylephrine | Adenosine | 130 ± 23* | 107 ± 3 | 123± 22* | 59 ± 3 |
| | Exercise | 147 ± 16* | 106 ± 4 | 142 ± 17* | 62 ± 3 |
| | ATP | 132 ± 23* | 105 ± 4 | 132 ± 23* | 58 ± 3 |
| Phenylephrine | Adenosine | 98 ± 17*† | 108 ± 3 | 93 ± 16*† | 60 ± 3 |
| | Exercise | 126 ± 14*†‡ | 107 ± 4 | 120 ± 16*†‡ | 63 ± 4* |
| | ATP | 129 ± 21*‡ | 104 ± 4 | 126 ± 21*‡ | 57 ± 4 |

| Table 2B. Forearm and Systemic Haemodynamics in Older Adults: Dexmedetomidine Trials | | | | | |
|---|---|---|---|---|---|
| Time | Condition | Forearm Blood Flow (ml $min^{-1}$) | Mean Arterial Pressure (mmHg) | Forearm Vascular Conductance (ml $min^{-1}$ 100 $mmHg^{-1}$) | Heart Rate (beats $min^{-1}$) |
| Baseline | Adenosine | 25 ± 3 | 103 ± 4 | 25 ± 3 | 61 ± 3 |
| | Exercise | 25 ± 4 | 104 ± 3 | 24 ± 4 | 61 ± 4 |
| | ATP | 28 ± 5 | 104 ± 4 | 27 ± 5 | 60 ± 3 |
| Pre-Dexmedetomidine | Adenosine | 132 ± 22* | 105 ± 4 | 127 ± 22* | 60 ± 3 |
| | Exercise | 149 ± 16* | 105 ± 4 | 143 ± 15* | 64 ± 4 |
| | ATP | 136 ± 20* | 104 ± 3 | 133 ± 21* | 60 ± 3 |
| Dexmedetomidine | Adenosine | 82 ± 10*† | 107 ± 4 | 79 ± 11*† | 61 ± 3 |
| | Exercise | 110 ± 10*†‡ | 109 ± 4 | 103 ± 10*†‡ | 63 ± 4 |
| | ATP | 132 ± 22*‡ | 105 ± 4 | 128 ± 23*‡ | 60 ± 3 |

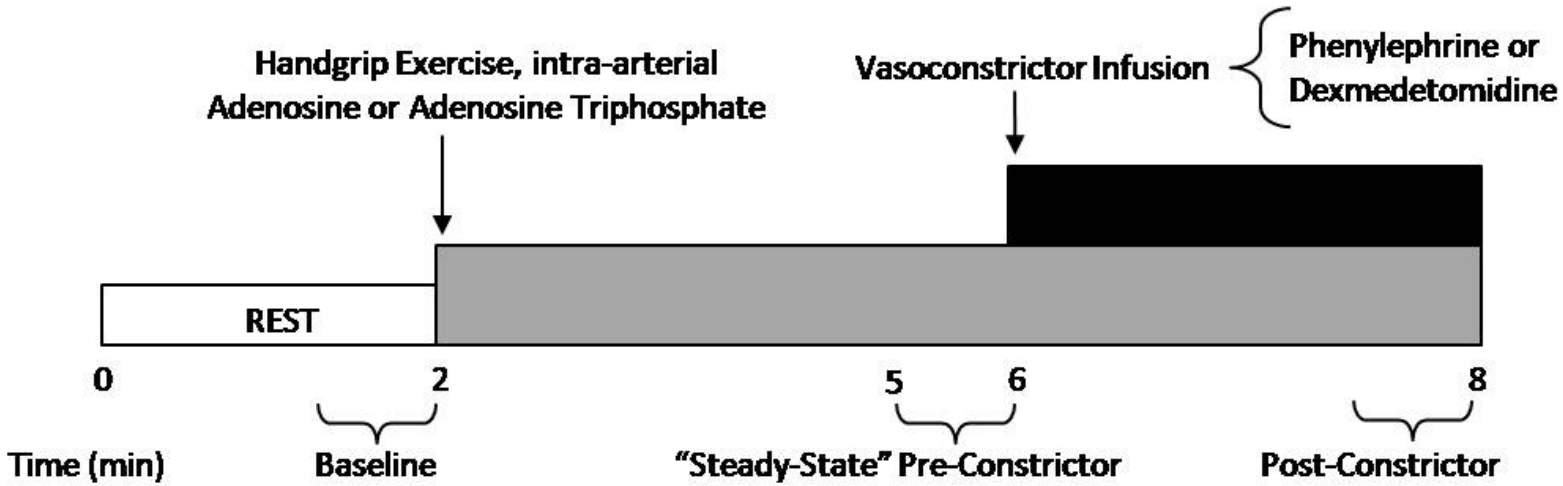
* $P<0.05$ vs baseline; † $P<0.05$ vs steady-state (pre-drug); ‡ $P<0.05$ vs adenosine

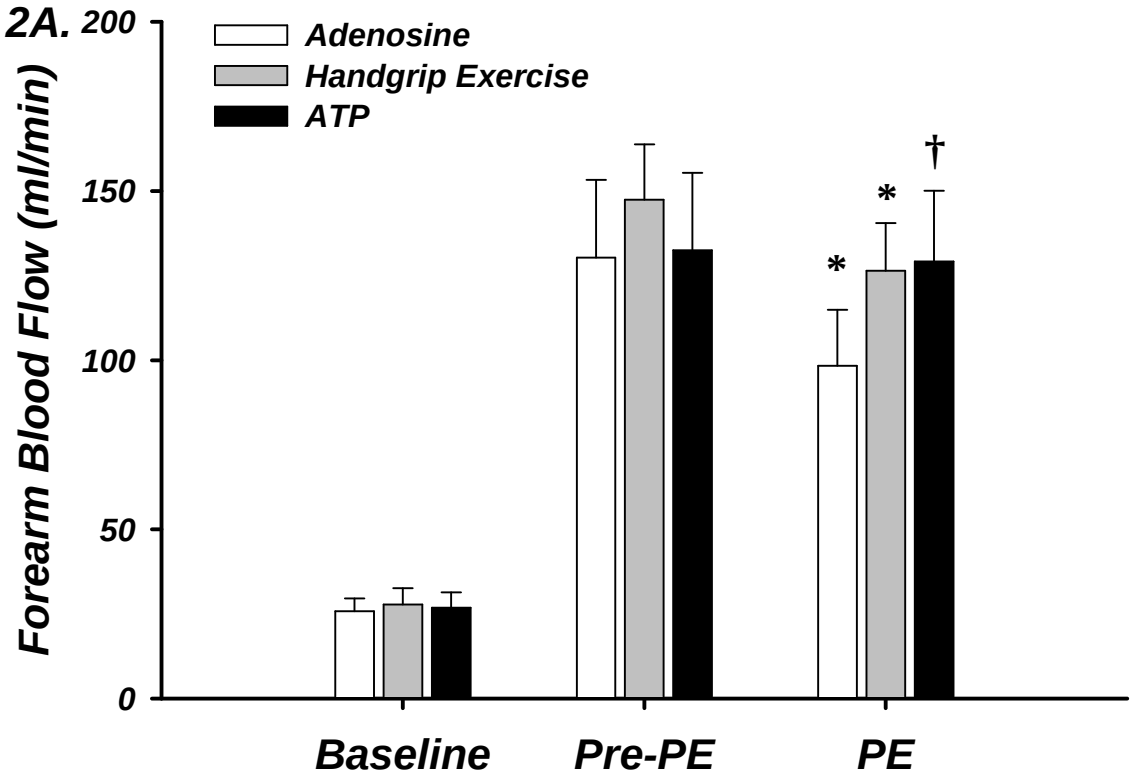
**Figure 1: General Experimental Trial.** Each trial consisted of a 2-minute baseline period. After this time period, subjects either began rhythmic handgrip exercise or received intra-arterial adenosine or adenosine triphosphate (ATP) to elevate resting forearm blood flow to levels observed during exercise. During minutes 5-6 (pre-constrictor), the doses of the $\alpha_1$- or $\alpha_2$-adrenoceptor agonists (phenylephrine or dexmedetomidine, respectively) were calculated on the basis of steady-state hyperaemic forearm blood flow and forearm volume. Subsequently, the α-agonist was infused for 2 minutes until minute 8. An average of forearm blood flow and mean arterial blood pressure during the final 30 seconds of α-agonist infusion was used to calculate the

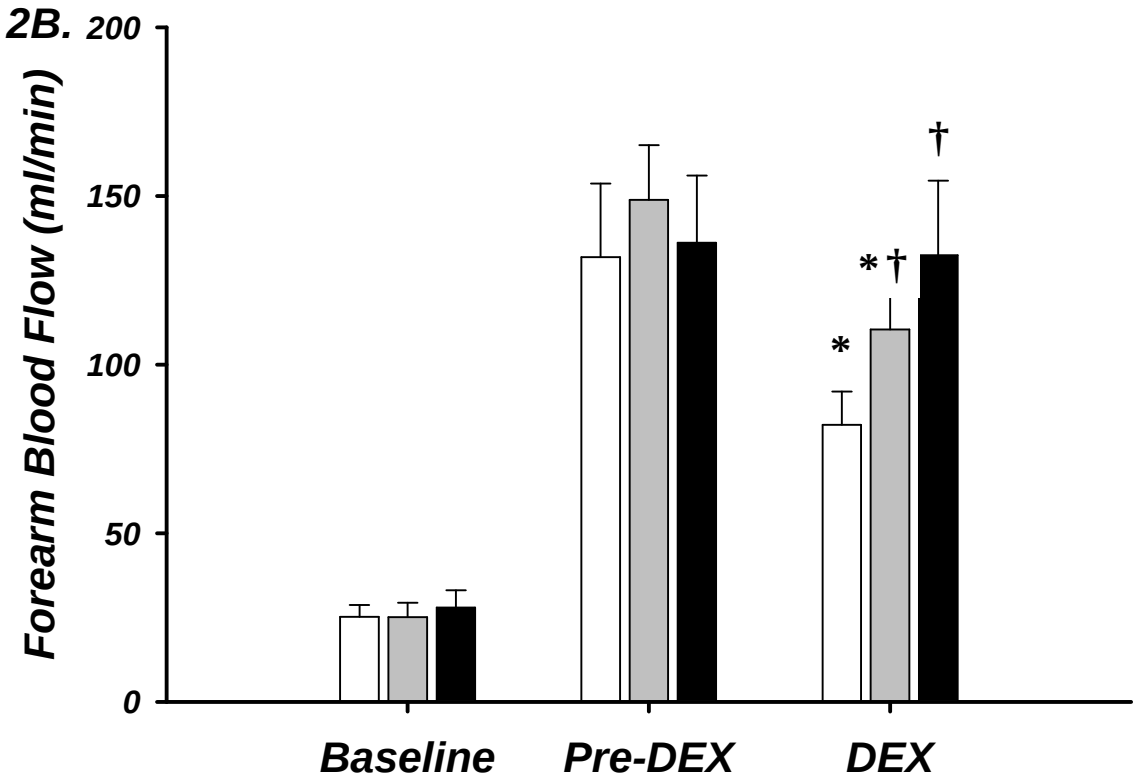
**Figure 2: Forearm blood flow at rest, during each hyperaemic condition, and during infusion of α-agonists in older adults.** Steady-state hyperaemia was similar during rhythmic handgrip exercise, adenosine, and ATP infusions for trials involving the $\alpha_1$-agonist phenylephrine (A; Pre-PE) and the $\alpha_2$-agonist dexmedetomidine (B; Pre-Dex). Forearm blood flow was reduced significantly with both α-agonists during adenosine and exercise, but the response was attenuated during exercise. In contrast, α-agonist infusion did not significantly reduce forearm blood flow during ATP. ***** $P$ <0.05 vs steady state (Pre-vasoconstrictor; PE/Dex) within condition; † $P < 0.05$ vs adenosine during α-agonist infusion.

