DISSERTATION

ROLE OF VASCULAR HYPERPOLARIZATION IN MUSCLE BLOOD FLOW REGULATION IN HEALTHY HUMANS

Submitted by

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In partial fulfillment of requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2013

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ABSTRACT

ROLE OF VASCULAR HYPERPOLARIZATION IN MUSCLE BLOOD FLOW REGULATION IN HEALTHY HUMANS

The following investigation composes a series of experiments with the overall aim of determining the role for vascular hyperpolarization via activation of inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+-ATPase in the regulation of vascular tone in response to muscle contractions and ischaemia in young, healthy humans. We tested the general hypothesis that activation of K_{IR} channels and Na^+/K^+-ATPase contributes in large part to the vasodilatory, hyperaemic, and sympatholytic responses observed in these conditions and this contribution is greater than that of other vasodilators, specifically nitric oxide (NO) and prostaglandins (PGs). The specific aims of each experiment were: 1) to determine whether K^+-stimulated vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase mediates contraction-induced rapid vasodilatation in the human forearm; 2) to determine whether vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase contributes to the hyperaemic response at the onset of repeated muscle contractions, as well as to steady-state forearm blood flow during rhythmic handgrip exercise; 3) to determine whether vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase contributes to the observed blunting of sympathetically-mediated vasoconstriction that occurs during moderate intensity rhythmic forearm exercise; and 4) to determine whether vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase contributes to the observed reactive hyperaemia that occurs in the human forearm following release of temporary ischaemia. Our collective findings

demonstrate a significant contribution of K_{IR} channels and Na^+/K^+-ATPase activation to rapid vasodilatation following a single muscle contraction, the onset of exercise hyperaemia in response to repeated muscle contractions, steady-state muscle blood flow during rhythmic handgrip exercise and reactive hyperaemia following temporary ischaemia. In contrast to our hypothesis, we did not observe a significant contribution of K_{IR} channels and Na^+/K^+-ATPase to the observed blunting of sympathetic α-adrenergic vasoconstriction that occurs during handgrip exercise. In all studies, any role of NO and PGs was modest, if present at all. Taken together, our findings indicate that during a variety of vasodilatory stimuli, there is a large contribution of pathways that are independent of NO and PGs, specifically activation of K_{IR} channels and Na^+/K^+-ATPase. Hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase represents a novel mechanistic pathway in the understanding of *in vivo* regulation of muscle blood flow in response to contractions and ischaemia. These findings may provide insight into understanding impaired vascular function in patient populations and as such, could represent a novel therapeutic target for reversing microvascular dysfunction.

ACKNOWLEDGEMENTS

We would like to thank the subjects who volunteered for these studies as well as Leora J. Garcia, Hannah Scott, Christopher M. Hearon Jr., and Devin V. Dinenno for their assistance in conducting these studies and preparation of contained manuscripts. All work was funded by NIH Awards HL102720, HL095573 (F.A.D.).

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CHAPTER I – INTRODUCTION AND EXPERIMENTAL AIMS

Skeletal muscle comprises the largest tissue mass in the human body and has a high capacity for increased metabolism. During exercise, muscle blood flow can increase up to 20 fold and receive a large proportion (~85%) of total cardiac output and thus can have a significant effect on total peripheral resistance. As mean arterial pressure is determined by total peripheral resistance and cardiac output, the understanding of muscle blood flow regulation is important in understanding overall cardiovascular control.

At the local tissue level, blood flow is the product of the pressure gradient across the tissue ($\Delta P = P_{arterial} - P_{venous}$) and the vascular conductance. According to Poiseuille's equation, given a constant viscosity, conductance is proportional to the radius of a vessel to the fourth power. Taken together, if perfusion pressure is fairly constant, it is the calibre of the resistance vessels that largely determines tissue blood flow. A given arteriolar diameter represents the integrative balance between a variety of vasodilating and vasoconstricting stimuli. These stimuli have diverse categorization, from neural, hormonal, metabolic, to mechanical, as well as varied origin, stemming from nerve, skeletal muscle, vascular smooth muscle, endothelial, and blood cell sources. Ultimately, the contractile state of vascular smooth muscle cells is determined by intracellular $[Ca^{2+}]$ with greater $[Ca^{2+}]_i$ resulting in contraction and vasoconstriction and decreased $[Ca^{2+}]_i$ resulting in relaxation and vasodilatation. Vascular smooth muscle cell $[Ca^{2+}]_i$ can be affected by changes in second messenger signaling [i.e. cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP)] and related kinases as well as direct alterations in $[Ca^{2+}]$ via calcium ion channel activity. Of most relevance, the open state of voltage-dependent calcium channels is determined by the membrane potential of vascular smooth muscle cells. Hyperpolarization of vascular smooth muscle cells inhibits voltage-

dependent calcium channels, decreasing $[Ca^{2+}]_i$ resulting in vascular smooth muscle cell relaxation and vasodilatation. Characteristic of hyperpolarization-induced vasodilatation is that it is conducted, and with respect to tissue blood flow control, spreads upstream from the site of stimulus and this is thought to be important in regulating profound changes in arteriolar resistance.

The vasoactive signaling pathways that contribute to vascular tone during a variety of conditions have been well-studied in humans. Many signaling pathways involve the innermost lining of blood vessels, a monolayer of cells critical in vascular control, known as the endothelium. An important role for endothelium-dependent vasodilating substances has been realized, and endothelial health relates to cardiovascular disease morbidity and mortality. Muscle contractions, or exercise, stimulate a variety of responses that span all of the regulatory levels (neural, hormonal, metabolic, mechanical) of muscle blood flow control. Attempts have been made to characterize this complex and integrative regulation, but our understanding of the specific signaling pathways involved remains unclear, particularly at the level of local vasodilator signaling. Investigations have utilized additional stimuli other than muscle contraction such as temporary ischaemia in order to experimentally 1) create a mismatch in oxygen delivery and demand, independent of muscle contractions and evoke local vasodilatation, and 2) serve as a marker of vascular health and responsiveness. However, similar to exercise, the underlying signaling pathways that contribute to the reactive hyperaemic response to arterial occlusion is also uncertain.

In addition to a variety of signals for vasodilatation and vascular smooth muscle cell relaxation, repeated muscle contractions present an additional challenge in understanding vascular regulation. In order to appropriately regulate mean blood pressure given the

aforementioned significant capacity to increase blood flow to skeletal muscle during exercise, sympathetic activation increases with progressive exercise intensity. Sympathetic activation results in increased noradrenaline release and subsequent binding to α-adrenergic receptors on vascular smooth muscle cells, eliciting vasoconstriction which in turn, limits the decrease in total peripheral resistance. Sympathetic vasoconstrictor activity increases throughout the body, including in the active muscle vasculature. However, contracting skeletal muscle has a unique ability to somewhat blunt the vasoconstrictor stimulus (as compared to the same stimulus at rest), as a means of preserving oxygen delivery to the active tissue. This phenomenon, classically termed "functional sympatholysis", is graded with exercise intensity and occurs through post-junctional modification of both α_1 and α_2-adrenergic receptors. The exact signaling pathways involved in the modulation of sympathetic constriction that occurs in contracting skeletal muscle are also largely undetermined in humans.

The development of various pharmacological agents has led to the *in vivo* ability to enzymatically inhibit the synthesis of two primary vascular signaling pathways, nitric oxide (NO) and prostaglandins (PGs) that predominantly stimulate vasodilatation by local changes in vascular smooth muscle cell $[Ca^{2+}]_i$ via alterations in cyclic-guanosine monophosphate dependent protein kinase (PKG) and cyclic-adenosine monophosphate dependent protein kinase (PKA), respectively. As stated, $[Ca2^{+}]_i$ can also be modulated by changes in smooth muscle cell membrane potential and this mechanism of vasodilatation has been challenging to investigate, particularly in humans. It has been shown in animal models that stimulation of inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+-ATPase leads to hyperpolarization, and we have recently demonstrated the ability to inhibit these pathways in the human forearm.

Therefore, this body of work comprises four specific hypotheses with the general aim of understanding the role of vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase in the regulation of vascular tone in human skeletal muscle in response to muscle contractions and temporary ischaemia.

Overall hypothesis: Vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase has a significant role in the control of vascular tone in young healthy humans. Specifically, these pathways contribute to rapid vasodilatation following a single muscle contraction and the hyperaemic response to repeated muscle contractions and ischaemia. Further, these pathways are able to modulate sympathetically-mediated vasoconstriction during repeated muscle contractions.

Specific Aims

Experiment #1: to determine whether vascular K^+-stimulated hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase mediates rapid vasodilatation in the forearm of young adults.

Experiment #2: to determine whether vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase contributes to the hyperaemic response at the onset of repeated muscle contractions, as well as to steady-state forearm blood flow during rhythmic handgrip exercise in young, healthy humans.

Experiment #3: to determine whether vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase contributes to the observed blunting of sympathetically-mediated vasoconstriction that occurs during moderate intensity rhythmic forearm exercise in young, healthy humans.

Experiment #4: to determine whether vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase contributes to the observed reactive hyperaemia that occurs in the forearm of young, healthy humans following release of temporary ischaemia.

To the best of our knowledge, this collection of work presents novel and significant insight into a highly-involved vascular signaling pathway in human skeletal muscle. We show that activation of K_{IR} channels and Na^+/K^+-ATPase significantly contributes to rapid vasodilatation following a single muscle contraction, the onset and steady-state hyperaemia observed with repeated muscle contractions and the reactive hyperaemic response to temporary ischaemia. Further, the contribution of NO and PGs is minimal to non-existent under these conditions. Contrary to our hypothesis, we did not observe a significant role for K_{IR} channel and Na^+/K^+-ATPase activation in the ability to modulate sympathetic vasoconstriction during muscle contractions, and thus the signaling pathways regulating functional sympatholysis remain undetermined. However, the collective evidence from the contained study and literature suggests that vascular hyperpolarization, beyond K_{IR} channels and Na^+/K^+-ATPase may be involved. Taken together, our findings and the profound magnitude of some of the observed effects of inhibiting K_{IR} channels and Na^+/K^+-ATPase activation indicate that vascular hyperpolarization is essential to vascular control in humans in response to vasoactive stimuli.

