DISSERTATION

ROLE OF THE ENDOTHELIUM IN MODULATING SYMPATHETIC VASOCONSTRICTION IN CONTRACTING SKELETAL MUSCLE OF YOUNG AND OLDER ADULTS

Submitted by

Christopher M. Hearon Jr.

Department of Health and Exercise Science

In partial fulfillment of requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2016

Doctoral Committee:

Advisor: Frank A. Dinenno

Gregory Amberg
Adam Chicco
Christopher Gentile

ABSTRACT

ROLE OF THE ENDOTHELIUM IN MODULATING SYMPATHETIC VASOCONSTRICTION IN CONTRACTING SKELETAL MUSCLE OF YOUNG AND OLDER ADULTS

Aerobic capacity is a powerful independent predictor of all-cause mortality in healthy and disease populations. Healthy (primary) ageing is associated with a decline in maximal aerobic capacity, exercise intolerance and elevated risk for ischemic cardiovascular disease. Specifically, ageing is characterized by impaired regulation of vascular tone during exercise, due in part to lower vasodilatory signaling and elevated sympathetic vasoconstrictor activity in the peripheral vasculature. Impaired regulation of peripheral vascular tone results in attenuated blood flow and oxygen delivery to contracting skeletal muscle during exercise and is a primary contributor to the age-associated decline in aerobic capacity. The overall aim of this dissertation is to determine the vascular signaling mechanisms responsible regulating sympathetic vasoconstrictor signaling during exercise in young healthy adults and translate these findings to improve vascular function during exercise in older adults.

The regulation of blood flow and oxygen delivery during exercise depends on the proper integration of local vasodilatation and neural sympathetic vasoconstriction. In healthy humans, the integration of these competing signals results in attenuation of sympathetic vasoconstriction, or “sympatholysis”, to ensure adequate blood flow to contracting skeletal muscle. The signaling mechanisms responsible for sympatholysis in healthy humans are unknown. To date, the only exogenous vasodilator shown to mimic exercise in its ability to attenuate sympathetic vasoconstriction in humans is adenosine triphosphate (ATP). The first aim of this dissertation is to determine if smooth muscle cell hyperpolarization (via activation of inwardly-rectifying potassium (K_{IR}) channels), the primary vasodilatory pathway of ATP, is responsible for ATP-mediated attenuation of sympathetic vasoconstriction. In contrast to smooth muscle specific

signaling, vasodilatory stimuli such as ATP and exercise can act through endothelium-dependent pathways. The second aim of this dissertation tests the hypothesis that endothelium-dependent signaling is capable of attenuating sympathetic vasoconstriction during exercise in young healthy humans. With age, impaired endothelial function and elevated sympathetic vasoconstrictor activity results in impaired functional sympatholysis. The third aim is to determine if augmentation of endothelium-dependent signaling during exercise improves age-associated impairments in functional sympatholysis.

The primary findings of this dissertation are that 1) similar to exercise, the ability of ATP to attenuate sympathetic vasoconstriction is independent of smooth muscle cell hyperpolarization via activation of K_{IR} channels, 2) activation of endothelium-dependent signaling during exercise significantly enhances the ability of contracting skeletal muscle to attenuate sympathetic vasoconstriction, and 3) that augmentation of endothelium-dependent signaling during exercise significantly improves functional sympatholysis in older adults. These findings are the first to identify endothelium-dependent modulation of sympathetic vasoconstriction in humans, and identifies vascular signaling pathways capable of improving the regulation of vascular tone during exercise in older adults. These findings are clinically significant for patient populations and disease states characterized by impaired functional sympatholysis including ageing, hypertension, and heart failure.

ACKNOWLEDGEMENTS

We would like to thank the subjects who volunteered for these studies, as well as Leora J. Garcia, Hannah K. Scott, and Devin V. Dinenno, Jennifer C. Richards, Brett S. Kirby, Anne R. Crecelius and Matthew L. Racine for their assistance in conducting these studies and preparation of contained manuscripts. All work was funded by NIH awards HL102720 and HL095573 (F.A.D)

TABLE OF CONTENTS

CHAPTER I – INTRODUCTION AND EXPERIMENTAL AIMS

Exercise capacity is a powerful independent predictor of all-cause mortality in healthy, and disease populations. A decline in exercise capacity occurs with healthy (primary) ageing, and is attributed to a number of age-associated alterations within the cardiovascular system including reductions in central cardiac pumping capacity, impaired peripheral vascular function, and elevated sympathetic nervous system activity. These alterations collectively result in impaired blood flow regulation and oxygen delivery to exercising skeletal muscle during both large (upright cycling) and small (single knee extensor, handgrip exercise) muscle mass exercise. While peripheral blood flow during maximal exercise may be limited in part due to decrements in maximal cardiac output, studies utilizing submaximal exercise or small muscle mass exercise clearly identify age-associated impairments in peripheral vascular signaling, independent of central cardiac limitations. The age-associated decline in muscle blood flow during exercise is due to impaired vascular conductance, resulting from impaired local production of vasodilatory and vasoconstrictor substances, as well as elevated sympathetic vasoconstrictor signaling within the skeletal muscle vasculature.

Within resistance vascular beds, elevated sympathetic vasoconstrictor activity is necessary to limit blood flow to splanchnic and inactive tissues and maintain mean arterial pressure (MAP) in the face of profound metabolic vasodilatation within contracting skeletal muscle. However, sympathetic outflow and subsequent noradrenaline (NA) release is also elevated within the vasculature of contracting skeletal muscle. Normally, sympathetic α-adrenergic vasoconstriction is significantly attenuated in contracting skeletal muscle relative to inactive tissues. This phenomenon, originally referred to as “functional sympatholysis”, is necessary to ensure adequate blood flow and oxygen delivery to active tissues despite elevated sympathetic vasoconstrictor nerve activity. Contracting skeletal muscle blunts vasoconstriction during a variety of stimuli including reflex activation of the sympathetic nervous system (e.g. via

cold pressor test, baroreceptor unloading), local intra-arterial administration of tyramine evoking endogenous NA release, as well as intra-arterial infusion of direct α_1- and α_2-adrenoceptor agonists. Collectively, these studies indicate that functional sympatholysis occurs post-junctionally at the level of the α-adrenoceptors, implicating specific signaling within the resistance vasculature is responsible attenuating vasoconstriction. The exact mechanism by which this phenomenon occurs in healthy humans is not well understood, and impaired modulation of sympathetic vasoconstriction is observed in ageing and hypertensive humans, as well as in an animal model of chronic myocardial infarction. As such, impaired sympatholysis may be an important contributor to the malperfusion of skeletal muscle and exercise intolerance observed in these populations, and thus it is of considerable interest to identify the vascular signaling pathways underlying this regulation.

Recent evidence suggest that adenosine triphosphate (ATP) may be an important contributor to sympatholysis and the haemodynamic response to exercise. In humans, the dual ability to induce vasodilatation and limit adrenergic-vasoconstriction is unique to ATP, as exogenous administration of ADP, adenosine, sodium nitroprusside (nitric oxide donor), potassium chloride, and acetylcholine all fail to attenuate adrenergic-vasoconstriction. Thus, the specific signaling pathways responsible for ATP-mediated vasodilatation are of particular interest in the context of exercise hyperaemia and functional sympatholysis. Data from our laboratory identified activation of inwardly rectifying K^+ (K_{IR}) channels as a primary contributor to ATP-mediated vasodilatation in the human forearm. Activation of K_{IR} channels can hyperpolarize vascular smooth muscle cells directly or amplify hyperpolarizing signals generated from the endothelium. Importantly, K_{IR} channel activation and subsequent vascular hyperpolarization is an important component of conducted vasodilatation, which is implicated in the coordination of blood flow responses during muscle contractions. Indeed, our laboratory has recently demonstrated that inhibition of K_{IR} channels attenuates the normal vasodilatory response to forearm handgrip exercise by ~30%. However, despite combined blockade of K_{IR}

channels, Na^+/K^+-ATPase activity, NO and PGs production resulting in greater than ~40% decrease in the normal vasodilatory response to exercise, the ability of contracting skeletal muscle to attenuate α_1-adrenergic vasoconstriction remained intact. Thus, the primary vasoactive pathways involved in ATP-mediated vasodilatation in humans are not obligatory to observe functional sympatholysis in contracting skeletal muscle.

The overall goal of this dissertation is to identify the signaling mechanisms responsible for sympatholysis in young healthy adults and translate these findings to improve sympatholysis in older adults.

Specific Aims

Experiment 1: to determine if the ability of ATP to attenuate α-adrenergic vasoconstriction is dependent upon activation of smooth muscle cell hyperpolarization via activation of inwardly-rectifying potassium channels in young adults

Experiment 2: to determine if augmentation of endothelium-dependent vasodilatory signaling enhances the ability of contracting skeletal muscle to attenuate α-adrenergic vasoconstriction in young adults.

Experiment 3: to determine if augmentation of endothelium-dependent signaling will improve functional sympatholysis in older adults.

These studies demonstrate that endothelium-independent vasodilatory pathways do not contribute to sympatholysis whereas endothelium-dependent vasodilatory pathways demonstrate a profound ability to modulate α-adrenergic vasoconstriction during exercise. To the best of our knowledge, these are the first studies in humans to demonstrate endothelium-dependent modulation of sympathetic vasoconstriction in humans. Further, despite classic

endothelial dysfunction, small augmentations in endothelium-dependent signaling during exercise restores functional sympatholysis in older adults. These findings provide evidence, that when stimulated appropriately, the endothelium of older adults is capable of attenuating sympathetic vasoconstriction similar to young adults. Importantly, these studies identify vascular signaling pathways capable of attenuating sympathetic vasoconstriction, even in populations that typically demonstrate endothelial dysfunction. This may hold significant therapeutic potential for patient populations demonstrating impaired functional sympatholysis, exercise intolerance, and reduced aerobic capacity.

CHAPTER II – MANUSCRIPT I

ATP Maintains the Ability to Blunt α_1-Adrenergic Vasoconstriction During Combined Blockade of Nitric Oxide, Prostaglandins, Na^+/K^+-ATPase, and K_{IR} Channels in Humans

Summary

Exercise and ATP elicit vasodilatation that is partially dependent on inwardly rectifying potassium (K_{IR}) channel activation, with a modest reliance on nitric oxide (NO) and prostaglandin (PG) production. The vasodilation to both ATP and exercise also has the ability to attenuate sympathetic vasoconstriction. Evidence suggests that activation of K_{IR} channels, and production of NO and PGs are not obligatory for contracting skeletal muscle to blunt α_1-adrenergic vasoconstriction. Given the importance of these pathways in ATP-mediated vasodilatation, we tested the hypothesis that inhibition of K_{IR} channels alone, or in combination with inhibition of Na^+/K^+-ATPase, NO and PG production, would not alter the ability of ATP to blunt α_1-adrenergic vasoconstriction. In healthy subjects, we measured forearm blood flow (Doppler ultrasound) and calculated changes in vascular conductance (FVC) to local intra-arterial infusion of phenylephrine (PE; α_1-agonist) during infusion of ATP or control vasodilators, before and after blockade of K_{IR} channels (barium chloride) alone (n=7); or combined blockade of K_{IR} channels, Na^+/K^+-ATPase (ouabain), NO (L-NMMA), and PGs (Ketorolac) (n=6). ATP significantly attenuated PE-mediated vasoconstriction relative to both sodium nitroprusside (SNP) and adenosine (ADO) (Δ FVC: ATP: -16±2; ADO: -38±6; SNP: -59±6%; $P<0.05$ vs. ADO and SNP). Blockade of K_{IR} channels alone, or in combination with Na^+/K^+-ATPase, NO, and PGs, significantly attenuated ATP-mediated vasodilatation (~-40% and -60% respectively; $P<0.05$ vs. control). Despite this, the ability of ATP to attenuate PE-mediated vasoconstriction remained intact (Δ FVC: K_{IR} blockade alone: -6±5%; combined blockade: -4±14%; $P>0.05$ vs. respective control). These studies highlight that while activation of K_{IR} channels, NO, and PGs

are contributors to the vasodilatory response to exercise and exogenous ATP infusions, these pathways are not obligatory to observe the unique ability of both stimuli to blunt sympathetic vasoconstriction in humans.

Introduction

The regulation of blood flow and oxygen delivery to contracting skeletal muscle is the result of a complex integration of local vasodilatory signals and sympathetic neural vasoconstriction, which enables adequate oxygen delivery to active tissues while maintaining mean arterial blood pressure (MAP) in the face of marked local vasodilatation. While sympathetic vasoconstriction is essential for the maintenance of MAP during large muscle mass or high intensity exercise (Marshall *et al.*, 1961; Rowell, 1997), sympathetic vasoconstriction is significantly attenuated in contracting skeletal muscle relative to resting tissues (Remensnyder *et al.*, 1962; Anderson & Faber, 1991; Thomas & Victor, 1998; Buckwalter *et al.*, 2001; Tschakovsky *et al.*, 2002; Dinenno & Joyner, 2003; Wray *et al.*, 2004) The ability of contracting skeletal muscle to attenuate sympathetic vasoconstriction, referred to as functional sympatholysis (Remensnyder *et al.*, 1962), is graded with exercise intensity. Whereas mild muscle contractions (<10% MVC, ~25% WR) do not interfere with sympathetic vasoconstriction, graded increases in contraction intensity result in progressively greater attenuation of sympathetic vasoconstriction (Thomas *et al.*, 1994; Buckwalter *et al.*, 2001; Tschakovsky *et al.*, 2002; Kirby *et al.*, 2005). The specific mechanisms responsible for functional sympatholysis remain unclear. Studies indicate that functional sympatholysis occurs post-junctionally at the level of the α-adrenoceptors, indicating specific signaling within the resistance vasculature is responsible attenuating vasoconstriction. Importantly, impaired sympatholysis is a common feature of vascular dysfunction in clinical populations such as ageing (Koch *et al.*, 2003; Dinenno *et al.*, 2005) and hypertension (Vongpatanasin *et al.*, 2011) and has been observed in an animal model of chronic myocardial infarction (Thomas *et al.*, 2001). Thus, there is

considerable interest in identifying the vascular signaling pathways responsible for this phenomenon.

Recent evidence suggest that adenosine triphosphate (ATP) may be an important contributor to the haemodynamic response to exercise (Crecelius *et al.*, 2015*a*). While a number of cell types release ATP as a signaling molecule, ATP release from red blood cells in response to progressive haemoglobin deoxygenation is a proposed mechanism coupling mismatches in oxygen delivery and demand via eliciting vasodilatation in proportion to the metabolic demand of the contracting muscle (McCullough *et al.*, 1997; Jagger *et al.*, 2001; González-Alonso *et al.*, 2002). Indeed, intra-arterial infusion of ATP induces vasodilatation sufficient to elevate blood flow to levels observed during maximal exercise (González-Alonso *et al.*, 2002; Rosenmeier *et al.*, 2004) and the concentration of ATP in the venous effluent of contracting skeletal muscle increases progressively during incremental exercise (González-Alonso *et al.*, 2002; Mortensen *et al.*, 2011; Kirby *et al.*, 2012). In addition to vasodilatation, ATP may co-ordinate blood flow responses within active tissue by modulating local sympathetic vasoconstriction (Rosenmeier *et al.*, 2004, 2008; Kirby *et al.*, 2008). Work by Rosenmeier and colleagues (2004) initially demonstrated that ATP infused intra-arterially to resting skeletal muscle attenuates vasoconstriction in response to tyramine-mediated endogenous noradrenaline release. Importantly, Kirby and colleagues (2008) demonstrated that circulating ATP attenuates direct post-junctional α_1- and α_2-adrenergic vasoconstriction in a graded manner, similar to that observed in contracting skeletal muscle. In humans, the dual ability to induce vasodilatation and limit adrenergic-vasoconstriction is unique to ATP, as exogenous administration of ADP, AMP (Rosenmeier *et al.*, 2008), adenosine (Rosenmeier *et al.*, 2003*a*, 2003*b*, 2004, 2008; Dinenno & Joyner, 2003, 2004; Kirby *et al.*, 2008; Crecelius *et al.*, 2015*b*), sodium nitroprusside (SNP) (nitric oxide donor) (Tschakovsky *et al.*, 2002; Rosenmeier *et al.*, 2003*b*), potassium chloride, and acetylcholine (Hearon *et al.*, in review) all fail to independently attenuate adrenergic-

vasoconstriction. Thus, the signaling pathways responsible for ATP-mediated vasodilatation are of particular interest in the context of exercise hyperaemia and functional sympatholysis.

It is well established that ATP elicits vasodilatation by binding purinergic (P2) receptors located on the endothelium (Burnstock & Kennedy, 1986; Winter & Dora, 2007), however until recently the downstream signaling pathways responsible for the vasodilatory response in humans were less clear. A number of investigations have identified a variable and modest contribution of nitric oxide (NO) and prostaglandins (PGs) (~20%) in response to intra-luminal ATP infusion in humans (Rongen *et al.*, 1994; van Ginneken *et al.*, 2004; Mortensen *et al.*, 2009; Crecelius *et al.*, 2011). Data from our laboratory identified activation of inwardly rectifying K^+ (K_{IR}) channels as a primary contributor to ATP-mediated vasodilatation in the human forearm (Crecelius *et al.*, 2012). Activation of K_{IR} channels can hyperpolarize vascular smooth muscle cells directly (Jackson, 2005) or amplify hyperpolarizing signals generated from the endothelium (Jantzi *et al.*, 2006). Importantly, K_{IR} channel activation and subsequent vascular hyperpolarization is an important component of conducted vasodilatation (Rivers *et al.*, 2001; Jantzi *et al.*, 2006; De Wit, 2010), which is implicated in the coordination of blood flow responses during muscle contractions (Wölfle *et al.*, 2009). Indeed, our laboratory has recently demonstrated that inhibition of K_{IR} channels attenuates the normal vasodilatory response to forearm handgrip exercise by ~30% (Crecelius *et al.*, 2014). However, despite combined blockade of K_{IR} channels, Na^+/K^+-ATPase activity, NO and PGs production resulting in greater than ~40% decrease in the normal vasodilatory response to exercise, the ability of contracting skeletal muscle to attenuate α_1-adrenergic vasoconstriction remained intact (Crecelius *et al.*, 2015*b*). Thus, the primary vasoactive pathways involved in ATP-mediated vasodilatation in humans are not obligatory to observe functional sympatholysis in contracting skeletal muscle.

Therefore, given the specific role of K_{IR} channels in ATP-mediated vasodilatation, the primary purpose of this study was to determine whether the ability of ATP to blunt α_1-adrenergic

vasoconstriction is dependent upon K_{IR} channel activity in humans. A secondary purpose was to determine the effect of combined inhibition of K_{IR} channels and Na^+/K^+-ATPase, as well as the synthesis of NO and PGs on the ability of ATP to modulate α_1-adrenergic vasoconstriction. We hypothesized that despite significant attenuation of the vasodilatory response to ATP by inhibition of K_{IR} channels alone or in combination with blockade of the Na^+/K^+-ATPase, that ATP would maintain the ability to attenuate α_1-adrenergic vasoconstriction in humans.

Methods

Subjects

With Institutional Review Board approval and after written informed consent, a total of 13 young healthy adults (7 men, 6 women; age = 22±1 years old; weight = 65.5±4.5 kg; height = 186±13 cm; body mass index = 22.6±0.7 kg/m^2; forearm volume (FAV) = 915±79 ml; means ± SEM) participated in the present study. All subjects were sedentary to moderately active, non-smokers, non-obese, normotensive and not taking any medications. Studies were performed after an overnight fast and 24 hour abstention from caffeine and exercise. The subjects were in the supine position with the experimental arm abducted to 90° and slightly elevated above heart level upon a tilt-adjustable table. Female subjects were studied during the early follicular phase of their menstrual cycle or placebo phase of oral contraceptive use to minimize any potential cardiovascular effects of sex-specific hormones. All studies were performed according to the *Declaration of Helsinki.*

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

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). The catheter was continuously flushed at 3 ml hr^{-1} with heparinized saline and connected to a pressure transducer to measure MAP, and a 3-port connector for local infusion of vasoactive drugs. Heart rate (HR) was

determined using a 3-lead electrocardiogram (Cardiocap/5, Datex-Ohmeda Louisville, CO, USA) (Crecelius *et al.*, 2012, 2015*b*).

Forearm blood flow and vascular conductance

A 12 MHz linear-array ultrasound probe (Vivid 7, General Electric, Milwaukee, WI, USA) was used to determine brachial artery mean blood velocity (MBV) and brachial artery diameter. The probe was placed over the brachial artery proximal to the catheter insertion site as previously described (Crecelius *et al.*, 2010). For blood velocity measurements, the probe insonation angle was maintained at <60 degrees and the frequency used was 5 MHz. The Doppler shift frequency spectrum was analyzed via a Multigon 500M TCD (Multigon Industries, Mt Vernon NY, USA) spectral analyzer from which mean velocity was determined as a weighted mean of the spectrum of Doppler shift frequencies. Brachial artery diameter was measured in triplicate at the end of rest, during steady-state hyperaemia prior to infusion of Phenylephrine (PE), at the end of the PE vasoconstrictor response (see Experimental Protocol below). Forearm blood flow (FBF) was calculated as: FBF = MBV × π (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}. All studies were performed in a cool (20-22°C) temperature-controlled environment with a fan directed toward the forearm to minimize the contribution of skin blood flow to forearm haemodynamics.

Vasoactive drug infusion

All drug infusions occurred via a brachial artery catheter to ensure the effects of experimental drugs remained localized to the forearm vasculature. In order to strictly control the vasoconstrictor stimulus across all conditions, phenylephrine (PE; selective α_1-agonist; Sandoz Inc., Princeton, NJ) was infused at 0.065 μg (dl forearm volume)$^{-1}$ min^{-1} in male subjects and

was doubled to 0.125 μg (dl forearm volume)$^{-1}$ min^{-1} in female subjects to account for reduced adrenergic responsiveness and elicit similar magnitude of vasoconstrictor response across subjects (Kneale *et al.*, 2000). The doses of PE were chosen based on our previous experience in eliciting robust vasoconstriction at rest and during vasodilator infusion, and was adjusted for the steady-state hyperaemic condition as previously described (see below)(Dinenno & Joyner, 2003).

ATP was infused at a dose of 4.6 μg (dl forearm volume)$^{-1}$ min^{-1} based on previous studies demonstrating that this dose of ATP significantly attenuates PE-mediated vasoconstriction (Crecelius *et al.*, 2012). Because ATP infusion elevates forearm blood flow, "control" vasodilatory agents were infused to match the hyperaemia observed during ATP infusion. Our lab has previously demonstrated that α_1- adrenergic vasoconstriction is maintained during control vasodilator infusion (Kirby *et al.*, 2008). Therefore, the control vasodilator infusions are used to create a "high flow" control state (Tschakovsky *et al.*, 2002).

Adenosine (ADO; Akorn, Lake Forest, IL, USA) and the NO donor, sodium nitroprusside (SNP; Hospira, Lake Forest, IL, USA) were chosen as control vasodilators based on previous studies demonstrating preserved α-adrenergic responsiveness during intra-arterial infusion of these agents (Tschakovsky *et al.*, 2002; Rosenmeier *et al.*, 2003*b*) (see specific protocol). For each, an initial dose (Ado: 400 μg (min)$^{-1}$; SNP: 8 μg (min)$^{-1}$) was infused and subsequently adjusted to match the observed or expected hyperaemia induced by ATP. In cases where ADO or SNP trials preceded ATP trials, we predicted the level of hyperaemia based on our experience with ATP responses in control and inhibited conditions.

A number of vasodilatory pathways were inhibited via direct intra-arterial infusion of pharmacological antagonists. N^G-Monomethyl-$_L$-arginine ($_L$-NMMA; Bachem, Weil am Rhein, Germany) and ketorolac (Hospira, Lake Forest, IL, USA) were co-administered to inhibit nitric oxide synthase and cyclooxygenase-mediated production of NO and PGs, respectively. Loading doses of $_L$-NMMA and ketorolac were 25 mg (5 mg min^{-1} for 5 min) and 6 mg (600 μg min^{-1} for

10 minutes) respectively. Subsequent trials were performed during infusion of $_{L}$-NMMA and ketorolac at maintenance doses of 1.25 mg min^{-1} and 150 µg min^{-1}, respectively(Crecelius *et al.*, 2015*b*). To inhibit vascular hyperpolarization via K_{IR} channel activation or Na^+/K^+-ATPase activity, loading doses of $BaCl_2$ (K_{IR} channel inhibitor; 10% w/v BDH3238, EMD Chemicals, Gibbstown, NJ, USA) and ouabain octahydrate (Na^+/K^+-ATPase inhibitor, Sigma-O3125; Sigma-Aldrich, St. Louis, MO, USA), were administered at 0.9 µmol (dl FAV)$^{-1}$ min^{-1} (absolute dose 8-10 µmol min^{-1} for 3 minutes) and 2.7 nmol min^{-1} (15 minutes), respectively, prior to the first vasodilator trial. These same doses were infused for 3 minutes prior to, and throughout the duration of the subsequent vasodilator trials (Crecelius *et al.*, 2014).

General experimental protocol

Figure 1 presents the general trial timelines (A and B) and the protocol for each individual trial(C). The total length of each trial was 8 min, consisting of 2 min of baseline conditions and 6 min total vasodilator infusion. Infusion of the vasodilators resulted in steady-state blood flow within 3 minutes, and haemodynamic measures were made between minutes 3 and 4 of vasodilator infusion. Once steady-state blood flow was achieved, the dose of PE was calculated based on forearm blood flow (FBF; Doppler ultrasound) and forearm volume and infused for the final 2 minutes of the trial. Haemodynamic measures were repeated during the final 30 seconds of PE infusion.

Protocol 1. Independent blockade of K_{IR} channels (n=7)

Given the large independent contribution of K_{IR} channels to ATP-mediated vasodilatation in humans (~40%) (Crecelius *et al.*, 2012), we first tested the effect of K_{IR} channel blockade alone, on the ability of ATP to blunt PE-mediated vasoconstriction. Following control trials assessing PE-mediated vasoconstriction during vasodilator conditions (ATP, SNP, ADO), $BaCl_2$ (K_{IR} channel inhibitor) was administered and the vasodilator trials repeated in the same order

they were performed prior to blockade. The order of control vasodilator infusion was counterbalanced between subjects under control conditions, and repeated in that order during the following blockade conditions.

Protocol 2. Combined blockade of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase (n=6)

Our lab has previously shown that NO and PGs contribute modestly to ATP-mediated vasodilatation in human forearm vasculature (~20%) (Crecelius *et al.*, 2011). Additionally, during handgrip exercise NO and PGs may interact with K_{IR} and Na^+/K^+-ATPase in a compensatory manner (Crecelius *et al.*, 2014). Thus, we investigated the effect of NO and PG blockade alone, and in combination with blockade of K_{IR} and Na^+/K^+-ATPase activity, on the ability of ATP to blunt PE-mediated vasoconstriction. Due to time constraints, SNP control trials were omitted and ADO was used as the control vasodilator. ATP and ADO trials were performed under control conditions, after administration of $_L$-NMMA and ketorolac, and following administration of $_L$-NMMA, ketorolac, $BaCl_2$, and ouabain to achieve combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase activity, respectively. The order of ATP and ADO infusion was counterbalanced between subjects under control conditions, and repeated in that order during the following blockade conditions.

Data Acquisition and Analysis

Data were collected and stored on computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Baseline FBF, heart rate, and MAP represent an average of the last 30 seconds of the resting time period, the steady-state hyperaemia (Pre PE; prior to PE infusion), and the effects of the α_1-adrenergic agonist (PE; after 2 minutes of PE infusion). We calculated percent reduction in FVC as our standard index to compare vasoconstrictor responses across conditions, as this appears to be the most appropriate comparison of vasoconstrictor responsiveness under conditions where there might

be differences in vascular tone (Lautt, 1989; O'Leary, 1991; Buckwalter & Clifford, 2001). The percent reduction in FVC during PE administration was calculated as: ((FVC during PE - FVC Pre PE)/(FVC Pre PE)) x 100. In an effort to be comprehensive, we have also presented absolute values of forearm haemodynamics for all conditions in tabular form (Table 1 and 2).

Statistics

All values are reported as means ± SEM. Given the complexity of our experimental design, many comparisons are possible. We have used a statistical approach that focuses on the comparisons that are most relevant to our experimental questions. Our primary interest was the effect of blockade on vasoconstrictor responsiveness to PE in each vasodilator condition. Vasoconstrictor responses (%Δ FVC) were compared with a two-way (trial (ATP, SNP or ADO) × condition (control, BaCl2 or control, L-NMMA + ketorolac, L-NMMA + ketorolac + BaCl2 + ouabain)) RM ANOVA. Due to the predictably large change from rest to Pre-PE (steady state) conditions with ATP, SNP or ADO infusion, resting forearm haemodynamics were analysed separately by two-way (condition x trial) RM ANOVA. Absolute forearm haemodynamics were assessed by two-way (time point (pre-PE or PE) × condition) repeated measures (RM) ANOVA inclusive of ATP, SNP and ADO trials. Systemic haemodynamics were evaluated by two-way RM ANOVA (time point × condition) inclusive of ATP, SNP, and ADO trials. Effect of blockade on ATP vasodilatation prior to PE (Pre-PE) was assessed using one-way RM ANOVA. Student–Newman–Keuls post hoc testing was performed to make pairwise comparisons. Significance was set at $P<0.05$.

Results

Systemic haemodynamics

Systemic haemodynamics in all experimental conditions for each protocol are presented in Tables 1 and 2. Small changes in MAP and heart rate occurred throughout the course of the experiment.

Protocol 1. Independent blockade of K_{IR} channels

As intended by experimental design, prior to infusion of PE (Pre-PE), FBF and FVC during infusion of SNP and ADO were matched to levels observed during ATP infusion in all experimental conditions (Figure 2A). During SNP and ADO trials, direct stimulation of α_1-adrenoceptors via infusion of PE induced robust vasoconstriction (ΔFVC: -59 ± 6 and -38 ± 6% respectively) that was significantly attenuated by ATP (ΔFVC: -16±2%, P<0.05 vs. SNP and ADO). Infusion of $BaCl_2$ to inhibit K_{IR} channels had no effect on resting haemodynamics, but significantly attenuated the vasodilatory response to ATP by ~35% (FVC: Control 189 ± 24 , $BaCl_2$: 147 ± 19 ml min^{-1} 100 $mmHg^{-1}$; P<0.05). After blockade, vasoconstrictor responses to PE were maintained during SNP infusion (ΔFVC: -60 ± 6%, P>0.05 vs control SNP) and significantly elevated during ADO infusion (ΔFVC:-54 ± 5%, P<0.05 vs control ADO). In contrast, ATP maintained the ability to attenuate α_1-adrenergic vasoconstriction (ΔFVC: -6 ± 5%, P<0.05 vs. SNP and ADO within condition) (Figure 2B).