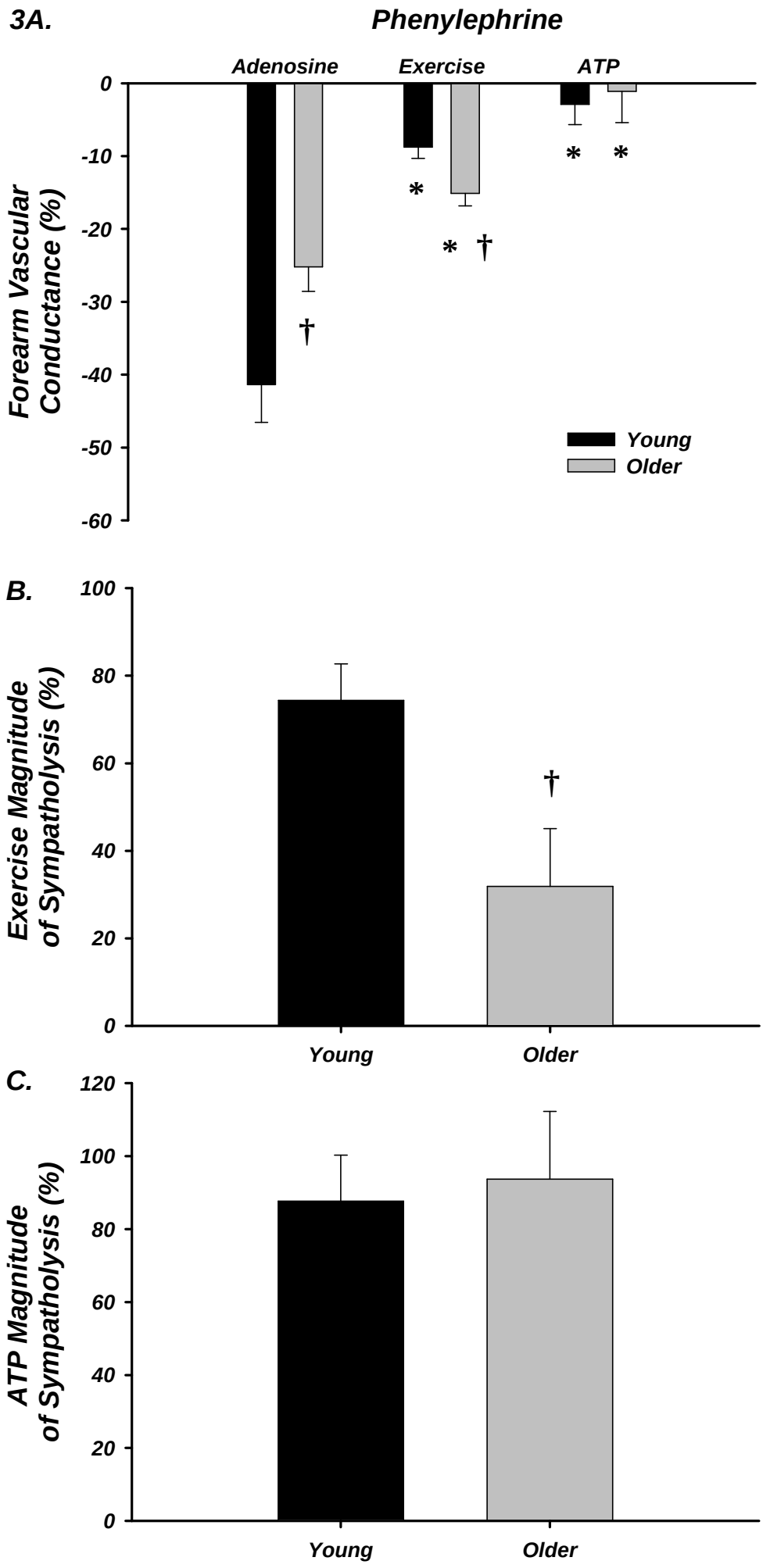
**Figure 3: Forearm vascular responses to $\alpha_1$-adrenoceptor stimulation.** (A) The vasoconstrictor responses to phenylephrine ($\alpha_1$-agonist) were significantly blunted in older compared with young during passive vasodilatation with adenosine, yet vasoconstriction was greater in older vs young adults during exercise. In both groups, the vasoconstrictor responses during exercise were blunted compared with adenosine, and abolished during ATP infusion. (B) Calculating the ability of exercise to blunt $\alpha_1$-adrenoceptor stimulation demonstrates a significant impairment in older compared to young adults, yet (C) no age-associated impairment in the sympatholytic properties of ATP was observed. Forearm vascular conductance was calculated as (forearm blood flow/mean arterial pressure) x 100. The percent magnitude of sympatholysis was calculated as [(% $\Delta$ $FVC_{constriction}$ Adenosine – % $\Delta$ $FVC_{constriction}$ Exercise)/ % $\Delta$ $FVC_{constriction}$ Adenosine] *100. * $P < 0.05$ vs adenosine within age group; † $P < 0.05$ vs young.

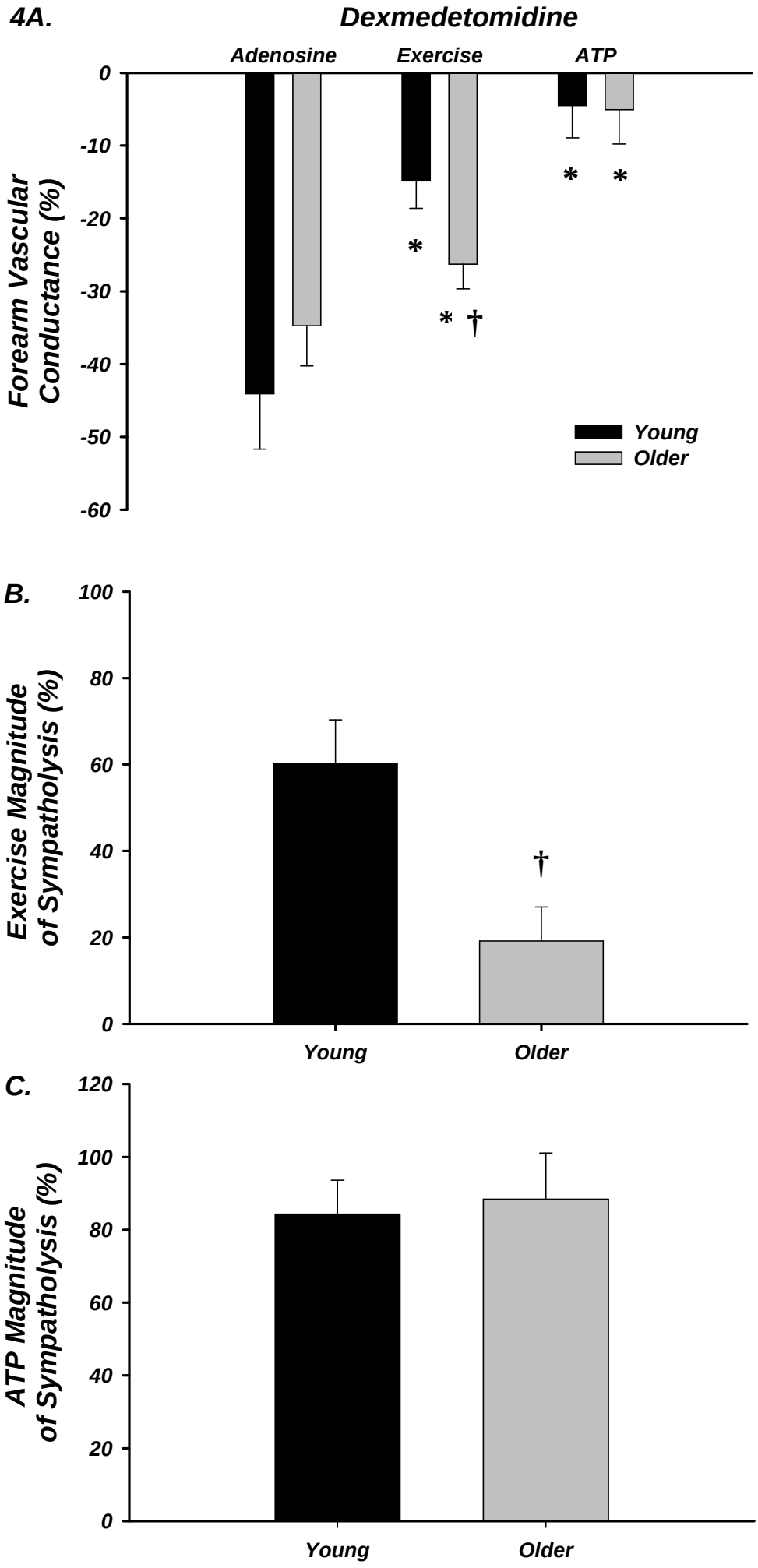
**Figure 4: Forearm vascular responses to $\alpha_2$-adrenoceptor stimulation.** (A) The vasoconstrictor responses to dexmedetomidine ($\alpha_2$-agonist) were significantly greater in older compared with young adults during exercise. In both groups, the vasoconstrictor responses during exercise were blunted, and abolished during ATP compared with adenosine infusions. (B) Calculating the ability of exercise to blunt $\alpha_2$-adrenoceptor stimulation demonstrates a significant impairment in older compared to young adults, yet (C) no age-associated impairment in the sympatholytic properties of ATP was observed. Forearm vascular conductance was calculated as (forearm blood flow/mean arterial pressure) x 100. The percent magnitude of sympatholysis was calculated as [(% $\Delta$ $FVC_{constriction}$ Adenosine – % $\Delta$ $FVC_{constriction}$ Exercise)/ % $\Delta$ $FVC_{constriction}$ Adenosine] *100. * $P < 0.05$ vs adenosine within age group; † $P < 0.05$ vs young.


Handgrip Exercise, intra-arterial
Adenosine or Adenosine Triphosphate
Vasoconstrictor Infusion
Phenylephrine or
Dexmedetomidine
REST
0
2
5
6
8
Time (min)
Baseline
"Steady-State" Pre-Constrictor
Post-Constrictor


2A.
Adenosine
Handgrip Exercise
ATP
Forearm Blood Flow (ml/min)
200
150
100
50
0
*
*
†
Baseline
Pre-PE
PE


2B.
Forearm Blood Flow (ml/min)
200
150
100
50
0
*
*†
†
Baseline
Pre-DEX
DEX


3A.
Phenylephrine
Adenosine
Exercise
ATP
0
-10
-20
-30
-40
-50
-60
Forearm Vascular Conductance (%)
*
*
†
*
*
†
Young
Older
B.
100
80
60
40
20
0
Exercise Magnitude of Sympatholysis (%)
†
Young
Older
C.
120
100
80
60
40
20
0
ATP Magnitude of Sympatholysis (%)
Young
Older


4A.
Dexmedetomidine
Adenosine
Exercise
ATP
0
-10
-20
-30
-40
-50
-60
Forearm Vascular Conductance (%)
*
*
†
*
*
Young
Older
B.
Exercise Magnitude of Sympatholysis (%)
100
80
60
40
20
0
†
Young
Older
C.
ATP Magnitude of Sympatholysis (%)
120
100
80
60
40
20
0
Young
Older

CHAPTER V – Manuscript IV

# Reduced Circulating ATP during Exercise in Older Adults: potential mechanism for impaired skeletal muscle blood flow with advancing age?

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Key Words: adenosine triphosphate, vascular tone, oxygen demand

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**Non-technical Summary**

Increasing blood flow during exercise helps to deliver appropriate amounts of oxygen to active muscle; however older adults appear to have a limited ability to do this. A naturally occurring substance in the circulation called ATP stimulates blood vessels to relax thereby facilitating increased blood flow. Normally, ATP in blood increases during exercise in young adults, yet whether this occurs in older adults is unknown. Our result show that ATP levels in the blood do not increase during exercise in aging humans and that this relates to reductions in blood flow and oxygen delivery during exercise. This knowledge helps to understand why exercise may be less tolerable for the aging population.

**Abstract**

Aging is associated with impaired control of skeletal muscle blood flow during exercise. In young adults, circulating adenosine triphosphate (ATP) aids in the control of muscle vascular tone during exercise. We tested the hypothesis that increases in venous plasma [ATP] and ATP release during forearm exercise are impaired in aging humans. We measured forearm blood flow (FBF; Doppler ultrasound) and [ATP], and calculated ATP release (FBF x [ATP]) during 5, 15, and 25% MVC rhythmic handgrip exercise in 14 young (22±1) and 12 older (63±2 yrs) adults. Deep v*enous blood samples were mixed in stop solution to preserve [ATP] for plasma measurement. FBF tended to be lower during 5% MVC* and was lower during 15 and 25% MVC in older adults, and forearm vasodilation was reduced at all exercise intensities in older adults ($P$<0.05). At rest, there were no age-group differences in [ATP] (Y=214±16; O=183±35 nmol/L; $P$=0.4) or ATP release. [ATP] increased above rest during all exercise intensities in young adults ($P$<0.05); in contrast there was no significant increase in [ATP] in older adults. ATP release increased in a graded fashion in both age groups, however this increase was blunted ~50-60% at all exercise intensities in older adults ($P$<0.05 vs young). ATP release was related to FBF during exercise in both groups ($r^2$=0.73). We conclude that the increase in venous plasma [ATP] and ATP release during forearm exercise is impaired in healthy older adults and relates to the observed declines in exercise hyperemia.

**Abbreviations List**

ATP, adenosine triphosphate; BMI, body mass index; $CtCO_2$, carbon dioxide content; $CtO_2$, oxygen content; DEXA, dual-energy X-ray absorptiometry; ECG, electrocardiogram; FBF, forearm blood flow; $FO_2Hb$, fraction of oxyhemoglobin; FVC, forearm vascular conductance; HCT, hematocrit; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MAP, mean arterial pressure; MBV, mean blood velocity; MVC, maximal voluntary contraction; NO, nitric oxide; NOS, nitric oxide synthase; $pCO_2$, partial pressure of carbon dioxide; PG, prostaglandin; $pO_2$, partial pressure of oxygen; pHb, plasma hemoglobin; RBC, red blood cell; SNO-Hb, *S*-nitrosohemoglobin; tHb, total hemoglobin.

## Introduction

Metabolically active tissue requires oxygen to generate energy and is thus largely dependent on effective convective oxygen transport. This metabolic demand for oxygen must be finely matched with oxygen delivery occurring primarily via the local modulation of vascular tone (Saltin *et al.*, 1998). With respect to skeletal muscle, local vasodilation and blood flow increases when oxygen demand is heightened during active muscle contractions (exercise) (Clifford & Hellsten, 2004; Saltin, 2007), and this response is further augmented in humans in the presence of systemic hypoxia indicating the sensitivity of blood flow regulatory mechanisms during mismatches in oxygen supply and demand (Rowell *et al.*, 1986; Crecelius *et al.*, 2009). Various lines of evidence suggest that innate properties *within* the vasculature and/or the extravascular tissue may sense and produce metabolites capable of evoking vasodilation; however a complete understanding of how these mechanisms respond to changes in oxygen requirements within the physiological range has yet to become clear (Marshall, 1999; Ellsworth, 2000). More recently, it has been proposed that local vascular perfusion may be coupled to oxygen carrying capacity of the blood itself. Given that vasodilation to metabolic stressors may be more closely related to the degree of hemoglobin saturation rather than oxygen tension ($pO_2$) (Gonzalez-Alonso *et al.*, 2001), the erythrocyte has been investigated for its capacity to 'sense' oxygen requirements and subsequently release vasodilators (such as nitric oxide and ATP) (Ellsworth *et al.*, 2009).