CHAPTER II – MANUSCRIPT I

Mechanisms of rapid vasodilatation following a brief contraction in human skeletal muscle[1]

Summary

A monophasic increase in skeletal muscle blood flow is observed following a brief single forearm contraction in humans, yet the underlying vascular signaling pathways contributing to this response remain largely undetermined. Evidence from experimental animals indicates an obligatory role of vasodilatation via smooth muscle hyperpolarization and human data suggests little-to-no independent role for nitric oxide (NO) or vasodilating prostaglandins (PGs). Recent *in situ* data suggest that potassium (K^+) release during a single muscle contraction activates both inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+-ATPase, and thus may initiate the observed vasodilatation. We tested the hypothesis that K^+-mediated vascular hyperpolarization

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occurring via activation of K_{IR} channels and Na^+/K^+-ATPase underlies the vasodilatation following a brief single contraction in humans and that combined inhibition of NO and PGs would have a minimal effect on this response. We measured forearm blood flow (Doppler ultrasound) and calculated vascular conductance 10 sec prior to, and for 30 sec after a single 1-sec dynamic forearm contraction at 10, 20, and 40% maximum voluntary contraction (MVC) in 16 young adults. Inhibition of K^+-mediated vasodilatation was achieved through combined inhibition of K_{IR} channels and Na^+/K^+-ATPase via intra-arterial infusion of barium chloride ($BaCl_2$) and ouabain, respectively. Combined enzymatic inhibition of NO and PG synthesis occurred via L-NMMA (NO synthase) and ketorolac (cyclooxygenase), respectively. In Protocol 1 (n=8), $BaCl_2$+ouabain reduced the magnitude of the peak vasodilator response at all intensities (P<0.05; range = 30-45%) and total post-contraction vasodilatation (area under the curve; AUC) was attenuated ~55-75% from control. Contrary to our hypothesis, L-NMMA+ketorolac further reduced peak vasodilatation and AUC by ~60% and ~80%, respectively, from control. Thus, in Protocol 2 (n=8), we first administered L-NMMA+ketorolac and observed a reduced peak vasodilatation (P<0.05; range = 25-35%) and AUC ~25-40% from control at all contraction intensities. $BaCl_2$+ouabain further attenuated the response and the total effect of all four inhibitors was similar to Protocol 1. We conclude that K^+-mediated hyperpolarization and NO and PGs, in combination, significantly contribute to contraction-induced rapid vasodilatation and inhibition of all four pathways nearly abolishes this phenomenon in humans.

Introduction

Regulation of skeletal muscle hyperaemia during muscle contractions is complex and involves a variety of signals that control both the arterio-venous perfusion pressure gradient and

arteriolar calibre (Clifford & Hellsten, 2004; Calbet & Joyner, 2010). In an attempt to isolate the local mechanisms underlying exercise hyperaemia, early experiments utilized a single brief muscle contraction to allow for contraction-induced hyperaemia without the continuous interruption of the blood flow response or further stimulus for hyperaemia, as occurs with repeated contractions (Corcondilas *et al.*, 1964). In this regard, the single contraction model can serve as a tool to examine feed-forward mechanisms of hyperaemia that are largely independent of changes in tissue oxidative metabolism (Mohrman *et al.*, 1973). The typical response is characterized by an intensity-dependent, rapid, monophasic increase in blood flow that occurs immediately (within one cardiac cycle) following contraction, achieves full magnitude in ~5 cardiac cycles, and then declines towards baseline. To date, the essential underlying mechanisms for this rapid hyperaemia, and thus feed-forward regulation of muscle blood flow, have yet to be determined in humans.

Given the rapid nature of single contraction-induced hyperaemia, some investigators have suggested that this response is due to changes in the arterio-venous perfusion pressure gradient (Laughlin, 1987; Sheriff *et al.*, 1993; Tschakovsky *et al.*, 1996); however, several lines of evidence now clearly demonstrate vasodilatation is obligatory to observe this hyperaemic phenomenon (Hamann *et al.*, 2003; Hamann *et al.*, 2004; Tschakovsky *et al.*, 2004) and a portion of this response is attributable to the mechanical compression of the vasculature that occurs during muscle contraction (Clifford *et al.*, 2006; Kirby *et al.*, 2007). Over the last century, a multitude of vasodilating factors have been suggested to contribute to contraction-induced vasodilatation, yet in humans, none have been found obligatory to observe a significant increase in muscle blood flow (Clifford, 2007; Joyner & Wilkins, 2007).

Interestingly, evidence in animals indicates that the potassium ion (K^+) released during muscle contractions activates both inwardly-rectifying K^+ (K_{IR}) channels and Na^+/K^+-ATPase to evoke vascular smooth muscle cell hyperpolarization and subsequent rapid vasodilatation (Armstrong *et al.*, 2007). These data substantiate early observations that changes in interstitial $[K^+]$ following brief muscle contraction has the appropriate magnitude and time course to have a significant involvement in the hyperaemic response (Mohrman & Sparks, 1974b; Duling, 1975; Murray & Sparks, 1978; Hazeyama & Sparks, 1979; Murray *et al.*, 1979; Kiens *et al.*, 1989) and that smooth muscle vascular hyperpolarization may be essential to observe rapid vasodilatation (Hamann *et al.*, 2004). Whether K^+-mediated vasodilatation via K_{IR} channel and Na^+/K^+-ATPase activation contributes to contraction-induced rapid vasodilatation in humans is unknown.

In an attempt to understand the contributing vasoactive pathways to rapid vasodilatation in human subjects, investigators have targeted acetylcholine released from motor nerves as well as "traditional" substances synthesized by the vascular endothelium. In this context, acetylcholine spillover from motor neurons is not obligatory to observe the vasodilator response, (Brock *et al.*, 1998), and further, independent inhibition of nitric oxide (NO) (Brock *et al.*, 1998) or vasodilating prostaglandin (PG) synthesis (Shoemaker *et al.*, 1996) does not impact rapid vasodilatation. However, despite evidence indicating considerable interaction between NO and PGs in the control of vascular tone in response to a variety of stimuli (Vanhoutte, 1992; Schrage *et al.*, 2004; Nicholson *et al.*, 2009; Markwald *et al.*, 2011), no studies to date have determined whether combined NO and PG inhibition reduces the rapid vasodilatation in response to single contractions.

Given this information as background, we sought to determine the underlying signaling mechanisms of muscle contraction-induced rapid vasodilatation in humans. We tested the hypothesis that K^+-stimulated vascular hyperpolarization mediated by activation of K_{IR} channels and Na^+/K^+-ATPase underlies the vasodilatation following a brief single contraction in humans and that combined inhibition of NO and PGs has a minimal effect on this response.

Methods

Subjects

With Institutional Review Board approval and after written informed consent, a total of 16 young healthy adults (13 men, 3 women; age = 23±1 years old; weight = 72.3±2.4 kg; height = 176±2 cm; body mass index = 23.4±0.5 kg m^{-2}; means ± SEM) participated in the present study. All subjects were sedentary to moderately active, non-smokers, non-obese, normotensive (resting blood pressure <140/90 mmHg), 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 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) for local administration of study

drugs and blood sampling. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml hr^{-1} with heparinized saline. The two side ports were used for drug infusions of vasoactive drugs (Kirby *et al.*, 2008; Crecelius *et al.*, 2010). Heart rate was determined using a 3-lead ECG (Cardiocap/5, Datex-Ohmeda Louisville, CO, USA).

Forearm Blood Flow and Vascular Conductance

A 4 MHz pulsed Doppler probe (Model 500M, Multigon Industries, Mt. Vernon, NY, USA) was used to measure brachial artery mean blood velocity (MBV) with the probe securely fixed to the skin over the brachial artery proximal to the catheter insertion site as previously described by our laboratory (Dinenno & Joyner, 2003; Kirby *et al.*, 2007). The probe insonation angle relative to the skin was 45 degrees. A linear 12 MHz echo Doppler ultrasound probe (GE Vingmed Ultrasound Vivid7, Horten, Norway) was placed in a holder securely fixed to the skin immediately proximal to the velocity probe to measure brachial artery diameter. Brachial artery diameter was measured in triplicate prior to any contractions under all experimental conditions, as we and others have shown that brachial diameter does not change in response to this stimulus (Tschakovsky *et al.*, 2004; Carlson *et al.*, 2008). Forearm blood flow 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 $mmHg^{-1}$.

Single Dynamic Forearm Contractions

Maximum voluntary contraction (MVC) was determined for the experimental arm as the average of three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3% of each other. Brief, dynamic forearm contractions were performed at 10, 20, and 40% of the subject's MVC using a handgrip pulley system attached to weights corresponding to each workload. The weight was lifted 4-5 cm over the pulley for a single, 1-second dynamic contraction as previously described (Carlson *et al.*, 2008). These mild-to-moderate contraction intensities were chosen to limit the contribution of systemic haemodynamics to forearm vasodilator responses and to eliminate reflex increases in sympathetic nervous system activity, and thus isolate the local effects of muscle contraction on vascular tone (Carlson *et al.*, 2008). At least 1.5 minutes of relaxation were given between each contraction to allow continuous measures of forearm haemodynamics post-contraction, as well as ample time for haemodynamics to return to baseline values (Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007; Carlson *et al.*, 2008) Workload intensity was randomized and counterbalanced across subjects to eliminate any order effect and trials were performed in triplicate to calculate an average response for each subject. Pilot studies in our laboratory have determined that MVC is not affected by any of the vasoactive substances, particularly barium chloride and ouabain, administered in these studies (n=12; pre: 41±2 kg *vs* post: 40±2 kg; P=0.43).

Vasoactive Drug Infusion

All drug infusions occurred via brachial artery catheter to create a local effect in the forearm. In order to inhibit K^+-mediated hyperpolarization, ouabain octahydrate (Sigma 03125, St. Louis, MO, USA) was infused at 2.7 nmol min^{-1} for 15 minutes as a loading dose to inhibit

Na^+/K^+-ATPase combined with barium chloride ($BaCl_2$; 10% w/v BDH3238, EMD Chemicals, Gibbstown, NJ, USA), infused at 0.45 μmol dl FAV^{-1} min^{-1} with a minimum dose of 4 μmol min^{-1} to a maximum dose of 5 μmol min^{-1} for 3 minutes as a loading dose to inhibit K_{IR} channels (Dawes *et al.*, 2002; Burns *et al.*, 2004; Dwivedi *et al.*, 2005; Jantzi *et al.*, 2006; Crecelius *et al.*, 2012). This dose of $BaCl_2$ has been adjusted to forearm volume as compared to our previous study (Crecelius *et al.*, 2012) in order to maximize efficacy while still remaining within doses safe for human administration and specific for K_{IR} channels (Dawes *et al.*, 2002; Jackson, 2005). Ouabain and $BaCl_2$ were prepared in saline and confirmed sterile and free of fungus/endotoxin and particulate matter with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS, USA) prior to use. To inhibit "traditional" endothelium-dependent vasodilatation, N^G-monomethyl-L-arginine (L-NMMA; Bachem, Germany) was administered to inhibit nitric oxide synthase-mediated production of NO in combination with ketorolac (Hospira, Lake Forest, IL, USA) to inhibit cyclooxygenase-mediated synthesis of PGs. Loading doses of L-NMMA and ketorolac were 25 mg (5 mg min^{-1} for 5 minutes) and 6 mg (600 μg min^{-1} for 10 min), respectively (Crecelius *et al.*, 2011b; Crecelius *et al.*, 2012). Depending on the protocol, maintenance doses of either $BaCl_2$ (0.45 μmol dl FAV^{-1} min^{-1}), ouabain (2.7 nmol min^{-1}), L-NMMA (1.25 mg min^{-1}), or ketorolac (150 μg min^{-1}) were infused for 3 minutes prior to each set of single contractions to ensure continuous blockade (see Experimental Protocol below). Forearm volume used for normalization for specific vasoactive drugs was determined from regional analysis of whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc, Bedford, MA, USA). Three single contractions at the respective workload were performed at 15, 30, and 45 seconds of the 3 min loading infusion prior to each set of single contractions in order to facilitate drug delivery to the active tissue.