Protocol 2. Combined blockade of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase

As intended by experimental design, prior to infusion of PE (Pre-PE), FBF and FVC during ADO infusion was matched to levels observed during ATP infusion in all experimental conditions. During control ADO, direct stimulation of α_1-adrenoceptors via infusion of PE induced robust vasoconstriction that was significantly attenuated by ATP (ΔFVC: -42 ± 2 and -14 ± 4% respectively; P<0.05). Co-infusion of $_L$-NMMA and ketorolac reduced resting FBF and

FVC by ~20%, and significantly attenuated ATP-mediated vasodilatation by ~20% (FVC: Control: 223 ± 60, $_L$-NMMA +ketorolac: 160 ± 53 ml min^{-1} 100 mmHg^{-1}; P<0.05), consistent with effective blockade of nitric oxide synthase and cyclooxygenase-mediated production of NO and PGs, respectively (Figure 3A). Vasoconstrictor responses to PE were maintained during ADO infusion and tended to be greater than those observed in control conditions (ΔFVC: Control: -42 ± 2; $_L$-NMMA + ketorolac: -54 ± 8%; P=0.09). As observed in control conditions, infusion of ATP significantly attenuated PE-mediated vasoconstriction relative to ADO (ΔFVC: ADO: -54 ± 8, ATP: -14 ± 9 %; P<0.05). Finally, combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase activity significantly attenuated ATP-mediated vasodilatation by ~60% (FVC: 114 ± 49 ml min^{-1} 100 mmHg^{-1}; P<0.05 vs. control ATP). Vasoconstrictor responses to PE infusion were maintained or tended to be elevated during ADO infusion after combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase activity relative to control (ΔFVC: -60 ± 10%; P=0.07 vs. control ADO). In contrast, Infusion of ATP significantly attenuated PE-mediated vasoconstriction (ΔFVC:-4 ± 14%, P<0.05 vs. ADO within condition) similar to control conditions (Figure 3B).

Discussion

The purpose of this investigation was to determine if activation of K_{IR} channel activation is necessary for ATP to modulate α_1-adrenergic vasoconstriction. The results confirm previous findings demonstrating a significant contribution of K_{IR} channels, NO and PGs to ATP-mediated vasodilatation. The primary novel finding of the present study is that ATP-mediated attenuation of α_1-adrenergic vasoconstriction is independent of K_{IR} channel activation. Additionally, ATP maintained the ability to blunt vasoconstriction despite a 60% reduction in the vasodilatory response during combined NO, PG, K_{IR} channel and Na^+/K^+-ATPase inhibition. This is in agreement with findings in contracting human skeletal muscle suggesting vascular signalling pathways beyond NO, PGs, and smooth muscle hyperpolarization are critical for both metabolic

and pharmacologic modulation of α-adrenergic vasoconstriction in humans (Crecelius *et al.*, 2015*b*).

Exercise and ATP-mediated vasodilatation

The vasculature within contracting skeletal muscle has the unique ability to attenuate sympathetic vasoconstriction; a phenomenon referred to as 'functional sympatholysis' (Remensnyder *et al.*, 1962). Attenuation of sympathetic vasoconstriction is critical to ensure adequate blood flow and oxygen delivery to contracting skeletal muscle during exercise when sympathetic nervous system activity is significantly elevated. While a number of vasodilatory substances have been investigated (Tschakovsky *et al.*, 2002; Rosenmeier *et al.*, 2003*b*, 2008; Hearon *et al.*, 2015), to date, the only exogenous vasodilator known to attenuate sympathetic vasoconstriction in a manner similar to exercise is ATP (Kirby *et al.*, 2008; Rosenmeier *et al.*, 2008). ATP is thought to contribute to exercise hyperaemia via direct vasodilatation and by modulating sympathetic vasoconstriction, thus there has been considerable interest in understanding the mechanisms responsible for ATP-mediated vasodilatation (Sprague & Ellsworth, 2012; Crecelius *et al.*, 2015*a*).

Studies in animal models have clearly demonstrated that ATP elicits a conducted vasodilatory response that is dependent upon elevations in endothelial cell calcium and activation of small- and intermediate-conductance Ca^{2+}-activated potassium channels (SK_{Ca} and IK_{Ca}, respectively) and subsequent endothelium derived hyperpolarization (EDH) (Duza & Sarelius, 2003; Winter & Dora, 2007; Dietrich *et al.*, 2012). Hyperpolarization of the endothelium serves as the initiating signal that is transduced, either directly through gap junctions, or by elevations in interstitial K^+, into a vasodilatory response by subsequent activation of K_{IR} channels and Na^+/K^+-ATPase located on vascular smooth muscle cells (Edwards *et al.*, 1998). In support these findings, our lab recently identified a primary role (~50%; range: 40-70%) for K_{IR} channel activation in mediating the vasodilatory response to intra-luminal ATP infusion in

humans (Crecelius *et al.*, 2012). Interestingly, while the vasodilatory response to handgrip exercise also relies heavily on activation of K_{IR} channels (~30%) (Crecelius *et al.*, 2015*b*), contracting skeletal muscle maintains the ability to attenuate adrenergic vasoconstriction after blockade of K_{IR} channels (Crecelius *et al.*, 2015*b*). This finding seemingly dissociates an essential component of ATP-mediated vasodilatation, activation of K_{IR} channels, and the ability to attenuate adrenergic vasoconstriction. Therefore, in protocol 1 we hypothesized that the ability of ATP to attenuate α_1-adrenergic vasoconstriction would be independent of K_{IR} channel activity.

Independent blockade of K_{IR} channels

Given the clear independent effect of barium alone on ATP-mediated vasodilatation, the first protocol aimed to identify an independent role of K_{IR} channel activation on ATP-mediated sympatholysis. The present findings support previous work showing a significant contribution of K_{IR} channels to ATP-mediated vasodilatation. A previous study by Crecelius et al. (2012) using a similar dose of ATP demonstrated a ~40% reduction in vasodilatation after blockade of K_{IR} channels, which is in line with the ~35% reduction in FVC observed in this study (Fig. 1A). In support of our hypothesis, α_1-adrenergic responsiveness during ATP infusion was similar before and after blockade of K_{IR} channels and remained substantially blunted relative to both ADO and SNP conditions (Fig. 1B). Thus despite K_{IR} channels being a critical part of the vasodilatory response to ATP, activation of these channels is not obligatory to observe attenuation of α-adrenergic vasoconstriction. This is consistent with previous observations that K_{IR} channels do not contribute to functional sympatholysis in contracting human skeletal muscle despite accounting for a significant portion of the hyperaemic response.

Interestingly, in control conditions α_1-adrenergic vasoconstriction was attenuated during ADO infusion relative to that observed during SNP, and was significantly augmented following blockade of K_{IR} channels (Fig. 1B). It is possible that under control conditions ADO-mediated

vasodilatation is relatively more resistant to α_1-adrenergic vasoconstriction than vasodilatation induced by exogenous NO administration. Similar to ATP, adenosine has been shown to elicit conducted vasodilatory responses (Rivers *et al.*, 2001; De Wit, 2010), however, the nature of the conducted response to ADO is distinct from that observed in response to ATP. Specifically, ADO relies primarily on activation of ATP-dependent K^+ (K_{ATP}) channels and subsequent hyperpolarization of smooth muscle (Kleppisch & Nelson, 1995; Hein & Kuo, 1999; De Wit, 2010), as opposed to ATP which relies on SK_{Ca}/IK_{Ca} channel activation and endothelium derived hyperpolarization (Winter & Dora, 2007; Dora, 2016). To date, relatively few studies have investigated the ability of K_{ATP} channels to modulate sympathetic vasoconstriction. In a rat hind limb preparation, pharmacological activation of K_{ATP} channels attenuated sympathetic vasoconstriction at rest, and inhibition of K_{ATP} channels augmented sympathetic vasoconstriction during muscle contractions (Thomas *et al.*, 1997). In humans, systemic administration of the K_{ATP} channel antagonist glyburide augmented baroreflex-mediated vasoconstriction in the leg at rest similar to animal models, in contrast the exercise-induced attenuation of sympathetic vasoconstriction remained relatively intact (Keller *et al.*, 2004). While it remains a possibility that ADO modulates adrenergic-vasoconstriction to some extent in resting tissues via activation of K_{ATP} channels, the ability of ADO to modulate α-adrenergic vasoconstriction is clearly much less potent than either ATP or exercise (fig 1) (Tschakovsky *et al.*, 2002; Kirby *et al.*, 2008).

Similar to previous studies, the vasoconstrictor responsiveness during ADO was augmented after blockade of K_{IR} channels, thus it could be interpreted the K_{IR} channel activation is in part responsible for limiting vasoconstriction during control ADO conditions. However, the current set of studies are not designed to test this hypothesis. Specifically, the dose of ADO was adjusted in each condition to match the match the blunted vasodilatory response to ATP (ADO dose: Control: 477 ± 55, $BaCl_2$: 299 ± 62 μg $(min)^{-1}$; P=0.07). Therefore, it is unclear whether the augmented vasoconstrictor response was due to a lower overall dose of ADO or an interaction between $BaCl_2$ and PE. Further, despite the potentially greater vasoconstrictor stimulus, there

was no similar effect of K_{IR} channel inhibition of ATP-mediated sympatholysis. This supports recent findings from our laboratory demonstrating that activation of K_{IR} channels via intra-arterial infusion of KCl fails to blunt α_1-adrenergic vasoconstriction in humans (Hearon) and in fact may even augment vasoconstrictor responses (Hearon, in review). Collectively, these investigations demonstrate that similar to exercise, the ability of ATP to blunt sympathetic vasoconstriction greatly exceeds that of ADO and SNP under control conditions and that while activation of K_{IR} channels may be an important contributor to the vasodilatation observed during ATP infusion, it does not contribute to the observed attenuation of adrenergic vasoconstriction.

Combined blockade of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase

In addition to K_{IR} channel activation, previous studies in humans have demonstrated a modest and variable contribution of NO and PGs to ATP-mediated vasodilatation (Rongen *et al.*, 1994; van Ginneken *et al.*, 2004; Mortensen *et al.*, 2009; Crecelius *et al.*, 2011). While one study in humans has identified a potential role for NO in mediating functional sympatholysis (Chavoshan *et al.*, 2002), the majority of studies employing both pharmacological inhibition of NO synthase, or direct NO donation (e.g. via infusion of sodium nitroprusside), suggest little independent involvement of NO in modulating sympathetic vasoconstriction at rest or during exercise (Tschakovsky *et al.*, 2002; Rosenmeier *et al.*, 2003*b*; Dinenno & Joyner, 2004; Crecelius *et al.*, 2015*b*). However there is significant overlap between NO and PG signaling (Schrage *et al.*, 2004; Dinenno & Joyner, 2004; Markwald *et al.*, 2011) and combined blockade of NO and PGs has been shown to elevate vasoconstrictor responsiveness in resting tissues (Dinenno & Joyner, 2004). Further, studies in both animal models (Bauersachs *et al.*, 1996; Sheng & Braun, 2007; Sheng *et al.*, 2009) and humans (Taddei *et al.*, 1999) indicate significant crosstalk between NO, PGs and vascular hyperpolarization. Therefore, in a follow-up study, we tested the effect of NO and PG inhibition alone as well as combined NO, PG, K_{IR} channel, and

Na^+/K^+-ATPase inhibition on the ability of ATP to blunt α_1-adrenergic vasoconstriction similar to previous studies investigating functional sympatholysis in human skeletal muscle.

Despite variability in the absolute vasodilatory responses to ATP, the effects of progressive blockade was consistent with findings from previous studies (Crecelius *et al.*, 2011, 2012). Inhibition of NO and PG production reduced ATP-mediated vasodilatation by approximately 20%, while addition of K_{IR} channel and Na^+/K^+-ATPase reduced vasodilatation by an additional 40%. In total, the combined blockade reduced ATP-mediated vasodilatation by ~60% indicating a robust and effective pharmacological blockade. In adenosine conditions the vasoconstrictor responsiveness tended to be elevated after blockade of NO and PGs and after combined blockade of NO, PG, K_{IR} channels, and Na^+/K^+-ATPase activity, as observed previously (Dinenno & Joyner, 2004; Crecelius *et al.*, 2015*b*). However, α_1-adrenergic vasoconstrictor responsiveness during ATP infusion was consistent across conditions and remained significantly attenuated relative to ADO, despite the significant reduction in vasodilatation. Given the clear lack of effect of blockade on the ability of ATP to blunt vasoconstriction, and considering the invasive nature of these protocols, we did not feel justified in extending this protocol beyond the initial cohort of six. These results are in agreement with previous studies demonstrating that attenuation of α_1-adrenergic vasoconstriction occurs independently of NO, PG, K_{IR} channels, and Na^+/K^+-ATPase activity during exercise in humans.

Potential Mechanisms

While the specific mechanisms responsible for the ability of ATP to blunt sympathetic vasoconstriction remain unclear, it is obvious that there are fundamental differences in the signaling mechanisms elicited by ATP that distinguish it from SNP and ADO. Perhaps most notably, is the reliance of ATP on endothelium-dependent signaling to elicit vasodilatation as opposed to SNP and ADO, which are not reliant on the endothelium to elicit vasodilatation (Prentice *et al.*, 1997; Tabrizchi & Bedi, 2001; De Wit, 2010). We propose that specific signaling

within the endothelium is responsible for modulating sympathetic vasoconstriction. Specifically, the primary mechanism of action for ATP is through the activation of $G_{q/11}$ protein coupled receptors (P_{2y}-receptors) and subsequent endothelium-derived hyperpolarization via activation of K_{Ca} channels (Winter & Dora, 2007). Endothelium-derived hyperpolarization can evoke a conducted vasomotor response resulting in vasodilatation and opposition of sympathetically-mediated vascular depolarization (Kurjiaka & Segal, 1995). The ability of ATP to elicit a conducted vasodilatory response is dependent upon activation of sK_{Ca} and iK_{Ca} channels (Winter & Dora, 2007), and specific localization of iK_{Ca} channels to myoendothelial gap junctions places them in unique position to modulate smooth muscle cell contractility (Sandow *et al.*, 2009). Indeed, iK_{Ca} channel activity has been identified as a critical component of myoendothelial feedback whereby endothelium-dependent signaling through gap junctions limits α-adrenergic vasoconstriction (Tran *et al.*, 2012). Similar to ATP, animal models have shown that K_{Ca} channel activation and conducted vasodilatation is a critical part of the vasodilatory response to muscle contractions (Segal & Jacobs, 2001; Milkau *et al.*, 2010). Further, in humans, our laboratory recently demonstrated that small increases in endothelium-dependent signaling via infusion of ATP or ACh during mild intensity muscle contractions significantly increases the ability of contracting skeletal muscle to attenuate sympathetic vasoconstriction (Hearon *et al.*, 2015). While current pharmacology to address these speculations in humans is limited, the prevailing evidence strongly suggests that the endothelium may be the critical site for the integration of vasodilatory and vasoconstrictor signals.

Experimental Limitations and Considerations

Functional sympatholysis occurs post-junctionally, and involves specific signaling within the skeletal muscle vasculature. In order to directly investigate post-junctional signaling, PE (α_1-adrenergic agonist) was infused to simulate sympathetic vasoconstriction. In contrast to tyramine which induces endogenous NA release or α_2-adrenergic agonists which have pre-

junctional effects on NA release, PE can be used to isolate post-junctional signaling in a highly controlled manner. ATP has been shown to attenuate vasoconstriction induced by both α_1- and α_2- adrenoceptor agonists, as well tyramine-mediated endogenous NA release (Kirby *et al.*, 2008; Rosenmeier *et al.*, 2008) and thus the selective use of PE should not limit the interpretation of the data as it relates to sympatholysis.

Considering the presence of residual vasodilatation following combined blockade of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase, the effectiveness of the various pharmacological antagonists utilized in this study deserves consideration. Briefly, we utilized standard doses of L-NMMA and ketorolac that have been shown previously to be effective in attenuating NO and PG production in humans and observed effects on haemodynamics at rest, and during vasodilator administration (Kirby *et al.*, 2010; Markwald *et al.*, 2011). Blockade of NO and PGs via $_L$-NMMA and ketorolac respectively reduces resting forearm blood flow by 20% which is consistent with effective blockade of NO and PGs in resting tissue (Dinenno & Joyner, 2003). Additionally, the effect of combined NO and PG blockade on ATP-mediated vasodilatation was similar to (Mortensen *et al.*, 2009; Crecelius *et al.*, 2011), or greater than (Rongen *et al.*, 1994; Crecelius *et al.*, 2012) that observed in previous investigations. In regards to $BaCl_2$ and ouabain, we utilized doses that have been shown to abolish vasodilatation to increasing doses of intra-arterial KCl infusion (Crecelius *et al.*, 2012). The effect of $BaCl_2$ alone on ATP-mediated vasodilatation tended to be slightly less effective than reported previously (Crecelius *et al.*, 2012). Repeated exposure to other vasodilatory and vasoconstrictor stimuli over the duration of the study may partially explain this finding. Repeated exposure to both ADO and ATP has been shown to modify the subsequent cellular signaling pathways recruited by each vasodilator (Kochukov *et al.*, 2014; Maimon *et al.*, 2014). However, this should not alter the interpretation of our data, as administration of $BaCl_2$ significantly attenuated ATP vasodilatation similar to what has been observed previously (33% vs 40%) suggesting little alteration in the reliance of ATP on K_{IR} channel activation to mediate vasodilatation. Thus, we conclude that the residual

vasodilatory response to ATP primarily represents alternate vasodilatory pathways independent of NO, PGs, K_{IR} channels, and Na^+/K^+-ATPase activity. Considering the profound effect of combined blockade on ATP-mediated vasodilatation, we do not believe that lack of inhibitor effectiveness can explain preserved attenuation of vasoconstrictor responsiveness during ATP infusion.

Perspectives and Conclusion

The ability of contracting skeletal muscle to blunt sympathetic vasoconstriction is critical to ensure adequate blood flow and oxygen delivery to contracting skeletal muscle. To date, ATP is the only exogenous vasodilatory substance with the ability to attenuate sympathetic vasoconstriction, and is suggested to play an important role in exercise hyperaemia due to its ability to induce vasodilatation and limit vasoconstriction. Recent findings have demonstrated that the primary vasodilatory pathways activated by ATP are not obligatory to observe functional sympatholysis in humans, seemingly dissociating ATP signaling and the ability to attenuate vasoconstriction.

This series of studies confirms that ATP relies heavily on activation of K_{IR} channels, as well as NO, PGs and Na^+/K^+-ATPase activity to elicit vasodilatation. However, similar to exercise, these important vasodilatory pathways are not obligatory to observe blunting of α-adrenergic vasoconstriction. Identification of the residual signaling pathways engaged by ATP represents an important area of future investigation. This is of special significance in the context of, ageing, diabetes, hypertension and heart failure, all of which are characterized by elevated sympathetic nervous system activity coupled with impaired endothelial function. The specific signaling mechanisms that underlie the ability of ATP to attenuate vasoconstriction represent a potentially important therapeutic target for the improvement of vascular function in these patient populations.

Table 1. Protocol 1 Forearm and Systemic Haemodynamics: $BaCl_2$ Blockade Trials

Condition	Time point	Trial	Forearm Blood Flow (ml min^{-1})	Mean Arterial Pressure (mmHg)*	Forearm Vascular Conductance (ml min^{-1} 100 $mmHg^{-1}$)*	Heart Rate (beats min^{-1})
Control	Baseline	SNP	30 ± 2	94 ± 2	32 ± 2	59 ± 4
		ADO	28 ± 2	97 ± 3	30 ± 2	59 ± 4
		ATP	28 ± 2	94 ± 2	30 ± 3	57 ± 3
	Pre-PE	SNP	180 ± 18	88 ± 3	204 ± 21	61 ± 4
		ADO	202 ± 27	96 ± 3	209 ± 25	59 ± 4
		ATP	208 ± 27	93 ± 3	223 ± 27	59 ± 3
	PE	SNP	71 ± 6 †	91 ± 3	78 ± 7 †	59 ± 4
		ADO	136 ± 26 †	102 ± 3	132 ± 23 †	59 ± 3
		ATP	179 ± 25 †	94 ± 3	189 ± 24 †	54 ± 2
$BaCl_2$	Baseline	SNP	26 ± 3	96 ± 4	28 ± 4	62 ± 5
		ADO	34 ± 8	93 ± 2	36 ± 8	60 ± 5
		ATP	30 ± 4	95 ± 2	31 ± 5	58 ± 4
	Pre-PE	SNP	146 ± 18	94 ± 2	154 ± 18	63 ± 5
		ADO	147 ± 24	93 ± 3	158 ± 23	59 ± 4
		ATP	149 ± 19 ‡	95 ± 2	156 ± 19 ‡	58 ± 3
	PE	SNP	57 ± 9 †	96 ± 3	59 ± 8 †	58 ± 4
		ADO	74 ± 21 †	97 ± 3	76 ± 19 †	59 ± 4
		ATP	141 ± 19	95 ± 2	147 ± 20	59 ± 3

*P<0.05, interaction Time point x condition
†P<0.05, vs. Pre-PE within Trial
‡P<0.05, vs. Control Condition
PE, Phenylephrine; $BaCl_2$, Barium Chloride; SNP, Sodium Nitroprusside, ADO, Adenosine, ATP, Adenosine Triphosphate

Table 2. Protocol 2 Forearm and Systemic Haemodynamics: Combined L-NMMA +Ketorolac + $BaCl_2$ + Oaubain

Condition	Time point	Trial	Forearm Blood Flow (ml min^{-1})	Mean Arterial Pressure (mmHg)*	Forearm Vascular Conductance (ml min^{-1} 100 $mmHg^{-1}$)*	Heart Rate (beats min^{-1})
Control	Baseline	ADO	30 ± 10	95 ± 3	31 ± 11	60 ± 2
		ATP	25 ± 6	96 ± 3	26 ± 6	59 ± 2
	Pre-PE	ADO	203 ± 53	97 ± 4	203 ± 48	60 ± 3
		ATP	203 ± 57	90 ± 3	223 ± 60	59 ± 3
	PE	ADO	125 ± 36 †	101 ± 4	118 ± 30 ‡	59 ± 4
		ATP	181 ± 50	94 ± 3	190 ± 50	59 ± 3
L-NMMA+ Ketorolac	Baseline	ADO	25 ± 11	96 ± 3	26 ± 11	61 ± 4
		ATP	21 ± 5	95 ± 4	22 ± 5	59 ± 3
	Pre-PE	ADO	163 ± 56	94 ± 3	172 ± 60	62 ± 4
		ATP	156 ± 55 ‡	94 ± 3	160 ± 53 ‡	57 ± 2
	PE	ADO	60 ± 13 †	97 ± 3	62 ± 13 †	61 ± 5
		ATP	129 ± 42	94 ± 4	133 ± 40	57 ± 3
L-NMMA+ Ketorolac+ $BaCl_2$+ Ouabain	Baseline	ADO	15 ± 3 ‡	99 ± 4	15 ± 3 ‡	63 ± 5
		ATP	17 ± 4 ‡	97 ± 3	18 ± 4 ‡	61 ± 4
	Pre-PE	ADO	95 ± 40	101 ± 4	91 ± 37	62 ± 4
		ATP	110 ± 45 ‡§	99 ± 2	114 ± 49 ‡§	61 ± 4
	PE	ADO	21 ± 5 †	102 ± 4	20 ± 4 †	64 ± 5
		ATP	92 ± 35	105 ± 2	90 ± 35	60 ± 4

*P<0.05, interaction Time point x condition
†P<0.05, vs. Pre-PE within Trial
‡P<0.05, vs. Control condition
§P<0.05, vs. L-NMMA+ Ketorolac
PE, Phenylephrine; $BaCl_2$, Barium Chloride; SNP, Sodium Nitroprusside, ADO, Adenosine, ATP, Adenosine Triphosphate

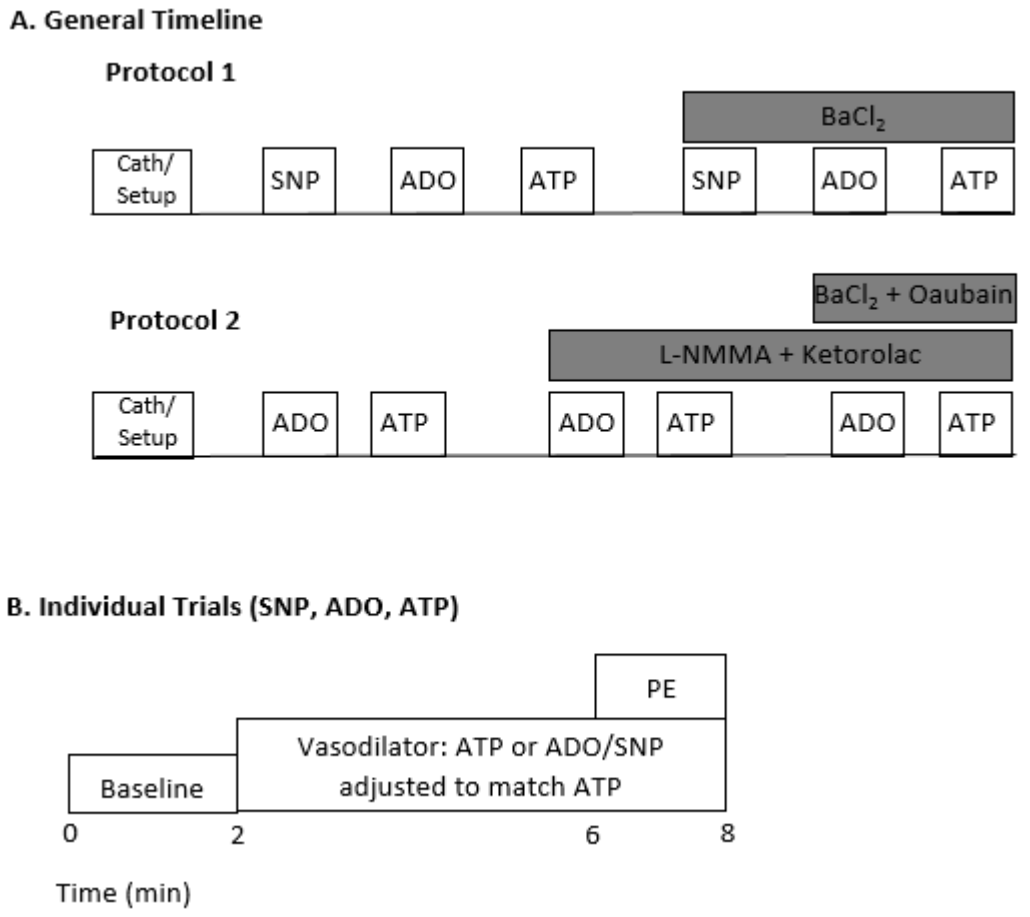

Figure 1. Experimental Protocol

A. After catheterization of the brachial artery (cath) and instrumentation, vasoconstrictor responses to phenylephrine (PE) were assessed during infusion of ATP, and during sodium nitroprusside (SNP) or adenosine (ADO) as high flow control conditions. In protocol 1, PE responses were assessed before and after independent blockade of inwardly rectifying potassium (K_{IR}) channels via infusion of barium chloride ($BaCl_2$). In protocol 2, PE responses were assessed before and after blockade of nitric oxide (NO; via L-NMMA) and prostaglandins (PGs; via ketorolac) alone, or combined blockade of NO, PG, K_{IR} channels, and the sodium-potassium ATPase (ouabain). **B**. After baseline measurements, steady-state the vasodilator was infused and forearm blood flow allowed to reach a steady state. PE responses were assessed in the final 2 minutes of vasodilator infusion and quantified as a percent decrease in forearm vascular conductance (FVC).