Three principal mechanisms have been proposed indicating a tight link between blood oxygenation and vascular control: (1) the deoxygenation of hemoglobin appears to act as a reductase where by nitrite is reduced to NO, (2) a hemoglobin bound NO-like

substance may be preserved and charioted via the RBC through the formation of *S*-nitrosohemoglobin (SNO-Hb), and (3) the RBC has been shown to release ATP in relation to the degree of hemoglobin saturation (Stamler *et al.*, 1997; Jagger *et al.*, 2001; Crawford *et al.*, 2006). With relevance to exercising muscle, our laboratory has taken interest in the role of ATP as a circulating vasodilator for a variety of reasons additional to mismatches in tissue oxygenation, as acidosis and hypercapnia have also been demonstrated to facilitate RBC ATP release (Bergfeld & Forrester, 1992; Ellsworth *et al.*, 1995). In addition, mechanical deformation of RBCs, as may be seen with a single passage through a capillary network or during muscle contractions, has also been shown to release ATP (Sprague *et al.*, 1998; Wan *et al.*, 2008). Moreover, exercise significantly increases blood flow increasing shear stress along endothelial cells which may further increase circulating [ATP] and further potentiated flow-mediated vasodilatory effects (Liu *et al.*, 2004; Yamamoto *et al.*, 2007). Collectively, a multitude of stimuli imposed during exercise appear to readily facilitate increases in circulating ATP. Accordingly, such findings have been observed in young adults since the late 1960's (Forrester & Lind, 1969).

In addition to evidence supporting the release of ATP in circulation during exercise, other characteristics further highlight the unique role that ATP has on vascular tone. Specifically, we and others have demonstrated in young adults that not only does ATP evoke substantial vasodilation by activating purinergic-$2_Y$ receptors, but can blunt sympathetic vasoconstriction in a dose-dependent fashion, unlike other endothelium-dependent vasodilators such as NO and adenosine (Dinenno & Joyner, 2003; Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008). Evidence also indicates that ATP can facilitate both

propagated ascending vasodilation and venular-arteriolar communication to aid in the full expression of the hyperemic response and $O_2$ delivery (Hester & Hammer, 2002; Winter & Dora, 2007). Taken together, circulating ATP not only aids in the control of muscle vascular control at rest and exercise but is unique when measured against other endothelium-dependent vasodilators.

Human aging is the predominant risk factor for cardiovascular disease and is characterized by reduced vasodilatory responsiveness to endothelium-dependent stimuli (endothelial dysfunction via intra-arterial acetylcholine or shear stress) (Celermajer *et al.*, 1994; Lloyd-Jones *et al.*, 2009). Maybe even more importantly, this decline in endothelium-dependent vasodilation strongly relates to attenuations in muscle blood flow commonly observed in aging humans during elevated oxygen demand situations such as exercise(Kirby *et al.*, 2009b). Given the preponderance of evidence *in vitro* indicating the endothelium dependence of ATP-mediated vasodilation (Ralevic & Burnstock, 1998; Winter & Dora, 2007), we recently tested the hypothesis that vasodilatory responsiveness to ATP would be impaired in older adults and lend insight into impaired vascular function during metabolic stress. To our surprise, ATP-mediated vasodilation was not impaired in the aged human forearm despite confirmation of endothelial dysfunction as evidenced by reduced acetylcholine dilator responsiveness (Kirby *et al.*, 2009a). Keeping in mind that a change in vessel caliber can be dictated by both receptor sensitivity as well as the concentration of the substrate stimulus, we postulated that an inability to increase circulating [ATP] may exist in aging humans. Therefore, in the present investigation we tested the hypothesis that circulating plasma [ATP] and ATP release is diminished during the metabolic stress of exercise in older healthy humans and may elucidate a means by

which exercise-induced vasodilation and hyperemia are impaired with advancing age. To the best of our knowledge, to date no information exists regarding circulating plasma [ATP] in older healthy humans at rest or during exercise.

## Methods

### *Subjects*

With Institutional Review Board approval and after written informed consent, a total of 14 young and 12 older healthy adult men and women participated in the present study. All subjects were normotensive and free from overt cardiovascular disease as assessed from casual blood pressure measurements and a medical history. Older subjects were further evaluated for clinical evidence of cardiopulmonary disease with a physical examination and resting and maximal exercise electrocardiograms. All subjects were sedentary to moderately active, non-smokers, not taking any medications including antioxidants, and studies were performed after a minimum of a 4-hour fast. Subjects provided written, informed consent after all potential risks and procedures were explained. This study was approved by the Human Research Committee of Colorado State University and was performed according to the Declaration of Helsinki.

### *Arterial Blood Pressure and Heart Rate*

Resting arterial blood pressure was measured non-invasively over the brachial artery of the control arm after 30 minutes of supine rest before any experimental trials, and just prior to each experimental trial after the study began (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA). Beat-by-beat arterial blood pressure was measured at

heart level by finger photoplethysmography (Finometer, FMS, Netherlands) on the middle finger of the control hand during all experimental trials. Heart rate was determined using a 3-lead ECG (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA).

### *Body Composition and Forearm Volume*

Body composition was determined by dual-energy X-ray absorptiometry (DEXA; Hologic, Inc; Bedford, MA, USA). Total forearm volume was calculated from regional analysis of the experimental forearm (from the proximal to distal radioulnar joint) from whole-body DEXA scans with QDR series software for normalization of individual drug doses. Body mass index was calculated as bodyweight (kg) divided by height (meters) squared.

### *Forearm Blood Flow and Vascular Conductance*

A 12MHz linear-array ultrasound probe (Vivid7, General Electric, Milwaukee, WI, USA) was used to measure brachial artery mean blood velocity (MBV) and brachial artery diameter and was placed in a holder securely fixed to the skin as previously described by our laboratory (Kirby *et al.*, 2009b). 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 500V TCD (Multigon Industries, Mt Vernon NY, USA) spectral analyzer from which mean velocity was determined as a weighted mean of the spectrum of Doppler shift frequencies. Brachial artery diameter measurements were made in triplicate in duplex mode at end-

diastole and between contractions during steady-state conditions. Forearm blood flow was calculated as:

$$FBF = MBV\ (cm \cdot s^{-1}) * \pi\ (\text{brachial artery diameter}/2)^2 * 60,$$

where the FBF is in ml $min^{-1}$, the MBV is in cm $s^{-1}$, the brachial diameter is in cm, and 60 is used to convert from ml $s^{-1}$ to ml $min^{-1}$. Forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml $min^{-1}$ (100 $mmHg^{-1}$).

***Rhythmic Handgrip Exercise***

Maximum voluntary contraction (MVC) was determined for each subject as the average of at least three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3 percent of each other. For the dynamic forearm exercise trials, weights corresponding to 5, 15, or 25% MVC were 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 minute) using audio and visual signals to ensure the correct timing. We chose these moderate workloads to limit the contribution of systemic haemodynamics to forearm vasodilator responses and to eliminate reflex increases in sympathetic nervous system activity, and thus isolate the local effects of muscle contraction on vascular tone (Kirby *et al.*, 2009b).

***Venous Catheterization***

An 18-gauge, 1.5-cm catheter was placed in retrograde fashion into a vein (that appeared to drain muscle rather than skin) of the non-dominant arm for blood sampling. The catheter was connected to a 3-way stopcock with one connection to an I.V. solution

set for continuous flushing with heparinized saline and the other to a 10 or 3cc syringe for blood sampling.

***ATP – Stop Solution/Blood Sampling***

Venous blood samples were drawn through an 18 gauge catheter directly into a pre-heparinized 10cc syringe to minimize hemolysis in which 2ml of blood was gently and at once expelled into a test tube containing 2.7mL of an 'ATP-stop solution' to equal a blood:diluent ratio of 1.35 as described by Gorman and colleagues (Gorman *et al.*, 2003; Gorman *et al.*, 2007). Briefly, the 'ATP-stop solution' (ethylenediaminetetraacetic acid (EDTA), NaCl, KCl, tricine buffer, nitrobenzyl thioinosine, forskolin, and isobutylmethylxanthine) was made as previously described and has been shown to inhibit degradation of ATP via ectonucleotidases and ATP production from other blood sources (such as platelets) for up to ~30 minutes (REF). The blood:diluent volume provided sufficient volume for plasma ATP measurements in triplicate and a plasma hemoglobin (pHb) measurement per sample. Blood:diluent samples were then immediately centrifuged at 4,000 rpm for 3 minutes at 22°C. Although this "ATP-stop solution" has been shown to maintain stable ATP values for up to ~30 minutes (Gorman *et al.*, 2003; Gorman *et al.*, 2007), to minimize any potential modulation of plasma [ATP], samples were analyzed for plasma [ATP] and plasma [Hb] directly following centrifugation. In addition, a 2mL blood sample was also drawn into a pre-heparinized 3cc syringe for co-oximetry blood gas parameters measured via blood gas analyzer (Siemens Healthcare Diagnostics, Deerfield, IL, USA).

***Measurement of Plasma [ATP] and [Hb]***

Directly following centrifugation of blood:diluent samples,100µL of supernatant was pipette into 3 individual microcentrifuge tubes for subsequent plasma [ATP] determination. Plasma ATP was measured via the luciferin-luciferase technique where light is generated by the reaction of ATP with luciferase and is dependent on [ATP](McElroy & DeLuca, 1983). First, 25µL of $Mg^{2+}$ solution (44.25 mmol/L, 40 mmol/L tricine buffer, pH 7.75) was automatically injected into the plasma supernatant to counteract the decrease of sample $Mg^{2+}$ concentration by EDTA. Two seconds later, 100µL of luciferase (ATP Bioluminescence Assay Kit CLS II: Roche Diagnostics) was automatically injected directly into the same 100µL plasma sample via an automated dual injector single tube luminometer in which relative light units (RLU) were collected (Turner BioSystems 20/20n, Sunnyvale, CA, USA). After three seconds, cumulative light output in RLUs was measured for ten seconds and averaged. An ATP standard curve was created on the day of the experiment prior to all experimental trials and in plasma medium from each subject studied. Specifically, a baseline blood:diluent sample was obtained and 90µL plasma samples were spiked with 10µL of varying concentrations of ATP standard (equating to 25, 51, 103, 206, 413, 826, 1650 nmol/L). The average standard curve $r^2$ value was 0.996. Any ATP standard in plasma medium that provided >10% variation in RLUs were discarded and reanalyzed. After accounting for background RLUs from an unspiked plasma sample, RLU's were plotted vs ATP and a least squares linear regression line was fit to the data. Plasma [ATP] was calculated as:

Final venous plasma [ATP] = $(ATP_{blood:diluent} - ATP_{hemolysis})*(1.35+1-HCT)/(1-HCT)$

To account for venous concentrations of ATP induced from hemolysis, 1mL of supernatant from the same blood:diluent sample used for plasma [ATP] measurements was pipette into a square 1.5mL methacrylate disposable spectrophotometer cuvette (Cole-Parmer No. U-06343-70) and analyzed for plasma Hb via spectrophotometry (Molecular Devices SpectraMax). Plasma Hb was calculated from absorbance (optical density) output of 3 wavelengths (415,380,450) using the Harboe method (Harboe, 1959; Malinauskas, 1997; Gorman *et al.*, 2007). This plasma [Hb] reading provided an indication of hemolysis (% Hemolysis = {(100 – HCT) * p[Hb]/t[Hb]}*100 ) and corresponded with a certain amount of ATP (nmol/L). ATP concentration (nmol/L) was plotted vs plasma [Hb] (range 0-25 mg/L) and data was fit with a linear regression line as described by Eric Feigl's laboratory in 3 young ($R^2$ = 0.98; y=22.145x) and 3 older ($R^2$ =0.99; y=44.202x) subjects as previously described (Farias *et al.*, 2005; Gorman *et al.*, 2007). Further, any blood:diluent sample containing more than 0.5% hemolysis was considered technical error and not used in the analysis of data.

## Experimental Protocols

### *General Experimental Protocol*

The purpose of this protocol was to determine endogenous plasma [ATP] and ATP release at rest and during mild, moderate, and moderately-heavy exercise in young and older healthy adults. Figure 1 is an example of the specific trial timeline. After 2 minutes of baseline measurements, subjects performed dynamic rhythmic handgrip exercise in the supine position with the non-dominant arm for a total of 15 minutes. This 15 minute trial was graded in exercise intensity whereby 5 minute segments of each 5,

15, and 25% MVC workloads were completed. Venous blood was sampled for blood gases as well as plasma [ATP] and [Hb] at the end of rest and the end of each exercise intensity segment. In addition, measurements for FBF calculation were also determined at rest and within each exercise intensity segment.

### *Data Acquisition and Analysis*

Data was collected and stored on a computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Baseline FBF, HR, and MAP represent an average of the last minute of the resting time period, and the steady-state hyperaemic values represent an average of the last minute of each exercise intensity (Kirby *et al.*, 2008). In an effort to be comprehensive, we have also presented absolute values of forearm hemodynamics for all conditions in tabular form (Table 2).

### *Statistics*

All values are reported as means ± S.E.M. Specific hypothesis testing within over time during exercise was performed using repeated measures ANOVA. In the case of a significant F value, Newman-Keuls method for multiple comparisons was used to determine where differences occurred. Statistical significance was set *a priori* at $P<0.05$. Comparison of the outcome variables at specific time points between the exercise conditions were made with unpaired t-tests, and the values within each condition with paired t-tests. Pearson Product Moment Correlation was used to determine significant

association between variables. SigmaPlot 11.0 (Systat Software, Inc., San Jose, CA, USA) was used for all analysis and significance was set at $P<0.05$.