Experimental Protocols

Two separate groups of 8 subjects were studied, with the primary difference being the order in which pharmacological inhibitors were administered. The experimental timeline is depicted in Figure 1. To establish control contraction-induced rapid vasodilatory responses, subjects performed single, brief forearm contractions in triplicate at 10, 20, or 40% MVC for 1-second with a minimum of 1.5 minutes of rest between contractions. Between contraction intensities, saline was infused for 3 minutes prior to the first contraction (Figure 1).

Protocol 1: To address our primary hypothesis regarding K^+-stimulated vascular hyperpolarization via K_{IR} channel and Na^+/K^+-ATPase activation as the predominant signaling pathway involved in rapid vasodilatation, in 8 subjects (MVC = 52±3 kg) following control responses, single contraction bouts were performed at each exercise intensity after combined infusion of $BaCl_2$ and ouabain. Next, in attempt to further elucidate the underlying signaling of rapid vasodilatation, we addressed endothelium-dependent vasodilators. The combined contribution of NO and PGs was assessed with combined administration of L-NMMA and ketorolac, respectively, and the single contractions were repeated.

Protocol 2: Given the unexpected findings from Protocol 1 regarding an effect of combined inhibition of NO and PGs on rapid vasodilation (see Results), in 8 different subjects (MVC = 42±4 kg), the order of inhibition was reversed so that after control responses were obtained, L-NMMA and ketorolac were infused to assess the combined contribution of NO and PGs, respectively, to contraction-induced rapid vasodilatation. The third set of single contractions was performed after subsequent infusion of $BaCl_2$ and ouabain to address K^+-mediated vascular

hyperpolarization via K_{IR} channel and Na^+/K^+-ATPase activation in the presence of combined NO and PG inhibition.

Control Experiments: In a subset of subjects (n=6), sodium nitroprusside (SNP; Nitropress, Hospira Inc.) was infused at 2 μg 100ml FAV^{-1} min^{-1} for 5 minutes (Kirby *et al.*, 2010) in control (saline) conditions and after prior administration of all four antagonists ($BaCl_2$, ouabain, L-NMMA and ketorolac) as a negative control to confirm intact capacity of the forearm resistance vasculature to vasodilate.

In a different subset of subjects (n=4), prior to any pharmacological inhibition, phenylephrine (PE; Baxter, Irvine, CA, USA) was infused at 6.25 ng 100ml FAV^{-1} min^{-1} for 2 minutes to pre-constrict the vasculature (Kirby *et al.*, 2008) prior to performance of a bout of 40% MVC single contractions to determine the impact of reduced basal vascular tone *per se* on forearm rapid vasodilatation. This dose of PE was selected in order to reduce basal forearm vascular conductance to a similar level (~20-30%) as we typically observe with infusion of the antagonists utilized in the experimental protocols (Crecelius *et al.*, 2011a; Crecelius *et al.*, 2012).

Time control experiments were not performed in this study as we have previously demonstrated that rapid vasodilatation to a single contraction is repeatable over the course of an experiment of similar duration (~2-3 hours) (Kirby *et al.*, 2009).

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, FVC, MAP, and HR represent an average of the last 10 seconds of the resting time period prior to

muscle contraction. The post-contraction data represent beat-by-beat analysis beginning with the first unimpeded cardiac cycle immediately after release of the contraction for a total of 30 cardiac cycles (Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007; Carlson *et al.*, 2008). The data presented for SNP trials represent an average of the final 30 seconds of pre-drug and post-drug infusion. Percent changes in FVC were calculated as: ((FVC post – FVC pre)/(FVC pre)) × 100 as this tracks changes in blood vessel radius independent of the initial level of vascular tone and is therefore the most appropriate index of changes in vasomotor tone (Buckwalter & Clifford, 2001). The total contraction-induced vasodilator response (area under the curve; AUC) was calculated as the sum of absolute FVC (ml min^{-1}) following contraction for 30 cardiac cycles minus pre-contraction FVC.

Statistics

All values are reported as means ± SEM. Baseline haemodynamics and total vasodilatation (AUC) values for each intensity (10, 20, 40% MVC) were assessed by one-way repeated measures ANOVA for drug condition. We chose to analyze each intensity separately due to differences in the magnitude of these values and to limit our analysis to only the relevant comparisons. For the change in peak vasodilatation, a two-way (condition × intensity) repeated measures ANOVA was used. Student-Newman-Keuls *post hoc* testing was performed when a significant *F* was observed. Comparisons in the control protocols were made with paired Student's t-tests. Significance was set at $P < 0.05$.

Results

Protocol 1

Baseline haemodynamics for both experimental protocols are presented in Table 1. Figure 2 presents the dynamic absolute forearm vascular conductance (*A, C, E)* and the vasodilatory response (%ΔFVC; *B, D, F)* to single muscle contractions at 10%, 20%, and 40% MVC. These responses followed the typical temporal pattern of vasodilatation, comprised of an immediate rise in FVC in all trials that peaked in an intensity-dependent manner within ~4-5 cardiac cycles and then returned to baseline levels. It is these dynamic responses from which we calculate our main variables of interest (peak vasodilatation and total vasodilatation). We have not performed statistical analysis on these dynamic curves, but present them in an effort to be comprehensive.

Infusion of $BaCl_2$+ouabain reduced resting forearm vascular conductance (Table 1; Figure 2), and significantly reduced the magnitude of the peak vasodilatory response at all intensities ($P<0.05$; Figure 3; range = 30-45%). Similarly, total post-contraction vasodilatation (AUC) was reduced from control following $BaCl_2$+ouabain for all intensities (10%: -74±8%; 20%: -59±10%; 40%:-55±4%; $P<0.05$; Figure 4).

Addition of L-NMMA+ketorolac tended to further reduce baseline forearm vascular conductance ($P=0.12$; Table 1; Figure 2) and contrary to our hypothesis, also attenuated the vasodilatory response following a single contraction (Figure 2). The peak post-contraction vasodilatory response was further reduced by L-NMMA and ketorolac for 20% and 40% MVC ($P< 0.05$; Figure 3), but only approached significance at 10% MVC ($P = 0.065$). Similarly, L-NMMA+ketorolac further reduced total post-contraction vasodilatation from the $BaCl_2$+ouabain condition at 20% and 40% ($P<0.05$) but not at 10% ($P=0.2$; Figure 4). The presence of all

inhibitors ($BaCl_2$+ouabain+L-NMMA+ketorolac) reduced the peak vasodilatation by ~60% and total vasodilatory response (AUC) by ~80% on average for the 3 contraction intensities, thus explaining nearly all of the rapid vasodilatation in response to a single muscle contraction. In all conditions and exercise intensities, systemic haemodynamics did not change post-contraction.

Protocol 2

Given the findings of Protocol 1 regarding an effect of combined inhibition of NO and PGs, we reversed the order of pharmacological inhibition in Protocol 2 in order to address the combined role of these pathways without prior inhibition of vascular hyperpolarization via K_{IR} channel and Na^+/K^+-ATPase activation. As anticipated, L-NMMA+ketorolac significantly reduced baseline FBF and FVC (Table 1). The dynamic absolute forearm vascular conductance (*A, C, E)* and the vasodilatory response (%ΔFVC *B, D, F)* to single muscle contractions at 10%, 20%, and 40% MVC for Protocol 2 are presented in Figure 5. Similar to Protocol 1, these responses followed the typical temporal pattern of vasodilatation observed and prior infusion of L-NMMA+ketorolac significantly reduced the magnitude of the peak vasodilatory response at all intensities (P<0.05; Figure 6; range = 27-34%). Total post-contraction vasodilatation (AUC) was also reduced from pre-blockade following L-NMMA+ketorolac at all intensities (10%: -34±14%; 20%: -25±12%; 40%:-40±7%; P<0.05; Figure 7).

Addition of $BaCl_2$+ouabain further reduced baseline vascular conductance (Table 1) and also attenuated the vasodilatory response following a single contraction (Figure 5). The peak post-contraction vasodilatory response was significantly impacted by the addition of $BaCl_2$+ouabain, approximately doubling the effect from L-NMMA+ketorolac at all contraction intensities (Figure 6). Similarly, $BaCl_2$+ouabain further reduced total post-contraction

vasodilatation from the L-NMMA+ketorolac condition at all intensities (Figure 7; $P< 0.05$). Remarkably similar to Protocol 1, the presence of all inhibitors ($BaCl_2$+ouabain+L-NMMA+ketorolac) reduced the peak vasodilatation by ~60% and total vasodilatory response (AUC) by ~80% on average for the 3 contraction intensities again explaining nearly all of the rapid vasodilatation in response to a single muscle contraction. In all conditions and exercise intensities, systemic haemodynamics did not change post-contraction.

Control Experiments

In order to confirm preserved vasodilator capacity after administration of $BaCl_2$+ouabain+L-NMMA+ketorolac, SNP was administered pre-blockade and at the end of the experimental protocol in a subgroup of 6 subjects. SNP caused significant vasodilatation that was unaffected by $BaCl_2$+ouabain+L-NMMA+ketorolac, despite a reduction in baseline FVC (Table 2).

As stated above and presented in Table 1, changes in baseline FVC were observed following administration of the experimental antagonists. In order to determine whether there is a direct effect of reduced baseline FVC *per se* on the vasodilator response to a single contraction, we pre-constricted the forearm vasculature with phenylephrine (α_1-adrenergic agonist) and had 4 subjects perform single contractions at 40% MVC and compared this to the control (pre-blockade) condition. As shown in Figure 8, we were successful in reducing baseline forearm vascular conductance to a similar extent as occurred in our experimental protocols (~30%; Panel A). In contrast to what was observed in our experimental conditions ($BaCl_2$+ouabain, L-NMMA+ketorolac, and $BaCl_2$+ouabain+L-NMMA+ketorolac), pre-constricting with phenylephrine did not reduce the dynamic vasodilator response to a single contraction (Figure

8*B*), nor did it impact the total post-contraction vasodilatation (Control: 1585±258 ml 100mmHg^{-1} vs Pre-Constricted: 1497±188 ml 100mmHg^{-1}; *P*=0.7).

Discussion

The purpose of the current study was to determine the primary vasodilator signaling pathways involved in response to a single muscle contraction. Specifically, based on prior work, we were interested in the contribution of K^+-stimulated vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase and the effect of combined inhibition of NO and PGs synthesis. The primary novel findings of the present study support a significant combined role of K_{IR} channel and Na^+/K^+-ATPase activation, likely stimulated by K^+ efflux from contracting skeletal muscle, in all facets of the rapid vasodilator response. Inhibition of K^+-mediated vascular hyperpolarization significantly reduces peak and total vasodilatation in response to three increasing intensities of single muscle contractions. Additionally, and contrary to our original hypothesis, we demonstrate that combined inhibition of NO and PGs synthesis significantly reduces the peak and total vasodilatation following a single contraction. When all four signaling pathways were inhibited, the peak and total vasodilatory responses were reduced remarkably by ~60 and ~80%, respectively compared with control conditions, thus explaining the majority of the response. Our collective findings identify for the first time that inhibition of K^+-mediated vascular hyperpolarization, along with NO and PG synthesis, nearly abolishes the rapid vasodilator response to a single muscle contraction and that these pathways largely explain this feed-forward aspect of muscle blood flow regulation in humans.