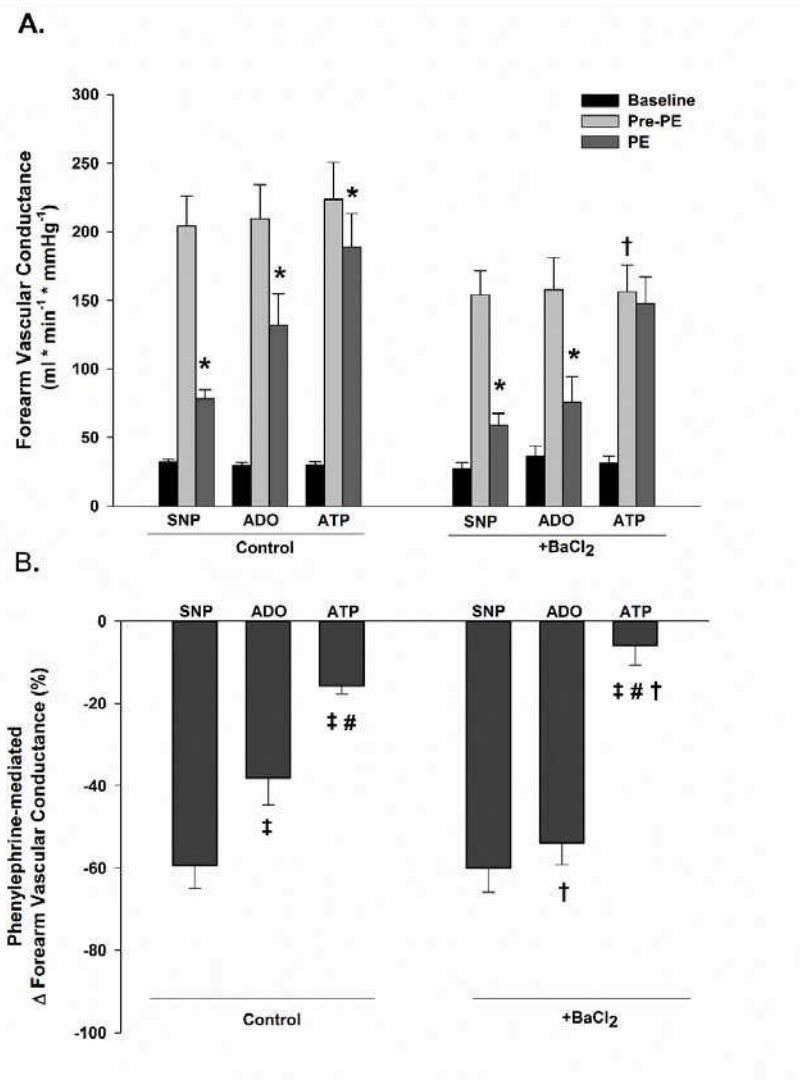

Figure 2. ATP-mediated sympatholysis is independent of inwardly rectifying potassium (K_{IR}) channel

A. Forearm vascular conductance (FVC) at baseline, steady state hyperaemia prior to infusion of phenylephrine (PE; Pre-PE), and at the end of 2 minutes of PE infusion (PE) , before and after blockade of K_{IR} channels via infusion of barium chloride ($BaCl_2$). $BaCl_2$ significantly reduced ATP-mediated vasodilatation by ~35%. **B.** ATP significantly attenuated PE-mediated vasoconstriction in control conditions. After blockade of K_{IR} channels, despite a significant reduction in the vasodilatory response to ATP, the ability of ATP to attenuate PE-mediated vasoconstriction remained intact. *P<0.05 vs. Pre-PE; †P<0.05 vs. time point in control condition; ‡P<0.05 vs. SNP within condition; #P<0.05 vs. ADO within condition

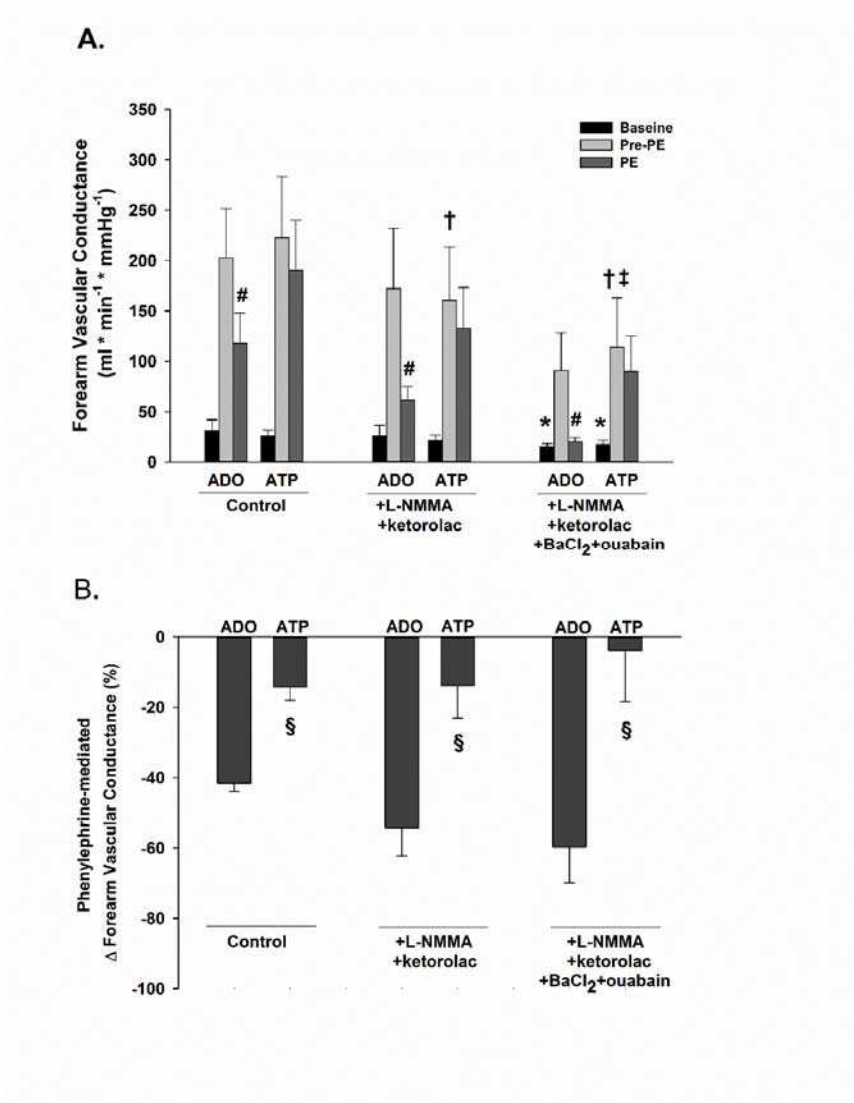

Figure 3. ATP-mediated sympatholysis is independent of nitric oxide (NO), prostaglandin (PG), inwardly rectifying potassium (K_{IR}) channel, and sodium-potassium ATPase (Na^+/K^+-ATPase) activity

A. Forearm vascular conductance (FVC) at baseline, steady state hyperaemia prior to infusion of phenylephrine (PE; Pre-PE), and at the end of 2 minutes of PE infusion (PE) , before and after blockade of NO and PGs (L-NMMA and ketorolac, respectively), or combined blockade of NO, PGs, K_{IR} channels via infusion of barium chloride ($BaCl_2$), and Na^+/K^+-ATPase (ouabain). Combined blockade significantly reduced ATP-mediated vasodilatation by ~60%. **B.** ATP significantly attenuated PE-mediated vasoconstriction in control conditions. After, blockade of NO and PGs, or combined blockade of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase despite a significant reduction in the vasodilatory response to ATP, the ability of ATP to attenuate PE-mediated vasoconstriction remained intact. *P<0.05 vs. Control #P<0.05 vs. Pre-PE; †P<0.05 vs. time point in control condition; ‡P<0.05 vs. time point in L-NMMA+ketorolac; §P<0.05 vs. ADO within condition

REFERENCES

Anderson KM & Faber JE (1991). Differential sensitivity of arteriolar alpha 1- and alpha 2-adrenoceptor constriction to metabolic inhibition during rat skeletal muscle contraction. *Circ Res* **69,** 174–184.

Bauersachs J, Popp R, Hecker M, Sauer E, Fleming I & Busse R (1996). Nitric Oxide Attenuates the Release of Endothelium-Derived Hyperpolarizing Factor. *Circulation* **94,** 3341–3347.

Buckwalter JB & Clifford PS (2001). The paradox of sympathetic vasoconstriction in exercising skeletal muscle. *Exerc Sport Sci Rev* **29,** 159–163.

Buckwalter JB, Naik JS, Valic Z & Clifford PS (2001). Exercise attenuates {alpha}-adrenergic-receptor responsiveness in skeletal muscle vasculature. *J Appl Physiol* **90,** 172–178.

Burnstock G & Kennedy C (1986). A dual function for adenosine 5'-triphosphate in the regulation of vascular tone. Excitatory cotransmitter with noradrenaline from perivascular nerves and locally released inhibitory intravascular agent. *Circ Res* **58,** 319–330.

Chavoshan B, Sander M, Sybert TE, Hansen J, Victor RG & Thomas GD (2002). Nitric oxide-dependent modulation of sympathetic neural control of oxygenation in exercising human skeletal muscle. *J Physiol* **540,** 377–386.

Crecelius AR, Kirby BS & Dinenno FA (2015*a*). Intravascular ATP and the regulation of blood flow and oxygen delivery in humans. *Exerc Sport Sci Rev* **43,** 5–13.

Crecelius AR, Kirby BS, Hearon CM, Luckasen GJ, Larson DG & Dinenno FA (2015*b*). Contracting human skeletal muscle maintains the ability to blunt α1 -adrenergic vasoconstriction during KIR channel and Na(+) /K(+) -ATPase inhibition. *J Physiol* **593,** 2735–2751.

Crecelius AR, Kirby BS, Luckasen GJ, Larson DG & Dinenno FA (2012). ATP-mediated vasodilatation occurs via activation of inwardly rectifying potassium channels in humans. *J Physiol* **590,** 5349–5359.

Crecelius AR, Kirby BS, Richards JC, Garcia LJ, Voyles WF, Larson DG, Luckasen GJ & Dinenno FA (2011). Mechanisms of ATP-mediated vasodilation in humans: modest role for nitric oxide and vasodilating prostaglandins. *Am J Physiol Heart Circ Physiol* **301,** H1302–H1310.

Crecelius AR, Luckasen GJ, Larson DG & Dinenno FA (2014). KIR channel activation contributes to onset and steady-state exercise hyperemia in humans. *Am J Physiol Heart Circ Physiol* **307,** H782–H791.

Dietrich HH, Horiuchi T, Xiang C, Hongo K, Falck JR & Dacey Jr. RG (2012). Mechanism of ATP-induced Local and Conducted Vasomotor Responses in Isolated Rat Cerebral Penetrating Arterioles. *J Vasc Res* **29,** 997–1003.

Dinenno FA & Joyner MJ (2003). Blunted sympathetic vasoconstriction in contracting skeletal muscle of healthy humans: is nitric oxide obligatory? *J Physiol* **553,** 281–292.

Dinenno FA & Joyner MJ (2004). Combined NO and PG inhibition augments alpha-adrenergic vasoconstriction in contracting human skeletal muscle. *Am J Physiol Heart Circ Physiol* **287,** H2576–H2584.

Dinenno FA, Masuki S & Joyner MJ (2005). Impaired modulation of sympathetic alpha-adrenergic vasoconstriction in contracting forearm muscle of ageing men. *J Physiol* **567,** 311–321.

Dora KA (2016). Conducted dilatation to ATP and K(+) and in rat skeletal muscle arterioles. *Acta Physiol (Oxf)*; DOI: 10.1111/apha.12656.

Duza T & Sarelius IH (2003). Conducted dilations initiated by purines in arterioles are endothelium dependent and require endothelial Ca2+. *Am J Physiol Heart Circ Physiol* **285,** H26–H37.

Edwards G, Dora KA, Gardener MJ, Garland CJ & Weston AH (1998). K+ is an endothelium-derived hyperpolarizing factor in rat arteries. *Nature* **396,** 269–272.

Van Ginneken EEM, Meijer P, Verkaik N, Smits P & Rongen G a (2004). ATP-induced vasodilation in human skeletal muscle. *Br J Pharmacol* **141,** 842–850.

González-Alonso J, Olsen DB & Saltin B (2002). Erythrocyte and the regulation of human skeletal muscle blood flow and oxygen delivery: role of circulating ATP. *Circ Res* **91,** 1046–1055.

Hearon CJ, Kirby B, Crecelius A, Luckasen G, Larson D & Dinenno F (2015). Augmentation of Functional Sympatholysis via Endothelial Signaling Is Independent of Nitric Oxide and Prostaglandins in Humans. *FASEB J* **29,** 649.9 – .

Hein TW & Kuo L (1999). cAMP-Independent Dilation of Coronary Arterioles to Adenosine : Role of Nitric Oxide, G Proteins, and KATP Channels. *Circ Res* **85,** 634–642.

Jackson WF (2005). Potassium channels in the peripheral microcirculation. *Microcirculation* **12,** 113–127.

Jagger JE, Bateman RM, Ellsworth ML & Ellis CG (2001). Role of erythrocyte in regulating local O2 delivery mediated by hemoglobin oxygenation. *Am J Physiol Heart Circ Physiol* **280,** H2833–H2839.

Jantzi MC, Brett SE, Jackson WF, Corteling R, Vigmond EJ & Welsh DG (2006). Inward rectifying potassium channels facilitate cell-to-cell communication in hamster retractor muscle feed arteries. *Am J Physiol Heart Circ Physiol* **291,** H1319–H1328.

Keller DM, Ogoh S, Greene S, Olivencia-Yurvati a & Raven PB (2004). Inhibition of KATP channel activity augments baroreflex-mediated vasoconstriction in exercising human skeletal muscle. *J Physiol* **561,** 273–282.

Kirby BS, Crecelius AR, Voyles WF & Dinenno F a. (2012). Impaired skeletal muscle blood flow control with advancing age in humans: Attenuated ATP release and local vasodilation during erythrocyte deoxygenation. *Circ Res* **111,** 220–230.

Kirby BS, Crecelius AR, Voyles WF & Dinenno FA (2010). Vasodilatory responsiveness to adenosine triphosphate in ageing humans. *J Physiol* **588,** 4017–4027.

Kirby BS, Markwald RR, Smith EG & Dinenno FA (2005). Mechanical effects of muscle contraction do not blunt sympathetic vasoconstriction in humans. *Am J Physiol Heart Circ Physiol* **289,** H1610–H1617.

Kirby BS, Voyles WF, Carlson RE & Dinenno FA (2008). Graded sympatholytic effect of exogenous ATP on postjunctional alpha-adrenergic vasoconstriction in the human forearm: implications for vascular control in contracting muscle. *J Physiol* **586,** 4305–4316.

Kleppisch T & Nelson M (1995). Adenosine activates ATP-sensitive potassium channels in arterial myocytes via A2 receptors and cAMP-dependent protein kinase. *Proc Natl …* **92,** 12441–12445.

Kneale BJ, Chowienczyk PJ, Brett SE, Coltart DJ & Ritter JM (2000). Gender differences in sensitivity to adrenergic agonists of forearm resistance vasculature. *J Am Coll Cardiol* **36,** 1233–1238.

Koch DW, Leuenberger UA & Proctor DN (2003). Augmented leg vasoconstriction in dynamically exercising older men during acute sympathetic stimulation. *J Physiol* **551,** 337–344.

Kochukov MY, Balasubramanian a., Abramowitz J, Birnbaumer L & Marrelli SP (2014). Activation of Endothelial Transient Receptor Potential C3 Channel Is Required for Small Conductance Calcium-Activated Potassium Channel Activation and Sustained Endothelial Hyperpolarization and Vasodilation of Cerebral Artery. *J Am Heart Assoc* **3,** e000913–e000913.

Kurjiaka DT & Segal SS (1995). Interaction Between Conducted Vasodilation and Sympathetic Nerve Activation in Arterioles of Hamster Striated Muscle. *Circ Res* **76,** 885–891.

Lautt WW (1989). Resistance or conductance for expression of arterial vascular tone. *Microvasc Res* **37,** 230–236.

Maimon N, Titus PA & Sarelius IH (2014). Pre-exposure to adenosine, acting via A(2A) receptors on endothelial cells, alters the protein kinase A dependence of adenosine-induced dilation in skeletal muscle resistance arterioles. *J Physiol* **592,** 2575–2590.

Markwald RR, Kirby BS, Crecelius AR, Carlson RE, Voyles WF & Dinenno FA (2011). Combined inhibition of nitric oxide and vasodilating prostaglandins abolishes forearm vasodilatation to systemic hypoxia in healthy humans. *J Physiol* **589,** 1979–1990.

Marshall RJ, Schirger a & Shepherd JT (1961). Blood pressure during supine exercise in idiopathic orthostatic hypotension. *Circulation* **24,** 76–81.

McCullough WT, Collins DM & Ellsworth ML (1997). Arteriolar responses to extracellular ATP in striated muscle. *Am J Physiol Hear Circ Physiol* **272,** H1886–H1891.

Milkau M, Köhler R & de Wit C (2010). Crucial importance of the endothelial K+ channel SK3 and connexin40 in arteriolar dilations during skeletal muscle contraction. *FASEB J* **24,** 3572–3579.

Mortensen SP, Bune LT, Saltin B & Pilegaard H (2009). ATP-induced vasodilation and purinergic receptors in the human leg : roles of nitric oxide , prostaglandins , and adenosine. 1140–1148.

Mortensen SP, Thaning P, Nyberg M, Saltin B & Hellsten Y (2011). Local release of ATP into the arterial inflow and venous drainage of human skeletal muscle: insight from ATP determination with the intravascular microdialysis technique. *J Physiol* **589,** 1847–1857.

O'Leary DS (1991). Regional vascular resistance vs. conductance: which index for baroreflex responses? *Am J Physiol* **260,** H632–H637.

Prentice DJ, Payne SL & Hourani SM (1997). Activation of two sites by adenosine receptor agonists to cause relaxation in rat isolated mesenteric artery. *Br J Pharmacol* **122,** 1509–1515.

Remensnyder JP, Mitchell JH & Sarnoff SJ (1962). Functional sympatholysis during muscular activity: Observations on influence of carotid sinus on oxygen uptake. *Circ Res* **11,** 370–380.

Rivers RJ, Hein TW, Zhang C & Kuo L (2001). Activation of Barium-Sensitive Inward Rectifier Potassium Channels Mediates Remote Dilation of Coronary Arterioles. *Circulation* **104,** 1749–1753.

Rongen GA, Smits P & Thien T (1994). Characterization of ATP-induced vasodilation in the human forearm vascular bed. *Circulation* **90,** 1891–1898.

Rosenmeier JB, Dinenno FA, Fritzlar SJ & Joyner MJ (2003*a*). Alpha1- and Alpha2-Adrenergic Vasoconstriction Is Blunted in Contracting Human Muscle. *J Physiol* **547,** 971–976.

Rosenmeier JB, Fritzlar SJ, Dinenno FA & Joyner MJ (2003*b*). Exogenous NO administration and alpha-adrenergic vasoconstriction in human limbs. *J Appl Physiol* **95,** 2370–2374.

Rosenmeier JB, Hansen J & González-Alonso J (2004). Circulating ATP-induced vasodilatation overrides sympathetic vasoconstrictor activity in human skeletal muscle. *J Physiol* **558,** 351–365.

Rosenmeier JB, Yegutkin GG & González-Alonso J (2008). Activation of ATP/UTP-selective receptors increases blood flow and blunts sympathetic vasoconstriction in human skeletal muscle. *J Physiol* **586,** 4993–5002.

Rowell LB (1997). Neural control of muscle blood flow: importance during dynamic exercise. *Clin Exp Pharmacol Physiol* **24,** 117–125.

Sandow SL, Haddock RE, Hill CE, Chadha PS, Kerr PM, Welsh DG & Plane F (2009). What's where and why at a vascular myoendothelial microdomain signalling complex. *Clin Exp Pharmacol Physiol* **36,** 67–76.

Schrage WG, Joyner MJ & Dinenno FA (2004). Local inhibition of nitric oxide and prostaglandins independently reduces forearm exercise hyperaemia in humans. *J Physiol* **557,** 599–611.

Segal SS & Jacobs TL (2001). Role for endothelial cell conduction in ascending vasodilatation and exercise hyperaemia in hamster skeletal muscle. *J Physiol* **536,** 937–946.

Sheng J, Ella S, Davis MJ, Hill MA & Braun AP (2009). Openers of SKCa and IKCa channels enhance agonist-evoked endothelial nitric oxide synthesis and arteriolar vasodilation. *FASEB J* **23,** 1138–1145.

Sheng J-Z & Braun AP (2007). Small- and intermediate-conductance Ca2+-activated K+ channels directly control agonist-evoked nitric oxide synthesis in human vascular endothelial cells. *Am J Physiol Cell Physiol* **293,** C458–C467.

Sprague RS & Ellsworth ML (2012). Erythrocyte-derived ATP and perfusion distribution: role of intracellular and intercellular communication. *Microcirculation* **19,** 430–439.

Tabrizchi R & Bedi S (2001). Pharmacology of adenosine receptors in the vasculature. *Pharmacol Ther* **91,** 133–147.

Taddei S, Ghiadoni L, Virdis A, Buralli S & Salvetti A (1999). Vasodilation to Bradykinin Is Mediated by an Ouabain-Sensitive Pathway as a Compensatory Mechanism for Impaired Nitric Oxide Availability in Essential Hypertensive Patients. *Circulation* **100,** 1400–1405.

Thomas GD, Hansen J & Victor RG (1997). Atp-sensitive potassium channels mediate contraction- induced attenuation of sympathetic vasoconstriction in rat skeletal muscle. *J Clin Invest* **99,** 2602–2609.

Thomas GD, Hansen JIM, Victor G, Gail D & Hansen J (1994). Inhibition of alpha 2-adrenergic vasoconstriction during contraction of glycolytic, not oxidative, rat hindlimb muscle. *Am J physiol* **266,** H920–H929.

Thomas GD & Victor RG (1998). Nitric oxide mediates contraction-induced attenuation of sympathetic vasoconstriction in rat skeletal muscle. *J Physiol* **506,** 817–826.

Thomas GD, Zhang W & Victor RG (2001). Impaired modulation of sympathetic vasoconstriction in contracting skeletal muscle of rats with chronic myocardial infarctions: role of oxidative stress. *Circ Res* **88,** 816–823.

Tran CHT, Taylor MS, Plane F, Nagaraja S, Tsoukias NM, Solodushko V, Vigmond EJ, Furstenhaupt T, Brigdan M & Welsh DG (2012). Endothelial Ca2+ wavelets and the induction of myoendothelial feedback. *Am J Physiol Cell Physiol* **302,** C1226–C1242.

Tschakovsky ME, Sujirattanawimol K, Ruble SB, Valic Z & Joyner MJ (2002). Is sympathetic neural vasoconstriction blunted in the vascular bed of exercising human muscle? *J Physiol* **541,** 623–635.

Vongpatanasin W, Wang Z, Arbique D, Arbique G, Adams-Huet B, Mitchell JH, Victor RG & Thomas GD (2011). Functional sympatholysis is impaired in hypertensive humans. *J Physiol* **589,** 1209–1220.

Winter P & Dora KA (2007). Spreading dilatation to luminal perfusion of ATP and UTP in rat isolated small mesenteric arteries. *J Physiol* **582,** 335–347.

De Wit C (2010). Different pathways with distinct properties conduct dilations in the microcirculation in vivo. *Cardiovasc Res* **85,** 604–613.

Wölfle SE, Schmidt VJ, Hoyer J, Köhler R & De Wit C (2009). Prominent role of KCa3.1 in endothelium-derived hyperpolarizing factor-type dilations and conducted responses in the microcirculation in vivo. *Cardiovasc Res* **82,** 476–483.

Wray DW, Fadel PJ, Smith ML, Raven P & Sander M (2004). Inhibition of alpha-adrenergic vasoconstriction in exercising human thigh muscles. *J Physiol* **555,** 545–563.

CHAPTER III – MANUSCRIPT II

Endothelium-dependent hyperpolarization modulates α_1-adrenergic vasoconstriction in contracting skeletal muscle of humans

Summary

Stimulation of α-adrenoceptors elicits vasoconstriction in resting skeletal muscle that is blunted during exercise in an intensity dependent manner. In humans, the underlying mechanisms remain unclear. We tested the hypothesis that augmenting endothelium-dependent vasodilatory signaling will enhance the ability of contracting skeletal muscle to blunt α_1-adrenergic vasoconstriction. Changes in forearm vascular conductance (FVC; Doppler ultrasound, brachial intra-arterial pressure via catheter) to local intra-arterial infusion of phenylephrine (PE; α_1-adrenoceptor agonist) were calculated during (1) infusion of endothelium-dependent (acetylcholine, ACh; adenosine triphosphate, ATP) or -independent vasodilators (sodium nitroprusside, SNP, potassium chloride, KCl) at rest; (2) mild or moderate intensity handgrip exercise; and (3) combined mild exercise + ACh, ATP, SNP, or KCl infusions in healthy adults. Robust vasoconstriction to PE was observed during vasodilator infusion alone and mild exercise, and this was blunted during moderate exercise (ΔFVC:-34±4 and -34±3 vs. -13±2% respectively, $P<0.05$). Infusion of ACh or ATP during mild exercise significantly attenuated PE vasoconstriction similar to levels observed during moderate exercise (ACh:-3±4; ATP:-18±4%). In contrast, infusion SNP or KCl during mild exercise did not attenuate PE-mediated vasoconstriction (-32±5 and -46±3%). To further study the role of endothelium-dependent hyperpolarization, ACh trials were repeated with combined nitric oxide synthase and cyclooxygenase inhibition. Here, PE-mediated vasoconstriction was blunted at rest (blockade: -20±5 vs control: -31±3% vs.; $P<0.05$) and remained blunted during exercise (blockade: -15±5 vs control: -14±5%). We conclude that stimulation of endothelium-dependent hyperpolarization can blunt α_1-adrenergic vasoconstriction in contracting skeletal muscle of humans.

Introduction

The onset of whole-body exercise requires highly coordinated central and peripheral cardiovascular adjustments to ensure adequate blood flow and oxygen delivery to contracting skeletal muscle. Elevation of sympathetic nervous system activity is an essential component of the hemodynamic response to exercise as it contributes to both the increase in cardiac output, and the maintenance of total peripheral resistance. Within resistance vascular beds, elevated sympathetic vasoconstrictor activity is necessary to limit blood flow to splanchnic and inactive tissues and maintain mean arterial pressure (MAP) in the face of profound metabolic vasodilatation within contracting skeletal muscle. However, sympathetic outflow and subsequent noradrenaline (NA) release is also elevated within the vasculature of contracting skeletal muscle (Savard *et al.*, 1987; Taylor *et al.*, 1992). Normally, sympathetic α-adrenergic vasoconstriction is significantly attenuated in contracting skeletal muscle relative to inactive tissues. This phenomenon, originally referred to as "functional sympatholysis" (Remensnyder *et al.*, 1962), is necessary to ensure adequate blood flow and oxygen delivery to active tissues despite elevated sympathetic vasoconstrictor nerve activity (Joyner & Thomas, 2003).

Functional sympatholysis was first identified by Remensnyder et al. in 1962 and has since been confirmed by multiple groups using diverse exercise modalities (Tschakovsky *et al.*, 2002; Keller *et al.*, 2003; Koch *et al.*, 2003; Kirby *et al.*, 2008). Contracting skeletal muscle blunts vasoconstriction during a variety of stimuli including reflex activation of the sympathetic nervous system (e.g. via cold pressor test, baroreceptor unloading) (Keller *et al.*, 2003; Koch *et al.*, 2003), local intra-arterial administration of tyramine evoking endogenous NA release (Tschakovsky *et al.*, 2002; Dinenno and Joyner 2003), as well as intra-arterial infusion of direct α_1- and α_2-adrenoceptor agonists (Rosenmeier *et al.*, 2003*a*). Collectively, these studies indicate that functional sympatholysis occurs post-junctionally at the level of the α-adrenoceptors, implicating specific signaling within the resistance vasculature is responsible attenuating vasoconstriction. The exact mechanism by which this phenomenon occurs in

healthy humans is not well understood, and impaired modulation of sympathetic vasoconstriction is observed in ageing (Koch *et al.*, 2003; Dinenno *et al.*, 2005) and hypertensive humans (Vongpatanasin *et al.*, 2011), as well as in a model of chronic myocardial infarction (Thomas *et al.*, 2001). As such, impaired sympatholysis may be an important contributor to the malperfusion of skeletal muscle and exercise intolerance observed in these populations (Saltin & Mortensen, 2012), and thus it is of considerable interest to identify the vascular signaling pathways underlying this regulation.

To date, nitric oxide (NO) has been the most extensively studied signaling pathway in regards to modulating sympathetic □-adrenergic vasoconstriction in contracting skeletal muscle in humans and experimental animals. While one study in healthy humans has identified a potential role for NO in mediating functional sympatholysis (Chavoshan *et al.*, 2002), the majority of studies employing pharmacological inhibition of NO synthase, or direct NO donation (e.g. infusion of sodium nitroprusside), suggest little involvement of NO in modulating sympathetic vasoconstriction during exercise (Tschakovsky *et al.*, 2002; Rosenmeier *et al.*, 2003*b*; Dinenno & Joyner, 2003; Crecelius *et al.*, 2015*b*). Additionally, studies investigating the role of ATP-sensitive potassium channels (K_{ATP})(Keller *et al.*, 2004), and recent work from our laboratory employing combined blockade of NO, prostaglandins (PGs), and smooth muscle hyperpolarization via blockade of inwardly rectifying potassium (K_{IR}) channels and the sodium/potassium pump (Na^+/K^+-ATPase)(Crecelius *et al.*, 2015*b*), all demonstrate increased vasoconstrictor responsiveness at rest, while the exercise-induced attenuation of vasoconstriction remains intact. Thus, the mechanisms capable of blunting sympathetic vasoconstriction in contracting human skeletal muscle have been difficult to elucidate and remain unclear.

Mounting evidence from animal models suggests that the endothelium serves as a key site for the integration of both vasodilatory and vasoconstrictor signaling within resistance vasculature (Kerr *et al.*, 2012). Independent of the production of vasodilatory autacoids (e.g. NO

and PGs), endothelium-dependent vasodilators can induce hyperpolarization of the endothelium that spreads along the length of the vessel via gap junctions, and directly to vascular smooth muscle via myoendothelial gap junctions (Dora *et al.*, 2003). This endothelium-derived hyperpolarization (EDH) opposes sympathetic vasoconstriction in the hamster cremaster muscle and is suggested to play a role in functional sympatholysis (Kurjiaka & Segal, 1995). Therefore, we tested the hypothesis that augmenting endothelium-dependent vasodilatory signaling will enhance the ability of contracting skeletal muscle to blunt α_1-adrenergic vasoconstriction, whereas augmenting endothelium-independent vasodilatory signaling will not attenuate α_1-adrenergic responsiveness. Further, we hypothesized that any effect of endothelium-dependent vasodilatory signaling will be independent of NO and PG production and thus support a role for EDH in mediating functional sympatholysis in humans.

Methods

Ethical Approval and Subjects

With Institutional Review Board approval and after written informed consent, 42 young healthy adults (23 men, 19 women; age = 23±0.6 years; weight = 70±1.4 kg; height = 172±1.3 cm; body mass index = 24±0.4 kg/m^2; percent body fat = 26±1.5%; and MVC of 39±1.7 kg; means±S.E.M) participated in the present study. This study was approved by the Human Research Committee of Colorado State University and was performed according to the Declaration of Helsinki.

Arterial Catheterization, Blood Pressure and Heart Rate

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 administration of

pharmacological agents and measurement of MAP. Heart rate was monitored using a 3-lead ECG (Cardiocap/5; Datex-Ohmeda, Louisville, CO).

Forearm Blood Flow and Vascular Conductance

A 12 MHz linear-array ultrasound probe (Vivid 7, General Electric, Milwaukee, WI, USA) was used to measure brachial artery mean blood velocity (MBV) and brachial artery diameter proximal to the catheter site as previously described by our laboratory (Crecelius *et al.*, 2010). Brachial artery diameter measurements were made in triplicate in duplex mode at end diastole, and between contractions (for exercise trials) during steady-state conditions. Forearm blood flow (FBF) was calculated as described previously (Crecelius *et al.*, 2015*b*). As an index of forearm vascular tone, forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml $(min)^{-1}$ 100 $mmHg^{-1}$. Studies were performed in a cool temperature-controlled environment with a fan directed toward the forearm to minimize the contribution of skin blood flow to forearm haemodynamics (Kirby *et al.*, 2012; Crecelius *et al.*, 2013).

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. Exercise trials were performed with weight corresponding to 5 or 15% MVC (~15 and 40% maximum work rate) (Richards *et al.*, 2014) attached to a pulley system and lifted 4-5 cm 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. These workloads were chosen to represent both a mild intensity exercise (5% MVC) that alone does not blunt sympathetic vasoconstriction and a moderate intensity exercise (15% MVC) that significantly attenuates, but does not abolish sympathetic vasoconstriction (Tschakovsky *et al.*, 2002; Kirby *et al.*, 2005).

Sympathetic α_1-Adrenergic Vasoconstriction

In order to strictly control the vasoconstrictor stimulus across all conditions, phenylephrine (PE; selective α_1-agonist; Sandoz Inc., Princeton, NJ) was infused at 0.125 μg (dl forearm volume)$^{-1}$ min^{-1}. The dose of PE was chosen based on our previous experience in eliciting robust vasoconstriction at rest and during handgrip exercise and was adjusted for the steady-state hyperaemic condition as described previously (Dinenno & Joyner, 2003).