## Results

### *Subject characteristics*

The mean age difference between the young and older adults was 41 years (Table 1). There were no significant age-group differences in forearm volume, MVC, HDL-cholesterol, or triglycerides. Older individuals had a greater BMI, body fat percentage and total- and

LDL-cholesterol ($P$ <0.05; Table 1), although these values were within normal levels.

### *Forearm and systemic hemodynamic responses at rest and during graded exercise*

Forearm and systemic hemodynamics are presented in Table 2. Resting FBF, FVC, and HR were not different with age. Although not considered hypertensive, older adults had significantly greater MAP at rest compared to younger adults ($P<0.05$). As expected, handgrip exercise at all intensities increased FBF and FVC from baseline in both young and older adults and these responses were greater for each graded increase in exercise intensity ($P<0.05$). Despite increases in FBF and FVC to exercise, older compared to young adults had a significantly lower hyperemic response at 15 and 25% MVC exercise as well as a significant reduction in forearm vasodilation during all exercise workloads (Figures 2A-B; $P<0.05$). Both young and older adults significantly increased MAP in response to exercise and older adults continued to have a greater

absolute MAP compared to young ($P$<0.05). Heart rate increased with exercise yet was not different between age groups.

***Venous plasma [ATP] and ATP release at rest and during progressive exercise intensity***

Venous plasma [ATP] was not different at rest between young and older adults (214±16 nmol/L vs 183±35 nmol/L; $P$=0.4). However during exercise, older adults had significantly lower plasma [ATP] concentrations compared to young at all exercise intensities ($P$<0.05) and did not increase [ATP] above baseline (5% = 157±24, 15% = 232±31, 25% = 248±34 nmol/L; Figure 3). In contrast, young adults had significantly greater [ATP] during all exercise intensities compared to rest conditions (5% = 329±41, 15% = 359±32, 25% = 407±39 nmol/L; Figure 3; $P$<0.05). To account for the impact changes in FBF have on [ATP] measurements, ATP release was calculated. Similar to [ATP], ATP release was not different with age during rest (8±1 nmol/min vs 6±1 nmol/min). Although both young and older adults had greater ATP release during exercise compared to rest, ATP release was significantly reduced with age (5% = 35±7, 15% = 81±11, 25% = 162±23 nmol/min vs 5% = 13±2, 15% = 39±5, 25% = 71±10 nmol/min, respectively, Figure 4; $P$<0.05). Additionally, forearm vasodilation and ATP release were plotted against each other to better visualize the impact of diminished ATP release on exercise-mediated vasodilation for both age groups (Figure 5) and for a given workload (Figure 6A-C).

***Effect of exercise intensity and age on blood gas measurements and RBC hemolysis indicators***

All blood gas measurements and RBC hemolysis indicators (pHb and % hemolysis) are presented in Table 3. Briefly, the venous blood gas measurements of pH, $pO_2$, $pCO_2$, $CtO_2$, $CtCO_2$, and $FO_2Hb$ all increased during exercise from rest in both young and older adults, however there was no difference with age ($P<0.05$). Both indicators of RBC hemolysis were increased at 15 and 25% MVC compared to rest in young and older adults, and a small but significant increase from rest was observed at 5% MVC in older adults, however between age group comparisons did not reveal significant differences at rest or any exercise intensity. As noted in the methods, samples with > 0.5% hemolysis were assumed to result from 'handling', discarded, and not utilized in data analysis; this included 2 samples from the 15% and 1 sample from 25% intensity exercise trials.

To assess the association between [ATP] and potential metabolic stimuli, data from rest and all exercise intensities was pooled for both young and older adults. In young adults, [ATP] was significantly correlated to the Hb deoxygenation ($FO_2Hb$) as well as pH (r= -0.28; r= -36, respectively, $P<0.05$). Conversely, these variables were not related to [ATP] in older adults (r= 0.17; r= -0.17; $P>0.05$)

**Discussion**

The primary findings from the present investigation are as follows. Older healthy adults have reduced forearm skeletal muscle blood flow during mild to moderate intensity exercise, and this is mediated by attenuations in forearm muscle vasodilation. While

venous plasma [ATP] increases during dynamic forearm exercise in young adults, healthy older humans do not increase plasma [ATP] and have significantly lower plasma [ATP] than young adults at all exercise intensities. ATP release (to account for changes in FBF) increases during graded intensity forearm exercise regardless of age, yet older adults have significantly attenuated ATP release during this metabolic stress. Importantly, during mild to moderate forearm exercise, older adults demonstrate considerably diminished ATP release which is associated with significant declines exercise-induced vasodilation. Collectively, these data suggest that blunted endogenous ATP release may be a mechanism by which skeletal muscle blood flow and oxygen delivery is impaired with advancing age in humans.

In the present investigation we tested the hypothesis that circulating plasma [ATP] and ATP release is diminished during the metabolic stress of exercise in older healthy humans and may elucidate a means by which exercise-induced vasodilation and hyperemia are impaired with advancing age. Our rationale for this investigation was two-fold. First, aging humans demonstrate impaired vascular control to endothelium dependent stimuli that predisposes this population to an increased risk for adverse cardiovascular events (Taddei *et al.*, 2001). With this in mind, we recently predicted that ATP-mediated vasodilation would be impaired in healthy aging humans, but in contrast found that ATP vasodilatory responsiveness was in fact unaltered compared to young adults despite substantially blunted vasodilation to ACH(Kirby *et al.*, 2009a). Second, muscle blood flow and oxygen delivery during exercise is closely associated with endothelial health and is known to decline with advancing age. In combination, previous literature suggests that exercise increases circulating concentrations of the endothelium-

dependent vasodilator ATP in young adults (Gonzalez-Alonso *et al.*, 2002). However to the best of our knowledge, no information exists to date regarding circulating plasma [ATP] in older healthy humans at rest or during exercise.

As expected, skeletal muscle vasodilation was reduced during exercise in aging humans at all exercise intensities and forearm blood flow was reduced at 15 and 25% exercise corroborating our recent findings. Although experiments in the lower limb have documented blunted exercise hyperemia in aging humans, current evidence in the upper limb is minimal regarding differing exercise workloads and is often limited to post-exercise assessment via venous occlusion plethysmography. Nevertheless, our data clearly demonstrate and support previous results in the lower limb that aging humans have impaired blood flow and oxygen delivery during mild to moderate intensity forearm exercise that is mediated by blunted local vasodilation (Kirby *et al.*, 2009b).

Collective evidence suggests that metabolic stressors along with the mechanical stress of exercise can induce ATP release into circulation (Gonzalez-Alonso *et al.*, 2002; Wan *et al.*, 2008; Ellsworth *et al.*, 2009). Repeated muscle contractions of dynamic exercise produce a metabolically altered milieu of *local* hypoxia, hypercapnia, and acidosis, as well as evoke increases in arteriolar wall shear stress resultant from hyperemia. In addition, a mechanical stimulus upon the tissue exists due to increases in muscle tension developed with exercise. In the present study, we demonstrate that [ATP] increases during exercise in young adults in a fairly graded fashion that is significantly greater than rest. Indeed, calculating ATP release (to account for changes in FBF that could dilute the concentration of ATP) in young adults even more clearly demonstrates a significant intensity dependent increase in circulating ATP compared to rest. In contrast,

older adults did not increase [ATP] from rest during exercise at any intensity, and had significantly blunted ATP release at each workload when compare to young adults. Based on data indicating that RBCs release ATP in response to Hb deoxygenation, decreases in pH, and mechanical deformation, and that endothelial cells progressively release ATP during graded elevations in shear stress (Bodin & Burnstock, 1995; Yamamoto *et al.*, 2007; Ellsworth *et al.*, 2009), we speculate that these functional ATP sources in young adults may be significantly impaired in older adults.

As noted previously, tissue metabolic demand for oxygen must be tightly regulated via changes in local vascular tone as the (principal) means of delivering oxygen, whereby impairments in vasodilation should closely reflect tissue oxygen delivery assuming arterial oxygen content is quite stable in these healthy populations (~20 mg/dL) (Rowell, 1993). If release of and consequent concentrations of the circulating vasodilator ATP are indeed blunted in aging humans, then one would predict that vasodilation would be effected similarly. Therefore, we determined the relationship between forearm vascular conductance (as an index of vascular tone) and ATP release across exercise intensity (Figure 5) and within each workload (Figure 6) for both young and older adults. We in fact did observe a significantly diminished ATP release in the older population compared to the young, although the relationship between vasodilation and ATP release was not different (i.e. the slope was unchanged; $P > 0.05$). Specifically, for a given endogenous concentration of ATP the corresponding vasodilation was similar between populations indirectly supporting our previous findings that ATP receptor responsiveness is not impaired in healthy aging humans. Collectively, these data may suggest that decrements in oxygen delivery that occur via reductions in blood flow during

exercise in aging humans are potentially due to a significant attenuation in the ability to sense/release ATP into circulation and effectively evoking endothelium-dependent vasodilation.

*Potential Mechanisms*

Ellsworth and colleagues aptly demonstrated that RBCs from older hamsters are unable to significantly release ATP to hypoxia or acidosis (Ellsworth *et al.*, 1995). Accordingly, our blood gas analysis comparison between young and older adults indicates that a significant correlation exists between [ATP] and pH (P=0.006) and $fO_2Hb$ (P=0.037) for young adults, but not in older adults (P=0.25, P=0.26; respectively) suggesting an impairment in the ability cells to respond to metabolic and/or mechanical stress. Although in a different diseased population that also exhibits altered blood flow responses, accumulating evidence reveals that RBCs from diabetic humans have altered signal transduction and consequently blunted ATP release when stimulated (Sprague *et al.*, 2006). Endothelial cells are also known to release ATP to a variety of stimuli that occurs during repeated muscle contractions. Interestingly, caudal artery segments and isolated endothelial cells from aged rats have blunted release of ATP (Hashimoto *et al.*, 1995). Furthermore, this impairment is exacerbated with higher levels of cholesterol, yet offset by modulating the membrane fatty acid composition and with exercise training (Hashimoto *et al.*, 1999). Despite these collective and intriguing findings, to date no assessment of these stimuli has occurred in cells from healthy aging humans, thus the mechanism underlying our observations is still unknown.

In addition, it is plausible that the observed diminished circulating [ATP] in older adults is due to an enhancement of ATP degradation enzymes. Although not directly studied in healthy aging humans, ectonucleotidase activity is suggested to be elevated in a variety of disease states that are closely linked with cardiovascular disease and are prevalent with advancing age (Duarte *et al.*, 2007; Lunkes *et al.*, 2008). In particular, this is seen in patients with elevated oxidized-LDL, which is typically observed in our aging population (Kirby *et al.*, 2009a; Kirby *et al.*, 2009b). In this manner, ATP would be more rapidly degraded to its down stream by-products minimizing its robust vasodilator action. In general, it is thought that ectonucleotides activity is enhanced as a means of mitigating prothrombotic signaling with disease (Schetinger *et al.*, 2007). Taken together, one may predict that a combination of impaired extracellular ATP release and enhanced ATP degradation may take place and therefore reduce circulating [ATP] in aging humans.

*Experimental Considerations*

A few points should be considered in the present investigation. First, although vasodilation is closely associated with circulating ATP during exercise, to what exact ATP contributes to vascular tone at rest and exercise is currently unknown. Accordingly, the use of a $P_{2Y}$ receptor antagonist would be ideal to answer such questions, yet no substance is currently available for human use. Second, with regard to venous plasma [ATP] values in humans, an extreme degree of variation has become existent in the literature ranging from as low as ~35nmol/L to upwards of ~2000nmol/L(Gorman *et al.*, 2007). This discrepancy may be related to how [ATP] is measured but maybe more importantly, how blood samples are handled, preserved, and stored. However, as

Gorman and colleagues have precisely lain out, we used an ATP "stop-solution" to mitigate additional ATP release and ATP degradation that occurs immediately during blood sampling as well as accounted for ATP due to hemolysis, even though in the present study we minimized hemolysis to < 0.5% (Gorman *et al.*, 2007). Nevertheless, our results demonstrate clear differences if calculated as a change from rest, which would be independent of the absolute venous plasma [ATP]. Finally, we believe the calculation of ATP release (FBF x [ATP]/1000) is of considerable importance as elevations in blood flow could perhaps 'dilute' and confound the interpretation of [ATP] alone. Possibly, one could argue that our findings of blunted ATP release in aging humans is due to a reduction in blood flow, however upon closer inspection this unlikely to be the case as [ATP] are also low despite elevations in blood flow above rest in aged humans.

***Conclusions***

In healthy young humans, oxygen delivery is effectively matched to changes in oxygen demand that occur during metabolic stress, however older adults demonstrated an altered control of the vasculature (impaired vasodilation) that may predispose this population to increased risk for cardiovascular disease and exercise intolerance. Specifically, the collective findings from the present investigation indicate that significantly reduced circulating venous plasma [ATP] in older adults may be an important mechanisms by which impaired vasodilation and attenuations in oxygen delivery during mild to moderate intensity exercise occur with advancing age.

**Author Contributions**

B.S.K contributed to the experimental design, data acquisition, data analysis, data interpretation, and drafting of the manuscript. A.R.C contributed to data acquisition and interpretation, and critical review of the manuscript. W.F.V provided clinical support, invasive methodology, and contributed to data acquisition and interpretation, as well as critical review of the manuscript. F.A.D. contributed to the conception and experimental design, data acquisition and interpretation, and critical review of the manuscript. All authors approved the final version of the manuscript.