Contraction-induced Rapid Vasodilatation and Contributing Signaling Pathways

Early studies investigating muscle blood flow regulation in response to muscle contractions appreciated the rapid nature with which hyperaemia occurs following even a brief contraction (Anrep & von Saalfeld, 1935). Many different theories of what contributed to the rapid response were put forth including contributions of a mechanical effect of the muscle pump to alter perfusion pressure (Folkow *et al.*, 1970; Folkow *et al.*, 1971; Laughlin, 1987), direct mechanically-induced vasodilatation via arteriole compression/distortion (Gray *et al.*, 1967; Mohrman & Sparks, 1974a; Clifford *et al.*, 2006; Kirby *et al.*, 2007), neurally-mediated vasodilatation (Buckwalter *et al.*, 1997; Welsh & Segal, 1997; Buckwalter *et al.*, 1998) and metabolic vasodilatation (Gorczynski *et al.*, 1978). Near the end of the 20th century, a key study by Tschakovsky and colleagues eloquently demonstrated that mechanical effects of a contraction and resultant changes in perfusion pressure could not fully explain the hyperaemic response and vasodilatation of the vasculature did in fact occur in response to a single contraction in humans (Tschakovsky *et al.*, 1996). These findings were confirmed by studies in experimental animals where changes in arteriolar diameter can be directly determined (Hamann *et al.*, 2004; Mihok & Murrant, 2004; VanTeeffelen & Segal, 2006; Armstrong *et al.*, 2007). Despite the acceptance of this immediate rapid vasodilatation, studies in humans designed to understand the signaling mechanisms have yielded largely negative results. Typically performed in the forearm, human studies have shown little-to-no independent role for acetylcholine (Brock *et al.*, 1998), NO (Brock *et al.*, 1998), or PGs (Shoemaker *et al.*, 1996) in mediating rapid vasodilatation. To date, the most convincing evidence regarding the mechanism of rapid vasodilatation comes from animal models and suggests that K^+ from muscle released during contraction diffuses to vascular smooth muscle to activate both K_{IR} channels and Na^+/K^+-ATPase and cause hyperpolarization

and subsequent rapid vasodilatation (Hamann *et al.*, 2004; Armstrong *et al.*, 2007). However, prior to the current study, this hypothesized mechanism of K^+-mediated vascular hyperpolarization had not been tested in humans.

Recently, we demonstrated the ability to abolish exogenous K^+-mediated (intra-arterial KCl) vascular hyperpolarization and vasodilatation with combined $BaCl_2$ to inhibit K_{IR} channels and ouabain to inhibit Na^+/K^+-ATPase (Crecelius *et al.*, 2012). Thus, building upon this established pharmacology and recent findings in experimental animals, we tested the hypothesis that K^+-stimulated vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase contributes to contraction-induced rapid vasodilatation in humans. In Protocol 1, we demonstrate that combined blockade of K_{IR} channels and Na^+/K^+-ATPase significantly reduces both the peak and total rapid vasodilatory response (Figures 2-4). In addition to a ~30-45% reduction in the peak change in vascular conductance (Figure 3), we observed a ~55-75% attenuation in the total vasodilatation following a single muscle contraction (Figure 4). Taken together, the impact of inhibiting both K_{IR} channel and Na/K^+-ATPase activation on the total vasodilatation occurring post-contraction was profound, and is by far the largest in magnitude of any prior pharmacological inhibition of the rapid vasodilatory response in humans (Shoemaker *et al.*, 1996; Brock *et al.*, 1998).

In Protocol 1, given prior observations in humans indicating little-to-no independent role for NO nor PGs in single contraction-induced rapid vasodilatation (Shoemaker *et al.*, 1996; Brock *et al.*, 1998), we were somewhat surprised to observe a significant reduction in the response with combined inhibition of these substance (Figures 3 and 4). Specifically, we demonstrate that combined inhibition of NO and PGs further reduced the peak dilatory response from the $BaCl_2$+ouabain condition (~55-65% from control; Figure 3) as well as the total

vasodilatation (~80% from control; Figure 4). These results contrast previous findings in response to brief muscle contractions when each substance was independently inhibited (Shoemaker *et al.*, 1996; Brock *et al.*, 1998), but on the other hand, are consistent with what has been demonstrated during continuous steady-state exercise (Schrage *et al.*, 2004; Mortensen *et al.*, 2007). Based on the findings from Protocol 1, we next sought to determine the combined role of NO and PGs to contraction-induced rapid vasodilatation prior to inhibition of K^+-mediated vascular hyperpolarization via K_{IR} channels and Na/K^+-ATPase.

In Protocol 2, we reversed the order of drug infusions and found that combined inhibition of NO and PGs reduced the peak response by ~25-35%, whereas the total vasodilatory response was reduced by ~20-40%. These data are the first to clearly demonstrate a combined active role for these substances in contraction-induced rapid vasodilatation in humans. When $BaCl_2$ and ouabain were infused after inhibition of NO and PGs, the peak response was further attenuated (~60-70% from control; Figure 6) as was the total vasodilatory response (~80% from control; Figure 7). Thus, in both Protocols 1 and 2, the impact of all four inhibitors on the rapid vasodilatory response was profound, and our data provide clear evidence for the combined importance of K^+-mediated vascular hyperpolarization, NO, and PGs in mediating this response in healthy human subjects.

The collective data from our experiments support K^+-mediated vascular hyperpolarization via stimulation of K_{IR} channels and Na^+/K^+-ATPase, as well as a combined role for the autocoids NO and PGs, in mediating the rapid dilatory response to a single muscle contraction. In general, the magnitude of reduction in the total vasodilatation appears to be somewhat greater when K^+-mediated vascular hyperpolarization is inhibited as compared to NO and PGs; however, we lack the power to make an appropriate statistical comparison between

these 2 different subject groups. It also appears that the order in which combined inhibition of K_{IR} channels and Na^+/K^+-ATPase or of NO and PGs occurs does not impact the magnitude of the effects on peak or total vasodilatation (Figure 9).

Potential Stimuli for Vasodilatation Following a Single Muscle Contraction

The evidence from the present study clearly supports a profound effect of combined inhibition of K_{IR} channels, Na^+/K^+-ATPase, NO and PGs on contraction-induced rapid vasodilatation; however, the exact stimulus for the activation of these signaling pathways is unclear. Armstrong and colleagues (2007) demonstrated that both $BaCl_2$ and ouabain can independently inhibit arteriolar vasodilatation observed in response to muscle contractions evoked via electrical stimulation and that these pathways were activated by K^+ efflux from skeletal muscle into the interstitial space. In this model, they were able to pharmacologically inhibit voltage-gated K^+ channels to specifically address skeletal muscle K^+ as the stimulus to activate K_{IR} channels and Na^+/K^+-ATPase. Unfortunately, in our human forearm model, we are not able inhibit K^+ efflux from contracting muscle, and thus base our conclusions related to K^+-mediated activation of K_{IR} channels and Na^+/K^+-ATPase on our previous observations that inhibition of K_{IR} channels and Na^+/K^+-ATPase via $BaCl_2$ and ouabain, respectively, abolishes KCl-mediated vasodilatation in humans (Crecelius *et al.*, 2012). The potassium ion is an attractive candidate for the stimulus of rapid vasodilatation as the timecourse is appropriate and it would serve as a feedforward mechanism that couples rapid vasomotor responses with muscle activation (Murray *et al.*, 1979; De Clerck *et al.*, 2003; Burns *et al.*, 2004). Additionally, some animal studies report that K^+-mediated vasodilation is most often transient in nature (Burns *et al.*, 2004), and this may in part contribute to the distinct temporal pattern of the response.

While K^+ is a strong candidate for stimulating K_{IR} channels and Na^+/K^+-ATPase and supports our primary hypothesis regarding the mechanisms of rapid vasodilatation, K^+ would not explain the unexpected combined involvement of NO and PGs that we observed (Crecelius *et al.*, 2012). Recently, extracellular production of adenosine, resultant from degradation of adenine nucleotides via ecto 5' nucleotidase, was shown to contribute to rapid vasodilatation following electrically stimulated contraction of the hamster cremaster muscle (Ross *et al.*, 2013). Previous evidence would also suggest that adenosine is capable of stimulating NO and PG production in humans (Mortensen *et al.*, 2009; Nyberg *et al.*, 2010). It is also possible that acetylcholine, released at the neuromuscular junction could diffuse to nearby capillary and stimulate NO and PG production (Domeier & Segal, 2007). However, data in humans utilizing atropine to inhibit muscarinic receptors have shown little role for acetylcholine in rapid vasodilatation (Brock *et al.*, 1998), and thus, this latter possibility is unlikely. Finally, endothelial cell changes in intracellular [Ca_2^+] may be directly sensitive to the mechanical compression/distortion of the vasculature or changes in shear stress resultant from muscle contraction and thus could also stimulate NO and PG production (Gray *et al.*, 1967; Koller *et al.*, 1994; Osanai *et al.*, 2000; Clifford *et al.*, 2006; Kirby *et al.*, 2007).

Experimental Considerations

In several experimental conditions, alterations in baseline forearm vascular tone occurred as a result of the antagonists we administered. While we present our primary data as a relative change from baseline, as this appropriately tracks changes in arteriolar calibre from conditions of altered baseline blood flow (Buckwalter & Clifford, 2001), we felt it was necessary to more directly address whether increased basal vascular tone *per se* may impact the vasodilator

response to a single contraction. In a subgroup of subjects, we pre-constricted the forearm vasculature to a similar magnitude as observed in our experimental conditions with local infusions of the α_1-adrenergic agonist phenylephrine. Despite starting from a reduced level of forearm vascular conductance, there was no impact of pre-constriction on the vasodilator response following a brief contraction at 40% MVC (Figure 8). Thus, although the mechanisms by which phenylephrine causes an increase in basal vascular tone may differ from those of our inhibitors, we do not believe our primary conclusions regarding vascular hyperpolarization, NO, and PGs are simply due to a direct effect of the inhibitors on basal vascular tone.

Given the magnitude of the effect on vasodilatation that we observed, it is reasonable to question whether vasodilator capacity *per se* was impaired in our subjects throughout the experimental trials. To address this potential concern, in a subgroup of subjects, we administered sodium nitroprusside, an endothelium-independent vasodilator and demonstrated a preserved response following administration of all of our antagonists (Table 2). This is consistent with recent findings from our laboratory demonstrating that combined $BaCl_2$ and ouabain administration does not impair acetylcholine-mediated vasodilatation in humans (Crecelius *et al.*, 2012). Thus, the present observations do not reflect a generalized impairment in the forearm vasculature to respond to vasodilator stimuli (i.e. our findings are specific to muscle contractions).