Experimental Protocols

General Experimental Protocol

Baseline measures were collected in the supine position for 2 minutes, after which, subjects began one of 4 experimental trials: (1) an intra-arterial infusion of a vasodilatory substance alone (either endothelium-dependent: acetylcholine (ACh; Miochol-E, Novartis, Basel, Switzerland), adenosine triphosphate (ATP; Sigma A7699, St Louis, MO, USA); or endothelium-independent: sodium nitroprusside (SNP; Hospira, Lake Forest, IL, USA), potassium chloride (KCl; Hospira, Lake Forest, IL, USA); see specific protocol below)), (2) a bout of dynamic handgrip exercise alone at either a mild (5% maximum voluntary contraction (MVC)) or (3) moderate (15%MVC) intensity, or (4) mild intensity handgrip exercise in combination with the vasodilator agent to augment specific vasodilatory signaling during exercise (Figure 1A). These workloads were chosen because mild intensity exercise alone does not blunt sympathetic vasoconstriction, whereas the moderate intensity exercise significantly attenuates, but does not abolish sympathetic vasoconstriction (Kirby *et al.*, 2008). Hyperaemic conditions were maintained for 6 minutes with steady-state blood flow measures made between minutes 5 and 6 of each trial. The dose of the α_1-agonist PE was calculated based on steady-state forearm blood flow (FBF; Doppler ultrasound) and forearm volume, as described previously (Tschakovsky *et al.*, 2002; Dinenno and Joyner 2003), and infused for the final 2 minutes of the trial (Figure 1B). Vasoconstrictor responses to PE were quantified as a percent

decrease in forearm vascular conductance (FVC = (FBF/MAP)*100) (Buckwalter & Clifford, 2001). The total time for each trial was 10 minutes followed by at least 15 minutes of quiet rest before the initiation of subsequent trials. The order of the hyperaemic conditions was counterbalanced across subjects.

Specific Experimental Protocols

Protocol 1: Augmentation of endothelium-dependent vasodilator signaling via ACh during α_1-adrenoceptor stimulation

Evidence from animal models suggests that ACh-mediated, endothelial-derived hyperpolarization can blunt sympathetic vasoconstriction(Kurjiaka & Segal, 1995). This protocol was designed to investigate the ability of ACh to modulate α_1-adrenergic vasoconstriction during exercise in humans. In 10 subjects (5 males, 5 females) vasoconstrictor responses to PE were assessed during (1) infusion of the endothelium-dependent vasodilator ACh alone, (2) during mild or moderate intensity exercise (5% and 15% MVC respectively), or (3) during mild intensity (5% MVC) exercise combined with ACh to augment endothelium-dependent vasodilatation during contractions. The steady-steady state blood flow response during control ACh infusion, and during the combined 5% exercise plus ACh infusion were intentionally matched to the hyperaemia observed during 15% exercise. ACh was initially infused at 8 μg (dl forearm volume)$^{-1}$ min^{-1} and the infusion rate was adjusted thereafter to reach the appropriate level of hyperaemia (final doses: control ACh: 12±4 μg (dl forearm volume)$^{-1}$ min^{-1}, and 5%+ACh: 4±2 μg (dl forearm volume)$^{-1}$ min^{-1}).

Protocol 2: Augmentation of endothelium-independent vasodilator signaling via SNP during α_1-adrenoceptor stimulation

To further investigate the ability of NO and endothelium-independent vasodilatation to blunt α_1-adrenergic vasoconstriction, vasoconstrictor responses to PE were assessed in 8

subjects (5 males, 3 females) during (1) infusion of the endothelium-independent vasodilator SNP alone, (2) during mild or moderate intensity exercise (5% and 15% MVC respectively), or (3) during mild intensity exercise combined with SNP to augment endothelium-independent vasodilatation during exercise. Similar to protocol 1, the steady-steady state blood flow response during control SNP infusion, and during the combined 5% exercise plus SNP infusion were intentionally matched to 15% exercise. SNP was initially infused at 4 μg (dl forearm volume)$^{-1}$ min^{-1}and the infusion rate was adjusted thereafter to achieve the desired FBF (final doses: control SNP: 4±1 μg (dl forearm volume)$^{-1}$ min^{-1}, and 5%+SNP: 2±1 μg (dl forearm volume)$^{-1}$ min^{-1}).

Protocol 3: Augmentation of endothelium-dependent vasodilator signaling via ATP during α_1-adrenoceptor stimulation

The endothelium-dependent vasodilator ATP is considered a physiological contributor to exercise hyperaemia (Crecelius *et al.*, 2015*a*). This protocol was designed to investigate the ability of a low dose of ATP to blunt sympathetic vasoconstriction during exercise. In 8 subjects (4 males, 4 females) vasoconstrictor responses to PE were assessed during (1) infusion of a low dose of ATP (1.282 μg (dl forearm volume)$^{-1}$ min^{-1}) alone, (2) during mild or moderate intensity exercise (5% and 15% MVC respectively), or (3) during mild intensity exercise combined with the same low dose of ATP to augment endothelium-dependent vasodilatory signaling during exercise. We have previously demonstrated that higher doses of ATP are capable of blunting sympathetic vasoconstriction (Kirby *et al.*, 2008), therefore an intentionally low dose of ATP was utilized throughout these trials and was held constant across all conditions. This dose of ATP has previously been shown to have little independent effect on PE-mediated vasoconstriction (Kirby *et al.*, 2008).

Protocol 4: Augmentation of endothelium-dependent hyperpolarization via ACh and combined NO and PG inhibition during α_1- adrenoceptor stimulation

The purpose of this protocol was to determine if the production of NO or PGs contribute to the ability of ACh to blunt α_1-adrenergic vasoconstriction during mild intensity exercise. Therefore, 10 subjects (6 males, 4 females) completed experimental trials similar to those described in protocol 1. Briefly, vasoconstrictor responses to PE were assessed during (1) ACh infusion alone, (2) 15% MVC exercise or (3) combined 5% exercise and ACh before and after co-administration of N^G-Monomethyl-L-arginine a NO synthase inhibitor (L-NMMA; Bachem, Weil am Rhein, Germany; loading dose: 25mg, Maintenance dose: 1.25 mg min^{-1}) and ketorolac a cyclooxygenase inhibitor (Hospira, Lake Forest, IL, USA; loading dose: 6 mg; maintenance dose: 150 µg min^{-1}) to inhibit the production of NO and vasodilating PGs, respectively (Crecelius *et al.*, 2010). The same doses of ACh that were used to match 15% exercise hyperaemia in control conditions were also used after blockade of NO and PGs (final doses: control ACh: 12±3 µg (dl forearm volume)$^{-1}$ min^{-1} and 5%+ACh: 4±1 µg (dl forearm volume)$^{-1}$ min^{-1}). The order of hyperaemic condition was counterbalanced across all subjects during control conditions and were conducted in the same order during blockade trials. Due to the length of this protocol and the clear lack of ability of mild exercise to blunt PE-mediated vasoconstriction (see Results), a 5% exercise condition alone was not performed.

Protocol 5: Augmentation of endothelium-independent hyperpolarization via KCl during α_1-adrenoceptor stimulation

In humans, a portion of ATP-mediated vasodilatation occurs through activation of K_{IR} channels and subsequent smooth muscle cell hyperpolarization (Crecelius *et al.*, 2012). To further clarify the role of K_{IR} channel and Na/K^+-ATPase activation (Dawes, 2002; Burns *et al.*, 2004) and smooth muscle cell hyperpolarization vs. endothelium-dependent hyperpolarization, KCl was administered as a direct endothelium-independent hyperpolarizing agent to determine

if smooth muscle cell hyperpolarization *per se,* in conjunction with mild intensity exercise would blunt α_1-adrenergic vasoconstriction. Therefore, in 6 subjects (3 males, 3 females) vasoconstrictor responses to PE were assessed during infusion of (1) KCl (0.2 mEq $(min)^{-1}$) alone, (2) during mild or moderate intensity exercise (5% and 15% MVC respectively), or (3) during mild intensity exercise combined with the same dose of KCl to augment endothelium-independent hyperpolarization during exercise. Due to concerns regarding subject safety and comfort, the dose of KCl was kept constant across conditions and was equal to the largest dose of KCl given by our lab previously without subject discomfort (Crecelius *et al.*, 2012). Trials utilizing a dose of ACh eliciting a similar vasodilatory response observed with KCl were also included to enable comparison to a similar dose of an endothelium-dependent vasodilator (control: 10±2 μg $(dl\ forearm\ volume)^{-1}\ min^{-1}$), 5%+ACh: 3±0.5 μg $(dl\ forearm\ volume)^{-1}\ min^{-1}$).

Data Acquisition and Analysis

Data were collected and stored on computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Baseline FBF, heart rate, and MAP represent an average of the last 30 seconds of the resting time period, the steady-state hyperaemia (prior to PE infusion; Pre PE), and the effects of the α_1-agonist (after 2 minutes of PE infusion; PE). We calculated percent reduction in FVC as our standard index to compare vasoconstrictor responses 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.(Buckwalter & Clifford, 2001) The percent reduction in FVC during vasoconstrictor administration was calculated as: ((FVC during PE - FVC Pre PE)/(FVC Pre PE)) x 100. In an effort to be comprehensive, we have also presented absolute values of forearm haemodynamics for all conditions in tabular form (Supplemental Tables 1-5).

Statistical Analysis

All values are reported as means ± SEM. Given the complexity of our experimental design, numerous comparisons are possible. We have focused our statistical approach on the comparisons most relevant to our experimental questions. Due to the predictably large increase in blood flow from rest to exercise and vasodilator infusion, resting haemodynamics were analysed separately by one-way repeated measures ANOVA (time point x trial). Comparison of absolute forearm haemodynamics were assessed by two-way (time point (pre-PE or PE) x trial) repeated measures ANOVA, inclusive of blockade condition where applicable (Protocol 4). Comparison of haemodynamic values at specific time points after blockade was made with paired t-tests. Vasoconstrictor responses (%Δ FVC) were compared with one-way repeated measures ANOVA (time point x trial) or two-way repeated measure ANOVA inclusive of blockade condition (Protocol 4). Systemic haemodynamics were evaluated by two-way repeated measures ANOVA inclusive of all time points (baseline, pre-PE, PE). In all cases, Student-Newman-Keuls post hoc pairwise comparisons were made with significance set a priori at $P<0.05$.

Results

Systemic haemodynamics during all experimental conditions for each protocol are presented in the Online Supplement (Supplemental Tables 1-5). Small increases in MAP and heart rate occurred with exercise and throughout the course of the experiment.

Protocol 1: Augmentation of endothelium-dependent vasodilator signaling via ACh during α_1-adrenoceptor stimulation

As intended by experimental design, ACh infusion, handgrip exercise performed at 15% MVC, and the combination of 5% MVC exercise + ACh evoked similar levels of FVC (pre PE) that were not significantly different from each other ($P>0.05$; Figure 2A). Mild intensity exercise

(5% MVC) evoked a significant increase in FBF that was smaller in magnitude compared to the other trials. Direct stimulation of α_1-adrenoceptors with PE during ACh alone and during 5% MVC handgrip exercise induced robust vasoconstriction (ΔFVC = -34±4 and -33±3%, respectively) that was blunted during 15% MVC handgrip exercise (ΔFVC = -13±2%; P<0.05). Remarkably, when 5% handgrip exercise was combined with ACh infusion to increase endothelium-dependent vasodilatation during exercise, while neither condition alone blunted PE-mediated vasoconstriction, the combination abolished the vasoconstrictor response to PE (ΔFVC = -3±4%; Figure 2B).

Protocol 2: Augmentation of endothelium-independent vasodilator signaling via SNP during α_1-adrenoceptor stimulation

As intended, infusion of SNP, 15% handgrip exercise, and the combination of 5% exercise + SNP evoked levels of steady-state FVC (pre PE) that were not significantly different from each other (P>0.05; Figure 3A). Mild intensity exercise (5% MVC) caused a significant increase FVC that was smaller in magnitude compared to the other trials. Infusion of PE during SNP alone and during 5% handgrip exercise induced robust vasoconstriction (ΔFVC = -35±3 and -33±3% respectively) that was significantly blunted during 15% handgrip exercise (ΔFVC = -15±3%, P<0.05). In contrast to results from protocol 1 utilizing ACh, combined 5% exercise + SNP infusion had no effect on PE-mediated vasoconstriction (ΔFVC = -34±6%; Figure 3B).

Protocol 3: Augmentation of endothelium-dependent vasodilator signaling via ATP during α_1-adrenoceptor stimulation

Infusion of ATP alone elicited a significant increase in FVC from rest that was smaller in magnitude than 15% MVC exercise (P<0.05; Figure 4A). In contrast to previous protocols the dose of ATP was not adjusted to match the hyperaemia observed during 15% MVC because higher doses of ATP are independently sympatholytic and thus would confound the

interpretation of the data. Therefore, the same low dose of ATP was used in both the control ATP infusion and the 5% MVC + ATP conditions. Infusion of PE induced a robust vasoconstriction during both control ATP infusion and 5% MVC exercise (ΔFVC = -30±3 and -31±1%, respectively) that was blunted during 15% MVC exercise (ΔFVC = -17±3%; P<0.05). When 5% MVC exercise was performed in combination with low dose ATP infusion the vasoconstrictor response to PE was significantly attenuated (ΔFVC = -18±4%; P<0.05 vs ATP alone) and was not different from the vasoconstrictor response observed during 15% MVC exercise (P=0.83; Figure 4B).

Protocol 4: Augmentation of endothelium-dependent hyperpolarization via ACh and combined NO and PG inhibition during α_1- adrenoceptor stimulation

Steady-state FVC was matched across control conditions (P>0.05; Figure 5A). Consistent with findings in protocol 1, infusion of PE significantly reduced FVC during ACh infusion alone (ΔFVC = -30±3%), an effect that was blunted during 15% MVC exercise (ΔFVC = -14±2%; P<0.05) and during combined 5% MVC exercise and ACh infusion (ΔFVC: -10±3%; P<0.05; Figure 5B). Blockade of NO and PG production reduced resting FBF and FVC in all conditions as well as steady-state FVC during control ACh infusion (P<0.05; Figure 5A), consistent with effective blockade of ACh-mediated dilatation. Blockade of NO and PGs did not significantly blunt FBF or FVC responses during 15% MVC exercise consistent with previous findings from our laboratory and others (Schrage *et al.*, 2004; Crecelius *et al.*, 2015*b*). Importantly, vasoconstrictor responses to PE after blockade were attenuated in all conditions relative to the vasoconstrictor response observed during ACh at rest prior to blockade of NO and PGs, (ΔFVC post blockade = ACh: -20±5%; 15% MVC: -15±5%; 5%MVC+ACH: -8±4%; all P<0.05 vs ACh prior to blockade; Figure 5B). Further, there was no effect of blockade on vasoconstrictor responses to PE during 15% MVC exercise or 5%MVC exercise + ACh (P>0.05 relative to respective control condition; Figure 5B).

Protocol 5: Augmentation of endothelium-independent hyperpolarization via KCl during α_1-adrenoceptor stimulation

Infusion of KCl resulted in a significant increase in FVC that was smaller in magnitude than that observed during 5 and15% MVC exercise ($P<0.05$; Figure 6A). In the interest of subject safety and comfort the dose of KCl was not increased beyond the dose given during the control condition. ACh, infused at a dose to match the hyperaemia observed during KCl alone and when combined with 5% MVC exercise, was utilized as a control to enable comparison of KCl responses to a similarly low dose of an endothelium-dependent vasodilator. As intended, there was no significant difference in FVC during KCl and ACh conditions at rest or when combined with 5% MVC exercise ($P>0.05$; Figure 6A). PE-mediated vasoconstriction was significantly augmented during KCl infusion relative to that observed during control ACh infusion (Δ FVC: -46±7 and -27±2% respectively; $P<0.05$; Figure 6B). As observed in previous protocols, PE-mediated vasoconstriction was similar between ACh and 5% MVC exercise (ΔFVC = -27±2 and -24±2% respectively; $P>0.05$) and was significantly attenuated during 15% MVC exercise (ΔFVC = -7±1%; $P<0.05$ vs ACh; Figure 6B). Additionally, combined 5% MVC exercise + ACh significantly blunted PE-mediated vasoconstriction (ΔFVC = -9±3%; $P<0.05$ vs ACh). In contrast, combined 5% MVC exercise + KCl did not impact PE-mediated vasoconstriction relative to KCl alone (ΔFVC = -43±3%; Figure 6B) and remained greater than all ACh and 5% MVC exercise conditions.

Discussion

The primary novel finding from this study is that augmenting endothelium-dependent vasodilatory signaling blunts α_1-adrenergic vasoconstriction in contracting human skeletal muscle. In direct contrast, augmenting endothelium-independent vasodilatory signaling does not modulate α_1-adrenergic vasoconstriction during exercise. To further elucidate the mechanisms underlying these observations, we performed follow-up studies utilizing ACh in combination with

NO and PG inhibition to isolate EDH, and KCl to isolate smooth muscle hyperpolarization. The findings from these experiments clearly indicate that augmenting EDH in contracting muscle blunts α_1-adrenergic vasoconstriction, whereas augmenting direct SMC hyperpolarization in resting or active skeletal muscle does not limit α-mediated vasoconstriction. Together, these findings specifically highlight the endothelium as a critical site for the integration of vasodilatory and vasoconstrictor signaling and further supports EDH as a primary signaling mechanism capable of modulating α-adrenergic vasoconstriction in human skeletal muscle. The present findings lend insight into the basic mechanisms of functional sympatholysis and have important implications for both primary ageing and clinical populations (hypertension, heart failure) where inefficient functional sympatholysis may be an important contributor to impaired blood pressure regulation and exercise intolerance.

Mechanisms of Functional Sympatholysis in Humans

The ability of contracting skeletal muscle to blunt sympathetic vasoconstriction is critical to ensure proper blood flow and oxygen delivery to contracting skeletal muscle. While the phenomenon of functional sympatholysis has been extensively studied in both animal models and humans, the underlying mechanisms remain unclear. Studies in healthy humans have been performed to determine the role of the autocoids NO and PGs, both independently and in combination, as well as K_{ATP} channels. In general, the majority of the data indicate that none of these pathways are obligatory for functional sympatholysis in humans (Tschakovsky *et al.*, 2002; Rosenmeier *et al.*, 2003*b*; Dinenno & Joyner, 2003; Crecelius *et al.*, 2015*b*; Nyberg *et al.*, 2015). In some studies, there is an augmented sympathetic vasoconstriction observed both at rest and during exercise with administration of pharmacological inhibitors of the respective pathway, however the ability of muscle contractions to modulate this vasoconstriction remains intact (Keller *et al.*, 2004; Dinenno & Joyner, 2004).

Recently, our laboratory attempted the most comprehensive pharmacological approach to inhibit functional sympatholysis in humans to date (Crecelius *et al.*, 2015*b*). Using a strategy similar to previous studies, vasoconstriction to intra-arterial infusion of PE was assessed during handgrip exercise and control adenosine infusion, before and after pharmacological inhibition of multiple putative vasodilatory pathways. In addition to blockade of the vasodilatory autacoids NO and PGs, pathways involved in smooth muscle cell hyperpolarization were inhibited by local infusions of barium chloride and ouabain to inhibit K_{IR} channels and Na^+/K^+-ATPase, respectively. Importantly, this potent combination of pharmacological inhibitors attenuates exercise hyperaemia by ~40-45%(Crecelius *et al.*, 2014, 2015*b*), and reduces reactive hyperaemia by ~90% (Crecelius *et al.*, 2013). However, contrary to our hypothesis, despite significant augmentation of PE-mediated vasoconstriction in resting skeletal muscle, there was absolutely no effect of the combined blockade on PE-mediated vasoconstriction during exercise. Thus, the majority of studies to date utilizing extensive pharmacological blockade have failed to identify the local factors or signaling mechanisms that contribute to functional sympatholysis in humans.

With these collective observations in mind, we adopted an alternate approach in the present study to identify the vascular pathways capable of mediating sympatholysis in humans. Instead of utilizing pharmacological antagonists to inhibit the ability of skeletal muscle to blunt α-adrenergic vasoconstriction, various agonists were administered in an attempt to increase the ability of contracting skeletal muscle to blunt vasoconstriction. Subjects performed mild intensity exercise (5% MVC; ~15% maximum work rate), which on its own does not blunt sympathetic vasoconstriction, combined with infusion of vasodilators that signal through distinct pathways in an attempt to identify those capable of increasing the ability of contracting skeletal muscle to blunt α-adrenergic vasoconstriction. We utilized specific endothelium-dependent and -independent agonists to investigate the potential of these dichotomous pathways to modulate α-adrenergic vasoconstriction in contracting skeletal muscle.

Endothelium-dependent vs -independent vasodilatation: impact on α_1-adrenergic vasoconstriction

In the first set of experiments, ACh was administered intra-arterially during control resting conditions to mimic the hyperaemia observed during moderate intensity exercise (15% MVC), and during mild intensity (5%MVC) exercise in order to augment endothelium-dependent vasodilatory signaling in contracting muscle. The vasoconstrictor responses to PE during ACh at rest, and during mild intensity exercise alone were robust, and similar in magnitude to what we have shown previously during control vasodilator infusions of adenosine in the human forearm (Kirby *et al.*, 2008; Crecelius *et al.*, 2015*b*). As expected the vasoconstrictor responses to PE were significantly blunted during moderate intensity exercise (Figure 2B). Remarkably, while neither ACh nor mild intensity exercise alone were capable of blunting PE-mediated vasoconstriction (ΔFVC ~35%), the combination of these two stimuli effectively abolished the vasoconstrictor response to PE (ΔFVC~3%), an effect that was greater than that observed during moderate intensity exercise (ΔFVC ~13%). In stark contrast, when this same experiment was performed using the endothelium-independent vasodilator SNP (protocol 2), there was no change in responsiveness to PE when mild exercise was combined with infusion of SNP (Figure 3B). These results demonstrate that the effect of ACh on α_1-adrenergic vasoconstriction during exercise is due to the specific vasodilatory pathways through which ACh signals and not simply an artifact of elevating general vasodilatory signaling or blood flow during exercise.

Despite the widespread use of ACh as an endothelium-dependent vasodilator, there are questions regarding the physiological role of ACh as a vasodilator in humans, particularly as it relates to the control of skeletal muscle vascular tone during exercise. Thus, we performed follow-up studies utilizing ATP infusions (protocol 3). ATP-mediated vasodilatation is endothelium-dependent, and intravascular ATP is thought to play an important role in modulating vascular tone in active and hypoxic skeletal muscle (Crecelius *et al.*, 2015*a*). In humans, we have previously demonstrated that infusion of low doses of ATP have no effect on

α-adrenergic vasoconstriction, however, as the dose of ATP increases there is a concomitant graded increase in the ability of ATP to blunt α-adrenergic vasoconstriction in resting skeletal muscle (Kirby *et al.*, 2008). This graded attenuation of vasoconstriction is reminiscent of the graded nature of functional sympatholysis (Buckwalter *et al.*, 2001; Tschakovsky *et al.*, 2002). Thus, in protocol 3, a low dose of ATP was used to test the hypothesis that increasing endothelium-dependent vasodilatory signaling during mild exercise via ATP augments the ability of contracting skeletal muscle to blunt α_1-adrenergic mediated vasoconstriction. Similar to protocol 1, PE-mediated vasoconstriction was robust during low dose ATP infusion similar to mild intensity exercise alone, and this response was significantly blunted during moderate intensity exercise (Figure 4B). Further, the addition of ATP during mild intensity exercise substantially blunted PE-mediated vasoconstriction and was similar to that observed during moderate intensity exercise. Thus, the collective findings from Protocols 1-3 clearly indicate that augmenting endothelium-dependent signaling (via ACh or ATP) during mild skeletal muscle contractions enhances the ability of the vasculature to blunt sympathetic vasoconstriction.

Endothelium-dependent vs -independent hyperpolarization: impact on α_1-adrenergic vasoconstriction

We next sought to determine the mechanisms underlying these observations. Endothelium-dependent vasodilatation, independent of NO and PG production, occurs primarily through hyperpolarization of the endothelium. Once initiated, EDH spreads along the endothelial cell layer through homocellular gap junctions, as well as directly to vascular smooth muscle cells through myoendothelial gap junctions, resulting in a conducted vasodilatory response (Emerson & Segal, 2000). In Protocol 4, we repeated similar experiments as in Protocol 1 with ACh before and after combined inhibition of NO and PGs to isolate EDH. Consistent with the previous observations, robust vasoconstriction to PE was observed during ACh infusions in resting muscle, and the vasoconstrictor responses were blunted during moderate intensity exercise and

the combination of ACh + mild intensity exercise. Interestingly, after inhibition of NO and PGs, ACh infused alone to resting skeletal muscle attenuated the vasoconstrictor response to PE by ~10% compared with control conditions. This is in direct contrast to studies where inhibition of NO and PGs augments PE-mediated vasoconstriction during adenosine infusion (Dinenno & Joyner, 2004). Importantly, the ability of moderate intensity exercise and the combination of ACh + mild exercise to blunt sympathetic vasoconstriction persisted during combined NO and PG inhibition, strongly implicating a role for EDH in modulating α_1-mediated vasoconstriction in active muscle.

In order to determine whether these observations were due to EDH or simply hyperpolarization of vascular smooth muscle cells, we conducted the same set of experiments as previously described using KCl. Infusion of KCl elevates interstitial [K^+] resulting in activation of Na^+/K^+-ATPase and K_{IR} channels and subsequent direct (i.e. endothelium-independent) smooth muscle cell hyperpolarization. In skeletal uscle, KCl-mediated vasodilatation is independent of the endothelium (Knot *et al.*, 1996; De Clerck *et al.*, 2003; Burns *et al.*, 2004), and in humans, is abolished by inhibition of Na^+/K^+-ATPase and K_{IR} channels via ouabain and barium chloride, respectively, confirming the selective action of KCl on these channels.(Dawes, 2002; Crecelius *et al.*, 2012) In protocol 5, we found that the vasoconstrictor responsiveness to PE in resting skeletal muscle was augmented in the presence of KCl relative to that observed during ACh alone. Importantly, this augmentation persisted during combined mild exercise and KCl, thus clearly demonstrating that increasing endothelium-independent hyperpolarization has no impact on the ability of contracting skeletal muscle to blunt sympathetic vasoconstriction. These findings are in agreement with recent findings from our laboratory demonstrating that blockade of K_{IR} and Na^+/K^+-ATPase during exercise does not impair the ability of contracting skeletal muscle to blunt PE-mediated vasoconstriction.(Crecelius *et al.*, 2015*b*) While the mechanism by which α_1-adrenergic vasoconstriction is potentiated in the presence of KCl is unclear, it is conceivable that hyperpolarization of vascular smooth muscle membrane potential

could increase the electrical driving force for calcium entry into the smooth muscle cell in response to PE, resulting in a greater vasoconstrictor stimulus. In support of this notion, our lab has previously shown that inhibition of K_{IR} channels (via the divalent cation barium chloride) also potentiates PE-mediated vasoconstriction. It is unlikely that the relatively low dose of KCl was a primary contributor to this phenomenon as normal vasoconstrictor responsiveness to PE was observed during an equally low dose of ACh.

Despite the lower dose of ACh used in the KCl protocol compared to protocols 1 and 2, it is important to note that there was still a pronounced attenuation of PE-mediated vasoconstriction when combined with 5% exercise, further distinguishing endothelium-dependent signaling as a potent modulator of □-adrenergic vasoconstriction. Additionally, this set of studies most clearly dissociates the magnitude of vasodilatation *per se* from the ability to blunt sympathetic vasoconstriction. Despite the fact that the same level of vasodilatory signaling in both KCl and ACh conditions was combined with the same vasoconstrictor stimulus (PE doses: 1.27±.004 and 1.22±.003 μg/dl FAV/min respectively; P=0.45), the integration of these vasomotor signals resulted in divergent responses to PE (responsiveness) and net vascular tone (FVC: 67±7 and 103±11 respectively; P<0.05; see supplemental Table 5). As such, it is important to recognize that the regulation of vascular tone reflects more than a mere balance of vasodilatory and vasoconstrictor activity, and further, the endothelium appears to be a major site for the integration of these signals during exercise.

Potential Mechanisms

While this series of investigations highlights an important interaction between endothelium-dependent vasodilatory signaling and sympathetic vasoconstriction in humans, the specific signals originating from the endothelium that result in sympatholysis remains unclear. However, a few similarities between ACh- and ATP-mediated signaling warrant discussion as potential underlying mechanisms. Both ACh and ATP activate $G_{q/11}$ protein coupled receptors

located on the endothelium resulting in subsequent activation of K_{Ca} channels (Winter & Dora, 2007; Wölfle *et al.*, 2009). In animal models, activation of small- and intermediate-calcium-activated K^+ channels (sK_{Ca} and iK_{Ca}) are the primary signal underlying EDH in response to ACh and ATP (Marrelli *et al.*, 2003; Crane *et al.*, 2003; Winter & Dora, 2007), and also contribute to contraction-induced dilatation (Milkau *et al.*, 2010). Further, IK_{Ca} channels localized within myoendothelial gap junctions are in unique position to modulate smooth muscle cell contractility and have been identified as a crucial component of myoendothelial feedback whereby endothelium-dependent signaling through gap junctions limits □-adrenergic vasoconstriction (Tran *et al.*, 2012). The results from this study, in conjunction with accumulating evidence in animal models, points to a critical role for activation of K_{Ca} channels and subsequent endothelium-dependent hyperpolarization in mediating both the normal vasodilatory response, and potentially the opposition of sympathetic vasoconstriction, observed in contracting skeletal muscle.

Experimental considerations

In order to isolate the contribution of local signaling mechanisms to skeletal muscle blood flow control, subjects performed mild-to-moderate dynamic handgrip exercise which elicits local metabolic vasodilatation without major changes in central haemodynamics. To more directly investigate post-junctional signaling within the vasculature, PE (α_1-adrenergic agonist) was infused to simulate sympathetic vasoconstriction. In contrast to tyramine which induces endogenous NA release or α_2-adrenergic agonists which have pre-junctional effects on NA release, PE can be used to isolate post-junctional signaling in a highly controlled manner. While recruitment of the sympathetic nervous system during exercise results in the release of a number of neurotransmitters including NE, neuropeptide Y and ATP(Holwerda *et al.*, 2014), it is thought that NA is the primary neurotransmitter involved in exercise-induced sympathetic vasoconstriction (Buckwalter & Clifford, 1999). Further, handgrip exercise blunts both post-

junctional α_1- and α_2-adrenergic vasoconstriction similarly in humans.(Rosenmeier *et al.*, 2003*a*) Therefore, we do not feel that the use of PE exclusively in this study limits the interpretation of our data as it pertains to functional sympatholysis.