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## Table 1: Subject Characteristics

| Variable | Young | Older |
|---|---|---|
| Male:Female | 13:1 | 11:1 |
| Age (years) | 22 ± 1 | 63 ± 2* |
| Body mass index (kg $m^{-2}$) | 24 ± 1 | 27 ± 1* |
| Body fat (%) | 17 ± 1 | 25 ± 2* |
| Forearm volume (ml) | 1077 ± 40 | 1121 ± 77 |
| MVC (kg) | 47 ± 3 | 45 ± 4 |
| Total cholesterol (mmol $l^{-1}$) | 3.8 ± 0.2 | 4.7 ± 0.2* |
| LDL cholesterol (mmol $l^{-1}$) | 3.4 ± 0.1 | 4.3 ± 0.2* |
| HDL cholesterol (mmol $l^{-1}$) | 0.9 ± 0.5 | 0.9 ± 0.9 |
| Triglycerides (mmol $l^{-1}$) | 0.9 ± 0.1 | 1.0 ± 0.1 |

MVC = Maximum voluntary contraction, LDL = Low density lipoprotein, HDL = High density lipoprotein. * $P<0.05$ vs young adults

**Table 2. Forearm and systemic hemodynamics at rest and during exercise**

| *Table 2.* | | FBF (ml min$^{-1}$) | FVC (ml min$^{-1}$ (100 mmHg)$^{-1}$) | MAP (mmHg) | HR (beats min$^{-1}$) |
|---|---|---|---|---|---|
| ***Rest*** | *Young* | 36 ± 5 | 38 ± 5 | 93 ± 2 | 58 ± 1 |
| | *Older* | 33 ± 3 | 34 ± 3 | 99 ± 2* | 58 ± 3 |
| ***5% MVC*** | *Young* | 98 ± 9 | 102 ± 8 | 96 ± 3 | 60 ± 2 |
| | *Older* | 82 ± 7 | 77 ± 7* | 103 ± 3* | 62 ± 3 |
| ***15% MVC*** | *Young* | 220 ± 16 | 228 ± 13 | 96 ± 2 | 62 ± 2 |
| | *Older* | 175 ± 14* | 161 ± 13* | 110 ± 3* | 64 ± 3 |
| ***25% MVC*** | *Young* | 382 ± 30 | 366 ± 27 | 104 ± 3 | 68 ± 2 |
| | *Older* | 299 ± 25* | 260 ± 23* | 116 ± 3* | 67 ± 3 |

FBF = Forearm blood flow, FVC = Forearm vascular conductance, MAP = Mean arterial pressure, HR = Heart rate, MVC = Maximum voluntary contraction. * $P<0.05$ vs young adults

## Table 3. Blood variables at rest and during exercise with age

| | Rest | | 5% MVC | | 15% MVC | | 25% MVC | |
|---|---|---|---|---|---|---|---|---|
| | ***Young*** | ***Older*** | ***Young*** | ***Older*** | ***Young*** | ***Older*** | ***Young*** | ***Older*** |
| **pH** | 7.37 ± 0.01 | 7.38 ± 0.01 | 7.35± 0.01* | 7.36 ± 0.01* | 7.32 ± 0.01* | 7.32 ± 0.01* | 7.28 ± 0.01* | 7.29 ± 0.01* |
| **$pO_2$ (mmHg)** | 29.2 ± 1.5 | 29.6 ± 1.0 | 21.2 ± 0.9* | 21.4 ± 1.1* | 22.6 ± 1.0* | 21.5 ± 0.8* | 24.7 ± 1.0* | 24.1 ± 0.6* |
| **$pCO_2$ (mmHg)** | 46.7 ± 1.1 | 44.0 ± 0.8 | 50.0 ± 0.7* | 48.3 ± 1.2* | 55.5 ± 1.1* | 52.8 ± 1.5* | 56.1 ± 2.6* | 58.5 ± 2.2* |
| **$CtO_2$ (ml $dl^{-1}$)** | 10.9 ± 0.9 | 10.6 ± 0.5 | 6.3 ± 0.4* | 6.2 ± 0.4* | 6.7 ± 0.5* | 6.0 ± 0.4* | 7.3 ± 0.5* | 6.9 ± 0.3* |
| **$CtCO_2$ (ml $dl^{-1}$)** | 27.6 ± 0.5 | 26.4 ± 0.6 | 28.8 ± 0.5* | 28.2 ± 0.9* | 29.3 ± 0.5* | 28.2 ± 0.8* | 29.1 ± 0.7* | 29.2 ± 0.7* |
| ***F*$O_2$Hb (%)** | 50.9 ± 3.5 | 52.1 ± 2.1 | 30.3 ± 1.6* | 30.3 ± 1.6* | 31.7 ± 1.8* | 29.9 ± 1.8* | 34.0 ± 1.9* | 34.0 ± 1.5* |
| **Hct (%)** | 44.4 ± 1.0 | 42.8 ± 1.1 | 43.5 ± 1.0 | 41.7 ± 1.0 | 43.9 ± 1.0 | 42.1 ± 1.0 | 44.3 ± 1.0 | 42.8 ± 1.0 |
| **tHb (g $dl^{-1}$)** | 15.0 ± 0.3 | 14.5 ± 0.4 | 15.4 ± 0.7 | 14.2 ± 0.3 | 14.9 ± 0.3 | 14.3 ± 0.3 | 15.1 ± 0.3 | 14.6 ± 0.3 |
| **pHb (mg $L^{-1}$)** | 4.5 ± 0.6 | 4.4 ± 0.6 | 6.3 ± 0.7 | 6.3 ± 0.7* | 6.7 ± 0.6* | 6.9 ± 0.8* | 6.6 ± 0.6* | 7.7 ± 0.9* |
| **Hemolysis (%)** | 0.17 ± 0.03 | 0.17± 0.02 | 0.24 ± 0.03 | 0.26 ± 0.03* | 0.25 ± 0.02* | 0.28 ± 0.03* | 0.25 ± 0.02* | 0.30 ± 0.03* |

All values are venous blood. * $P < 0.05$ *vs* rest

**Figure 1: Experimental Timeline.** All subjects rested for two minutes prior to exercise. Dynamic rhythmic handgrip exercise was performed in graded fashion at 5, 15, and 25% of the subjects' maximum voluntary contraction using a handgrip pulley system attached to an external load. Dynamic contractions were performed over a range of 4-5 cm at a duty cycle of 1 sec contraction/2 sec relaxation. Each bout of exercise intensity was performed for 5 minutes totaling a15 minutes for the entire trial. At the end of rest and each progressive exercise intensity, forearm blood flow (FBF), forearm vascular conductance (FVC), [ATP], and blood gases were determined.

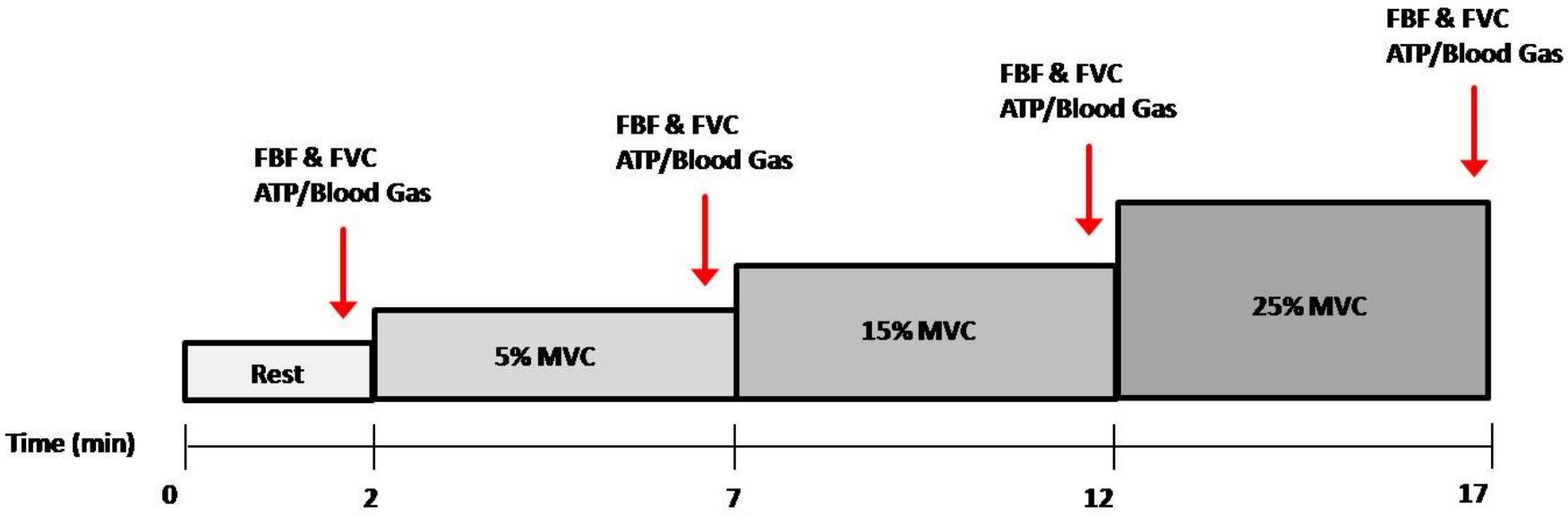
**Figure 2A-B: Forearm vascular responses to graded intensity rhythmic forearm exercise.** Forearm blood flow (FBF) was greater than rest at each exercise intensity regardless of age. Older adult had significantly reduced FBF at 15 and 25% intensity compared to young adults (A). Forearm vasodilation represented as forearm vascular conductance (FVC) increased greater than rest for both age groups at all exercise intensities. Significant age-associated reductions in FVC were observed for at all exercise intensities (B). * $P$ <0.05 vs young.

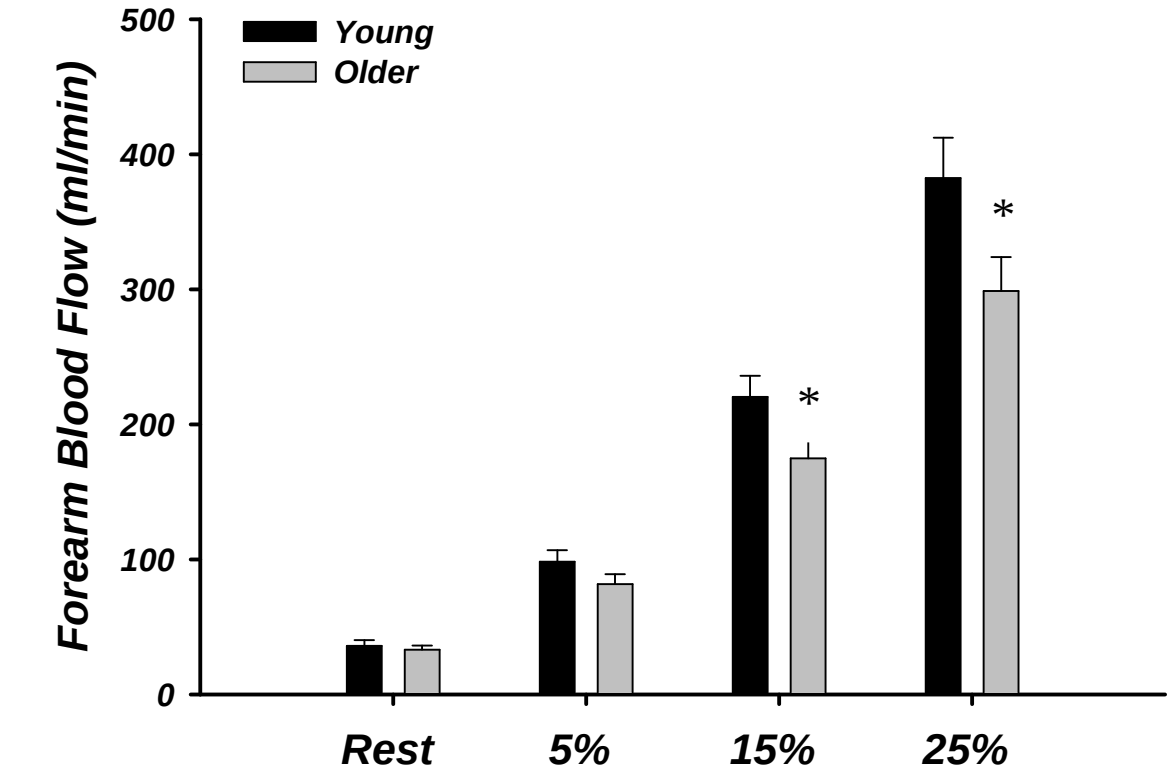
**Figure 3: Venous [ATP] to graded intensity rhythmic handgrip exercise.** In young adults, $[ATP]_v$ was significantly greater than rest at all exercise intensities. In contrast, older adults did not significantly increase $[ATP]_v$ for any exercise intensity. Older adults had significantly attenuated [ATP] compared to younger adults for all intensities, however this was not present at rest. † $P$ <0.05 vs rest; * $P$ < 0.05 vs young.