While we have previously shown that $BaCl_2$ and ouabain inhibit KCl-mediated vasodilatation (Crecelius *et al.*, 2012) and a multitude of work supports that activation of K_{IR} channels and Na^+/K^+-ATPase leads to vascular hyperpolarization *in vitro* (Nelson & Quayle, 1995; Edwards *et al.*, 1998; Burns *et al.*, 2004), we are inherently limited in our human *in vivo* model in that we are unable to directly determine cellular membrane potential and thus

demonstrate vascular hyperpolarization nor are we able to inhibit K^+ release from contracting muscle. In this same regard, it is possible that although we administer $BaCl_2$ at a concentration within the range to specifically block K_{IR} channels (Dawes *et al.*, 2002; Jackson, 2005), we cannot definitively exclude the possibility that we may be inhibiting other K^+ channels, mostly likely K_{ATP} channels. It should also be acknowledged that although we did not determine the efficacy of our inhibitors, we have clearly demonstrated efficacy of all drugs in various previous studies in the human forearm (Dinenno *et al.*, 2003; Schrage *et al.*, 2004; Crecelius *et al.*, 2012). It is possible that any potential intensity-dependent differences in the magnitude of the observed responses was due to incomplete blockade and the ability of high intensity (i.e. 40% MVC) contractions to somewhat override our inhibitors. This is particularly true for $BaCl_2$ which can be overridden by high concentrations of K^+ (Armstrong & Taylor, 1980; Jantzi *et al.*, 2006). Similarly, the remaining minimal vasodilatation observed could be explained by a lack of complete blockade or alternatively, other additional mechanisms that may contribute to rapid vasodilatation in response to a brief muscle contraction.

Perspectives

As recognized in early studies and clearly appreciated in more recent investigations, the model of a single muscle contraction allows for the investigation of feed-forward mechanisms of exercising blood flow regulation. Whereas much work has been done attempting to understand the underlying vasomotor signaling pathways of the metabolic feedback mechanisms of exercise hyperaemia, less attention has been paid to the feed-forward response (Clifford & Tschakovsky, 2008). We have previously shown that rapid vasodilatation is impaired in older healthy adults (Carlson *et al.*, 2008; Kirby *et al.*, 2009) and recent evidence suggests that obese individuals also

demonstrate attenuated rapid vasodilatation (Blain *et al.*, 2012). The underlying mechanisms of this impairment in these populations are currently unknown, but based on the present findings are most likely related to decreased K^+-mediated hyperpolarization and/or potentially attenuated production of NO and PGs.

Conclusions

Rapid vasodilatation occurs following a brief skeletal muscle contraction and, to date, the underlying vasomotor signaling pathways involved in this response have yet to be determined. In the present study, we demonstrate that K^+-stimulated vascular hyperpolarization via K_{IR} channel and Na^+/K^+-ATPase activation significantly contributes to contraction-induced rapid vasodilatation as do NO and PGs, in combination. Collective blockade of K_{IR} channels, Na^+/K^+-ATPase, NO and PGs nearly abolishes this phenomenon in humans, thus remarkably explaining the vast majority of rapid vasodilatation. Future studies designed to determine whether vascular hyperpolarization via these pathways, independently and in tandem with NO and PGs, continues to regulate exercise hyperemia when muscle contractions are repeated in humans are warranted.

Table 1. Baseline haemodynamics

* $P<0.05$ *vs* Pre-Blockade; † $P<0.05$ *vs* BaCl+Ouab; ‡ $P<0.05$ *vs* LNMMA+Ket

Protocol 1: n=8; Protocol 2: n=8; $BaCl_2$=Barium chloride; Ouab=ouabain; Ket=ketorolac

	Heart Rate (beats min^{-1})	**Mean Arterial Pressure (mmHg)**	**Forearm Blood Flow (ml min^{-1})**	**Forearm Vascular Conductance (ml min^{-1} 100mmHg^{-1})**
Protocol 1: $BaCl_2$+Ouab First				
Pre-Blockade	56±2	88±2	30.5±3.8	34.5±4.0
$BaCl_2$+Ouab	54±2	92±2*	22.6±1.4*	24.4±1.4*
$BaCl_2$+Ouab+L-NMMA+Ket	53±1	97±1*†	20.2±0.8*	19.4±1.4*
Protocol 2: L-NMMA+Ket First				
Pre-Blockade	55±3	87±3	21.1±2.5	24.1±2.8
L-NMMA+Ket	51±2	88±2	17.0±1.3*	19.2±1.5*
L-NMMA+Ket+$BaCl_2$+Ouab	52±3	91±3*	14.1±1.6*‡	15.5±1.7*‡

Table 2. Subgroup: Forearm haemodynamics during sodium nitroprusside infusion
**P*<0.05 *vs* Pre-Blockade

n=6; $BaCl_2$=Barium chloride; FAV=forearm volume; FVC=forearm vascular conductance (ml min^{-1} 100$mmHg^{-1}$); Ket=ketorolac; Ouab=ouabain; SNP=sodium nitroprusside

Condition	**Baseline FVC**	**SNP 2 µg dl FAV^{-1} min^{-1} FVC**	**ΔFVC (%)**
Pre-Blockade	16.2±2.4	138.5±25.5	877±276
L-NMMA+Ket+$BaCl_2$+Ouab	12.2±1.4*	117.5±12.6	912±124

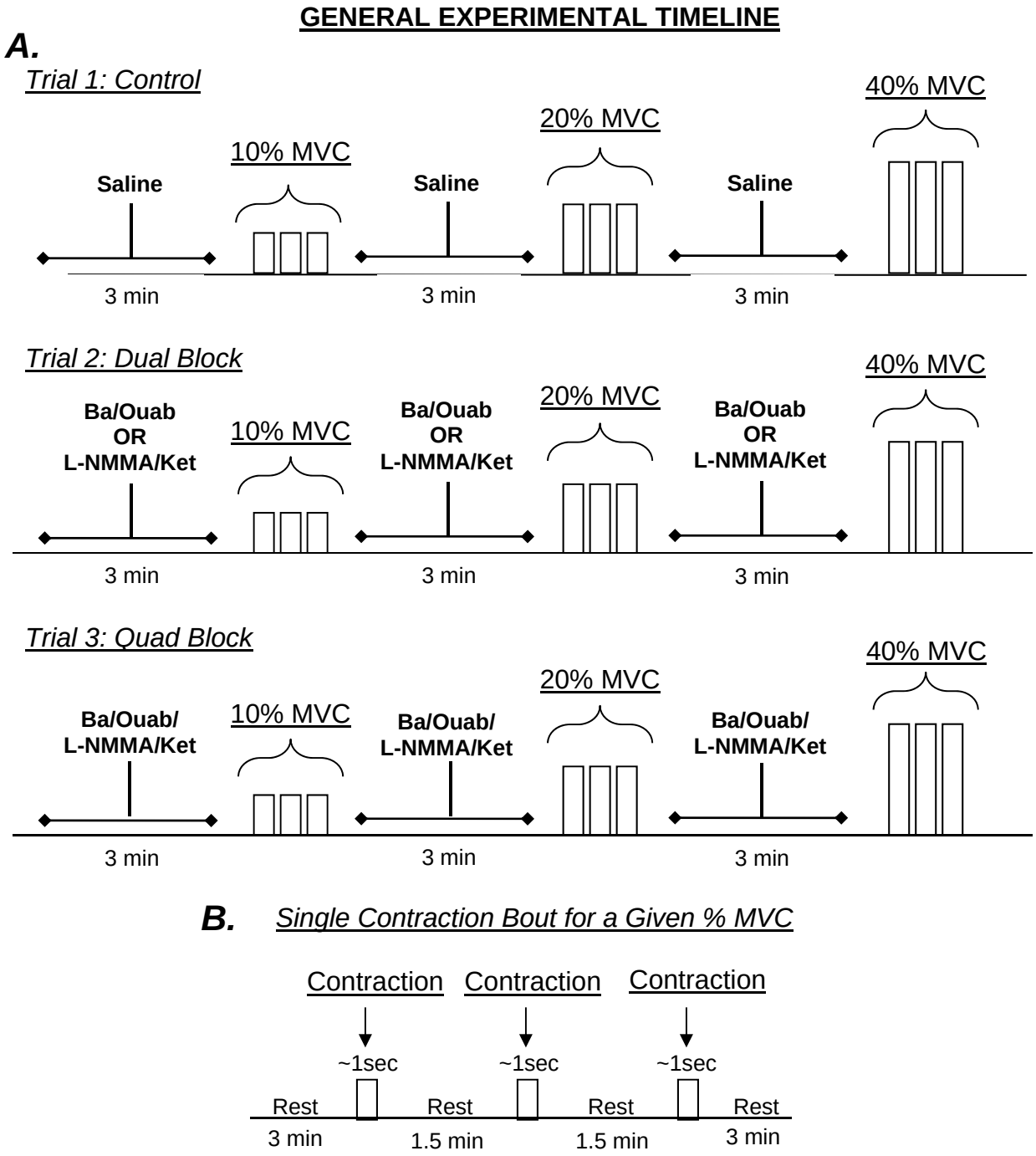

Figure 1. Experimental Timeline

A. Each experimental protocol consisted of 3 trials of single dynamic forearm contractions at 3 different intensities [10%, 20%, 40% maximal voluntary contraction (MVC)], all performed in triplicate. In the first trial, saline was infused via brachial artery catheter for 3 minutes prior to each set of contractions. In the second trial, depending on the experimental protocol, either $BaCl_2$+ouabain (n=8; Protocol 1) or L-NMMA+ketorolac (n=8; Protocol 2) was administered for 3 min prior to contractions. In the third trial, all subjects received all antagonists prior to each set of contractions. *B.* For each intensity, three contractions were performed, each lasting ~1 sec. Between each contraction, at least 1.5 min of rest was provided.