While combined blockade of NO and PG production using L-NMMA and ketorolac, respectively, did not reduce on the ability of ACh to blunt vasoconstriction in contracting skeletal muscle, both resting FBF and the hyperaemic response to ACh were significantly reduced by approximately ~35%, indicating effective inhibition of NO and PG production.(Dinenno & Joyner, 2003) Additionally, blockade of NO and PGs significantly enhanced the ability of ACh alone to blunt α_1-adrenergic vasoconstriction, potentially demonstrating greater reliance on vasodilatory pathways that are resistant α-adrenergic vasoconstriction. Taken together, we utilized standard doses of L-NMMA and ketorolac that have previously been shown to be effective in attenuating NO and PG production in humans and observed effects on haemodynamics at rest, and during both vasodilator and vasoconstrictor stimuli. Therefore, lack of inhibitor effectiveness cannot explain the present findings.

Where possible vasodilators were administered to match flows observed during moderate 15% MVC handgrip exercise. However, as described in the methods section, the doses of KCl and ATP were intentionally limited and as such did not reach the hyperaemic levels observed during 15% MVC exercise. We do not believe this impacts the interpretation of our data for two primary reasons. First, the magnitude of blood flow or shear stress *per se* was shown previously to have no effect on α-adrenergic vasoconstriction (Rosenmeier *et al.*, 2003*b*). Second, ACh served as a flow control for KCl demonstrating that the differential effect on vasoconstriction observed between these two vasodilators was due to their respective mechanism of action rather than simply an effect of different levels of vasodilatory signaling.

Perspectives

Age-associated cardiovascular disease is often characterized by exaggerated sympathetic nervous system activity coupled with impaired vasodilatory responses to

exercise.(Proctor *et al.*, 1998; Richards *et al*., 2014). Impaired functional sympatholysis is a common feature of age-associated cardiovascular disease including hypertension (Vongpatanasin *et al.*, 2011) and chronic myocardial infarction (Thomas *et al.*, 2001), and may represent a primary contributor to exercise intolerance in these populations (Saltin & Mortensen, 2012). Therefore, identifying the vascular signaling pathways responsible for limiting sympathetic vasoconstriction, both at rest and during exercise, is of clear importance. The findings from the current study clearly indicate that not all vasodilatory pathways are equal in their ability to blunt sympathetic vasoconstriction. In fact, endothelium-dependent vasodilatory signaling, independent of NO and PG production, has a clear and remarkable ability to modulate α_1-adrenergic vasoconstriction during muscle contractions. Interestingly, responsiveness to endothelium-dependent agonists that rely primarily on hyperpolarization (ATP, bradykinin) have been demonstrated to be intact in ageing humans (DeSouza *et al.*, 2002; Kirby *et al.*, 2010). Thus, EDH represents a vasoactive pathway with preserved responsiveness in older adults, and the dual ability to cause vasodilatation while simultaneously blunting adrenergic vasoconstriction. Increasing the availability of endothelium-dependent vasodilators that work via EDH, such as ATP, during exercise may be an effective strategy for the treatment of age-associated peripheral vascular dysfunction (Kirby *et al.*, 2012).

Conclusions

Functional sympatholysis is an important component of the integrative response to exercise that ensures proper perfusion of contracting skeletal muscle in the face of elevated sympathetic nervous system activity. This study supports findings in animal models that EDH is capable of blunting α-adrenergic vasoconstriction and is potentially the key signaling event underlying functional sympatholysis in humans. Further, a small elevation in endothelium-dependent signaling during muscle contraction has a profound ability to blunt α_1-adrenergic vasoconstriction. This finding highlights the endothelium as a critical and potent regulator of α-

adrenergic tone in humans, particularly in active skeletal muscle. Altogether, these studies provide novel insight into the basic mechanisms that contribute to functional sympatholysis in humans and identify EDH as a unique target for the improvement of blood flow, oxygen delivery, and potentially exercise intolerance in conditions associated with elevated sympathetic nervous system activity.

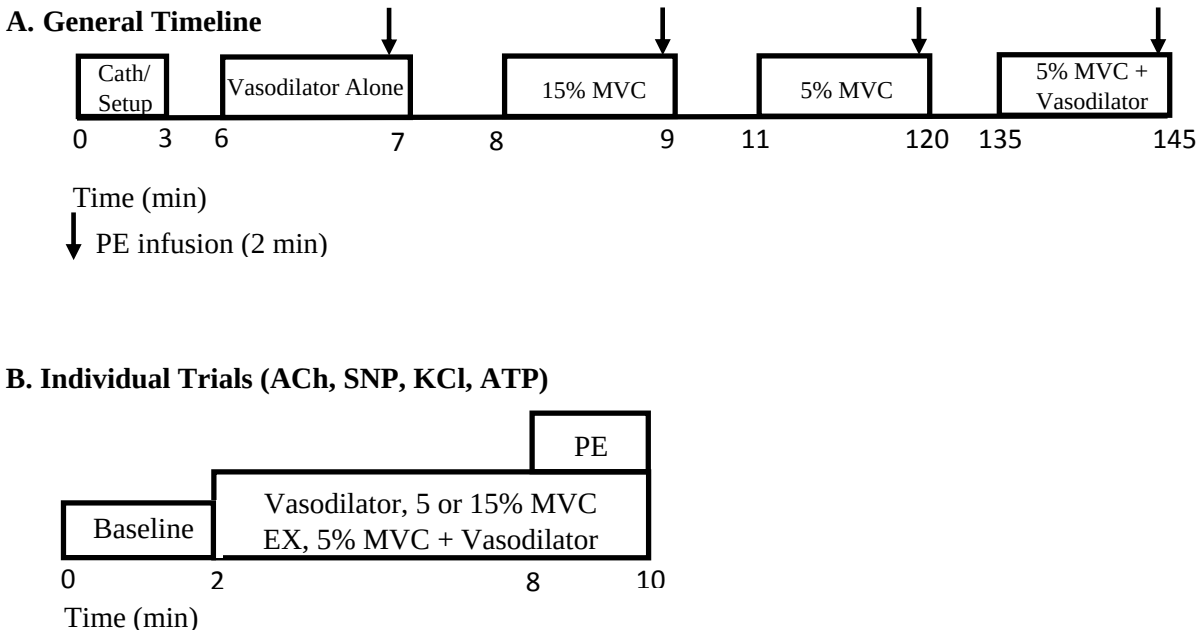

Figure 1. Experimental Protocol

A. After catheterization of the brachial artery (cath) and instrumentation, phenylephrine (PE) responsiveness was assessed during four different hyperaemic conditions. **B.** After baseline measurements, steady-state hyperaemia was achieved by infusion of a vasodilator alone (acetylcholine (ACh), sodium nitroprusside (SNP), adenosine triphosphate (ATP), or potassium chloride (KCl)), exercise alone (5 or 15% maximal voluntary contraction (MVC)) or combined 5% MVC exercise and vasodilator infusion to augment vasodilatory signaling during mild intensity muscle contractions. PE responsiveness was assessed in the final 2 minutes of each trial and quantified as a percent decrease in forearm vascular conductance (FVC).

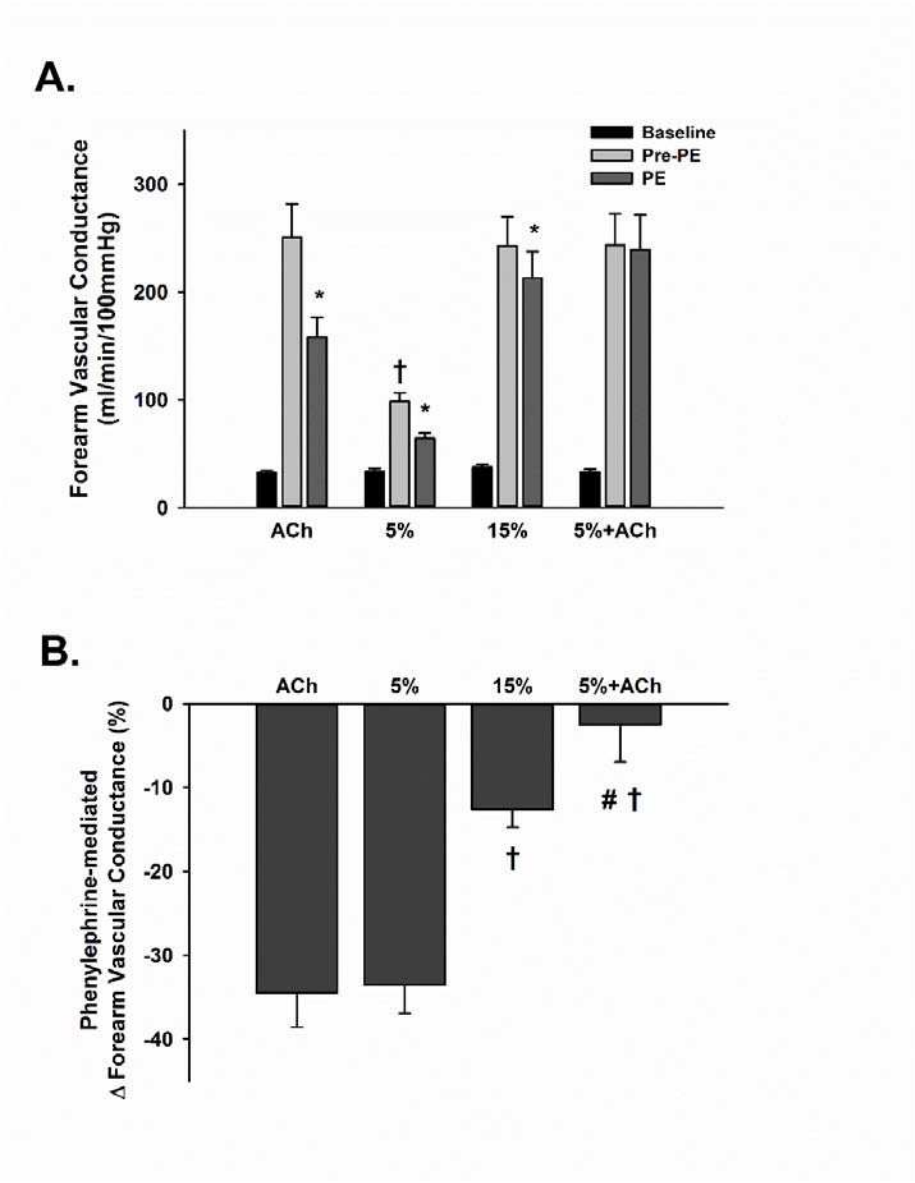

Figure 2. Acetylcholine (ACh) attenuates α_1-adrenergic vasoconstriction in contracting skeletal muscle

A. Forearm vascular conductance (FVC) is presented at baseline, steady-state hyperaemia prior to infusion of phenylephrine (PE; Pre-PE), and at the end of 2 minutes of PE infusion (PE). PE significantly reduced FVC during ACh infusion alone, and during 5 and 15% MVC handgrip exercise. **B.** PE-mediated vasoconstriction was similar during infusion of ACh alone and during 5% handgrip exercise. As expected, 15% handgrip exercise significantly attenuated PE-mediated vasoconstriction. Infusion of ACh during 5% exercise (5%+ACh) to augment endothelium-dependent signaling during exercise significantly attenuated PE-mediated vasoconstriction. *P<0.05 vs. Pre-PE; †P<0.05 vs. ACh; #P<0.05 vs. 15%.

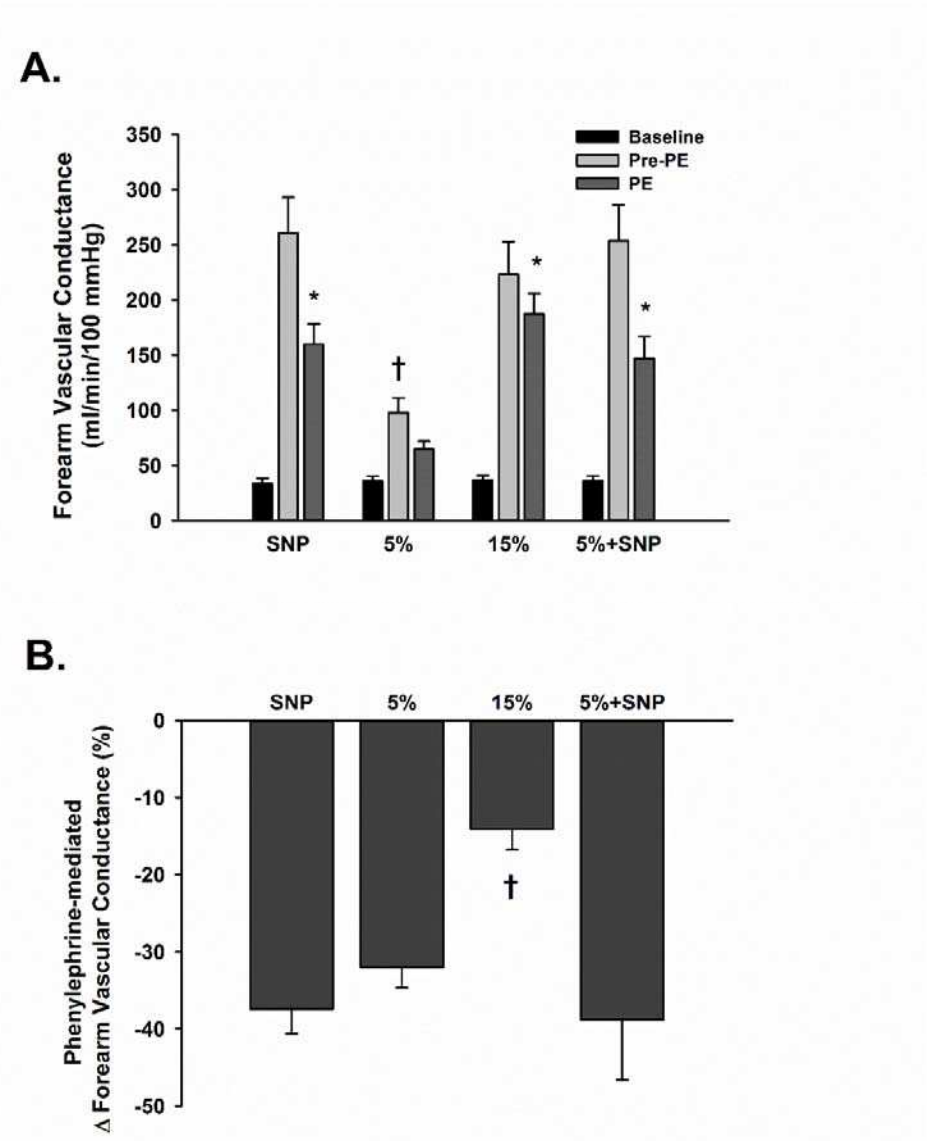

Figure 3. Sodium nitroprusside (SNP) does not attenuate α_1-adrenergic vasoconstriction in contracting skeletal muscle

A. Phenylephrine (PE) significantly reduced forearm vascular conductance (FVC) in all conditions. **B.** PE-mediated vasoconstriction was similar during infusion of SNP alone and during 5% MVC exercise. As expected 15% MVC exercise significantly attenuated PE-mediated vasoconstriction. In contrast to protocol 1, infusion of SNP during 5% exercise (5%+ACh) to augment endothelium-independent signaling during exercise did not attenuate PE-mediated vasoconstriction. *P<0.05 vs. Pre-PE; †P<0.05 vs. SNP.

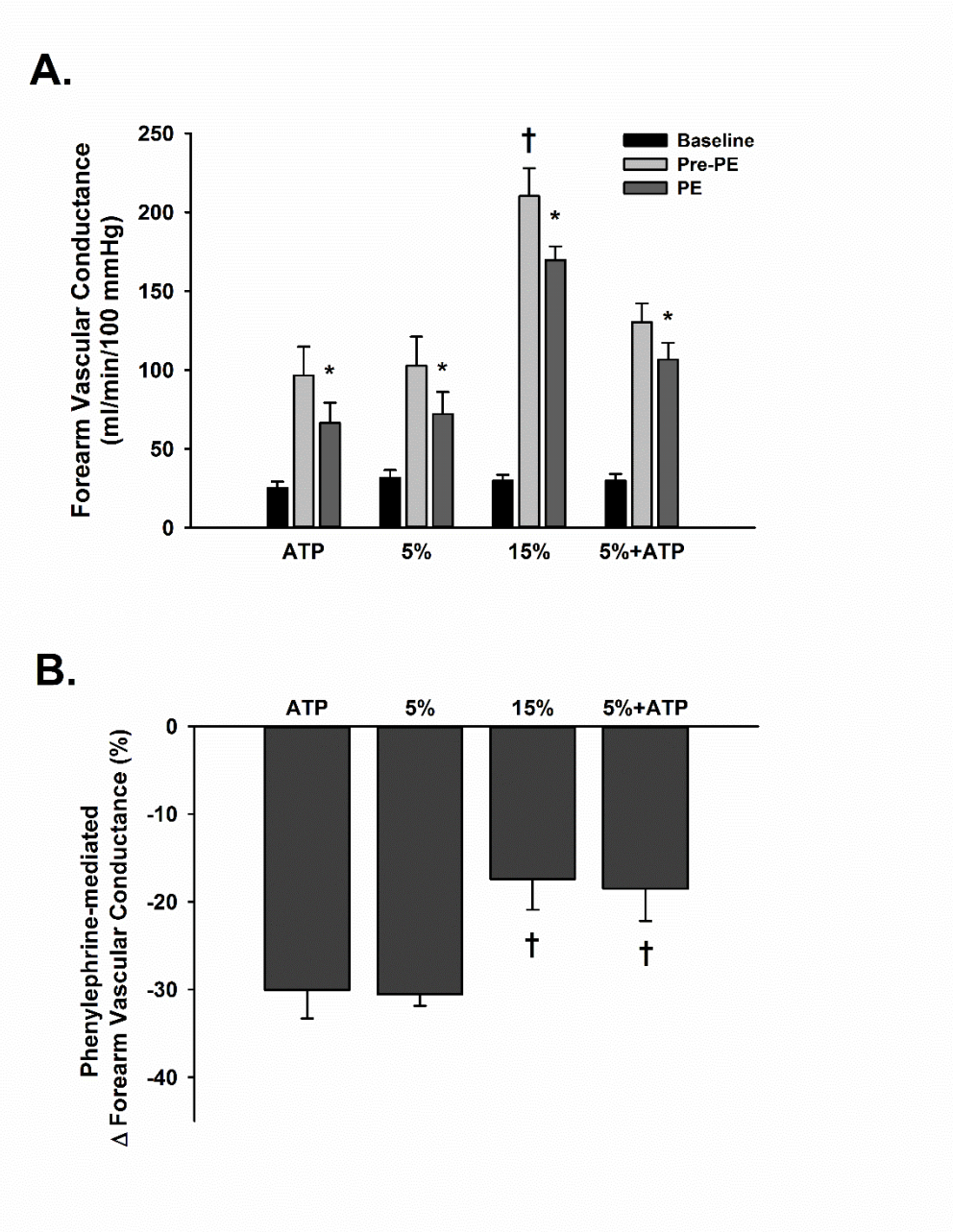

Figure 4. Adenosine triphosphate (ATP) attenuates α_1-adrenergic vasoconstriction in contracting skeletal muscle

A. Phenylephrine (PE) significantly reduced forearm vascular conductance (FVC) in all conditions. **B.** PE-mediated vasoconstriction was similar during infusion of ATP alone and during 5% MVC handgrip exercise. As expected, 15% MVC exercise significantly attenuated PE-mediated vasoconstriction. Infusion of ATP during 5% exercise (5%+ATP) to augment endothelium-dependent signaling during exercise significantly attenuated PE-mediated vasoconstriction. *P<0.05 vs. Pre-PE; †P<0.05 vs. ATP.

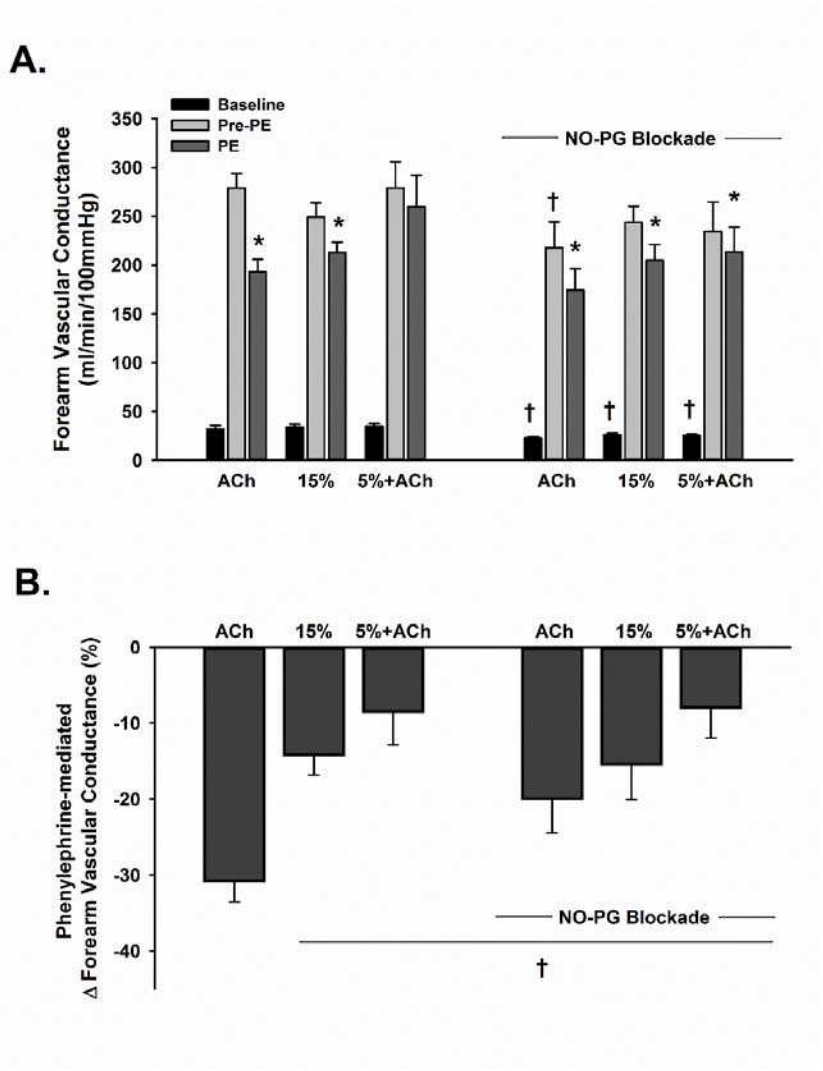

Figure 5. Endothelium-dependent hyperpolarization (EDH) attenuates α_1-adrenergic vasoconstriction

A. Similar to Protocol 1, Phenylephrine (PE) significantly reduced forearm vascular conductance (FVC) during infusion of acetylcholine (ACh) alone and during 15% exercise. Inhibition of nitric oxide (NO) and prostaglandin (PG) synthesis significantly reduced FVC at baseline and in response to ACh infusion. After blockade, PE significantly reduced FVC in all conditions. **B.** As shown in the left panel, PE-mediated vasoconstriction was significantly attenuated during 15% exercise and combined 5% exercise + ACh infusion compared with Ach alone. After blockade of NO and PG synthesis (to isolate EDH), PE-mediated vasoconstriction was attenuated during infusion of Ach at rest, and remained attenuated during both 15% exercise and combined 5%+ACh infusion. *P<0.05 vs. Pre-PE; †P<0.05 vs. control ACh.

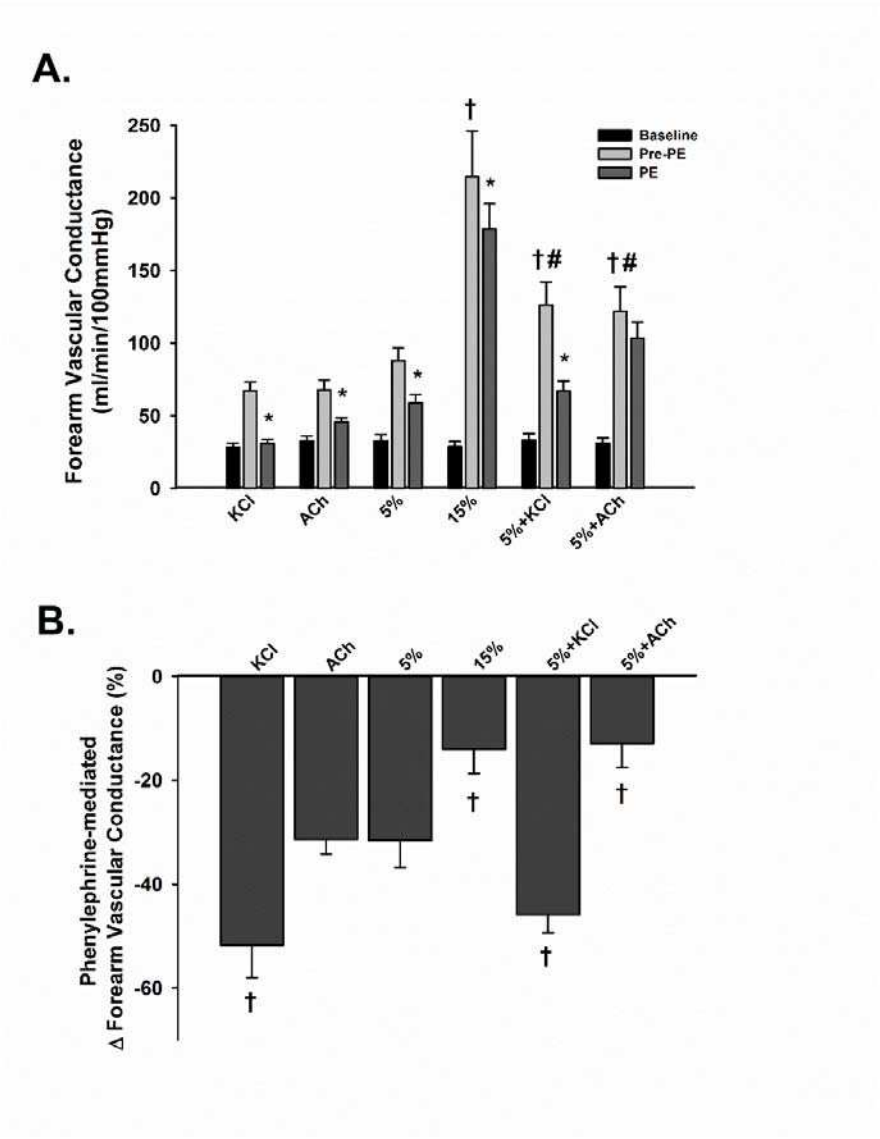

Figure 6. Endothelium-independent hyperpolarization does not attenuate α_1-adrenergic vasoconstriction

A. As intended, steady-state FVC during acetylcholine (ACh) trials was matched with potassium chloride (KCl) trials (Pre-PE). Phenylephrine (PE) reduced forearm vascular conductance (FVC) in all conditions except combined 5%+ACh. **B.** Similar to protocol 1, PE-mediated vasoconstriction was similar during infusion of ACh aloneand during 5% exercise, and was attenuated during 15% exercise and combined 5%+ACh infusion. PE-mediated vasoconstriction was greater during infusion of KCl alone compared with ACh. During combined 5% exercise and KCl infusion (5%+KCl) to augment endothelium-independent hyperpolarization during exercise, PE-mediated vasoconstriction remained significantly greater. *P<0.05 vs. Pre-PE; †P<0.05 vs. ACh; #P<0.05 vs. 15%.

REFERENCES

Buckwalter JB & Clifford PS (1999). alpha-adrenergic vasoconstriction in active skeletal muscles during dynamic exercise. *Am J Physiol* **277,** H33–H39.

Buckwalter JB & Clifford PS (2001). The paradox of sympathetic vasoconstriction in exercising skeletal muscle. *Exerc Sport Sci Rev* **29,** 159–163.

Buckwalter JB, Naik JS, Valic Z & Clifford PS (2001). Exercise attenuates {alpha}-adrenergic-receptor responsiveness in skeletal muscle vasculature. *J Appl Physiol* **90,** 172–178.

Burns WR, Cohen KD & Jackson WF (2004). K+-induced dilation of hamster cremasteric arterioles involves both the Na+/K+-ATPase and inward-rectifier K+ channels. *Microcirculation* **11,** 279–293.

Chavoshan B, Sander M, Sybert TE, Hansen J, Victor RG & Thomas GD (2002). Nitric oxide-dependent modulation of sympathetic neural control of oxygenation in exercising human skeletal muscle. *J Physiol* **540,** 377–386.

De Clerck I, Boussery K, Pannier J-L & Van De Voorde J (2003). Potassium potently relaxes small rat skeletal muscle arteries. *Med Sci Sports Exerc* **35,** 2005–2012.

Crane GJ, Gallagher N, Dora KA & Garland CJ (2003). Small- and intermediate-conductance calcium-activated K+ channels provide different facets of endothelium-dependent hyperpolarization in rat mesenteric artery. *J Physiol* **553,** 183–189.

Crecelius AR, Kirby BS & Dinenno FA (2015*a*). Intravascular ATP and the regulation of blood flow and oxygen delivery in humans. *Exerc Sport Sci Rev* **43,** 5–13.

Crecelius AR, Kirby BS, Hearon CM, Luckasen GJ, Larson DG & Dinenno FA (2015*b*). Contracting human skeletal muscle maintains the ability to blunt α1 -adrenergic vasoconstriction during KIR channel and Na(+) /K(+) -ATPase inhibition. *J Physiol* **593,** 2735–2751.

Crecelius AR, Kirby BS, Luckasen GJ, Larson DG & Dinenno FA (2012). ATP-mediated vasodilatation occurs via activation of inwardly rectifying potassium channels in humans. *J Physiol* **590,** 5349–5359.

Crecelius AR, Kirby BS, Voyles WF & Dinenno FA (2010). Nitric oxide, but not vasodilating prostaglandins, contributes to the improvement of exercise hyperemia via ascorbic acid in healthy older adults. *Am J Physiol Heart Circ Physiol* **299,** H1633–H1641.

Crecelius AR, Luckasen GJ, Larson DG & Dinenno FA (2014). KIR channel activation contributes to onset and steady-state exercise hyperemia in humans. *Am J Physiol Heart Circ Physiol* **307,** H782–H791.

Crecelius AR, Richards JC, Luckasen GJ, Larson DG & Dinenno FA (2013). Reactive hyperemia occurs via activation of inwardly rectifying potassium channels and Na+/K+-ATPase in humans. *Circ Res* **113,** 1023–1032.

Dawes M (2002). Barium Reduces Resting Blood Flow and Inhibits Potassium-Induced Vasodilation in the Human Forearm. *Circulation* **105,** 1323–1328.