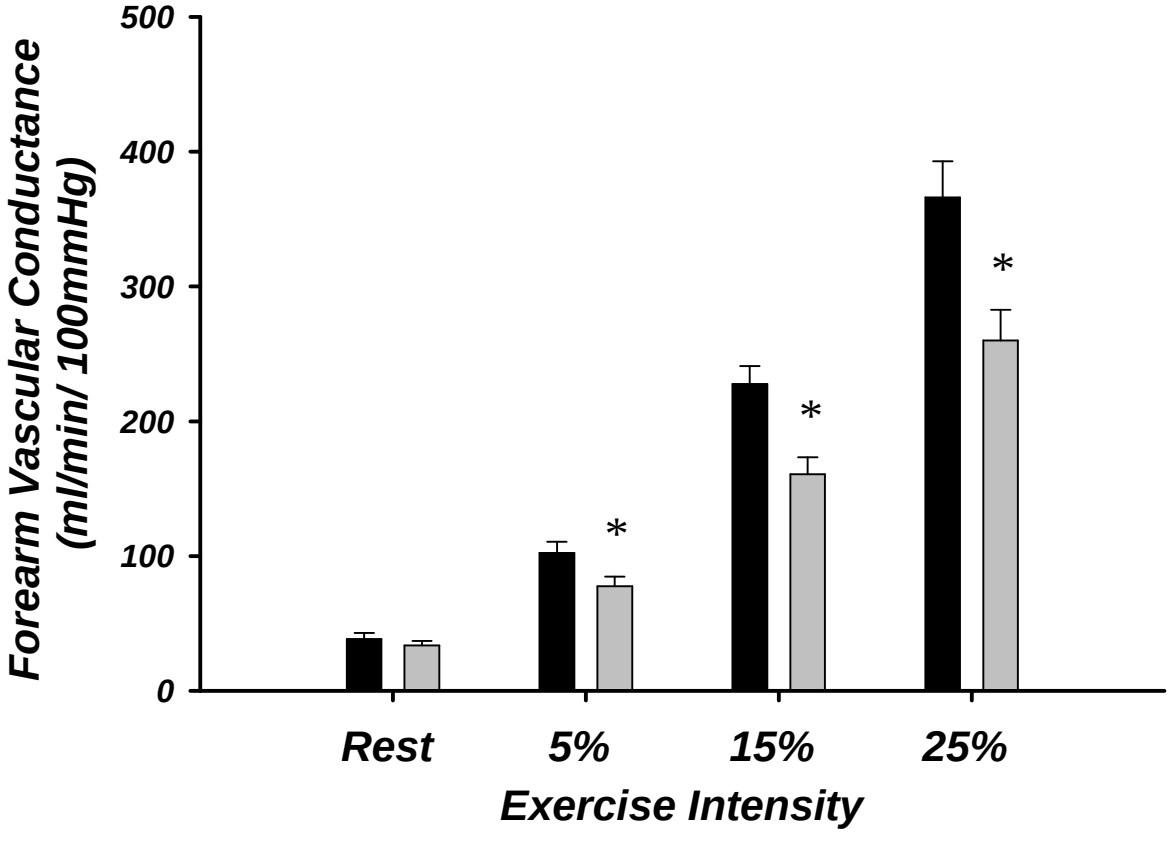
**Figure 4: [ATP] release into circulation during graded intensity rhythmic handgrip exercise.** To account for elevations in FBF that occur during exercise, ATP release was calculated. ATP release increased from rest during all exercise intensity for both young and older adults. Despite this, a significant age-associated decrease in ATP release was observed in older adults for each exercise intensity. * $P$ <0.05 vs young.

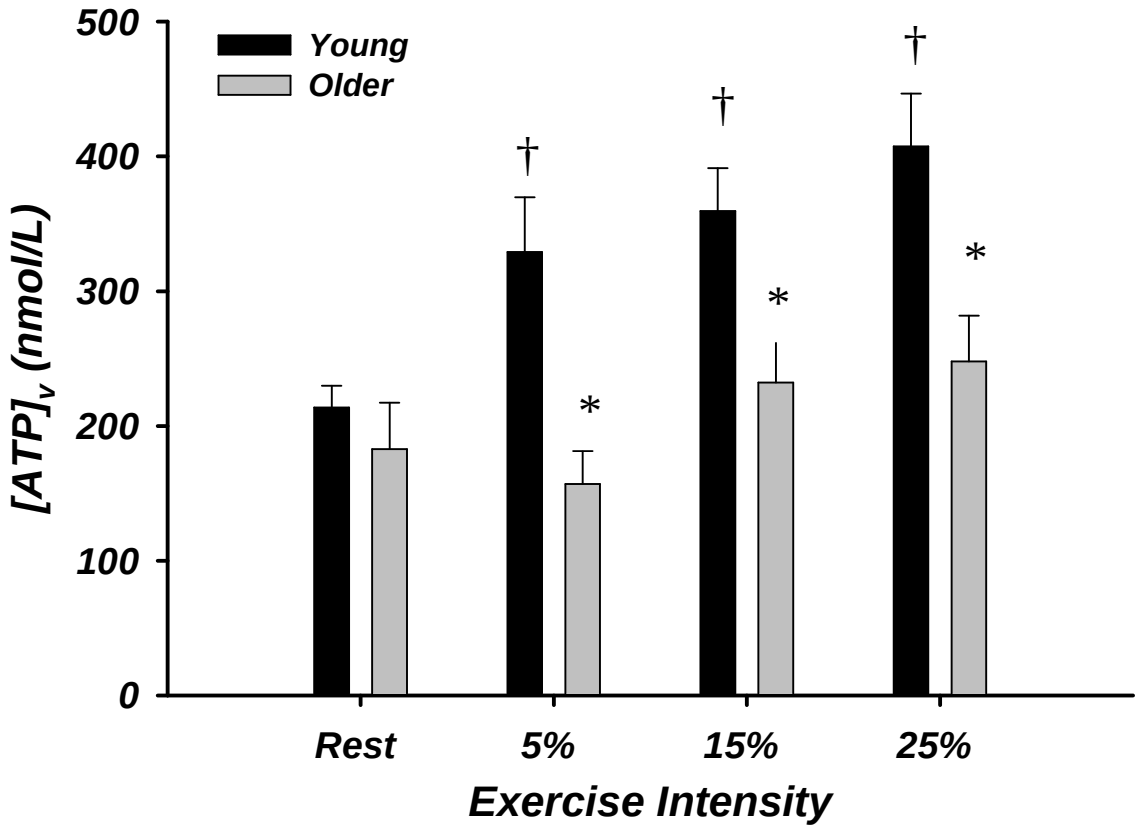
**Figure 5: Association between ATP release and vasodilatation in young and older adults.** Progressive increases in ATP release relate significantly to greater forearm vasodilation in both young and older adults.

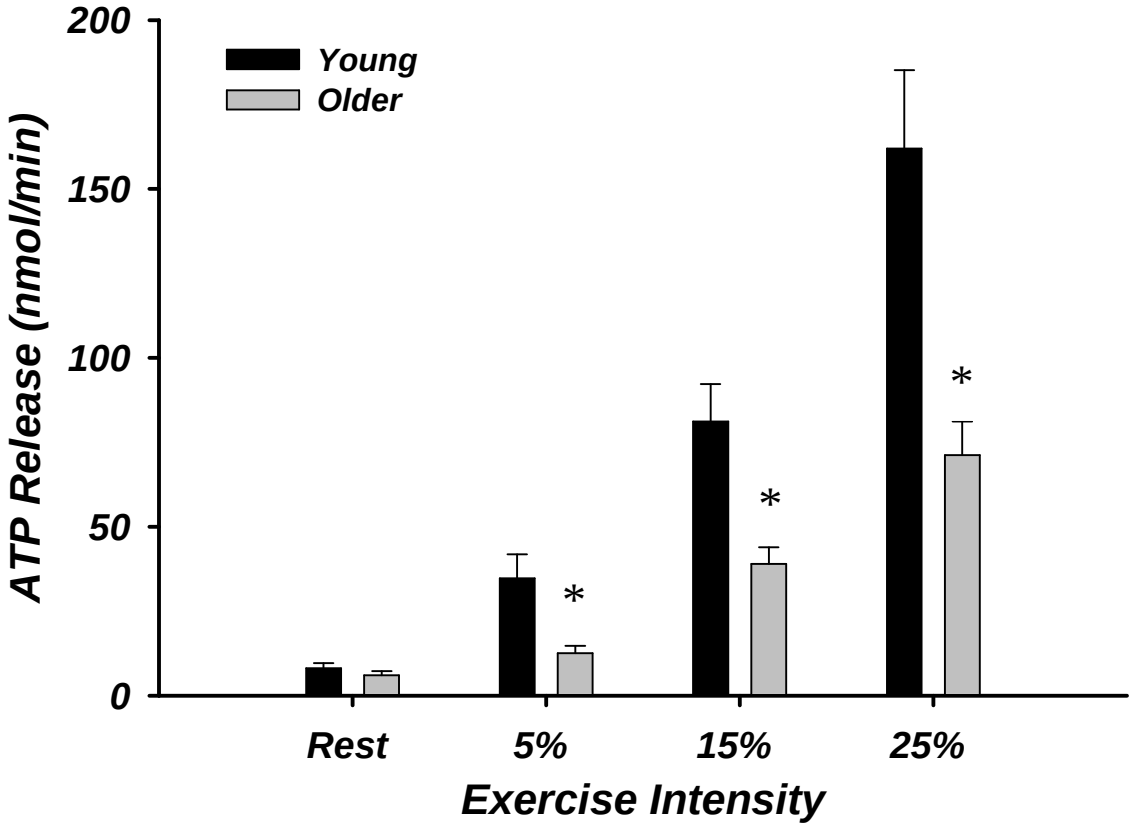
**Figure 6: Relationship between ATP release and vasodilatation for each exercise intensity in young and older adults.** Older adults demonstrate a lower ATP release that is related to reduced forearm vasodilatation compared to young adults during (A) 5%, (B) 15%, and (C) 25% MVC handgrip exercise.

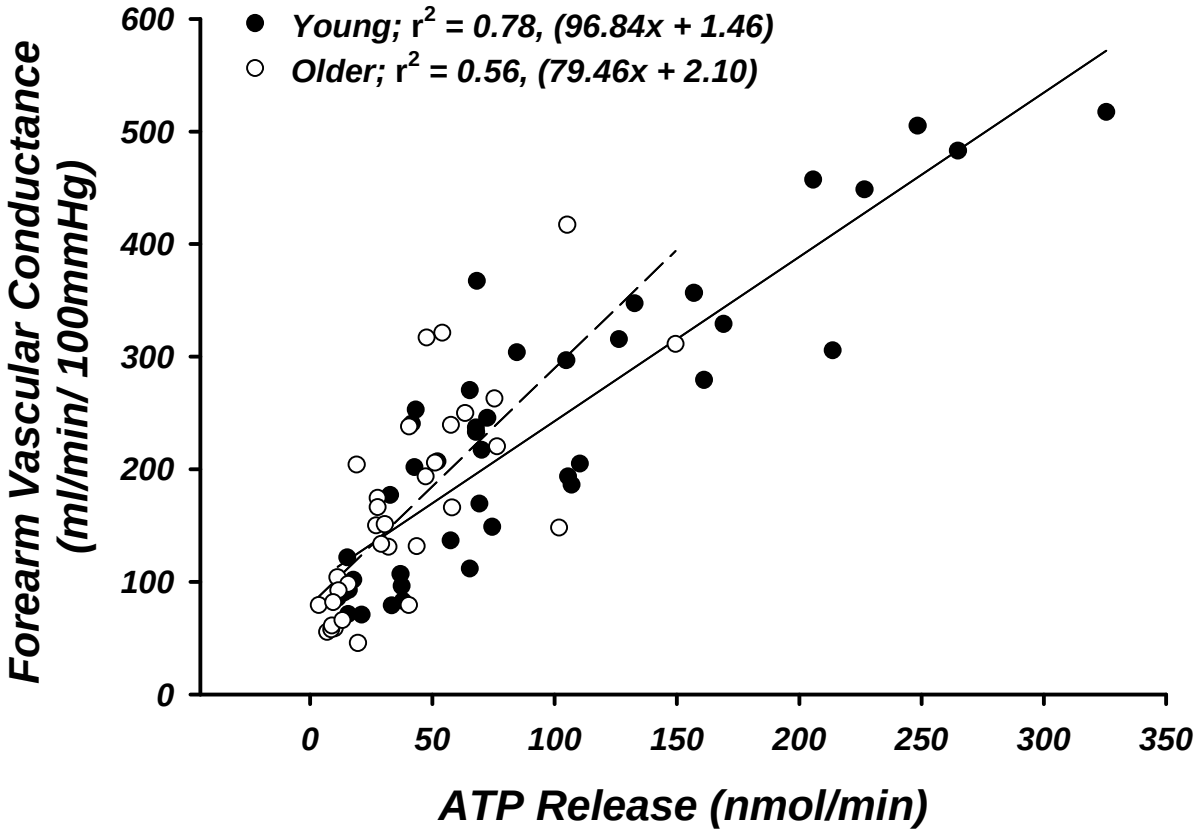

FBF & FVC
ATP/Blood Gas
FBF & FVC
ATP/Blood Gas
FBF & FVC
ATP/Blood Gas
FBF & FVC
ATP/Blood Gas
Rest
5% MVC
15% MVC
25% MVC
Time (min)
0
2
7
12
17