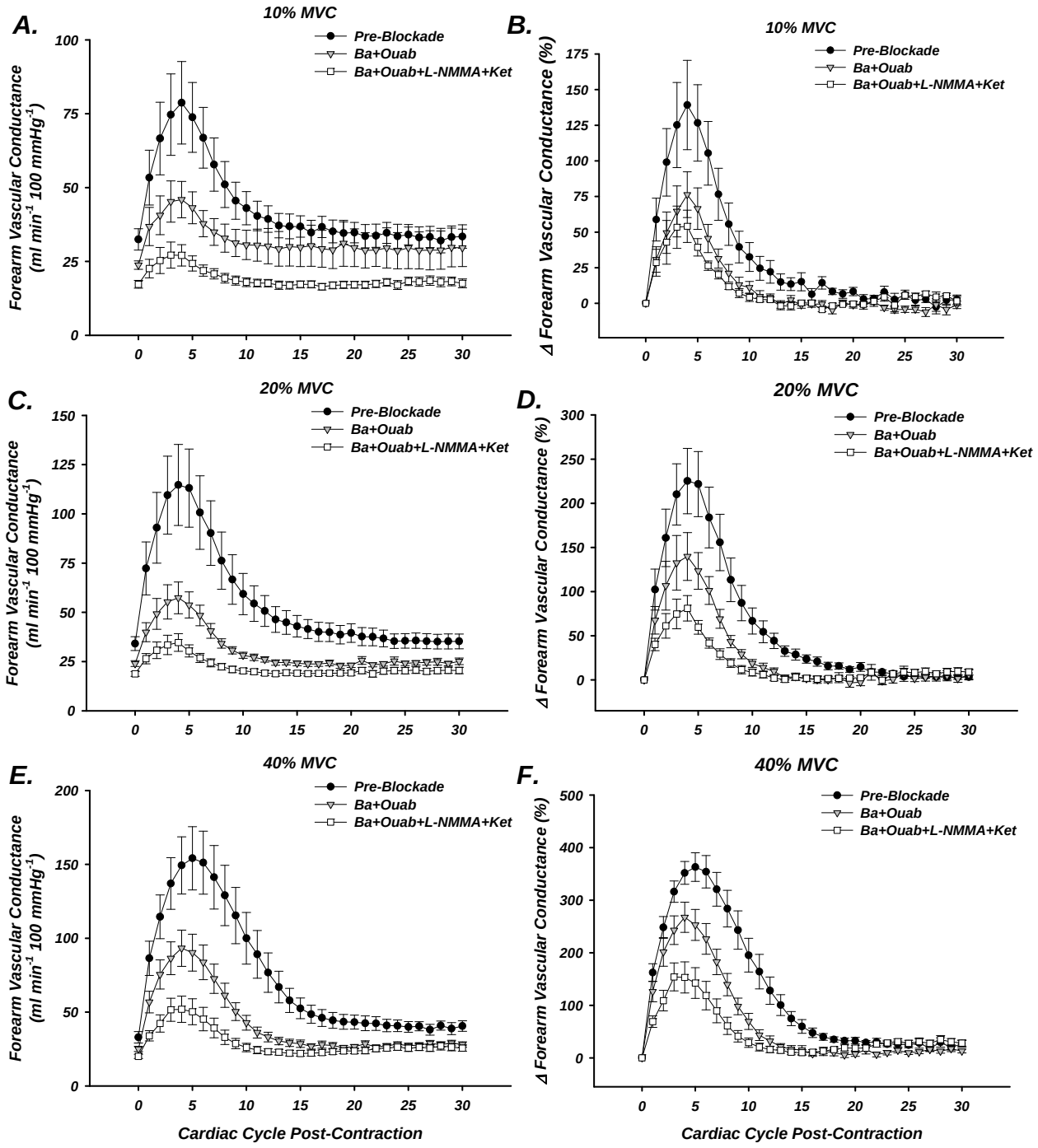

Figure 2. Protocol 1: Effect of $BaCl_2$+ouabain and $BaCl_2$+ouabain+L-NMMA+ketorolac on dynamic vasodilator responses to single contractions

Absolute forearm vascular conductance (*A, C, E*) and relative changes in forearm vascular conductance (*B, D, F*) are presented for 10%, 20%, and 40% MVC single contractions, respectively. Across all intensities, prior infusion of $BaCl_2$+ouabain (grey triangles) reduced the post-contraction vasodilatory response as compared to the pre-blockade (saline; black circles) condition. Additional infusion of L-NMMA+ketorolac (open squares) further inhibited these responses.

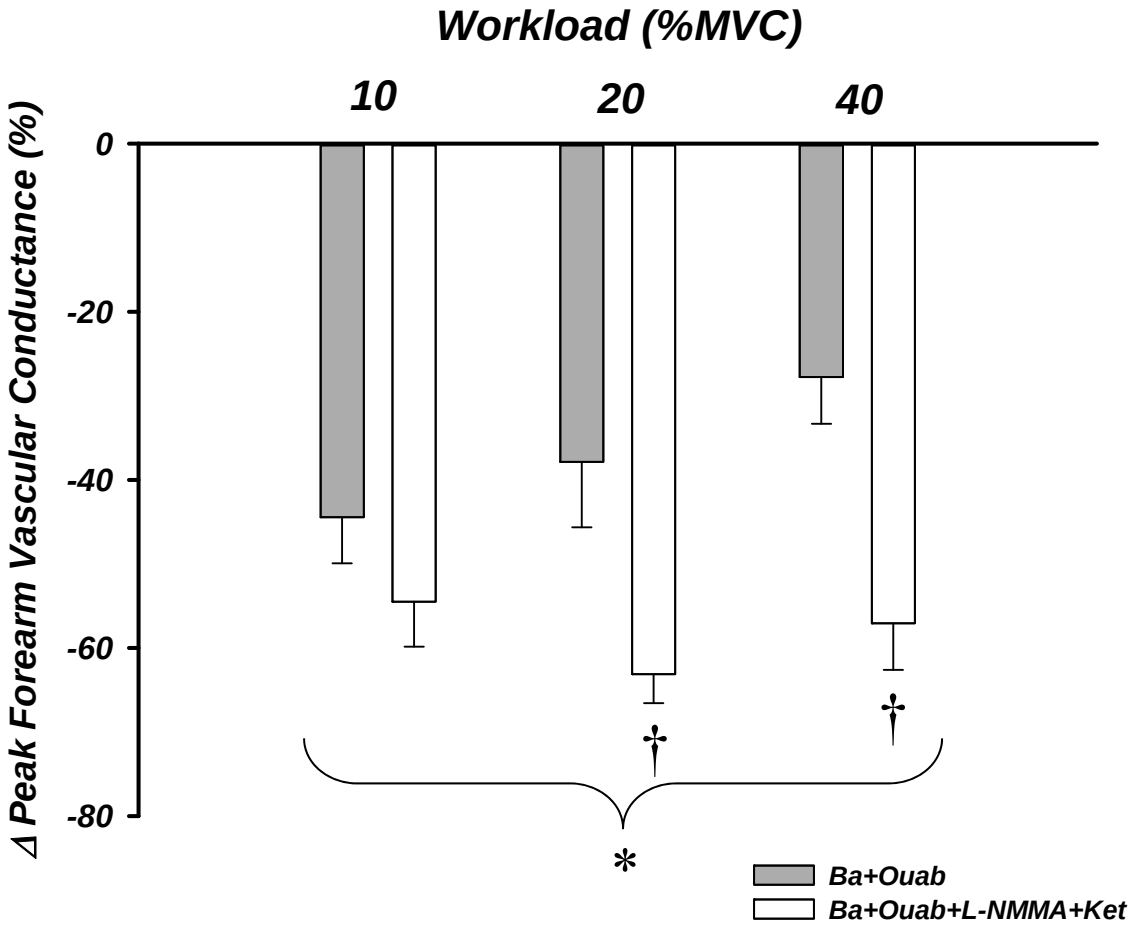

Figure 3. Protocol 1: Effect of $BaCl_2$+ouabain and $BaCl_2$+ouabain+L-NMMA+ ketorolac on peak post-contraction vasodilator response
Infusion of $BaCl_2$+ouabain (grey bars) significantly reduced the peak post-contraction vascular conductance ($P<0.05$ *vs* zero) at all contraction intensities. Addition of L-NMMA+ketorolac (open bars) further inhibited this response at 20% and 40% MVC. * $P<0.05$ *vs* zero; † $P<0.05$ *vs* Ba+Ouab.

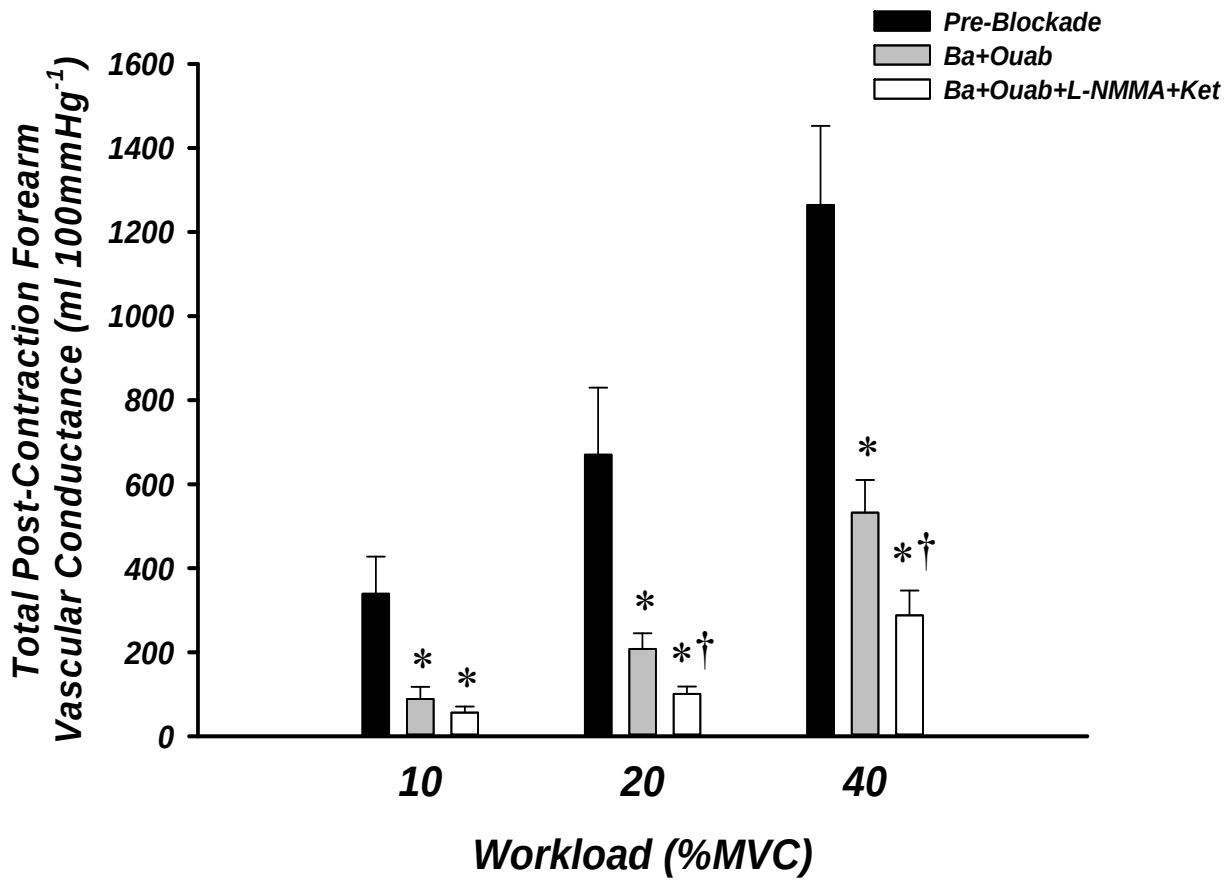

Figure 4. Protocol 1: Effect of $BaCl_2$+ouabain and $BaCl_2$+ouabain+L-NMMA+ketorolac on total post-contraction vasodilatation

The area under the dynamic response curves was calculated to determine the total post-contraction FVC. Infusion of $BaCl_2$+ouabain (grey bars) significantly reduced the total vasodilatory response from pre-blockade conditions (black bars) at all contraction intensities. Addition of L-NMMA+ketorolac (open bars) further attenuated this response at 20% and 40% MVC contractions, but not at 10% MVC. * $P<0.05$ *vs* Pre-Blockade. † $P<0.05$ *vs* Ba+Ouab.