DeSouza CA, Clevenger CM, Greiner JJ, Smith DT, Hoetzer GL, Shapiro LF & Stauffer BL (2002). Evidence for agonist-specific endothelial vasodilator dysfunction with ageing in healthy humans. *J Physiol* **542,** 255–262.

Dinenno FA & Joyner MJ (2003). Blunted sympathetic vasoconstriction in contracting skeletal muscle of healthy humans: is nitric oxide obligatory? *J Physiol* **553,** 281–292.

Dinenno FA & Joyner MJ (2004). Combined NO and PG inhibition augments alpha-adrenergic vasoconstriction in contracting human skeletal muscle. *Am J Physiol Heart Circ Physiol* **287,** H2576–H2584.

Dinenno FA, Masuki S & Joyner MJ (2005). Impaired modulation of sympathetic alpha-adrenergic vasoconstriction in contracting forearm muscle of ageing men. *J Physiol* **567,** 311–321.

Dora KA, Sandow SL, Gallagher NT, Takano H, Rummery NM, Hill CE & Garland CJ (2003). Myoendothelial gap junctions may provide the pathway for EDHF in mouse mesenteric artery. *J Vasc Res* **40,** 480–490.

Emerson GG & Segal SS (2000). Electrical coupling between endothelial cells and smooth muscle cells in hamster feed arteries: role in vasomotor control. *Circ Res* **87,** 474–479.

Holwerda SW, Restaino RM & Fadel PJ (2014). Adrenergic and non-adrenergic control of active skeletal muscle blood flow: Implications for blood pressure regulation during exercise. *Auton Neurosci*; DOI: 10.1016/j.autneu.2014.10.010.

Joyner MJ & Thomas GD (2003). Having it both ways? Vasoconstriction in contracting muscles. *J Physiol* **550,** 333.

Keller DM, Ogoh S, Greene S, Olivencia-Yurvati a & Raven PB (2004). Inhibition of KATP channel activity augments baroreflex-mediated vasoconstriction in exercising human skeletal muscle. *J Physiol* **561,** 273–282.

Keller DM, Wasmund WL, Wray DW, Ogoh S, Fadel PJ, Smith ML & Raven PB (2003). Carotid baroreflex control of leg vascular conductance at rest and during exercise. *J Appl Physiol* **94,** 542–548.

Kerr PM, Tam R, Ondrusova K, Mittal R, Narang D, Tran CHT, Welsh DG & Plane F (2012). Endothelial feedback and the myoendothelial projection. *Microcirculation* **19,** 416–422.

Kirby BS, Crecelius AR, Voyles WF & Dinenno F a. (2012). Impaired skeletal muscle blood flow control with advancing age in humans: Attenuated ATP release and local vasodilation during erythrocyte deoxygenation. *Circ Res* **111,** 220–230.

Kirby BS, Crecelius AR, Voyles WF & Dinenno FA (2010). Vasodilatory responsiveness to adenosine triphosphate in ageing humans. *J Physiol* **588,** 4017–4027.

Kirby BS, Markwald RR, Smith EG & Dinenno FA (2005). Mechanical effects of muscle contraction do not blunt sympathetic vasoconstriction in humans. *Am J Physiol Heart Circ Physiol* **289,** H1610–H1617.

Kirby BS, Voyles WF, Carlson RE & Dinenno FA (2008). Graded sympatholytic effect of exogenous ATP on postjunctional alpha-adrenergic vasoconstriction in the human forearm: implications for vascular control in contracting muscle. *J Physiol* **586,** 4305–4316.

Knot HJ, Zimmermann PA & Nelson MT (1996). Extracellular K(+)-induced hyperpolarizations and dilatations of rat coronary and cerebral arteries involve inward rectifier K(+) channels. *J Physiol* **492 (Pt 2,** 419–430.

Koch DW, Leuenberger UA & Proctor DN (2003). Augmented leg vasoconstriction in dynamically exercising older men during acute sympathetic stimulation. *J Physiol* **551,** 337–344.

Kurjiaka DT & Segal SS (1995). Interaction Between Conducted Vasodilation and Sympathetic Nerve Activation in Arterioles of Hamster Striated Muscle. *Circ Res* **76,** 885–891.

Marrelli SP, Eckmann MS & Hunte MS (2003). Role of endothelial intermediate conductance KCa channels in cerebral EDHF-mediated dilations. *Am J Physiol Heart Circ Physiol* **285,** H1590–H1599.

Milkau M, Köhler R & de Wit C (2010). Crucial importance of the endothelial K+ channel SK3 and connexin40 in arteriolar dilations during skeletal muscle contraction. *FASEB J* **24,** 3572–3579.

Nyberg M, Piil P, Egelund J, Sprague RS, Mortensen SP & Hellsten Y (2015). Effect of PDE5 inhibition on the modulation of sympathetic α-adrenergic vasoconstriction in contracting skeletal muscle of young and older recreationally active humans. *Am J Physiol Heart Circ Physiol* **309,** H1867–H1875.

Proctor DN, Shen PH, Dietz NM, Eickhoff TJ, Lawler LA, Ebersold EJ, Loeffler DL & Joyner MJ (1998). Reduced leg blood flow during dynamic exercise in older endurance-trained men. *J Appl Physiol* **85,** 68–75.

Remensnyder JP, Mitchell JH & Sarnoff SJ (1962). Functional sympatholysis during muscular activity: Observations on influence of carotid sinus on oxygen uptake. *Circ Res* **11,** 370–380.

Richards JC, Luckasen GJ, Larson DG & Dinenno F a. (2014). Role of α-adrenergic vasoconstriction in regulating skeletal muscle blood flow and vascular conductance during forearm exercise in ageing humans. *J Physiol* **592,** 4775–4788.

Rosenmeier JB, Dinenno FA, Fritzlar SJ & Joyner MJ (2003*a*). Alpha1- and Alpha2-Adrenergic Vasoconstriction Is Blunted in Contracting Human Muscle. *J Physiol* **547,** 971–976.

Rosenmeier JB, Fritzlar SJ, Dinenno FA & Joyner MJ (2003*b*). Exogenous NO administration and alpha-adrenergic vasoconstriction in human limbs. *J Appl Physiol* **95,** 2370–2374.

Saltin B & Mortensen SP (2012). Inefficient functional sympatholysis is an overlooked cause of malperfusion in contracting skeletal muscle. *J Physiol* **590,** 6269–6275.

Savard G, Strange S, Kiens B, Richter EA, Christensen NJ & Saltin B (1987). Noradrenaline spillover during exercise in active versus resting skeletal muscle in man. *Acta Physiol Scand* **131,** 507–515.

Schrage WG, Joyner MJ & Dinenno FA (2004). Local inhibition of nitric oxide and prostaglandins independently reduces forearm exercise hyperaemia in humans. *J Physiol* **557,** 599–611.

Taylor JA, Hand GA, Johnson DG & Seals DR (1992). Augmented forearm vasoconstriction during dynamic exercise in healthy older men. *Circulation* **86,** 1789–1799.

Thomas GD, Zhang W & Victor RG (2001). Impaired modulation of sympathetic vasoconstriction in contracting skeletal muscle of rats with chronic myocardial infarctions: role of oxidative stress. *Circ Res* **88,** 816–823.

Tran CHT, Taylor MS, Plane F, Nagaraja S, Tsoukias NM, Solodushko V, Vigmond EJ, Furstenhaupt T, Brigdan M & Welsh DG (2012). Endothelial Ca2+ wavelets and the induction of myoendothelial feedback. *Am J Physiol Cell Physiol* **302,** C1226–C1242.

Tschakovsky ME, Sujirattanawimol K, Ruble SB, Valic Z & Joyner MJ (2002). Is sympathetic neural vasoconstriction blunted in the vascular bed of exercising human muscle? *J Physiol* **541,** 623–635.

Vongpatanasin W, Wang Z, Arbique D, Arbique G, Adams-Huet B, Mitchell JH, Victor RG & Thomas GD (2011). Functional sympatholysis is impaired in hypertensive humans. *J Physiol* **589,** 1209–1220.

Winter P & Dora KA (2007). Spreading dilatation to luminal perfusion of ATP and UTP in rat isolated small mesenteric arteries. *J Physiol* **582,** 335–347.

Wölfle SE, Schmidt VJ, Hoyer J, Köhler R & De Wit C (2009). Prominent role of KCa3.1 in endothelium-derived hyperpolarizing factor-type dilations and conducted responses in the microcirculation in vivo. *Cardiovasc Res* **82,** 476–483.

CHAPTER IV – MANUSCRIPT III

Augmentation of endothelium-dependent vasodilatory signaling improves functional sympatholysis in contracting muscle of older adults

Summary

Sympathetic α-adrenergic vasoconstriction is blunted within contracting skeletal muscle, and this 'functional sympatholysis' is impaired with age and may contribute to reduced blood flow during exercise in older adults. We have previously demonstrated that increasing endothelium-dependent vasodilatory signaling during exercise in young individuals can augment sympatholysis. The purpose of this study was to determine if augmentation of endothelium-dependent signaling in older adults can improve sympatholysis. In 15 older individuals, we measured forearm blood flow (Doppler ultrasound) and calculated changes in vascular conductance (FVC) to local intra-arterial infusion of phenylephrine (PE; α_1-agonist) during 1) infusion of the endothelium-dependent vasodilator acetylcholine (ACh) or low dose adenosine triphosphate (ATP), 2) mild (5% maximum voluntary contraction; MVC) or moderate (15% MVC) intensity handgrip exercise, and 3) mild or moderate exercise + ACh or ATP infusion to augment endothelium-dependent signaling during exercise. Vasoconstriction to PE during control vasodilator infusions (ΔFVC: ACh: -31±3 and ATP: 30±4%) was similar to vasoconstriction observed during mild (ΔFVC: 5% MVC: -30±4 and -32±2%; P>0.05 vs. control ACh and ATP, respectively) moderate intensity exercise (ΔFVC: 15% MVC: -31±3 and -33±3%; P>0.05 vs. control ACh and ATP, respectively), indicating impaired sympatholysis. Augmentation of endothelium-dependent signaling via ACh or ATP infusion during mild intensity exercise did not attenuate PE-mediated vasoconstriction (ΔFVC: 5% MVC+ACh: -29±2, 5% MVC+ATP: -26±3%; P>0.05 vs control ACh and ATP, respectively), whereas augmentation of endothelium-dependent signaling during moderate intensity exercise significantly attenuated vasoconstriction (ΔFVC: 15% MVC+ACh: -13±1, 15% MVC+ATP: -19±5%; P<0.05 vs all conditions). Our

findings demonstrate that the endothelium of older adults is capable of attenuating sympathetic vasoconstriction when endothelium-dependent signaling is elevated during exercise. This study identifies viable vascular signaling pathways capable of improving sympatholysis, and thus tissue blood flow and oxygen delivery, in older adults.

Introduction

Exercise capacity is a powerful independent predictor of all-cause mortality in healthy, and disease populations (Kokkinos *et al.*, 2008; Kokkinos, 2012). A decline in exercise capacity occurs with healthy (primary) ageing, and is attributed to a number of age-associated alterations within the cardiovascular system including reductions in central cardiac pumping capacity, impaired peripheral vascular function, and elevated sympathetic nervous system activity (Seals *et al.*, 1994; Betik & Hepple, 2008; Calbet & Lundby, 2012). These alterations collectively result in impaired blood flow regulation and oxygen delivery to exercising skeletal muscle during both large (upright cycling) and small (single knee extensor, handgrip exercise) muscle mass exercise. While peripheral blood flow during maximal exercise may be limited in part due to decrements in maximal cardiac output (Proctor *et al.*, 2004), studies utilizing submaximal exercise (Proctor *et al.*, 1998, 2003; Poole *et al.*, 2003) or small muscle mass exercise (Lawrenson *et al.*, 2003; Donato *et al.*, 2006; Parker *et al.*, 2008; Kirby *et al.*, 2009; Barrett-O'Keefe *et al.*, 2014) clearly identify age-associated impairments in peripheral vascular signaling, independent of central cardiac limitations. The age-associated decline in muscle blood flow during exercise is due to impaired vascular conductance, as a result of impaired local production of vasodilatory (Kirby *et al.*, 2009) and vasoconstrictor signaling (Barrett-O'Keefe *et al.*, 2014), as well as elevated sympathetic vasoconstriction within the skeletal muscle vasculature (Taylor *et al.*, 1992; Proctor *et al.*, 1998).

In healthy individuals, the regulation of blood flow and oxygen delivery to contracting skeletal muscle is the result of a complex integration of local vasodilatory signaling and neural

sympathetic vasoconstriction. The proper integration of these competing signals results in attenuation of sympathetic vasoconstriction, or “functional sympatholysis” (Remensnyder *et al.*, 1962). Functional sympatholysis is necessary to maintain adequate blood flow to contracting skeletal muscle while maintaining blood pressure in the face of profound metabolic vasodilatation (Marshall *et al.*, 1961). With age, there is a pronounced ~2-3 fold increase in sympathetic nervous system activity directed to the skeletal muscle at rest (Sundlöf & Wallin, 1978), and greater than two-fold increase in noradrenaline (NA) spillover during whole body exercise (Proctor *et al.*, 1998). To compound the issue, our laboratory and others have shown impaired modulation of sympathetic vasoconstriction within contracting skeletal muscle of older adults (i.e impaired functional sympatholysis), resulting in exaggerated sympathetic vasoconstrictor responsiveness (Koch *et al.*, 2003; Dinenno *et al.*, 2005) and significant metabolic dysfunction during exercise (Mortensen *et al.*, 2012). Mortensen and colleagues (2012) showed that sedentary older adults demonstrating impaired sympatholysis have a lower $\dot{V}O_2$, higher lactate and elevated blood pressure during knee extensor exercise, relative to older endurance trained adults with intact functional sympatholysis. Interestingly, these metabolic impairments occurred despite similar levels of bulk blood flow between the groups, highlighting the importance of functional sympatholysis not only in the regulation of bulk blood flow to active tissues, but also in the distribution of blood flow within contracting skeletal muscle (Saltin & Mortensen, 2012).

The role of numerous vasodilatory substances and pathways have been investigated in the context of sympatholysis, including nitric oxide (NO) (Tschakovsky *et al.*, 2002; Chavoshan *et al.*, 2002; Rosenmeier *et al.*, 2003; Dinenno & Joyner, 2003), prostaglandins (PGs) (Dinenno & Joyner, 2004), adenosine (Kirby *et al.*, 2008; Rosenmeier *et al.*, 2008), ATP-sensitive potassium (K_{ATP}) channels (Keller *et al.*, 2004), and recent work from our laboratory investigating inwardly rectifying potassium (K_{IR}) channels and sodium-potassium ATPase (Na^+/K^+ATPase) alone or in combination with NO and PG blockade (Crecelius *et al.*, 2015*b*).

However to date, the signaling mechanisms responsible for functional sympatholysis in humans remain unclear. The only vasodilator shown to attenuate sympathetic vasoconstriction independently in humans is adenosine triphosphate (ATP) (Rosenmeier *et al.*, 2008; Kirby *et al.*, 2011). In a manner similar to exercise, intra-arterial infusion of ATP evokes significant vasodilatation that is capable of attenuating sympathetic vasoconstriction. Whereas lower doses of ATP are not sympatholytic, higher doses of ATP result in progressive attenuation of α-adrenergic vasoconstriction (Kirby *et al.*, 2008), reminiscent of the intensity-dependent nature of functional sympatholysis in contracting skeletal muscle (Tschakovsky *et al.*, 2002). Studies have identified ATP as an important signaling molecule released from a host of different tissues, during a variety of physiological situations (Burnstock, 2014). During exercise, release of ATP from red blood cells (RBC) in response to haemoglobin deoxygenation elevates circulating plasma ATP levels in healthy humans (Jagger *et al.*, 2001; González-Alonso *et al.*, 2002). This mechanism couples elevated oxygen consumption with release of a sympatholytic vasodilator, and is thought to be an important contributor to the vasodilatory response to exercise and ultimately the regulation of blood flow distribution within active skeletal muscle.

In the context of ageing, RBC-mediated ATP release is impaired, and the rise in plasma ATP concentration during exercise is attenuated (Kirby *et al.*, 2012). Despite a reduced ability to elevate plasma ATP levels during exercise, intra-arterial infusion of exogenous ATP maintains the ability to attenuate α-adrenergic vasoconstriction in older adults (Kirby *et al.*, 2011). Interestingly, this occurs despite the presence of classical endothelial dysfunction (impaired NO availability), and even impaired sympatholysis during muscle contractions (Mortensen *et al.*, 2012). Together these findings suggest that age-associated impairments in sympatholysis may result from loss of a functional signal (i.e. reduced plasma ATP) as opposed to an inability of the vasculature to respond to sympatholytic stimuli. Further, it seems that the vascular signaling pathways responsible for sympatholysis may be preserved with age and are independent of NO availability.

Along these lines, our lab recently showed that augmenting endothelium-dependent signaling during muscle contractions significantly enhances the ability of contracting skeletal muscle to attenuate α-adrenergic vasoconstriction (Hearon *et al.*, 2015). Importantly, the effect of endothelium-dependent signaling on sympatholysis is independent of NO production, and thus relies on alternative endothelium-dependent pathways to attenuate vasoconstriction (Hearon *et al.*, 2015). With these collective observations in mind, we hypothesized that the endothelium of older adults is capable of attenuating α-adrenergic vasoconstriction given a sufficient stimulus, and that augmenting endothelium-dependent signaling during muscle contractions would improve age-associated impairments in functional sympatholysis. To test this hypothesis, we assessed vasoconstrictor responses to direct α_1-adrenergic vasoconstriction during handgrip exercise with and without infusion of acetylcholine (ACh) or ATP to increase endothelium-dependent signaling during exercise.

Methods

Subjects

With Institutional Review Board approval and after written informed consent, 15 older healthy adults (7 men, 8 women; age = 68±2.1 years; weight = 74±4.2 kg; height = 167±4.4 cm; body mass index = 26±1.1 kg/m^2; percent body fat = 35±2.1%; and MVC of 28±2.6 kg; means±S.E.M) participated in the present study. All were non-smokers, non-obese, normotensive, sedentary to moderately active, free from overt cardiovascular disease and not taking any medications. All studies were performed in the Human Cardiovascular Laboratory located at Colorado State University after a 12 hour fast. This study was approved by the Human Research Committee of Colorado State University and was performed according to the *Declaration of Helsinki*.

Arterial Catheterization, Blood Pressure and Heart Rate

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 administration of pharmacological agents and measurement of MAP. Heart rate was monitored using a 3-lead ECG (Cardiocap/5; Datex-Ohmeda, Louisville, CO). (Richards *et al.*, 2014; Crecelius *et al.*, 2015*b*)

Body composition and forearm volume

Body composition was determined by dual-energy X-ray absorptiometry (DEXA; Hologic, INC; Bedford, MA, USA). Total forearm volume (FAV) 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. (Crecelius *et al.*, 2010)

Forearm Blood Flow and Vascular Conductance

A 12 MHz linear-array ultrasound probe (Vivid 7, General Electric, Milwaukee, WI, USA) was used to measure brachial artery mean blood velocity (MBV) and brachial artery diameter proximal to the catheter site as previously described by our laboratory (Crecelius *et al.*, 2010). Brachial artery diameter measurements were made in triplicate in duplex mode at end diastole, and between contractions (for exercise trials) during steady-state conditions. Forearm blood flow (FBF) was calculated as described previously (Crecelius *et al.*, 2015*b*). As an index of forearm vascular tone, forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml (min)-1 100 mmHg-1. Studies were performed in a cool temperature-controlled environment with a fan directed toward the forearm to minimize the contribution of skin blood flow to forearm haemodynamics (Kirby *et al.*, 2012; Crecelius *et al.*, 2013).

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. Exercise trials were performed with weight corresponding to 5 or 15% MVC (~15 and 40% maximum work rate)(Richards *et al.*, 2014) attached to a pulley system and lifted 4-5 cm 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. These workloads were chosen to represent both a mild intensity exercise (5% MVC) that alone does not blunt sympathetic vasoconstriction and a moderate intensity exercise (15% MVC) that in young healthy adults significantly attenuates, but does not abolish sympathetic vasoconstriction (Tschakovsky *et al.*, 2002; Kirby *et al.*, 2005).

Sympathetic α_1-Adrenergic Vasoconstriction

In order to strictly control the vasoconstrictor stimulus across all conditions, phenylephrine (PE; selective α_1-agonist; Sandoz Inc., Princeton, NJ) was infused at 0.250 μg (dl forearm volume)$^{-1}$ min^{-1}, and was flow-adjusted for the steady-state hyperaemic condition as described previously (see below) (Dinenno & Joyner, 2003). Previous studies using lower doses of PE in young and older subjects demonstrate attenuated α_1-adrenergic responsiveness at rest in older subjects (Dinenno *et al.*, 2005). Despite the relatively smaller vasoconstrictor stimulus in older subjects, vasoconstrictor responsiveness is maintained or exaggerated during exercise (Dinenno *et al.*, 2005; Kirby *et al.*, 2011). In order to normalize the vasoconstrictor stimulus being delivered to skeletal muscle, and provide greater resolution to observe any improvements in the ability to attenuate vasoconstriction, the dose of PE used in this study is twice the highest dose utilized previously in studies investigating sympatholysis in the forearm of older adults (Dinenno *et al.*, 2005; Kirby *et al.*, 2011; Richards *et al.*, 2014). During control trials, a vasodilating agent (see specific protocol below) was infused to elevate resting FBF to similar

levels observed during moderate intensity exercise. Our lab has previously demonstrated that vasoconstrictor responses to direct α_1- and α_2-adrenoceptor stimulation are maintained during a control vasodilator infusion (Kirby *et al.*, 2008). Therefore, the control dilator conditions are used to create a "high flow" control state (Dinenno & Joyner, 2003). Additionally, these trials confirm that the dilatory agents used to elevate endothelium-dependent signalling during exercise are not independently sympatholytic.

General Experimental Protocols

Experimental Protocol

Baseline measures were collected in the supine position for 2 minutes after which subjects began one of 5 experimental trials: (1) an intra-arterial infusion of an endothelium-dependent vasodilatory substance alone (either acetylcholine (ACh; Miochol-E, Novartis, Basel, Switzerland) or adenosine triphosphate (ATP; Sigma A7699, St Louis, MO, USA); (2) a bout of dynamic handgrip exercise alone at a mild (5% maximum voluntary contraction (MVC)) or moderate (15%MVC) intensity, or (3) mild or moderate intensity handgrip exercise in combination with the control vasodilator to elevate endothelium-dependent vasodilatory signaling during exercise (Figure 1A). Hyperaemic conditions were maintained for 6 minutes with steady-state blood flow measures made between minutes 5 and 6 of each trial. During combined 15% MVC+ vasodilator conditions, steady-state forearm blood flow was elevated 10-20% to match the deficit in blood flow typically observed during exercise in older subjects (Kirby *et al.*, 2009; Crecelius *et al.*, 2010; Richards *et al.*, 2014, 2015). The dose of the α_1-agonist PE was calculated based on steady-state forearm blood flow (FBF; Doppler ultrasound) and forearm volume as described previously (Tschakovsky *et al.*, 2002) and infused for the final 2 minutes of the trial (Figure 1B). Vasoconstrictor responses to PE were assessed as a percent decrease in forearm vascular conductance (FVC = (FBF/MAP)*100) (Buckwalter & Clifford, 2001). The total time for each trial was 10 minutes followed by at least 15 minutes of quiet rest

before the initiation of subsequent trials. The order of the hyperaemic conditions was counterbalanced across subjects.

Specific Experimental Protocols

Protocol 1: Augmentation of endothelium-dependent vasodilator signaling via ACh during α_1-adrenoceptor stimulation

This protocol was designed to investigate if increasing endothelium-dependent signaling during exercise, via infusion of ACh, improves the ability of older adults to attenuate α_1-adrenergic vasoconstriction. In 8 subjects (4 males, 4 females) vasoconstrictor responses to PE were assessed during (1) infusion of the endothelium-dependent vasodilator ACh alone, (2) during mild or moderate intensity exercise (5% and 15% MVC respectively), or (3) during mild or moderate intensity exercise combined with ACh to augment endothelium-dependent signaling during contractions. The steady-steady state blood flow response during control ACh infusion, and during the combined 5% MVC+ACh infusion were intentionally matched to the hyperaemia observed during 15% exercise. During combined 15% MVC + ACh infusion, blood flow was increased by approximately 10-20% over the hyperaemia observed during 15% exercise alone. ACh was initially infused at 40 μg (min^{-1}) and the infusion rate was adjusted thereafter to reach the appropriate level of hyperaemia (final doses: control ACh: 5.1±1.4, 5%+ACh: 0.9±0.2, 15%+ACh: 0.8±0.3 μg (dl forearm volume)$^{-1}$ min^{-1}; $P<0.05$ control vs. exercise + ACh conditions).

Protocol 2: Augmentation of endothelium-dependent vasodilator signaling via ATP during α_1-adrenoceptor stimulation

This protocol was designed to investigate if increasing ATP availability to contracting skeletal muscle vasculature improves the ability of older adults to attenuate α_1-adrenergic vasoconstriction during exercise. In 7 subjects (3 males, 4 females) vasoconstrictor responses

to PE were assessed during (1) infusion of a low dose of ATP (1.282 μg (dl forearm volume)$^{-1}$ min^{-1}) alone, (2) during mild or moderate intensity exercise (5% and 15% MVC respectively), or (3) during mild or moderate intensity exercise combined with low dose ATP to augment endothelium-dependent signaling. We have previously demonstrated that doses of ATP higher than those used here are capable of blunting sympathetic vasoconstriction in both young and older subjects (Kirby *et al.*, 2008), therefore intentionally low doses of ATP were utilized throughout these trials. The dose of ATP during control conditions, and during the combined 5% exercise plus ATP infusion was held constant at 1.282 μg (dl forearm volume)$^{-1}$ min^{-1} to avoid independent sympatholytic effects of higher ATP doses. During combined 15% exercise and ATP infusion, blood flow was increased by approximately 10-20% over the hyperaemia observed during 15% exercise alone (final dose: 15%+ATP: 0.58±0.1 μg (dl forearm volume)$^{-1}$ min^{-1}; $P<0.05$ vs. Control ATP). This range of ATP doses has been shown to have little independent effect on PE-mediated vasoconstriction (Kirby *et al.*, 2008).

Data Acquisition and Analysis

Data were collected and stored on computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Baseline FBF, heart rate, and MAP represent an average of the last 30 seconds of the resting time period, the steady-state hyperaemia (Pre PE; prior to PE infusion), and the effects of the α_1-agonists (PE; after 2 minutes of PE infusion). We calculated percent reduction in FVC as our standard index to compare vasoconstrictor responses across conditions, as this is the most appropriate way to compare vasoconstrictor responsiveness under conditions where there might be differences in vascular tone.(Buckwalter & Clifford, 2001) The percent reduction in FVC during vasoconstrictor administration was calculated as: ((FVC during PE - FVC Pre PE)/(FVC Pre PE)) x 100. In an effort to be comprehensive, we have also presented absolute values of forearm haemodynamics for all conditions in tabular form (Tables 1 and 2).

Statistical Analysis

All values are reported as means ± SEM. Given the complexity of our experimental design, numerous comparisons are possible. We have focused our statistical approach on the comparisons most relevant to our experimental questions. Due to the predictably large increase in blood flow from rest to exercise or vasodilator infusion, resting haemodynamics were analysed separately by one-way repeated measures ANOVA. Comparison of absolute forearm haemodynamics were assessed by two-way (time point (pre-PE or PE) x trial) repeated measures ANOVA. Vasoconstrictor responses (%Δ FVC) were compared with one-way repeated measures ANOVA. Systemic haemodynamics were evaluated by two-way repeated measures ANOVA inclusive of all time points (baseline, pre-PE, PE X trial). In all cases, Student-Newman-Keuls post hoc pairwise comparisons were made with significance set a priori at $P<0.05$.

Results

Systemic haemodynamics in all experimental conditions for each protocol are presented in Tables 1 and 2. Small increases in MAP and heart rate occurred with exercise and throughout the course of the experiment.

Protocol 1: Augmentation of endothelium-dependent vasodilator signaling via ACh during α_1-adrenoceptor stimulation

As intended by experimental design, ACh infusion, handgrip exercise performed at 15% MVC, and the combination of 5% MVC+ACh evoked similar levels of FVC (pre PE) that were not significantly different from each other ($P>0.05$; Figure 2A). Mild intensity exercise (5% MVC) evoked a significant increase in FVC that was smaller in magnitude compared to the other trials. Infusion of ACh during 15% MVC exercise elevated FVC by ~16% ($P<0.05$ vs. 15% MVC). Direct stimulation of α_1-adrenoceptors via PE infusion significantly reduced FBF and FVC in all

conditions. Infusion of PE induced robust vasoconstriction during control ACh, and 5% MVC exercise (ΔFVC: ACh: -31±3, 5%:-30±4%; P=0.88, Figure 2B) that was not attenuated during 15% MVC exercise (ΔFVC: -31±3%; P=0.89 vs. ACh) indicating impaired functional sympatholysis. Infusion of ACh to augment endothelium-dependent signaling during 5% MVC handgrip exercise did not augment the ability of contracting skeletal muscle to attenuate vasoconstriction (ΔFVC: 5%+ACh: -29±2%; P=0.70 vs. ACh). In contrast, augmentation of endothelium-dependent signaling via ACh infusion during 15% MVC exercise significantly attenuated PE-mediated vasoconstriction (ΔFVC = -13±1%; P<0.05 Figure 2B).

Protocol 2: Augmentation of endothelium-dependent vasodilator signaling via ATP during α_1-adrenoceptor stimulation

ATP infusion, handgrip exercise performed at 5% MVC, and the combination of 5% MVC +ATP evoked levels of FVC (pre PE) that were significantly lower than 15% MVC exercise (P<0.05; Figure 3A). Mild intensity exercise (5% MVC) evoked a significant increase in FVC that was smaller in magnitude compared to the other trials. Combined 15% MVC+ATP increased FVC by ~10% (P<0.05 vs. 15% MVC). Direct stimulation of α_1-adrenoceptors via PE infusion significantly reduced FBF and FVC in all conditions. PE infusion induced robust vasoconstriction during control ATP, and 5% MVC exercise (ΔFVC: ATP: -31±3, 5%:-30±4%; P=0.77, Figure 2B) that was not attenuated during 15% MVC exercise (ΔFVC:
-31±3%; P=0.61 vs. ATP) indicating impaired functional sympatholysis. Infusion of ATP to augment endothelium-dependent signaling during 5% MVC handgrip exercise did not improve the ability of contracting skeletal muscle to attenuate vasoconstriction (ΔFVC: 5%+ATP: -26±4; P=0.32 vs. ATP). In contrast, augmentation of endothelium-dependent signaling during 15% MVC exercise significantly attenuated PE-mediated vasoconstriction (ΔFVC = -19±5%; P<0.05 Figure 2B).