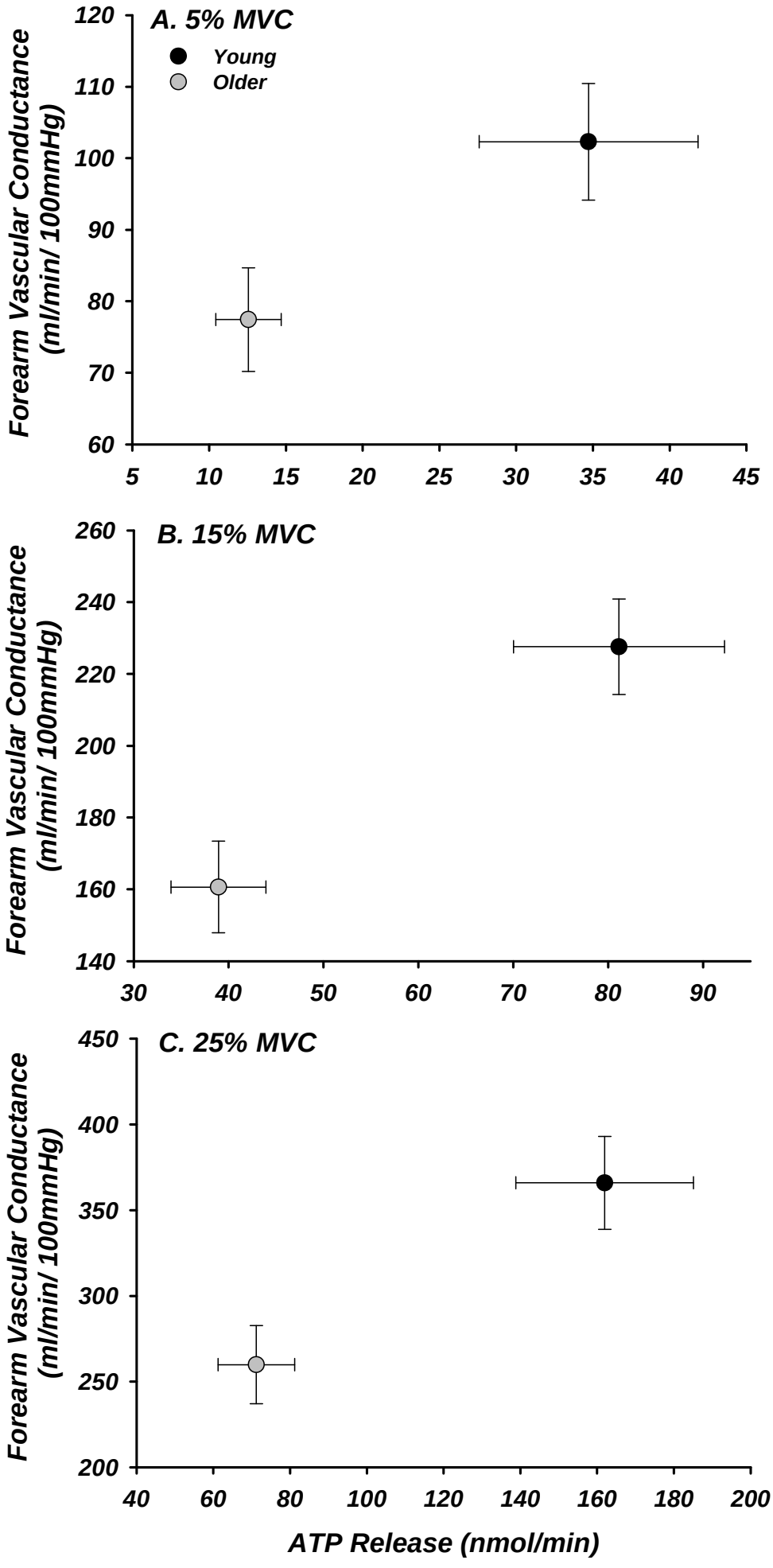

Young
Older
Forearm Blood Flow (ml/min)
500
400
300
200
100
0
*
*
Rest
5%
15%
25%


Forearm Vascular Conductance
(ml/min/ 100mmHg)
500
400
300
200
100
0
*
*
*
Rest
5%
15%
25%
Exercise Intensity


500
400
300
200
100
0
[ATP]v (nmol/L)
Young
Older
†
†
†
*
*
*
Rest
5%
15%
25%
Exercise Intensity


200
150
100
50
0
ATP Release (nmol/min)
Young
Older
*
*
*
Rest
5%
15%
25%
Exercise Intensity


Young; r2 = 0.78, (96.84x + 1.46)
Older; r2 = 0.56, (79.46x + 2.10)
Forearm Vascular Conductance (ml/min/ 100mmHg)
600
500
400
300
200
100
0
0
50
100
150
200
250
300
350
ATP Release (nmol/min)


A. 5% MVC
Young
Older
Forearm Vascular Conductance (ml/min/ 100mmHg)
120
110
100
90
80
70
60
5
10
15
20
25
30
35
40
45
B. 15% MVC
Forearm Vascular Conductance (ml/min/ 100mmHg)
260
240
220
200
180
160
140
30
40
50
60
70
80
90
C. 25% MVC
Forearm Vascular Conductance (ml/min/ 100mmHg)
450
400
350
300
250
200
40
60
80
100
120
140
160
180
200
ATP Release (nmol/min)

# CHAPTER VI – OVERALL CONCLUSIONS

Human aging is recognized as a primary risk factor for the development of cardiovascular disease. In particular, older adults typically demonstrate an impaired ability to increase blood flow (and oxygen delivery) in response to various stimuli including exercise, and this is largely a result of impaired vasodilation. An entire host of metabolic and chemical substances are known to evoke vasodilation and have been proposed to mediate the vasodilatory response observed during muscle contractions. In spite of this, although the study of exercise hyperemia has accrued for over a century, the discovery of any one obligatory factor has yet to take place thus creating difficulty when trying to pinpoint a specific substance that may explain age-associated impairments in active muscle blood flow. Nonetheless, we have recently turned our attention the postulate that circulating nucleotides have an explicit role in the control of vascular tone during exercise and a deficit in either the responsiveness or the circulating content of this molecule may take place with advancing age.

In specific, the purine nucleotide ATP is recognized to be released extracellularly from endogenous sources into the human circulation from a large host of cells. As such, *in vitro* evidence indicates that circulating ATP may result from deoxygenation and mechanical strain upon endothelial cells as well as erythrocytes and platelets, although other cells are recognized to have this ability as well. However, how the cellular release of ATP from these specific sources is altered with human aging is not yet known. Even so, for many decades, nucleotides have been recognized to reside within the circulation of

humans and to have robust vasodilator action when infused intra-arterially. More recently, evidence indicates that ATP has powerful vasodilatory action in that it can evoke elevations in blood flow similar to that observed during maximal exercise and results in the ability to significantly blunt (and in some instances abolish) sympathetically-mediated vasoconstriction in a dose-response fashion similar to that seen with graded intensity exercise in young adults. In addition, although heavily touted as an endothelium-dependent vasodilator, ATP appears to evoke vasodilation independent of nitric oxide, vasodilating prostaglandins, as well as adenosine, and may chiefly cause vasodilation via spreading hyperpolarization yet unexplored in humans. Interestingly, these vasomotor properties are specific to ATP and are typically not observed with the downstream adenine nucleotide, AMP, or nucleoside, adenosine.

To the best of our knowledge, the noted collection of experiments provides novel and significant insight into the understanding of how circulating ATP assists in vascular control in aging humans. The collective findings from these studies indicate that alterations in the contribution of ATP to vascular tone in aging humans exist and may in part be a potential mechanism by which aged adults have reductions in oxygen delivery to active skeletal muscle. The specific key findings from the present body of work are as follows: 1) exogenous ATP has the ability to blunt α-adrenergic vasoconstriction in young adults similar to that observed during exercise, 2) in contrast to our hypothesis, the vasodilatory responsiveness as well as 3) the sympatholytic properties of exogenous ATP remain intact in aging humans, yet 4) older adults demonstrate reduced venous plasma [ATP] and impaired ATP release during graded mild-to-moderate handgrip exercise which is associated with attenuations in skeletal muscle vasodilation and blood flow.

Taken together, it is our belief that the typically observed impairments in skeletal muscle vasodilation and the inability to offset sympathetic vasoconstrictor tone during exercise is in part due to diminished endogenous levels of circulating ATP. On the whole, ATP appears to be a significant regulator of vascular control in humans, and may act as a potential mechanism which in part explains the typically observed reductions in skeletal muscle blood flow and oxygen delivery to active tissue in aged humans thereby predisposing this population to an elevated risk for cardiovascular diseases, age-related declines in exercise capacity/tolerance, and an overall decline in quality of life in this population.

Colorado State University

Office of Regulatory Compliance
Office of Vice President for Research
Fort Collins, CO 80523-2011
(970) 491-1553
FAX: 491-2293

# Notice of Human Research Amendment Approval

| | |
|---|---|
| **Principal Investigator:** | Frank Dinenno, HES, 1582 |
| **Title:** | Regional Blood Flow Control and Vascular Function: Effects of Aging and Regular Physical Activity |
| **Protocol #:** | 04-151H |
| **Committee Action:** | **Amendment Approved:** December 7, 2006 |
| **HRC Administrator:** | Janell Meldrem |

The Human Research Committee reviewed and approved your request to amend the above-referenced project. The approved amendments are below.

**Amendment(s):**

- to increase the number of particpants by 60 - accepted.
- to add the grant title: Aging, Endothelial Dysfunction, and ATP-mediated Vasodilation
- to change the title on the consent form to reflect both grants to: Regional Blood Flow Control and Vascular Function: Effects of Aging and Regular Physical Activity

**Investigator Responsibilities:**

- It is the responsibility of the PI to immediately inform the Committee of any serious complications, unexpected risks, or injuries resulting from this research.
- It is also the PI's responsibility to notify the Committee of any changes in experimental design, participant population, consent procedures or documents. This can be done with a memo describing the changes and submitting any altered documents.
- Students serving as Co-Principal Investigators may not alter projects without first obtaining PI approval. The PI is ultimately responsible for the conduct of the project.

This approval is issued under Colorado State University's OHRP Federal Wide Assurance 00000647.

If you have questions, please contact me at 1-1655 or janell.meldrem@colostate.edu.

attachment Date of Correspondence 12/8/06

**Consent to Participate in a Research Study**
**Colorado State University**

**TITLE OF STUDY:** Regional Blood Flow Control and Vascular Function: Effects of Aging and
Regular Physical Activity
**PRINCIPAL INVESTIGATOR:** *Frank A. Dinenno, Ph.D. 491-3203*
**CO-PRINCIPAL INVESTIGATORS:** *Matt Hickey, Ph.D. 491-5727*
*Wyatt Voyles, M.D. 663-3107*

**WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH?** *You are a man or woman between the ages of 18-35 or 55-90 years. You are either 1) not exercising vigorously and regularly, or 2) have exercised vigorously and regularly for a number of years. 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., and Matt Hickey, Ph.D. of the Health and Exercise Science Department, and also by Wyatt Voyles, M.D., of the Heart Center of the Rockies. Trained graduate students, undergraduate students, research assistants, or research associates are assisting with the research. These studies are paid for by the National Institute on Aging, a part of the US Government.*

**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, in different muscle groups (forearm, thigh, calf), as well as in the neck. The makeup of muscle fibers is also being studied.*

**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 either:*
_____ *1) one or two visits over a several day period, or* _____ ***(your initials)***
_____ *2) several visits over a few to several weeks.* _____ ***(your initials)***

**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. Many potential procedures are described in the section below. However, the procedures that you will be asked to do for this part of the study have a check mark next to them. The check marks were put there by one of the researchers. The time associated with each procedure reflects the amount of time you will spend performing or undergoing the procedure, not the total time of the study. 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.*

_____ ***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)***

_____ ***Pregnancy Test.*** *If you are female you will be required to have a sample of your urine tested for the presence of human chronic gonadotropin (HCG), a hormone which indicates whether you may be pregnant. This will require approximately 1 cup of your urine. If you are pregnant or the test indicates that you are pregnant you will not be able to participate in this study. (~10 minutes)* _____ ***(your initials)***

_____ ***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)***

_____ ***Graded Exercise Test.*** *If you are in the 55-90 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 measured during and immediately after the test.(~1 hour)*

_____ ***(your initials)***

_____ ***Maximal Oxygen Consumption.*** *$VO_{2max}$ testing will be performed on a treadmill while you are walking or running and the steepness of the treadmill is increased until you can't exercise any more. You will be asked to put your mouth around a scuba-like mouthpiece and wear a nose clip to prevent breathing through your nose. The amount of oxygen your body uses for energy will be determined from the oxygen and carbon dioxide you breathe in and out during the exercise. Your heart rate will be measured using a heart rate monitor. Body mass and height will be measured on a medical beam scale. (~30 - 45 min)* _____ ***(your initials)***

____ ***Body Composition.*** *The fat, muscle, and bone in your body will be measured using an x-ray device (dual-energy x-ray absorptiometer) 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)***

_____ ***Forearm Volume.*** *The volume of your forearm will be measured via water displacement. You will place your forearm in a large water-filled cylinder, and the spillover of this water is collected in a large graduated cylinder to determine the volume. (~5 minutes)*

_____ ***(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)***

_____ ***Calf Exercise.*** *You will sit in a special chair and squeeze your calf muscles (similar to standing on your toes) while your arms, hips, and shoulders 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)***

_____ ***Knee Extensor Exercise.*** *You will sit in a special chair and squeeze your thigh muscles while your hips and shoulders are comfortably secured. Your feet will be secured in specially designed boots and you will be asked to extend your leg against resistance until your ankle is about at the height of your knee, relax back to a regular seated position, and then repeat. 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)***

_____ ***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. (~ 20 minutes)*
_____ ***(your initials)***

_____ ***Exercise Training.***

*<u>Forearm</u>: You will be instructed to exercise five times per week, for a total of four weeks. You will be given a special exercise device and will be instructed to exercise with your non-dominant forearm squeezing your muscles 12 times per minute at 30-35% of your maximum until you can't exercise any more. When you are able to exercise at this initial workload for 30 minutes, the workload will be increased. You will need to visit the laboratory once per week to adjust the training workload as your performance improves.*
_____ ***(your initials)***

*<u>Calf</u>: You will be instructed to exercise five times per week, for a total of four weeks. You will be instructed to exercise with your calf muscles and squeeze this muscle 12 times per minute at 30-35% of your maximum until you can't exercise any more. You will be instructed to perform calf extension exercise in the upright position with added weight (if necessary) to achieve the pre-determined workload. When you are able to exercise at this initial workload for 30 minutes, the workload will be increased. You will need to visit the laboratory once per week to adjust the training workload as their performance improves.*
_____ ***(your initials)***

*Knee extensor: You will be instructed to exercise 3 times per week, for a total of eight weeks. You will be required to perform the training studies in the laboratory under supervision. Each training session will be 60 minutes. The first two weeks will consist of short (5-10 min) high intensity exercise bouts, whereas the second two weeks will consist of long (15-45 min) low intensity exercise bout. This pattern of training will be repeated to attain a total training period of eight weeks. As your exercise performance improves, the training workload will be adjusted accordingly.* _____ ***(your initials)***