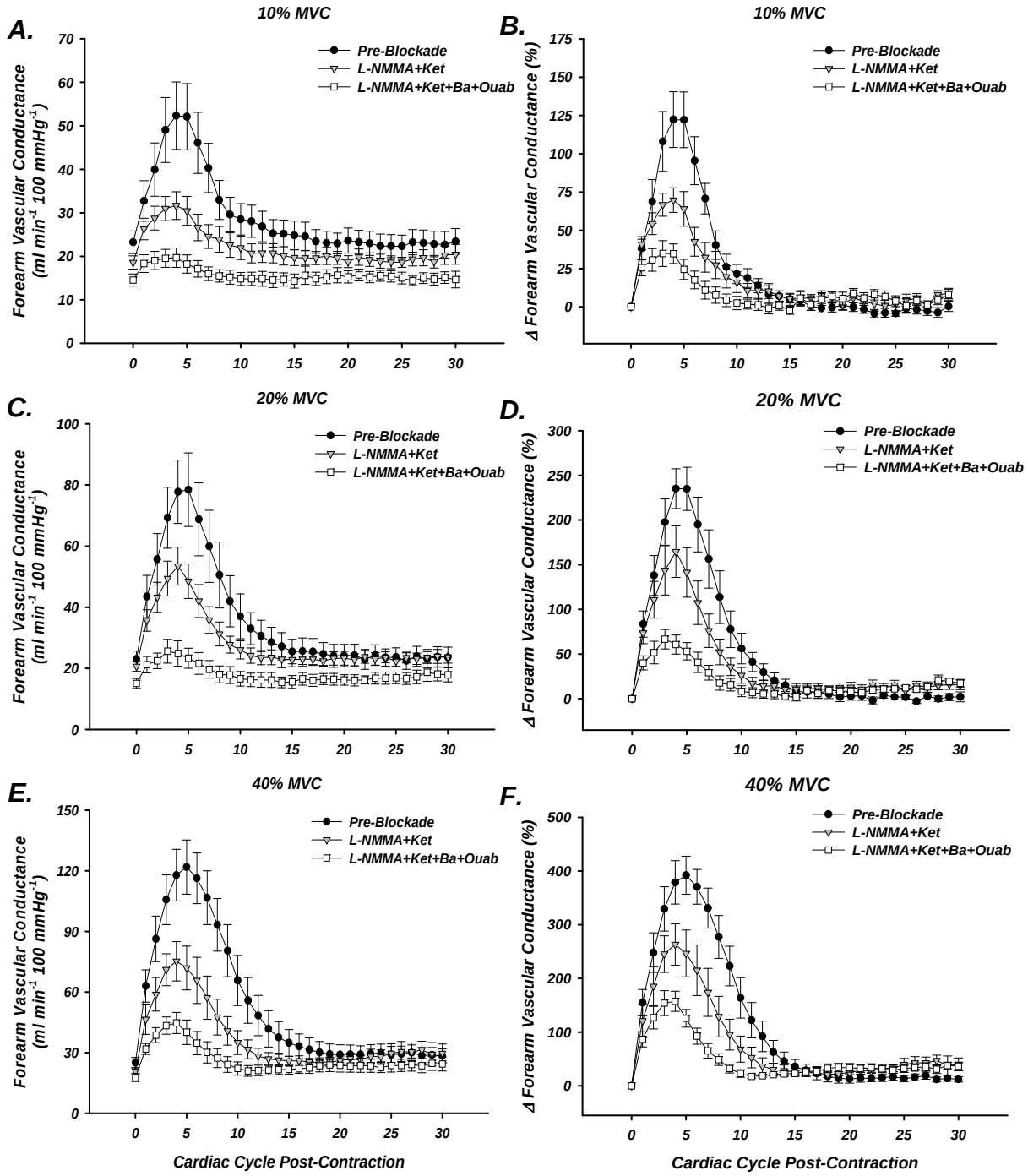

Figure 5. Protocol 2: Effect of L-NMMA+ketorolac and L-NMMA+ketorolac+ $BaCl_2$+ouabain on dynamic vasodilator responses to single contractions
Absolute forearm vascular conductance (*A, C, E*) and relative changes in forearm vascular conductance (*B, D, F*) are presented for 10%, 20%, and 40% MVC single contractions, respectively. Across all intensities, prior infusion of L-NMMA+ketorolac (grey triangles) reduced the post-contraction vasodilatory response as compared to the pre-blockade (saline; black circles) condition. Additional infusion of $BaCl_2$+ouabain (open squares) further inhibited these responses.

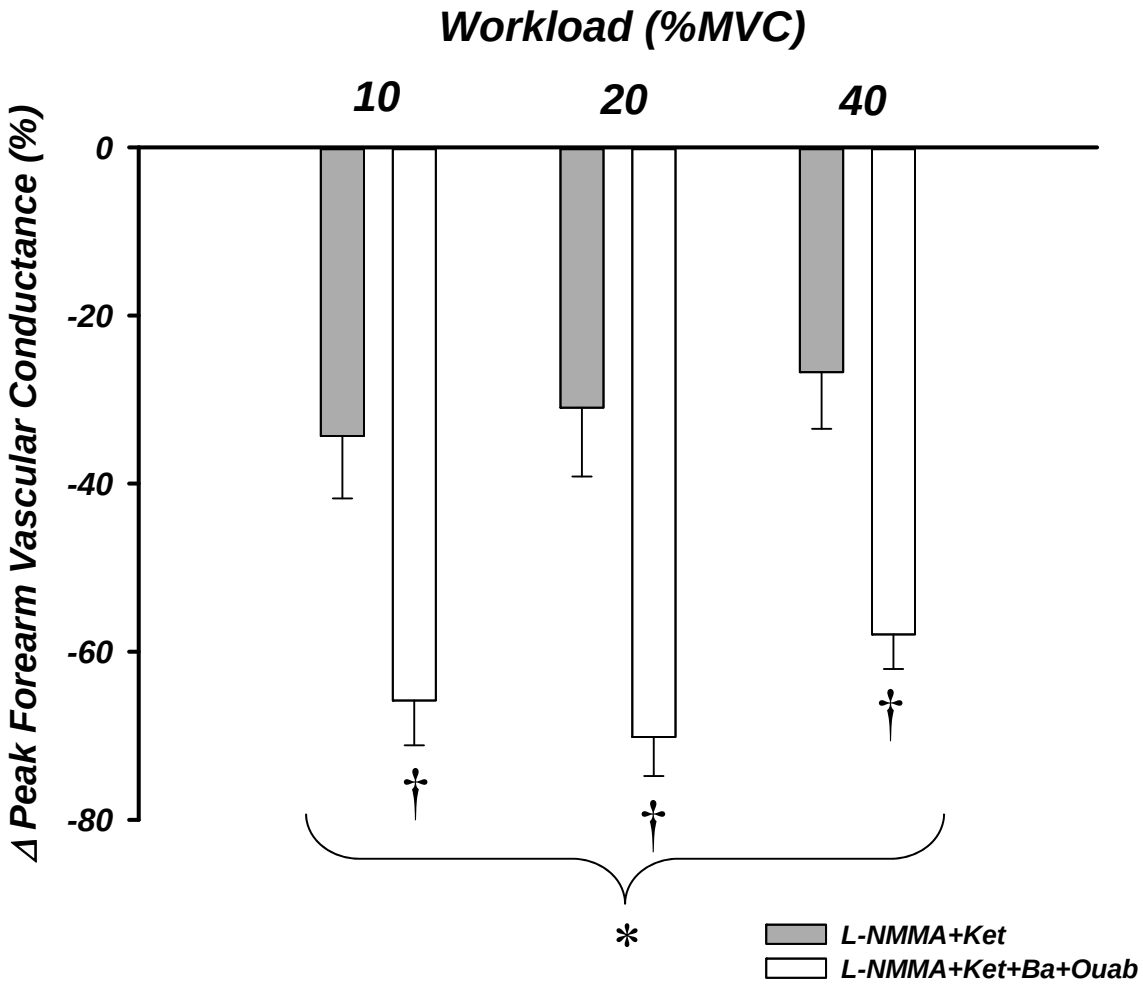

Figure 6. Protocol 2: Effect of L-NMMA+ketorolac and L-NMMA+ketorolac +$BaCl_2$+ouabain on peak post-contraction vasodilator response
Infusion of L-NMMA+ketorolac (grey bars) significantly reduced the peak post-contraction vascular conductance (P<0.05 *vs* zero) at all contraction intensities. Addition of $BaCl_2$+ouabain (open bars) significantly augmented this inhibition at all contraction intensities. * P<0.05 *vs* zero; † P<0.05 *vs* L-NMMA+ket.

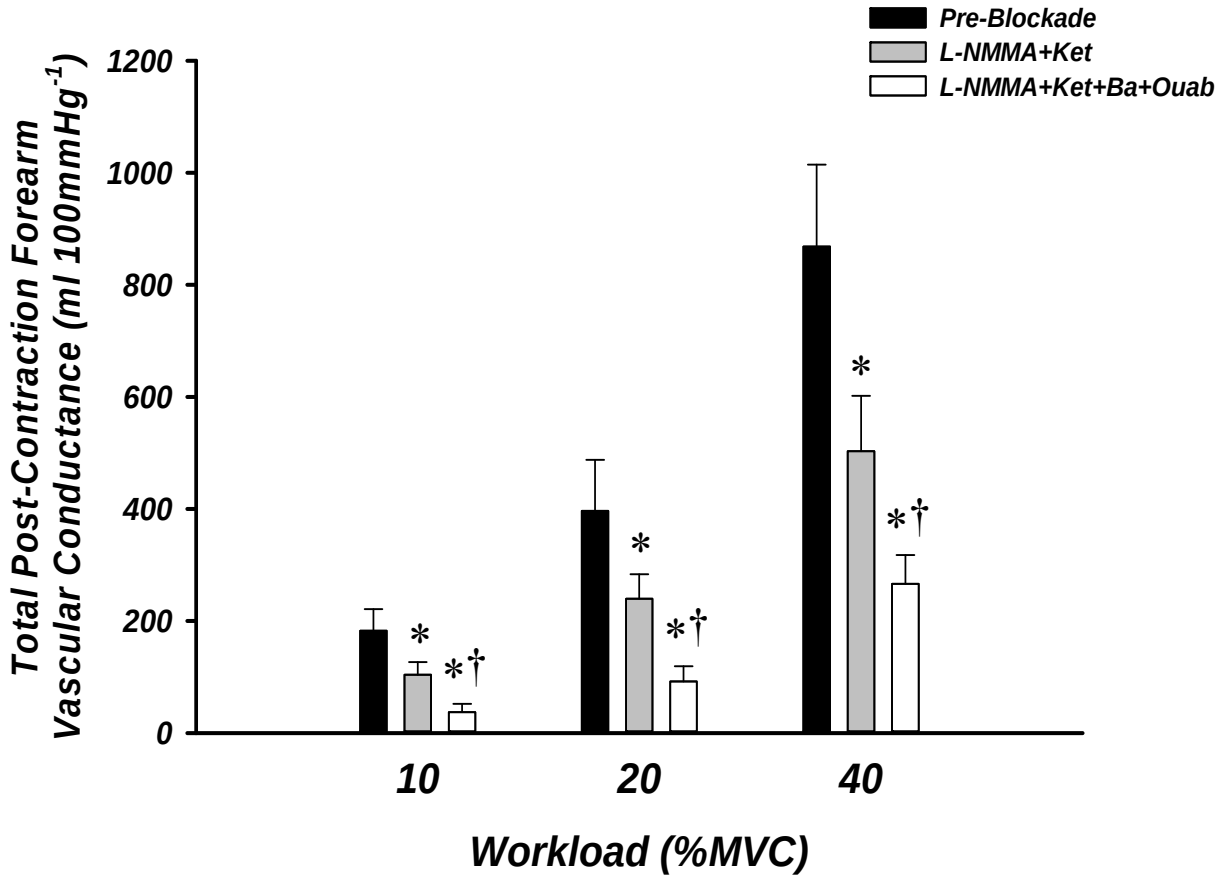

Figure 7. Protocol 2: Effect of L-NMMA+ketorolac and L-NMMA+ketorolac +$BaCl_2$+ouabain on total post-contraction vasodilatation