Discussion

The findings from this investigation confirm previous studies demonstrating impaired modulation of α_1-adrenoceptor mediated vasoconstriction during moderate intensity exercise, characteristic of impaired functional sympatholysis in older adults (Koch *et al.*, 2003; Dinenno *et al.*, 2005; Kirby *et al.*, 2011). The primary novel finding of the present investigation is that slight increases in endothelium-dependent signaling during moderate intensity exercise, via infusion of ACh or ATP, significantly enhanced the ability of contracting skeletal muscle to attenuate α_1-adrenergic vasoconstriction. These results support findings from previous studies suggesting that the impairment in functional sympatholysis may be secondary to reduced concentration of sympatholytic factors or sympatholytic signaling during exercise (i.e. lower plasma ATP) as opposed to an inability of the vasculature to respond to sympatholytic stimuli (Kirby *et al.*, 2010, 2012; Mortensen *et al.*, 2012). Collectively these studies provide evidence that the signaling pathways capable of modulating sympathetic vasoconstriction are intact in older adults, and that strategies aimed at increasing endothelium-dependent signaling during exercise may be an effective therapeutic strategy to improve blood flow and oxygen delivery to contracting skeletal muscle in older individuals.

Impaired Functional Sympatholysis with Age

The peripheral haemodynamic response to exercise is the result of a complex integration of local vasodilatory signaling and sympathetic vasoconstrictor signaling, which under normal conditions, results in the attenuation of sympathetic vasoconstriction termed “functional sympatholysis” (Remensnyder *et al.*, 1962). This phenomenon is important for the proper regulation of blood flow to contracting skeletal muscle. With age, alterations in peripheral vasculature signaling result in impaired blood flow and oxygen delivery to skeletal muscle during exercise. Notably, there is a marked increase in sympathetic vasoconstrictor activity (Sundlöf & Wallin, 1978; Taylor *et al.*, 1992; Proctor *et al.*, 1998), and attenuated vasodilatory signaling

(Kirby *et al.*, 2009), which together result in “malperfusion” of metabolically-active tissues and result in reduced oxidative capacity in older individuals (Saltin & Mortensen, 2012).

Koch and colleagues (2003) first identified impaired functional sympatholysis as an underlying mechanism contributing to reduced blood flow during exercise in older adults. Using a cold pressor test to elevate sympathetic nervous system activity during moderate-intensity cycle ergometer exercise, they observed a greater decline in leg blood flow in older adults. These findings have since been confirmed in both men and women (Fadel *et al.*, 2004; Dinenno *et al.*, 2005), across absolute and relative workloads in multiple vascular beds (Kirby *et al.*, 2011; Mortensen *et al.*, 2012), and utilizing diverse methods of sympathetic and α-adrenergic stimulation (Koch *et al.*, 2003; Fadel *et al.*, 2004; Dinenno *et al.*, 2005; Kirby *et al.*, 2011; Richards *et al.*, 2015). Recently, Mortensen and colleagues (2012) demonstrated that impaired functional sympatholysis in older sedentary individuals, was associated with reduced VO_2 and elevated blood lactate, despite similar levels of bulk leg blood flow when compared to exercise trained older adults with preserved sympatholysis. Thus, impairments in functional sympatholysis are associated with significant impairments in aerobic capacity, and training status is an important modulator of age-associated alterations in functional sympatholysis.

Currently, the specific mechanisms underlying functional sympatholysis in humans are unclear. A number of studies investigating various vasodilatory pathways including nitric oxide, prostaglandins, K_{ATP} channel activation, inwardly rectifying K^+ channels and Na^+/K^+-ATPase activity, have failed to identify mechanisms obligatory to observe functional sympatholysis (Hearon & Dinenno, 2015). However, precursory observations in animal models have identified endothelium-derived hyperpolarization (EDH) as a potential signaling mechanism capable of attenuating sympathetic vasoconstriction. Specifically, ACh-mediated conducted vasodilatory responses secondary to hyperpolarization of the endothelium has been shown to attenuate sympathetically induced depolarization (vasoconstriction) (Kurjiaka & Segal, 1995). Additional studies in anesthetized rodents investigating electrical and chemical coupling between

endothelial cells and vascular smooth muscle cells through gap junctions, have identified the endothelium as a critical site for the regulation of sympathetic vasoconstrictor signaling (Kerr *et al.*, 2012; Nausch *et al.*, 2012; Tran *et al.*, 2012). Recently, our laboratory demonstrated that increasing endothelium-dependent signaling during exercise, via infusion of ACh, significantly enhanced the ability of contracting skeletal muscle to attenuate α_1-adrenergic vasoconstriction in young healthy adults (Hearon *et al.*, 2015). Importantly, the effect of increasing endothelium-dependent signaling was independent of NO and PGs, thus supporting EDH as a mechanism contributing to functional sympatholysis in humans.

Endothelium-dependent signaling enhances sympatholysis in older adults

With these observations in mind, in the present study we attempted to extend these findings to older individuals with vascular dysfunction. In young healthy individuals, the ability of handgrip exercise to attenuate adrenergic vasoconstriction is graded with exercise intensity (Tschakovsky *et al.*, 2002). Handgrip exercise equal to 5% MVC (~15% WR max) has little effect on adrenergic responsiveness, whereas graded increases in exercise intensity above 10% MVC (~25% WR max) consistently induce robust attenuation of adrenergic vasoconstriction. In older adults, moderate intensity handgrip exercise fails to attenuate sympathetic vasoconstriction, and this impairment occurs independent of changes in lean mass, or maximal voluntary contraction (Kirby *et al.*, 2011). In line with previous investigations, moderate intensity (15% MVC) handgrip exercise failed to attenuate α_1-adrenergic vasoconstriction in older adults relative to control ACh or mild (5% MVC) handgrip exercise (ΔFVC: -31±3, -31±3, -30±4%, respectively $P>0.05$) confirming impaired functional sympatholysis in the forearm of older adults (Fadel *et al.*, 2004; Dinenno *et al.*, 2005; Kirby *et al.*, 2011). While the combination of mild intensity exercise and ACh infusion (5%+ACh) failed to modulate sympathetic vasoconstriction, augmentation of endothelium-dependent signaling during moderate intensity exercise (15%+ACh) significantly enhanced the ability of contracting

skeletal muscle to attenuate α_1-adrenergic vasoconstriction (Figure 2B). The failure of combined mild intensity exercise and ACh infusion to attenuate vasoconstriction was unexpected, considering a previous study using the same vasodilatory stimulus in young adults (5%MVC+ACh) nearly abolished α_1-adrenergic vasoconstriction (Hearon *et al.*, 2015). The observation that augmenting vasodilatation during mild intensity exercise in older adults failed to significantly attenuate vasoconstriction emphasizes the fact that simply elevating forearm blood flow, flow-mediated shear, or general vasodilatory signaling is not sufficient to attenuate vasoconstriction in this model. Thus, the attenuation of α_1-adrenergic vasoconstriction observed during 15% MVC+ACh is due to a specific interaction between ACh-mediated signaling and PE-mediated vasoconstriction, not simply an artifact of elevating blood flow *per se*. Interestingly, the ACh dose used during 15%+ACh trials that attenuated vasoconstriction was significantly smaller than that used in control conditions where robust vasoconstriction was observed (final doses: control ACh: 5.1±1.4 vs. 15%+ACh: 0.8±0.3 μg (dl forearm volume)$^{-1}$ min^{-1}; $P<0.05$). These findings again dissociate the dose of ACh and the magnitude of vasodilatation from the ability to attenuate vasoconstriction. These findings are similar to our findings in young subjects showing that very small increases in endothelium-dependent signaling during exercise are capable of modulating sympathetic vasoconstriction (Hearon *et al.*, 2015). Finally, it is clear that the sympatholytic properties of ACh are altered or enhanced in the presence of muscle contractions. Although it is presently unknown how this occurs, one potential explanation is that muscle contractions can alter the pathways through which agonists elicit vasodilatation. Increasing the reliance of any given vasodilator on potentially sympatholytic vasodilatory pathways, would be an effective way to ensure efficient redistribution of blood flow to contracting skeletal muscle. However, more investigation is needed to determine if muscle contraction does indeed alter the signaling pathways through which vasodilators operate.

Increased ATP delivery enhances sympatholysis

While a physiological role for ACh in coordinating haemodynamic responses to exercise remains controversial, ATP is an important physiological contributor to the haemodynamic adjustments to exercise (Crecelius *et al.*, 2015*a*). Studies from our laboratory and others demonstrate that venous plasma [ATP] draining contracting skeletal muscle increases in an intensity dependent manner in healthy young subjects (González-Alonso *et al.*, 2002; Kirby *et al.*, 2012), and elicits vasodilatation that is capable of attenuating sympathetic vasoconstriction in a graded manner (Kirby *et al.*, 2008). With age, venous plasma [ATP] levels are reduced during exercise relative to young healthy subjects. However, vasodilatation to exogenous ATP is preserved in the forearm vasculature of older adults (Kirby *et al.*, 2010), and there is no impairment in the ability of exogenous ATP to attenuate sympathetic vasoconstriction in the leg or forearm vasculature of older adults (Kirby *et al.*, 2011; Mortensen *et al.*, 2012). Therefore, lower ATP-mediated signaling (i.e. lower plasma [ATP]) may be a contributor to impaired functional sympatholysis with age (Kirby *et al.*, 2012). Accordingly, we tested the hypothesis that augmenting ATP-mediated signaling during exercise would improve functional sympatholysis in older subjects.

In contrast to ACh in protocol 1, the dose of ATP was intentionally restricted to avoid doses of ATP that would be independently sympatholytic. The results from this study confirm previous findings that low doses of ATP do not attenuate α_1-adrenergic vasoconstriction, indicated by the highly consistent vasoconstrictor responses observed across control vasodilator conditions, and during 5% MVC (ΔFVC range: -30-32% across ATP, ACh and 5% MVC exercise) (Figures 2 and 3). Similar to ACh, combined 5% MVC exercise and ATP infusion did not significantly attenuate α_1-adrenergic vasoconstriction, whereas increasing ATP-mediated signaling during 15% MVC exercise significantly enhanced functional sympatholysis in older subjects (ΔFVC: 15% MVC: -33±3%, 15% MVC+ATP: -19±5%; $P<0.05$). Importantly, the dose of ATP used during combined 15% MVC+ATP trials to elevate blood flow ~10% above steady-state exercise levels, was significantly less than the standardized dose of ATP used in the

control ATP and 5% MVC+ATP trials (0.58±0.1 μg (dl forearm volume)$^{-1}$ min^{-1} vs. 1.282 μg (dl forearm volume)$^{-1}$ min^{-1}; respectively). Despite the very low dose, and a slight ~6% increase in FBF and FVC, there was a significant ~40% attenuation of α_1-adrenergic vasoconstriction. This observation, once again, dissociates the vasodilatory action of ATP *per se*, with its ability to attenuate sympathetic vasoconstriction. Further supporting the assertion that lower sympatholytic signaling, not merely lower responsiveness to vasodilatory substances is responsible for age-associated impairments in functional sympatholysis. These findings demonstrate that small elevations in ATP delivery to contracting skeletal muscle during moderate intensity exercise improves functional sympatholysis in older adults.

Mechanisms

Endothelium-dependent signaling during handgrip exercise attenuates α_1-adrenergic vasoconstriction similarly in both young (Hearon *et al.*, 2015) and older adults. However, the effects of ACh and ATP on sympatholysis are observed during mild intensity exercise in young subjects, whereas enhanced sympatholysis occurred only during moderate intensity exercise in older adults. Assuming ACh and ATP elevate endothelium-dependent signaling during exercise to a level high enough to attenuate α_1-adrenergic vasoconstriction, we speculate that there may be a threshold level of signaling required to observe functional sympatholysis. In this scenario, 5% MVC exercise alone in young healthy individuals would not reach the required threshold of sympatholytic signaling, whereas sympatholytic signaling during moderate intensity exercise would be sufficiently high to independently attenuate vasoconstriction. Thus, the addition of ACh or ATP to mild intensity exercise would be sufficient to elevate total signaling above this threshold in young subjects. In older subjects, total sympatholytic signaling during moderate intensity exercise may be reduced below threshold, and infusion of ACh or ATP is sufficient to restore normal levels of endothelium-dependent signaling during exercise. It remains to be seen if this type of “threshold” regulation of sympathetic vasoconstriction exists.

The specific signaling mechanisms responsible for the improvement in functional sympatholysis remain unclear. Ageing is associated with impaired endothelial function characterized by reduced NO production in response to endothelium-dependent agonists due to scavenging by reactive oxygen species (Taddei *et al.*, 1995; Lakatta, 2003). While oxidative stress has been linked with impaired modulation of sympathetic vasoconstriction in healthy humans (Fadel *et al.*, 2012), the majority of evidence indicates that impaired NO availability is not a primary contributor to impaired functional sympatholysis in older adults. A study by Mortensen and colleagues (2012) demonstrated that lifelong physical activity can preserve functional sympatholysis in older adults despite the continued presence of endothelial dysfunction. Along these lines, acute ingestion of the antioxidant ascorbic acid (Richards *et al.*, 2015) or administration of sildenafil (phosphodiesterase 5 inhibitor) (Nyberg *et al.*, 2015), improves the vasodilatory response to exercise in older adults via improved NO availability (Crecelius *et al.*, 2010), without a concomitant improvement in functional sympatholysis. Additionally, studies in young healthy adults demonstrate that augmentation of sympatholysis via infusion of ACh during exercise is independent of NO and PG production (Hearon *et al.*, 2015). Collectively these findings indicate improving NO availability does not necessarily translate into improved functional sympatholysis, and that ACh augments functional sympatholysis through an alternate endothelium-dependent pathway.

Both ACh and ATP rely on activation of $G_{q/11}$ coupled receptors and subsequent activation of small- and intermediate- Ca^{2+} activated K^+ channels (SK_{Ca} and IK_{Ca}; K_{Ca}) causing potassium efflux from the endothelial cell and membrane hyperpolarization (EDH)(Busse *et al.*, 2002). This EDH is transmitted to vascular smooth muscle cells and has been shown to attenuate sympathetically-mediated depolarization (Kurjiaka & Segal, 1995). Interestingly, the vasodilatory responses to substances that rely primarily on hyperpolarization, such as ATP and bradykinin (Shiramoto *et al.*, 1997; Dwivedi *et al.*, 2005; Schrage *et al.*, 2005; Crecelius *et al.*, 2012), are preserved with ageing (DeSouza *et al.*, 2002; Kirby *et al.*, 2010). Collectively, these

data suggest endothelium-derived hyperpolarization is capable of enhancing functional sympatholysis and that these pathways remain intact in older subjects.

Experimental Considerations and Limitations

Elevated sympathetic nervous system activity results in the release of a number of neurotransmitters that contribute to vasoconstriction including neuropeptide Y, ATP and NA. In animal models, the contribution of the individual peptides varies depending on arteriolar branch order and nerve stimulation frequency (Lundberg *et al.*, 1994). Much less is known about the specific contributions of individual peptides in the regulation of skeletal muscle blood flow in humans. However, NA is considered the primary neurotransmitter involved in mediating exercise related sympathetic vasoconstriction (Buckwalter & Clifford, 1999). Additionally, α-adrenergic restraint of skeletal muscle blood flow is elevated at rest in the legs older subjects (Dinenno *et al.*, 2001) and during exercise there is a dramatic increase in NA spillover in within skeletal muscle of the arm and leg in older adults (Taylor *et al.*, 1992; Proctor *et al.*, 1998). These findings highlight alterations in α-adrenergic signaling as a primary contributor to impaired sympathetic regulation of vascular tone with age. In the context of sympatholysis, ageing impairs metabolic regulation of both α_1- and α_2-adrenergic signaling, however, the impairment may be relatively greater for α_1-adrenergic signaling (Dinenno *et al.*, 2005). Thus, identifying mechanisms to improve age-associated alterations in α_1-adrenergic signaling is of particular importance. Additionally, in contrast to tyramine which induces endogenous NA release or α_2-adrenergic agonists which have pre-junctional effects on NA release, PE can be used to isolate post-junctional signaling in a highly controlled manner.

Previous studies investigating functional sympatholysis in older individuals have clearly shown preserved vasoconstrictor responsiveness to PE during exercise despite attenuated vasoconstrictor responsiveness during rest (Dinenno *et al.*, 2002, 2005; Smith *et al.*, 2007). It was unknown if the lack of metabolic modulation of α_1-adrenergic vasoconstriction during

exercise was due to the smaller vasoconstrictor stimulus being delivered to the vasculature. Thus, in this study the vasoconstrictor stimulus was normalized to what might be observed in younger subjects (roughly 2x greater responsiveness) (Dinenno *et al.*, 2005) by utilizing a larger dose of PE than has been used previously in older subjects. This served to increase the vasoconstrictor stimulus at rest and provide greater resolution to observe metabolic or pharmacological attenuation of vasoconstriction. The findings that moderate intensity exercise failed to attenuate PE-mediated vasoconstriction are in line with previous studies using smaller doses of PE. Further, the present findings highlight the powerful ability of endothelium-dependent signaling to modulate a substantial vasoconstrictor stimulus, even in older individuals.

We did not specifically quantify or characterize endothelial dysfunction in this cohort of older subjects due to the time constraints of the present protocol. There is a multitude of evidence indicating endothelial dysfunction in populations similar to the one used in this study, including studies from our laboratory using the same inclusion and exclusion criteria that were used here (Kirby *et al.*, 2009, 2010). Additionally, the subjects clearly demonstrated vascular dysfunction in the form of impaired functional sympatholysis. Therefore, we feel that quantifying endothelial dysfunction specifically in this cohort of older subjects was not necessary to test the hypothesis that augmenting endothelium-dependent signaling would improve sympatholysis in older adults.

Perspectives and Conclusion

Impaired functional sympatholysis is a common feature of cardiovascular ageing, hypertension and heart failure (Saltin & Mortensen, 2012). In many cases, reduced endothelial function, specifically impaired NO availability, is a primary contributor to age-associated impairments in vascular function. However, the overwhelming majority of evidence (Tschakovsky *et al.*, 2002; Rosenmeier *et al.*, 2003; Mortensen *et al.*, 2012; Crecelius *et al.*,

2015*b*; Richards *et al.*, 2015; Nyberg *et al.*, 2015) indicates that NO does not contribute to functional sympatholysis in humans, and emerging evidence suggests that endothelium-dependent signaling independent of NO production may be important for the regulation of α-adrenergic vasoconstriction in humans (Hearon *et al.*, 2015). In older adults, these endothelium-dependent pathways may be preserved, and retain the ability to attenuate sympathetic vasoconstriction when activated. Indeed, this study demonstrates that the endothelium of older adults is capable of attenuating sympathetic vasoconstriction when a sufficient level of endothelium-dependent signaling is present. The preserved sympatholytic capacity of skeletal muscle vasculature, coupled with impaired plasma [ATP] during exercise (Kirby *et al.*, 2012), suggests lower sympatholytic signaling as the primary cause of impaired sympatholysis, as opposed to a dysfunction primarily of vascular origin in older adults. Importantly, these studies identify vascular signaling pathways capable of attenuating sympathetic vasoconstriction, even in populations that typically demonstrate endothelial dysfunction. Therefore, strategies aimed at improving ATP availability *in vivo* may be effective in improving functional sympatholysis in older adults. More specifically, the red blood cell is a particularly attractive therapeutic target as it is the primary source of intravascular ATP during exercise (Kirby *et al.*, 2013), and ageing is associated with impaired erythrocyte-mediated ATP release (Kirby *et al.*, 2012). Thus, strategies aimed at improving erythrocyte-mediated ATP release in response to deoxygenation would provide a targeted approach to improve vasodilatory and sympatholytic signaling only in tissues under metabolic stress. This may hold significant therapeutic potential for patient populations demonstrating impaired functional sympatholysis, exercise intolerance, and reduced aerobic capacity.

Table 1. Protocol 1 Forearm and Systemic Haemodynamics: Acetylcholine (ACh) Trials

Time point	Trial	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	**ACh**	39 ± 4	103 ± 2	38 ± 4	58 ± 3
	5%	33 ± 5	109 ± 2	31 ± 5	60 ± 2
	15%	36 ± 7	103 ± 2	35 ± 6	60 ± 2
	5%+ACh	38 ± 5	107 ± 3	35 ± 4	59 ± 2
	15%+ACh	36 ± 3	105 ± 1	35 ± 3	60 ± 2
Pre-PE	**ACh**	158 ± 16	104 ± 1	152 ± 15	61 ± 3
	5%	90 ± 9†	110 ± 2	83 ± 9†	62 ± 3
	15%	165 ± 17	105 ± 1	156 ± 16	63 ± 3
	5%+ACh	159 ± 16	108 ± 2	146 ± 14	62 ± 3
	15%+ACh	190 ± 20†	107 ± 2	1760 ± 17†	64 ± 3
PE	**ACh**	111 ± 13‡	104 ± 2	106 ± 12‡	57 ± 3
	5%	63 ± 5‡	112 ± 1	56 ± 4‡	65 ± 3
	15%	117 ± 13‡	110 ± 1	106 ± 11‡	62 ± 3
	5%+ACh	114 ± 11‡	110 ± 2	102 ± 9‡	62 ± 3
	15%+ACh	173 ± 18‡	112 ± 2	153 ± 16‡	62 ± 4

*P<0.05, effect of time point
†P<0.05, vs. ACh
‡P<0.05, vs. Pre-PE within Trial
PE, Phenylephrine

Table 2. Protocol 2 Forearm and Systemic Haemodynamics: ATP trials

Time point	Trial	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	ATP	35 ± 3	102 ± 2	35 ± 3	50 ± 3
	5%	40 ± 5	101 ± 1	39 ± 5	42 ± 7
	15%	41 ± 5	99 ± 4	41 ± 5	50 ± 3
	5%+ATP	35 ± 3	103 ± 2	34 ± 3	51 ± 3
	15%+ATP	33 ± 4	100 ± 2	34 ± 4	52 ± 3
Pre-PE	ATP	108 ± 19	100 ± 2	106 ± 16	50 ± 3
	5%	94 ± 10	103 ± 1	91 ± 9	53 ± 3
	15%	193 ± 14‡	104 ± 4	185 ± 12‡	55 ± 3
	5%+ATP	161 ± 22‡§	105 ± 2	151 ± 18‡§	53 ± 3
	15%+ATP	211 ± 19‡	106 ± 3	197 ± 14‡	53 ± 4
PE	ATP	77 ± 15#	102 ± 3	74 ± 12#	48 ± 3
	5%	65 ± 6#	105 ± 2	62 ± 5#	51 ± 4
	15%	133 ± 12#	107 ± 4	124 ± 8#	55 ± 3
	5%+ATP	128 ± 23#	110 ± 3	115 ± 18#	56 ± 2
	15%+ATP	173 ± 20#	107 ± 3	159 ± 15#	56 ± 3

*P<0.05, effect of time point
†P<0.05, effect of condition
‡P<0.05, vs. ATP within time point
§P<0.05, vs. 15% time point
#P<0.05, vs. Pre-PE
PE, Phenylephrine

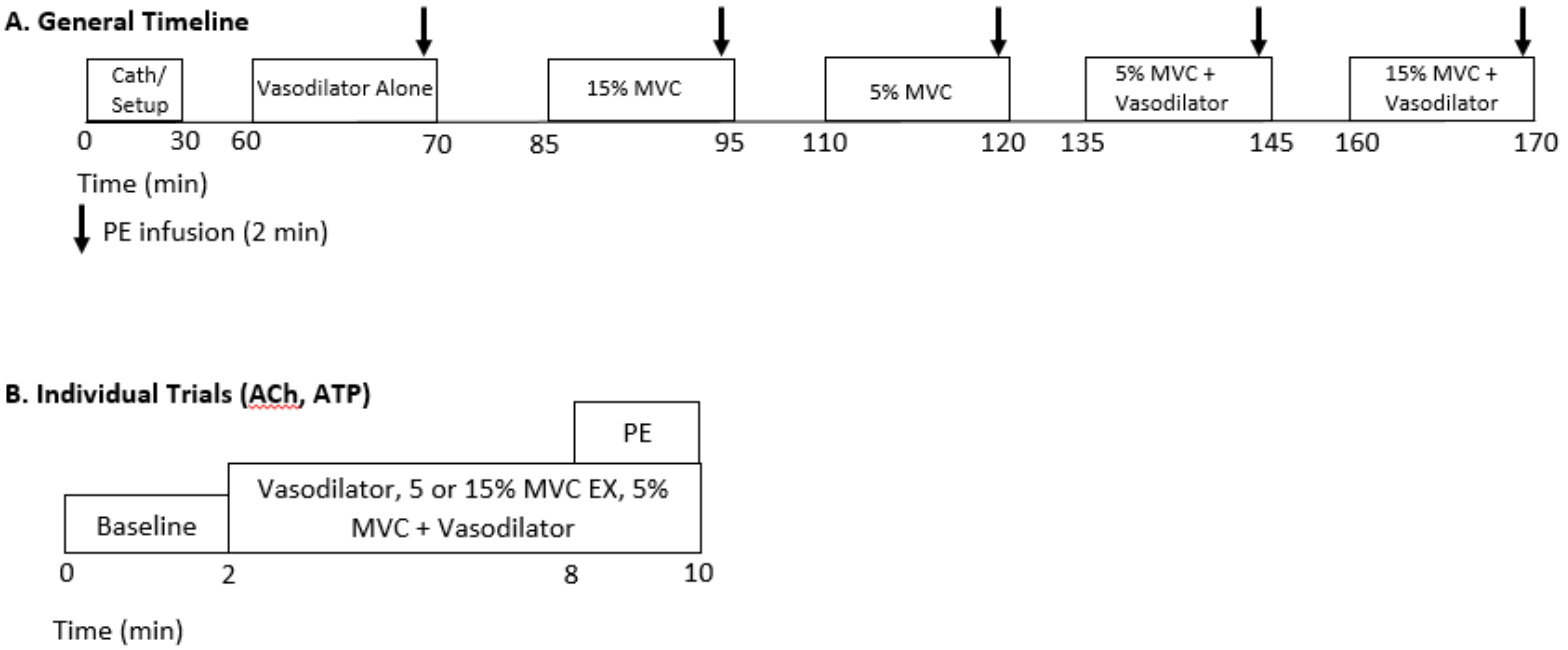

Figure 1. Experimental Protocol
A. After catheterization of the brachial artery (cath) and instrumentation, phenylephrine (PE) responsiveness was assessed during four different hyperaemic conditions. **B.** After baseline measurements, steady-state hyperaemia was achieved by infusion of a vasodilator alone (acetylcholine (ACh), or adenosine triphosphate (ATP)), exercise alone (5 or 15% maximal voluntary contraction (MVC)) or combined exercise and vasodilator infusion to augment vasodilatory signaling during muscle contractions (5%+Vasodilator). PE responsiveness was assessed in the final 2 minutes of each trial and quantified as a percent decrease in forearm vascular conductance (FVC).

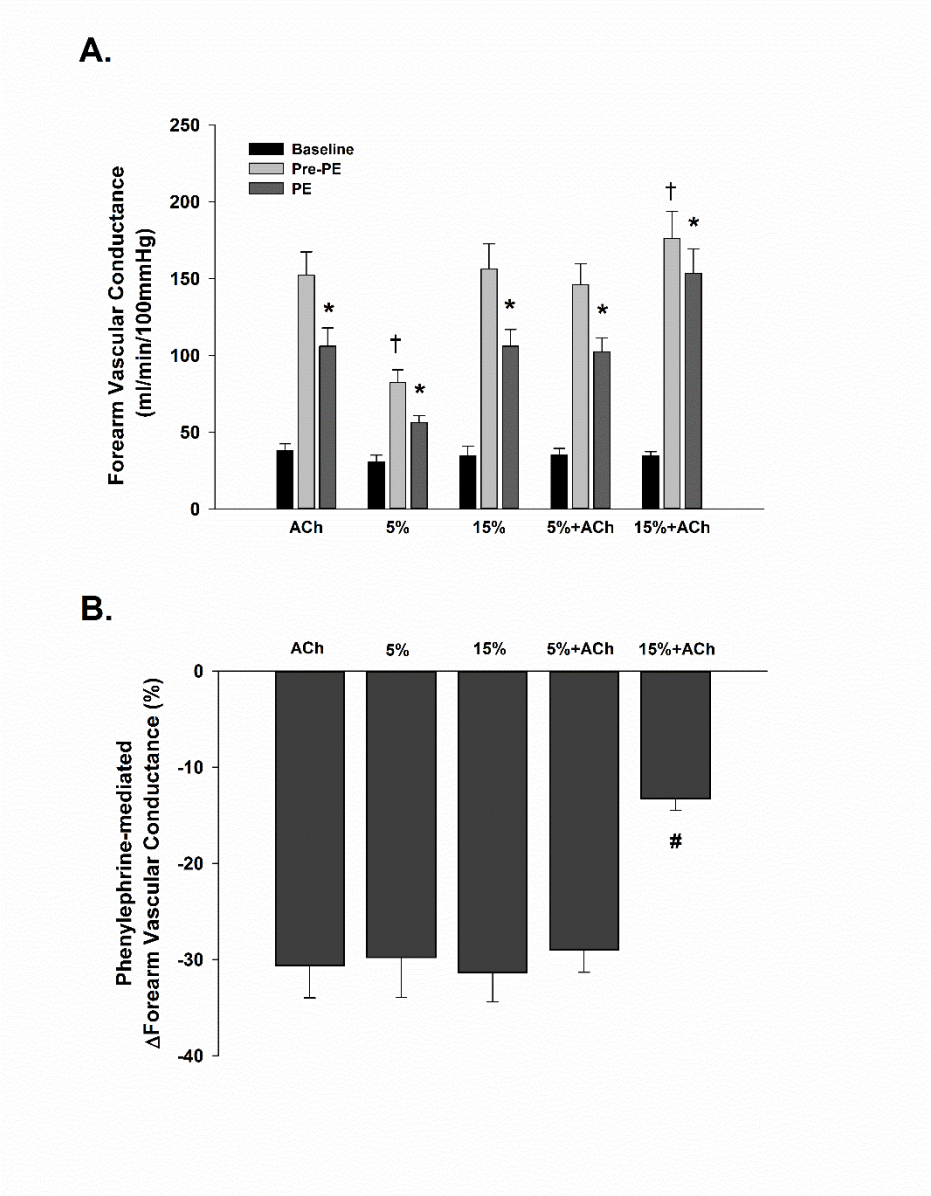

Figure 2. Acetylcholine (ACh) attenuates α_1-adrenergic vasoconstriction in contracting skeletal muscle of older adults

A. Forearm vascular conductance (FVC) is presented at baseline, steady-state hyperaemia prior to infusion of phenylephrine (PE; Pre-PE), and at the end of 2 minutes of PE infusion (PE). **B.** PE-mediated vasoconstriction was similar during infusion of ACh alone, 5% and 15% handgrip exercise indicating impaired functional sympatholysis. Combined ACh infusion and 5% exercise (5%+ACh) did not significantly attenuate sympathetic vasoconstriction whereas infusion of ACh to augment endothelium-dependent signaling during 15% exercise (15%+ACh) significantly attenuated PE-mediated vasoconstriction.*P<0.05 vs. Pre-PE;†P<0.05 vs. ACh; # P<0.05 vs. all conditions.