*Whole-body: You will be instructed to exercise 5 times per week, 40-50 minutes per exercise session at 60-85% of your maximum heart rate, for a total of twelve weeks. You will be asked to cycle, walk, jog, or run during this training period. You will be taught how to use heart rate monitors (provided by the lab) in order to train at the proper intensity as well as to record your exercise sessions.* _____ ***(your initials)***

____ ***Ischemic Exercise.*** *You will exercise your calf or forearm with a blood pressure cuff on your thigh or upper arm that is inflated very tightly to temporarily block the blood flowing to your muscle. 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. (20 – 30 minutes)* _____ ***(your initials)***

____ ***Cold Pressor Test.*** *You will place your hand or foot in ice water for 2-3 minutes on several occasions. (~10 minutes)* _____ ***(your initials)***

____ ***Lower Body Negative Pressure.*** *You will be placed in a sealed wooden chamber while you are laying 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 15 minutes at a time. (~ 1 hour)*

_____ ***(your initials)***

____ ***Up-right or head-down tilting.*** *You will be lying on a bed that is specially designed to be tilted ~60 degrees upright, or tilted downward ~10 degrees. This mimics what happens when you go from laying to standing up, and vice versa. (~ 1 hour)*

_____ ***(your initials)***

____ ***Forearm Negative/Positive Pressure.*** *You will place your forearm in a sealed chamber up to your elbow. Application of suction (like a vacuum) increases blood flow to your arm, whereas the opposite pressure reduces blood flow to your arm. (1-2 hours)*

_____ ***(your initials)***

____ ***Brachial Artery Compression.*** *A special device that is mounted to a frame above your forearm will be placed over your brachial artery at the elbow. When this device presses down on your arm, it will temporarily reduce the amount of blood to your forearm. This will be performed for approximately 5 minutes at a time, and will occur several times throughout the study. (1-2 hours)* _____ ***(your initials)***

____ ***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. 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)***

____ ***Venous Occlusion Plethysmography.*** *The blood flow in your forearm or calf will be measured by the use of blood pressure cuffs around your upper arm or thigh, and around your wrist or ankle. These cuffs will be inflated and deflated periodically. A sensitive gauge (similar to a rubber band) will also be placed around the maximum circumference of your forearm or calf. (2-3 hours)* _____ ***(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 ho* _____ ***(your initials)***

____ ***Reactive Hyperemia.*** *A blood pressure cuff will be placed on your upper arm or thigh and inflated really tight to temporarily block the blood to your forearm or calf. After 5, 10, or 15 minutes, the cuff will be released and the blood flow in your forearm or calf will be measured. This test is a measure of how much your blood vessels can relax and will be repeated several times throughout the study. (1- 1.5 hours)*

_____ ***(your initials)***

____ ***Flow-Mediated Vasodilation.*** *A blood pressure cuff will be placed on your forearm or your calf and inflated really tight to temporarily block the blood to your hand or foot. After 5, 10, or 15 minutes, the cuff will be released and the diameter changes of the blood vessels in your arm or leg will be measured using Doppler ultrasound. In some cases, your hand or foot will be warmed up for 15 minutes and the changes in blood vessel diameter will be measured. This will be repeated several times throughout the study. (1-1.5 hours)* _____ ***(your initials)***

____ ***Sympathetic Nervous System Activity.*** *The measurement of sympathetic nervous system activity involves measuring the activity of one of your nerves on the side of your knee. Two small microelectrodes (small needles) will be placed through your skin. The position of one of the electrodes will be moved back and forth through your skin while a very small electrical impulse (1-2 volts) is passed through the electrode. This search procedure will continue until the electrode being moved causes your foot to twitch. This procedure will take between 5-60 minutes. When a foot or hand twitch is observed, measurement of the activity of the sympathetic nervous system will begin. (2-3 hours)* _____ ***(your initials)***

____ ***Blood Sample.*** *Up to 100 ml (approximately 7 tablespoons) 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)***

***** For Arterial Catherization, Venous Catheterization, or Muscle Biopsy:*** *<u>If you are allergic to lidocaine or novacaine, or have had a negative reaction to medicines injected while at the dentist, you should notify us immediately and not have any of these procedures done.</u>*

_____ ***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. In some cases, a local anesthetic might be used to reduce any discomfort. (~2-4 hours)*

_____ ***(your initials)***

_____ ***Brachial Artery Catheterization.*** *Your skin will be cleaned and a local anesthetic will be given with a small needle to numb the area where the catheter will be placed (front side of your elbow). The catheter (plastic needle) will then be inserted and secured to the skin. (~2-4 hours)* _____ ***(your initials)***

_____ ***Femoral Artery Catheterization.*** *Your skin will be cleaned and a local anesthetic will be given with a small needle to numb the area where the catheter will be placed (about half way between your hip bone and groin on the front side of your leg). The catheter (plastic needle) will then be inserted and secured to the skin. (~2-4 hours)*

_____ ***(your initials)***

_____ ***Drug Administration (~ 2 - 4 hours).*** *The administration of one of more of the following drugs might occur several times throughout the study.*

*Vasoconstrictors – cause temporary narrowing of the blood vessels (minutes)*
___ *Tyramine*
___ *Norepinephrine*
___ *Phenylephrine*
___ *Clonidine*
___ *Dexmedetomidine*
___ *L-NAME*
___ *Aspirin*
___ *Ketorolac*

*Vasodilators – temporarily relax the blood vessels (minutes)*
___ *Acetylcholine*
___ *Adenosine*
___ *Sodium Nitroprusside*
___ *L-Arginine*
___ *Phentolamine*
___ *Adenosine Triphosphate (ATP)*

*No major effects*
___ *Ascorbic Acid (Vitamin C)*
___ *Propranolol*

_____ ***(your initials)***

_____ ***Muscle Biopsy.*** *A sample of muscle will be taken from a muscle on the outside of your thigh. This will take place under the supervision of a medical doctor in the Hartshorn Health Center on the CSU campus. Your skin will be temporarily numb using lidocaine, a medicine similar to novacaine. After deadening the skin, a ¼ inch incision, or cut, is made in the skin over the muscle using a sterilized scalpel. The sample is obtained using a sterilized sampling needle. The muscle sample obtained is usually about ½ the size of the eraser on the end of a pencil. You will not have to reduce your activity afterwards, but should not perform any unusual or extremely vigorous activity for*

*a few days. You will receive written instructions regarding care of the incision, and a telephone number to contact if you have any questions. (30 - 45 minutes)*
_____ ***(your initials)***

**FUTURE USE OF BLOOD OR MUSCLE SAMPLES**
*It is possible that we may want to use any extra blood or muscle tissue for future research not described in this consent form. For example, this may include determination of certain gene expressions that relate to various measures of cardiovascular function measured as part of this study. This information will remain private as will all of the data collected from the study.*
*Only choose one of the following:*

_____ *I give permission for the use of my blood or muscle tissue collected as part of the current study only.* _____ ***(your initials)***

_____ *I give permission for the use of my blood or muscle tissue for the current study as well as for future studies.* _____ ***(your initials)***

**ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?**
*If you are not 18-35 or 55-90 years of age, are pregnant, are a regular smoker, or have any diseases 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.*

**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)***

- _____ *Maximal Oxygen Consumption – There is the possibility of fatigue, muscle strains, heart rhythm abnormality, and change in blood pressure. There is the possibility of falling off of the treadmill. Incidence of myocardial infarction (MI) is also a risk. 1 in 10,000 individuals with cardiovascular disease may die and 4 in 10,000 may have abnormal heart rhythms or chest pain.* _____ ***(your initials)***

- _____ *Body composition (DEXA) scan – the risks associated with the DEXA are very low. The radiation you will receive is less than 1/3000$^{th}$ of the Food and Drug Administration (FDA) limit for annual exposure. The FDA is a government organization responsible for medical safety. In other words, you could receive 3000 DEXA scans in a single year and still not meet the FDA limit for radiation exposure. In this study you will receive one scan. The more radiation you receive over the course of your life, the greater the risk of having cancerous tumors or of inducing changes in genes. The radiation in this study is not expected to greatly increase*

*these risks, but the exact increase in such risks is not known. Women who are pregnant or could be pregnant should receive no unnecessary radiation and should not participate in this study.* _____ ***(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)***

- _____ *Exercise training – 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 and should get progressively less as training continues.* _____ ***(your initials)***

- _____ *Ischemic Exercise – There is a risk of temporary discomfort and possible cramping in the forearm or calf during and after the exercise. These symptoms will be relieved when the exercise stops.* _____ ***(your initials)***

- _____ *Cold Pressor Test – There is a risk of temporary discomfort of the hand or foot. In rare cases, subjects might feel light-headed or nauseous. These symptoms will be relieved when the hand or foot is removed from the ice water and wrapped in a blanket.* _____ ***(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)***

- _____ *Up-right or Head-down Tilting – Small risk of feeling nausea or fainting during up-right tilt. These symptoms will be relieved when the table is tilted back and the subject is lying supine. There are no known risks for head-down tilt.* _____ ***(your initials)***

- _____ *Forearm Positive/Negative Pressure – There is a small risk of slight discomfort or cramping if performing forearm exercise at the same time.* _____ ***(your initials)***

- _____ *Brachial Artery Compression – There is a risk of slight discomfort at the site of compression (elbow). There is also a risk of slight discomfort or cramping if performing forearm exercise at the same time.* _____ ***(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 Occlusion Plethysmography- There is a risk of temporary discomfort of the hand or foot when the blood pressure cuffs are inflated.* _____ ***(your initials)***

- _____ *Reactive Hyperemia/Flow-Mediated Vasodilation- There is a risk of temporary discomfort of the upper arm or thigh when the blood pressure cuffs are inflated. The discomfort might be greater the longer the cuffs are inflated.*

  _____ ***(your initials)***

- _____ *Sympathetic Nervous System Activity – Some subjects experience a temporary (seconds) pain and discomfort while the microelectrodes are being inserted. After the procedure there is a small risk of numbness, pins and needles type sensations, or pain which lasts 1-3 days. In very rare cases, numbness, pins and needles type sensations, or pain in the leg or arm has lasted several weeks or months (1-3 in 1000). These problems can be minimized by only having experienced individuals perform this technique. In addition, by minimizing the time to find the nerve to less than 60 minutes, the risk of unpleasant after-effects is reduced even more.*

  _____ ***(your initials)***

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

  _____ ***(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)***

- _____ *Arterial Catheterization – The risk of allergic reaction to lidocaine is extremely low. There is a risk that pain or discomfort may be experienced when the catheter is inserted in the artery, and local soreness after the study. In about 1 in 10 cases a small amount of bleeding under the skin will cause a bruise. There is about a 1 in 1,000 risk of infection or significant blood loss. In about 1 in 4,000 damage may occur to the artery requiring surgery.* _____ ***(your initials)***

- _____ *Drug Administration - The risks associated with drug administration include temporary increases or decreases in blood pressure and heart rate. In the case of clonidine and dexmedetomidine, you might experience mild drowsiness. These symptoms should resolve when the drug stops. With any of the vasoconstrictor drugs, there is a slight risk that ischemia (lack of blood to the tissues) could occur. Risks of these effects are minimized by calculating the amount of drug given relative to the size of your forearm or leg, and not the entire body. Finally, there is a potential risk of an allergic reaction to vasoactive drug administration. If you are allergic to aspirin, you should not participate.* _____ ***(your initials)***

- _____ *Muscle Biopsy – The risks associated with the muscle sample procedure include discomfort, soreness in that muscle, bruising, infection, and minor scarring. The discomfort and localized soreness are likely, but generally last only 24-48 hours. Temporary scarring is also expected. How wounds heal over time is different between people. The scar will only be about ¼ inch long, and is usually difficult to distinguish 8-12 months after the procedure. The risk of bruising is low, and infections are extremely rare.* _____ ***(your initials)***

- 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.

**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. For example, the law may require us to show your information to a court or to the Human Research Committee at CSU.*

**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.*

**WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY?** *For experiments that involve the blood sample, muscle sample, fine wire electrodes, and arterial or venous catheterization, you will be paid $25/hour.*

**WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH?** *Please be aware that for this study the University has made special arrangements to provide initial medical coverage for any injuries that are **directly related** to your participation in this research project. The research project will provide for the coverage of reasonable expenses for emergency medical care related to the treatment of research-related injuries, if necessary.*

*LIABILITY:*
*Because Colorado State University is a publicly-funded, state institution, it may have only limited legal responsibility for injuries incurred as a result of participation in this study under a Colorado law known as the Colorado Governmental Immunity Act*

*(Colorado Revised Statutes, Section 24-10-101, et seq.). In addition, under Colorado law, you must file any claims against the University within 180 days after the date of the injury.*

*In light of these laws, you are encouraged to evaluate your own health and disability insurance to determine whether you are covered for any physical injuries or emotional distresses you might sustain by participating in this research, since it may be necessary for you to rely on your individual coverage for any such injuries. Some health care coverages will not cover research-related expenses. If you sustain injuries, which you believe were caused by Colorado State University or its employees, we advise you to consult an attorney.*

*Questions concerning treatment of subjects' rights may be directed to Celia S. Walker at (970) 491-1563.*

**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 fdinenno@cahs.colostate.edu. If you would like to ask a medical doctor about your participation in the study, you may contact Wyatt Voyles, M.D. at 663-3107. If you have any questions about your rights as a volunteer in this research, contact Celia Walker, Director of Regulatory Compliance, at 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 9 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**

| | |
|---|---|
| Wyatt Voyles, M.D. | Work: 970-221-1000 (24 hours a day) |
| Poudre Valley Hospital Emergency | 970-297-6250 |
| Frank A. Dinenno, Ph.D. | Work: 970-491-3203 |
| | Home: 970-266-1719 |