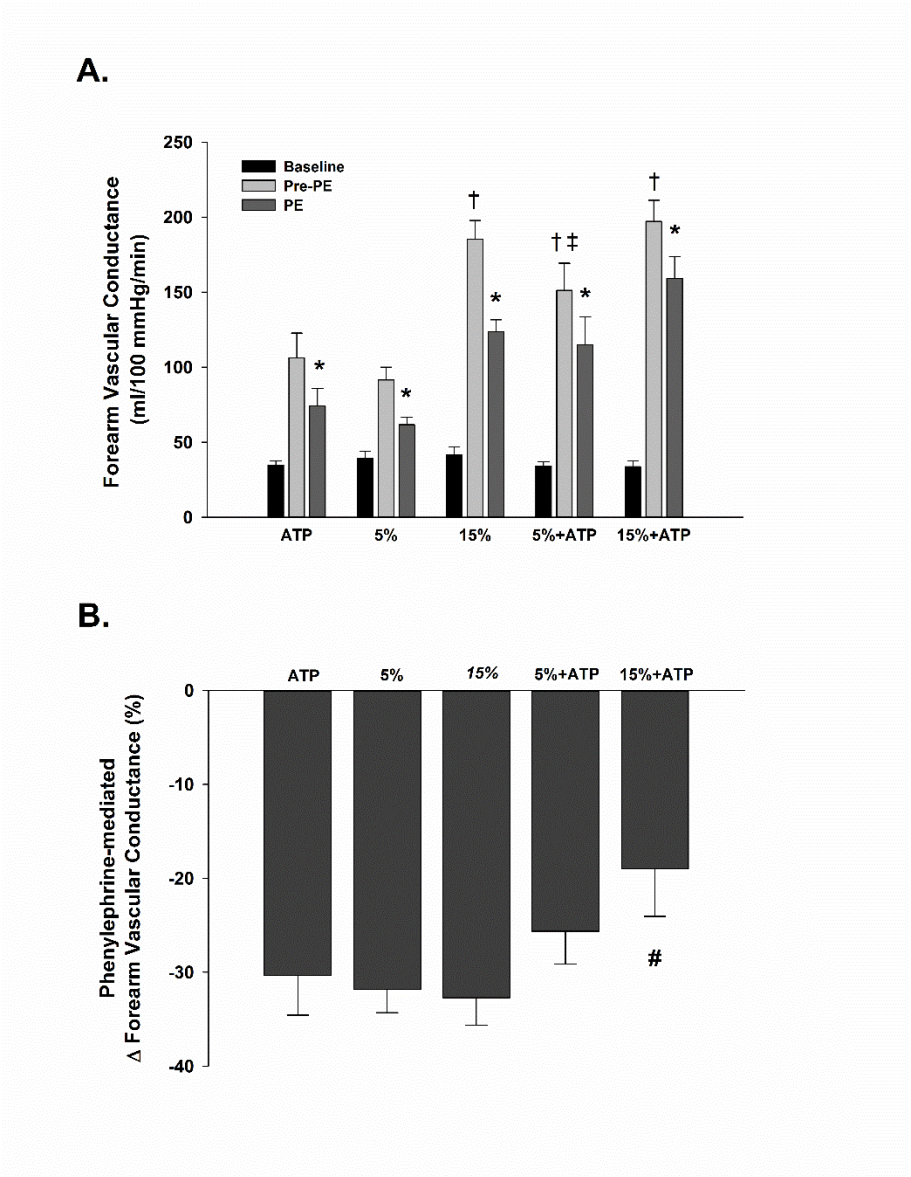

Figure 3. ATP attenuates α_1-adrenergic vasoconstriction in contracting skeletal muscle of older adults

A. Forearm vascular conductance (FVC) is presented at baseline, steady-state hyperaemia prior to infusion of phenylephrine (PE; Pre-PE), and at the end of 2 minutes of PE infusion (PE). **B.** PE-mediated vasoconstriction was similar during infusion of ATP alone, 5% and 15% handgrip exercise indicating impaired functional sympatholysis. Combined ATP infusion and 5% exercise (5%+ATP) did not significantly attenuate sympathetic vasoconstriction whereas infusion of ATP to augment endothelium-dependent signaling during 15% exercise (15%+ATP) significantly attenuated PE-mediated vasoconstriction.*P<0.05 vs. Pre-PE; †P<0.05 vs. ATP; ‡P<0.05 vs. 15%; #P<0.05 vs. ATP, 5%, and 15%.

REFERENCES

Barrett-O'Keefe Z, Ives SJ, Trinity JD, Morgan G, Rossman MJ, Donato AJ, Runnels S, Morgan DE, Gmelch BS, Bledsoe AD, Richardson RS & Wray DW (2014). Endothelin-A-Mediated Vasoconstriction During Exercise With Advancing Age. *J Gerontol A Biol Sci Med Sci* **70,** 554–565.

Betik AC & Hepple RT (2008). Determinants of VO2 max decline with aging: an integrated perspective. *Appl Physiol Nutr Metab* **33,** 130–140.

Buckwalter JB & Clifford PS (1999). alpha-adrenergic vasoconstriction in active skeletal muscles during dynamic exercise. *Am J Physiol* **277,** H33–H39.

Buckwalter JB & Clifford PS (2001). The paradox of sympathetic vasoconstriction in exercising skeletal muscle. *Exerc Sport Sci Rev* **29,** 159–163.

Burnstock G (2014). Purinergic signalling: from discovery to current developments. *Exp Physiol* **99,** 16–34.

Busse R, Edwards G, Félétou M, Fleming I, Vanhoutte PM & Weston AH (2002). EDHF: Bringing the concepts together. *Trends Pharmacol Sci* **23,** 374–380.

Calbet J a L & Lundby C (2012). Skeletal muscle vasodilatation during maximal exercise in health and disease. *J Physiol* **590,** 6285–6296.

Chavoshan B, Sander M, Sybert TE, Hansen J, Victor RG & Thomas GD (2002). Nitric oxide-dependent modulation of sympathetic neural control of oxygenation in exercising human skeletal muscle. *J Physiol* **540,** 377–386.

Crecelius AR, Kirby BS & Dinenno FA (2015*a*). Intravascular ATP and the regulation of blood flow and oxygen delivery in humans. *Exerc Sport Sci Rev* **43,** 5–13.

Crecelius AR, Kirby BS, Hearon CM, Luckasen GJ, Larson DG & Dinenno FA (2015*b*). Contracting human skeletal muscle maintains the ability to blunt α1 -adrenergic vasoconstriction during KIR channel and Na(+) /K(+) -ATPase inhibition. *J Physiol* **593,** 2735–2751.

Crecelius AR, Kirby BS, Luckasen GJ, Larson DG & Dinenno FA (2012). ATP-mediated vasodilatation occurs via activation of inwardly rectifying potassium channels in humans. *J Physiol* **590,** 5349–5359.

Crecelius AR, Kirby BS, Voyles WF & Dinenno FA (2010). Nitric oxide, but not vasodilating prostaglandins, contributes to the improvement of exercise hyperemia via ascorbic acid in healthy older adults. *Am J Physiol Heart Circ Physiol* **299,** H1633–H1641.

Crecelius AR, Richards JC, Luckasen GJ, Larson DG & Dinenno FA (2013). Reactive hyperemia occurs via activation of inwardly rectifying potassium channels and Na+/K+-ATPase in humans. *Circ Res* **113,** 1023–1032.

DeSouza CA, Clevenger CM, Greiner JJ, Smith DT, Hoetzer GL, Shapiro LF & Stauffer BL (2002). Evidence for agonist-specific endothelial vasodilator dysfunction with ageing in healthy humans. *J Physiol* **542,** 255–262.

Dinenno FA, Dietz NM & Joyner MJ (2002). Aging and forearm postjunctional alpha-adrenergic vasoconstriction in healthy men. *Circulation* **106,** 1349–1354.

Dinenno FA & Joyner MJ (2003). Blunted sympathetic vasoconstriction in contracting skeletal muscle of healthy humans: is nitric oxide obligatory? *J Physiol* **553,** 281–292.

Dinenno FA & Joyner MJ (2004). Combined NO and PG inhibition augments alpha-adrenergic vasoconstriction in contracting human skeletal muscle. *Am J Physiol Heart Circ Physiol* **287,** H2576–H2584.

Dinenno FA, Masuki S & Joyner MJ (2005). Impaired modulation of sympathetic alpha-adrenergic vasoconstriction in contracting forearm muscle of ageing men. *J Physiol* **567,** 311–321.

Dinenno FA, Tanaka H, Stauffer BL & Seals DR (2001). Reductions in basal limb blood flow and vascular conductance with human ageing: role for augmented alpha-adrenergic vasoconstriction. *J Physiol* **536,** 977–983.

Donato AJ, Uberoi A, Wray DW, Nishiyama S, Lawrenson L & Richardson RS (2006). Differential effects of aging on limb blood flow in humans. *Am J Physiol Heart Circ Physiol* **290,** H272–H278.

Dwivedi R, Saha S, Chowienczyk PJ & Ritter JM (2005). Block of inward rectifying K+ channels (KIR) inhibits bradykinin-induced vasodilatation in human forearm resistance vasculature. *Arterioscler Thromb Vasc Biol* **25,** e7–e9.

Fadel PJ, Farias Iii M, Gallagher KM, Wang Z & Thomas GD (2012). Oxidative stress and enhanced sympathetic vasoconstriction in contracting muscles of nitrate-tolerant rats and humans. *J Physiol* **590,** 395–407.

Fadel PJ, Wang Z, Watanabe H, Arbique D, Vongpatanasin W & Thomas GD (2004). Augmented sympathetic vasoconstriction in exercising forearms of postmenopausal women is reversed by oestrogen therapy. *J Physiol* **561,** 893–901.

González-Alonso J, Olsen DB & Saltin B (2002). Erythrocyte and the regulation of human skeletal muscle blood flow and oxygen delivery: role of circulating ATP. *Circ Res* **91,** 1046–1055.

Hearon CJ, Kirby B, Crecelius A, Luckasen G, Larson D & Dinenno F (2015). Augmentation of Functional Sympatholysis via Endothelial Signaling Is Independent of Nitric Oxide and Prostaglandins in Humans. *FASEB J* **29,** 649.9 – .

Hearon CM & Dinenno FA (2015). Regulation of skeletal muscle blood flow during exercise in ageing humans. *J Physiol*; DOI: 10.1113/JP270593.

Jagger JE, Bateman RM, Ellsworth ML & Ellis CG (2001). Role of erythrocyte in regulating local O2 delivery mediated by hemoglobin oxygenation. *Am J Physiol Heart Circ Physiol* **280,** H2833–H2839.

Keller DM, Ogoh S, Greene S, Olivencia-Yurvati a & Raven PB (2004). Inhibition of KATP channel activity augments baroreflex-mediated vasoconstriction in exercising human skeletal muscle. *J Physiol* **561,** 273–282.

Kerr PM, Tam R, Ondrusova K, Mittal R, Narang D, Tran CHT, Welsh DG & Plane F (2012). Endothelial Feedback and the Myoendothelial Projection. *Microcirculation* **19,** 416–422.

Kirby BS, Crecelius AR, Richards JC & Dinenno FA (2013). Sources of intravascular ATP during exercise in humans: critical role for skeletal muscle perfusion. *Exp Physiol* **98,** 988–998.

Kirby BS, Crecelius AR, Voyles WF & Dinenno F a. (2012). Impaired skeletal muscle blood flow control with advancing age in humans: Attenuated ATP release and local vasodilation during erythrocyte deoxygenation. *Circ Res* **111,** 220–230.

Kirby BS, Crecelius AR, Voyles WF & Dinenno FA (2010). Vasodilatory responsiveness to adenosine triphosphate in ageing humans. *J Physiol* **588,** 4017–4027.

Kirby BS, Crecelius AR, Voyles WF & Dinenno FA (2011). Modulation of postjunctional α-adrenergic vasoconstriction during exercise and exogenous ATP infusions in ageing humans. *J Physiol* **589,** 2641–2653.

Kirby BS, Markwald RR, Smith EG & Dinenno FA (2005). Mechanical effects of muscle contraction do not blunt sympathetic vasoconstriction in humans. *Am J Physiol Heart Circ Physiol* **289,** H1610–H1617.

Kirby BS, Voyles WF, Carlson RE & Dinenno FA (2008). Graded sympatholytic effect of exogenous ATP on postjunctional alpha-adrenergic vasoconstriction in the human forearm: implications for vascular control in contracting muscle. *J Physiol* **586,** 4305–4316.

Kirby BS, Voyles WF, Simpson CB, Carlson RE, Schrage WG & Dinenno FA (2009). Endothelium-dependent vasodilatation and exercise hyperaemia in ageing humans: impact of acute ascorbic acid administration. *J Physiol* **587,** 1989–2003.

Koch DW, Leuenberger UA & Proctor DN (2003). Augmented leg vasoconstriction in dynamically exercising older men during acute sympathetic stimulation. *J Physiol* **551,** 337–344.

Kokkinos P (2012). Physical activity, health benefits, and mortality risk. *ISRN Cardiol* **2012,** 718789.

Kokkinos P, Myers J, Kokkinos JP, Pittaras A, Narayan P, Manolis A, Karasik P, Greenberg M, Papademetriou V & Singh S (2008). Exercise capacity and mortality in black and white men. *Circulation* **117,** 614–622.

Kurjiaka DT & Segal SS (1995). Interaction Between Conducted Vasodilation and Sympathetic Nerve Activation in Arterioles of Hamster Striated Muscle. *Circ Res* **76,** 885–891.

Lakatta EG (2003). Arterial and Cardiac Aging: Major Shareholders in Cardiovascular Disease Enterprises: Part I: Aging Arteries: A "Set Up" for Vascular Disease. *Circulation* **107,** 139–146.

Lawrenson L, Poole JG, Kim J, Brown C, Patel P & Richardson RS (2003). Vascular and metabolic response to isolated small muscle mass exercise: effect of age. *Am J Physiol Heart Circ Physiol* **285,** H1023–H1031.

Lundberg JM, Franco-Cereceda A, Lou YP, Modin A & Pernow J (1994). Differential release of classical transmitters and peptides. *Adv Second Messenger Phosphoprotein Res* **29,** 223–234.

Marshall RJ, Schirger a & Shepherd JT (1961). Blood pressure during supine exercise in idiopathic orthostatic hypotension. *Circulation* **24,** 76–81.

Mortensen SP, Nyberg M, Winding K & Saltin B (2012). Lifelong physical activity preserves functional sympatholysis and purinergic signalling in the ageing human leg. *J Physiol* **590,** 6227–6236.

Nausch LWM, Bonev AD, Heppner TJ, Tallini Y, Kotlikoff MI & Nelson MT (2012). Sympathetic nerve stimulation induces local endothelial Ca2+ signals to oppose vasoconstriction of mouse mesenteric arteries. *Am J Physiol Heart Circ Physiol* **302,** H594–H602.

Nyberg M, Piil P, Egelund J, Sprague RS, Mortensen SP & Hellsten Y (2015). Effect of PDE5 inhibition on the modulation of sympathetic α-adrenergic vasoconstriction in contracting skeletal muscle of young and older recreationally active humans. *Am J Physiol Heart Circ Physiol* **309,** H1867–H1875.

Parker BA, Smithmyer SL, Pelberg JA, Mishkin AD & Proctor DN (2008). Sex-specific influence of aging on exercising leg blood flow. *J Appl Physiol* **104,** 655–664.

Poole JG, Lawrenson L, Kim J, Brown C & Richardson RS (2003). Vascular and metabolic response to cycle exercise in sedentary humans: effect of age. *Am J Physiol Heart Circ Physiol* **284,** H1251–H1259.

Proctor DN, Koch DW, Newcomer SC, Le KU & Leuenberger UA (2003). Impaired leg vasodilation during dynamic exercise in healthy older women. *J Appl Physiol* **95,** 1963–1970.

Proctor DN, Koch DW, Newcomer SC, Le KU, Smithmyer SL & Leuenberger UA (2004). Leg Blood Flow and VO2 during Peak Cycle Exercise in Younger and Older Women. *Med Sci Sports Exerc* **36,** 623–631.

Proctor DN, Shen PH, Dietz NM, Eickhoff TJ, Lawler LA, Ebersold EJ, Loeffler DL & Joyner MJ (1998). Reduced leg blood flow during dynamic exercise in older endurance-trained men. *J Appl Physiol* **85,** 68–75.

Remensnyder JP, Mitchell JH & Sarnoff SJ (1962). Functional sympatholysis during muscular activity: Observations on influence of carotid sinus on oxygen uptake. *Circ Res* **11,** 370–380.

Richards JC, Crecelius AR, Larson DG & Dinenno FA (2015). Acute ascorbic acid ingestion increases skeletal muscle blood flow and oxygen consumption via local vasodilation during graded handgrip exercise in older adults. *Am J Physiol Heart Circ Physiol* **309,** H360–H368.

Richards JC, Luckasen GJ, Larson DG & Dinenno F a. (2014). Role of α-adrenergic vasoconstriction in regulating skeletal muscle blood flow and vascular conductance during forearm exercise in ageing humans. *J Physiol* **592,** 4775–4788.

Rosenmeier JB, Fritzlar SJ, Dinenno FA & Joyner MJ (2003). Exogenous NO administration and alpha-adrenergic vasoconstriction in human limbs. *J Appl Physiol* **95,** 2370–2374.

Rosenmeier JB, Yegutkin GG & González-Alonso J (2008). Activation of ATP/UTP-selective receptors increases blood flow and blunts sympathetic vasoconstriction in human skeletal muscle. *J Physiol* **586,** 4993–5002.

Saltin B & Mortensen SP (2012). Inefficient functional sympatholysis is an overlooked cause of malperfusion in contracting skeletal muscle. *J Physiol* **590,** 6269–6275.

Schrage WG, Dietz NM, Eisenach JH & Joyner MJ (2005). Agonist-dependent variablity of contributions of nitric oxide and prostaglandins in human skeletal muscle. *J Appl Physiol* **98,** 1251–1257.

Seals DR, Taylor JA, Ng A V & Esler MD (1994). Exercise and aging: autonomic control of the circulation. *Med Sci Sports Exerc* **26,** 568–576.

Shiramoto M, Imaizumi T, Hirooka Y, Endo T, Namba T, Oyama J, Hironaga K & Takeshita A (1997). Role of nitric oxide towards vasodilator effects of substance P and ATP in human forearm vessels. *Clin Sci (Lond)* **92,** 123–131.

Smith EG, Voyles WF, Kirby BS, Markwald RR & Dinenno FA (2007). Ageing and leg postjunctional alpha-adrenergic vasoconstrictor responsiveness in healthy men. *J Physiol* **582,** 63–71.

Sundlöf G & Wallin BG (1978). Human muscle nerve sympathetic activity at rest. Relationship to blood pressure and age. *J Physiol* **274,** 621–637.

Taddei S, Virdis A, Mattei P, Ghiadoni L, Gennari A, Fasolo CB, Sudano I & Salvetti A (1995). Aging and Endothelial Function in Normotensive Subjects and Patients With Essential Hypertension. *Circulation* **91,** 1981–1987.

Taylor JA, Hand GA, Johnson DG & Seals DR (1992). Augmented forearm vasoconstriction during dynamic exercise in healthy older men. *Circulation* **86,** 1789–1799.

Tran CHT, Taylor MS, Plane F, Nagaraja S, Tsoukias NM, Solodushko V, Vigmond EJ, Furstenhaupt T, Brigdan M & Welsh DG (2012). Endothelial Ca2+ wavelets and the induction of myoendothelial feedback. *Am J Physiol Cell Physiol* **302,** C1226–C1242.

Tschakovsky ME, Sujirattanawimol K, Ruble SB, Valic Z & Joyner MJ (2002). Is sympathetic neural vasoconstriction blunted in the vascular bed of exercising human muscle? *J Physiol* **541,** 623–635.

CHAPTER V – LIMITATIONS AND PERSPECTIVES

General Experimental Limitations

In the first set of investigations, there was no significant effect of combined pharmacological blockade of NO, PGs, K_{IR} channels, and Na^+/K^+-ATPase on the ability of ATP to modulate α_1-adrenergic vasoconstriction. This could call into question the efficacy of the pharmacological agents used in this investigation. Very briefly, the method of administration and doses of all pharmacological inhibitors used in these protocols are well established within our laboratory to induce effective and robust blockade of the specific pathways we intend to manipulate in humans. Additionally, considering the profound effect of combined blockade on ATP-mediated vasodilatation (~60% attenuation of ATP-mediated vasodilatation), it is highly unlikely that lack of inhibitor effectiveness can explain the present findings.

The exercise studies contained within this dissertation utilize a dynamic handgrip exercise model to investigate the signaling mechanisms governing blood flow control during muscle contraction. The limitation to this model is that the forearm is a small muscle mass, thus extrapolating of these findings to large muscle mass, whole body dynamic exercise warrants caution. However, the forearm handgrip model offers distinct advantages given our interest in understanding the interaction between *local* vasodilatory, and *local* sympathetic vasoconstrictor signaling. Specifically, forearm handgrip exercise facilitates investigation of local metabolic vasodilatory signaling without engaging systemic reflex changes in hemodynamics that occur during whole body exercise, including elevated heart rate and mean arterial pressure, that can independently influence blood flow to peripheral tissues and confound the interpretation of the primary experimental variables of interest. Additionally, the forearm model, in conjunction with brachial arterial catheterization, facilitates utilization of unique pharmacological approaches to investigate signaling mechanisms in humans. Direct arterial access to a small muscle mass enables our laboratory to administer pharmacological agents at a doses adjusted for forearm

volume. This reduces the likelihood of pharmacological infusions having systemic effects, and allows safe utilization of highly novel pharmacological agents that otherwise cannot be administered systemically in humans.

In contrast to the handgrip exercise modality utilized in these studies, whole body exercise results in reflex increases in sympathetic nervous system activity and release of a number of neurotransmitters peripherally including noradrenaline, neuropeptide Y, and adenosine triphosphate. NA-mediated α-adrenergic vasoconstriction is considered the primary contributor to exercise induced sympathetic vasoconstriction, as such, we used infusions of PE (α_1-adrenergic agonist) to specifically isolate post-junctional adrenergic signaling, while avoiding pre-junctional effects associated with other sympathetic agonists such as tyramine (stimulates endogenous NA release), and dexmeditomidine (α_2 -adrenergic agonist). Importantly, muscle contractions are capable of blunting both α_1- and α_2-adrenergic agonists, tyramine, and reflex-mediated sympathetic vasoconstriction. Therefore, we do not expect our results to be specific only to α_1-adrenergic vasoconstriction. In the context of ageing, alterations in α-adrenergic signaling is a primary contributor to impaired sympathetic regulation of vascular tone. Further, ageing impairs metabolic regulation of both α_1- and α_2-adrenergic signaling, however, the impairment may be relatively greater for α_1-adrenergic signaling. Thus, identifying mechanisms to improve age-associated alterations in α_1-adrenergic signaling is of particular importance. Finally, the PE-mediated vasoconstrictor stimulus delivered to the exercising tissue can be strictly regulated across hyperaemic conditions using the pharmacological approached detailed within these studies.

Perspectives

John Remensnyder and colleagues first identified the ability of contracting skeletal muscle to attenuate sympathetic vasoconstriction in 1962. Functional sympatholysis is now understood to be a critical component of the normal hemodynamic adjustments to exercise. Further, impaired functional sympatholysis is a common characteristic of cardiovascular ageing and disease, including hypertension and heart failure. Despite decades of study, the specific mechanisms underlying functional sympatholysis in humans remain unclear. Approximately 20 years ago, work in animal models suggested that endothelium-derived hyperpolarization associated with acetylcholine-mediated vasodilatation, and later ATP-mediated vasodilatation, was capable of attenuating sympathetic vasoconstriction, and that these vasodilatory pathways may explain the phenomenon of functional sympatholysis in humans. However, since these initial studies in animals, there has been a relative paucity of data to support these conclusions regarding sympatholysis in humans, especially in contracting skeletal muscle. These studies are the first in humans to demonstrate the role of the endothelium in functional sympatholysis, and point to a primary role for endothelium-derived hyperpolarization in the modulation of sympathetic vasoconstriction during exercise. Further, endothelium-derived hyperpolarization represents a vasoactive pathway with the dual ability to cause vasodilatation while simultaneously blunting adrenergic vasoconstriction and preserved responsiveness in older adults. Therefore, EDH represents a unique target for the improvement of blood flow, oxygen delivery, and potentially exercise intolerance in conditions associated with elevated sympathetic nervous system activity and impaired vasodilatory function.

APPENDIX A – HUMAN SUBJECTS APPROVAL

Colorado State University
Knowledge to Go Places

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

NOTICE OF APPROVAL FOR HUMAN RESEARCH

DATE:	December 04, 2013
TO:	Dinenno, Frank, Health & Exercise Science
	Israel, Richard, Health & Exercise Science, Richards, Jennifer, 1582 Dept Hlth & Exer Sci, Scott, Hannah, 1570 Human Dev & Fam Stds
FROM:	Barker, Janell, Coordinator, CSU IRB 1
PROTOCOL TITLE:	Aging and Sympathetic Vasoconstriction: Rest vs. Exercise
FUNDING SOURCE:	Funding - Grants/Contracts , Other Funding
PROTOCOL NUMBER:	09-1186H
APPROVAL PERIOD:	Approval Date: November 18, 2013 Expiration Date: November 17, 2014

The CSU Institutional Review Board (IRB) for the protection of human subjects has reviewed the protocol entitled: Aging and Sympathetic Vasoconstriction: Rest vs. Exercise. The project has been approved for the procedures and subjects described in the protocol. This protocol must be reviewed for renewal on a yearly basis for as long as the research remains active. Should the protocol not be renewed before expiration, all activities must cease until the protocol has been re-reviewed.

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

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

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

Janell Barker, Senior IRB Coordinator - (970) 491-1655 Janell.Barker@Colostate.edu
Evelyn Swiss, IRB Coordinator - (970) 491-1381 Evelyn.Swiss@Colostate.edu

Barker, Janell

Janell Barker

Barker, Janell

Approval is to continue to recruit the remaining 201 participants with the approved recruitment and consent material. This also reflects an approved minor amendment to increase the total approved to recruit from 940 to 1,040. With a total of 839 already recruited, the balance remaining to recruit is 201. The above-referenced project was approved by the Institutional Review Board with the condition that the approved consent form is signed by the subjects and each subject is given a copy of the form. NO changes may be made to this document without first obtaining the approval of the IRB.

Approval Period:	November 18, 2013 through November 17, 2014
Review Type:	FULLBOARD
IRB Number:	00000202
Funding:	National Institute on Aging, National Institute of Health , Monfort Professor
	DEXA Scan, ECGs, Ultrasound, 2-MHz pulsed flat transcranial probe, 7.5 MHz linear transducer, 7.5 MHz linear echo probe, automated oscillometric technique, anesthesia monitor, Teflon catheters, biopsy needle, peroxidase diaminobenzidine reaction kit, coated tubes

APPENDIX B – CONSENT FORM

Consent to Participate in a Research Study
Colorado State University

TITLE OF STUDY: Regional Blood Flow Control and Vascular Function in Humans

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

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 of the Health and Exercise Science Department, and also by Dennis Larson, M.D. and Gary Luckasen, M.D., of the Heart Center of the Rockies (University of Colorado Health). Trained graduate students, undergraduate students, research assistants, or research associates are assisting with the research. These studies are paid for by the National Heart Lung and Blood Institute, a part of the US Government (National Institutes of Health).*

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

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST? *This whole research project will take place over a period of approximately five years. However, your part of this study will be:*
_____ *1) one or two visits over a several day period* _____ ***(your initials)***

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

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

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

_____ ***(your initials)***

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

_____ ***(your initials)***

_____ ***Maximum Voluntary Contraction.*** *This will consist of 3-4 trials where you will squeeze your muscles (either forearm, calf, or thigh) and generate as much force as you can. You will be asked to generate as much force over the course of ~3 seconds and hold this force another 5 seconds. After a 2-3 minute rest period, you will be asked to do this again. This is typically used to determine how heavy of exercise you perform so everybody is exercising at similar percentages of their maximum. (~ 15 minutes)*

_____ ***(your initials)***

FOR OLDER ADULTS ONLY:

_____ ***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)***

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

_____ ***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)***

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

_____ ***(your initials)***

____ ***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 hours)*

_____ ***(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)***

_____ ***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)***

CSU#: 14-5392H
APPROVED: 11/19/2014 * EXPIRES: 10/28/2015

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

____ ***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)***

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

_____ ***(your initials)***

____ ***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)***

____ ***Drug Administration (~ 2 - 4 hours).*** *The administration of one of more of the following drugs might occur several times throughout the study by way of the arterial catheter. If you do not respond to a certain drug, the investigators may need to change the drugs administered to achieve the desired responses. If this is the case, the changes will be explained to you as in the original consent process. If you choose to continue with the study, the changes will be noted on this consent form and you will be asked to initial and date next to the specific changes. If you do not choose to continue, the study will end after these initial tests.*

Vasoconstrictors – cause temporary narrowing of the blood vessels (minutes)

___ *Norepinephrine*
___ *Phenylephrine*
___ *Dexmedetomidine*
___ *L-NMMA*
___ *Ketorolac*
___ *Barium Chloride*
___ *Ouabain*

Vasodilators – temporarily relax the blood vessels (minutes)

___ *Acetylcholine*
___ *Adenosine*
___ *Sodium Nitroprusside*
___ *Phentolamine*
___ *Adenosine Triphosphate (ATP)*
___ *Potassium Chloride (K^+)*
___ *Isoproteronol*

No major effects

___ *Ascorbic Acid (Vitamin C)*
___ *Propranolol*
___ *Aminophylline*
___ *Pyridoxine*

_____ ***(your initials)***

- _____ *Reactive Hyperemia- 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)***

- _____ *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/Supplement Administration - The risks associated with drug administration include temporary increases or decreases in blood pressure and heart rate. In the case of 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)***

- 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 and arterial or venous catheterization, you will be paid $15/hour.*
Your identity/record of receiving compensation (NOT your data) may be made available to CSU officials for financial audits.

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

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

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

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

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

_____ _____
Name of person providing information to participant Date

_____

Signature of Research Staff

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

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