The area under the dynamic response curves was calculated to determine the total post-contraction FVC. Infusion of L-NMMA+ketorolac (grey bars) significantly reduced the total vasodilatory response from pre-blockade conditions (black bars) at all contraction intensities. Addition of $BaCl_2$+ouabain (open bars) further attenuated this response at all contraction intensities. * $P<0.05$ *vs* Pre-Blockade. † $P<0.05$ *vs* L-NMMA+Ket

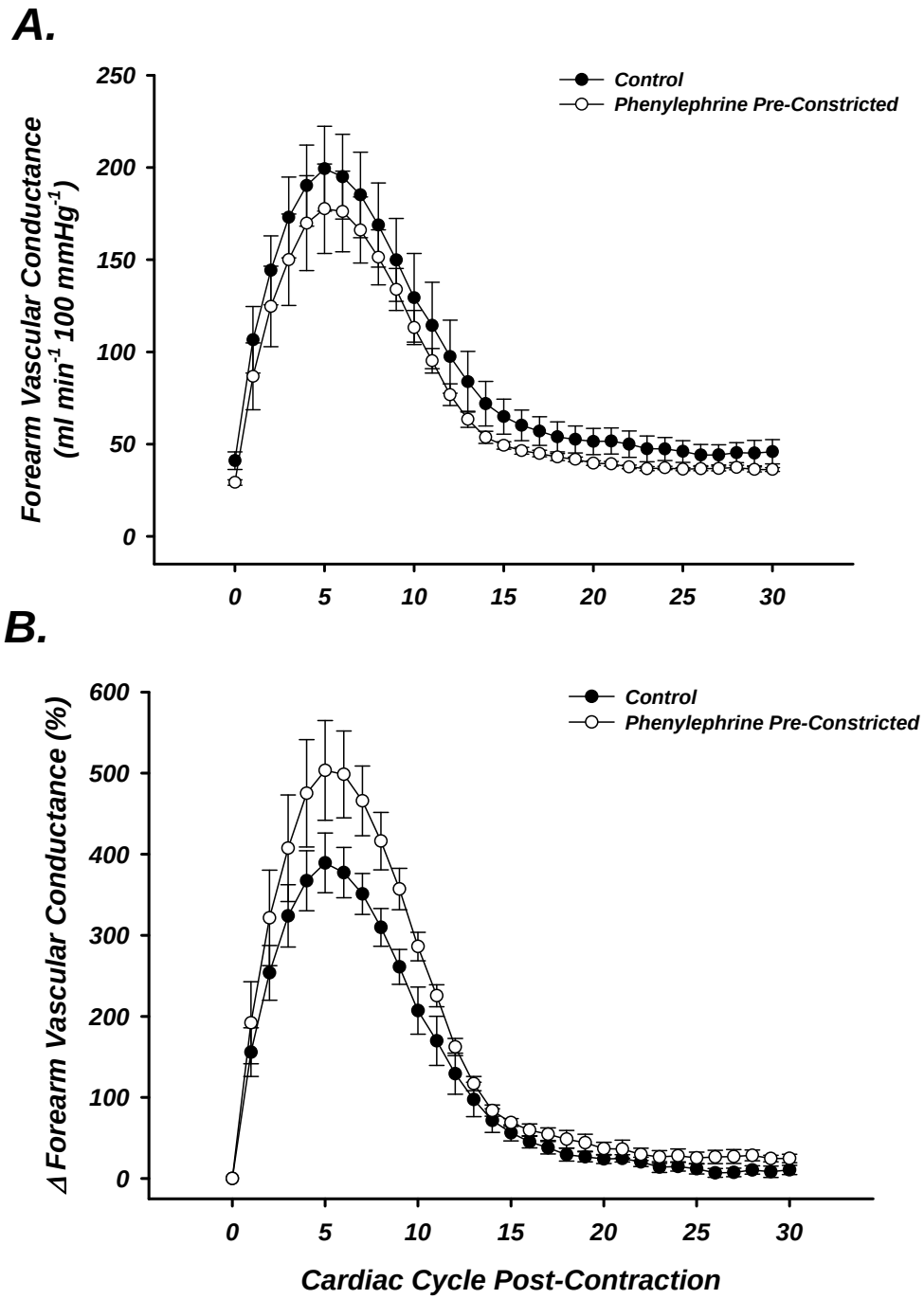

Figure 8. Subgroup Study: Effect of reducing baseline forearm vascular tone on dynamic vasodilator responses to a single contraction at 40% MVC.
A. Absolute forearm vascular conductance (FVC) is presented and demonstrates that infusion of the vasoconstrictor phenylephrine (α_1-adrenergic agonist; open circles) reduces forearm vascular tone prior to contraction (cardiac cycle 0) as compared to the control (saline; black circles) condition. The post-contraction changes in vascular conductance mimic those of the control condition, but are shifted to a lower absolute FVC. *B.* The relative vasodilator response (%ΔFVC) following a single contraction is unaffected by pre-constriction with phenylephrine as compared to control.

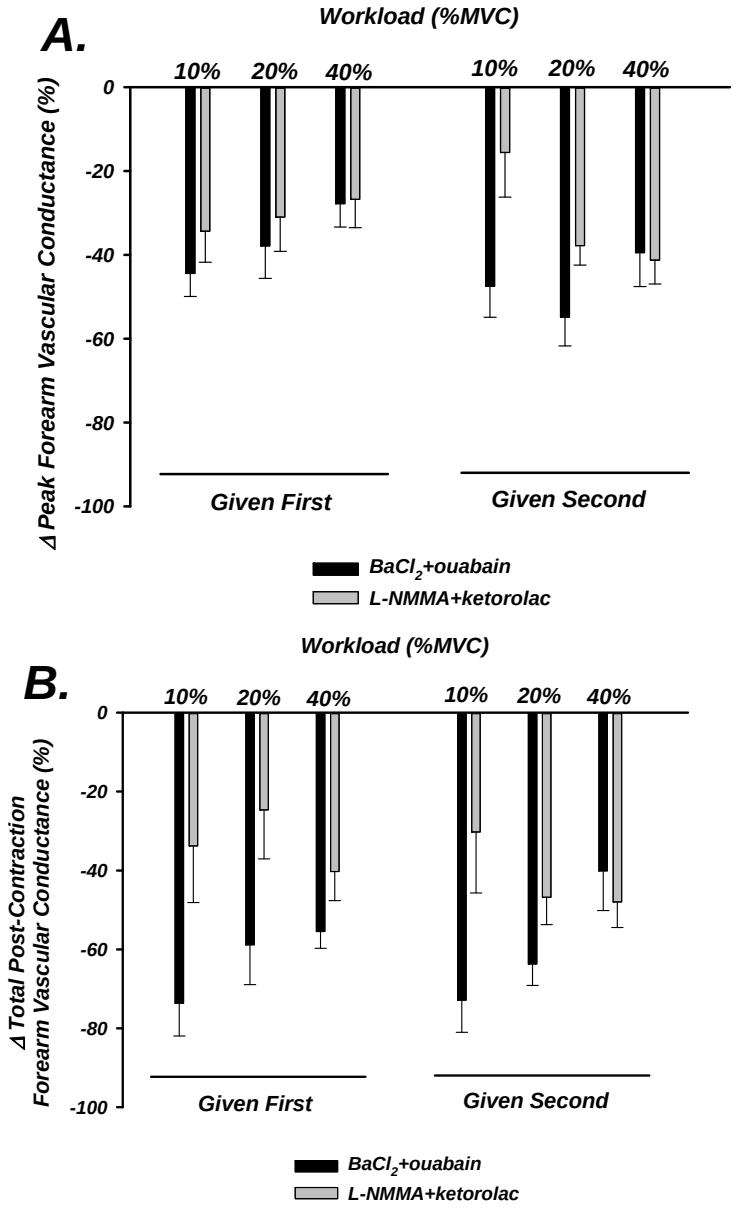

Figure 9. Protocols 1 and 2: Comparison of the effects of $BaCl_2$+ouabain and L-NMMA+ketorolac

A. Peak forearm vasodilatation (%Δ forearm vascular conductance) was attenuated by the inhibitors, in all conditions. There did not appear to be any appreciable and consistent differences between $BaCl_2$+ouabain and L-NMMA+ketorolac in terms of the magnitude of the effect on peak vasodilatation. Additionally, the effects were not impacted by the order of inhibitor administration [note: order is in reference to $BaCl_2$ + ouabain given first (Protocol 1) or given second (Protocol 2)]. *B.* Total post-contraction vasodilatation (area under the curve; AUC) was attenuated by the inhibitors in all conditions. It appears as though the magnitude of the effect on AUC of $BaCl_2$+ouabain was consistently greater than that of L-NMMA+ketorolac. Similar to the effects on peak vasodilatation, the impact of the inhibitors was not affected by the order of administration. Due to a lack of statistical power for unpaired comparisons, statistical testing of these data was not performed.

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CHAPTER III – MANUSCRIPT II

Vascular hyperpolarization via inwardly-rectifying potassium channels and Na^+/K^+-ATPase contributes to onset and steady-state exercise hyperaemia in humans[2]

Summary

The signaling mechanisms that contribute to exercise hyperaemia at the onset of muscle contractions and during steady-state conditions are yet to be fully elucidated in humans. Evidence in animal models suggests a role for vascular hyperpolarization and most data, including that in humans, suggests a modest role for the endothelial autocoids nitric oxide (NO) and prostaglandins (PGs). We tested the hypothesis that vascular hyperpolarization via activation of inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+-ATPase contributes to both the onset and steady-state hyperaemic response to exercise. Further, we determined whether a role for NO and PG would exist in the presence of inhibition of vascular hyperpolarization. In Protocol 1 (n=11), forearm blood flow (FBF; Doppler ultrasound) was measured during rhythmic handgrip exercise at 10% maximal voluntary contraction for 5 minutes under control conditions (intra-arterial saline; T1), after combined inhibition of K_{IR}

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channels and Na^+/K^+-ATPase alone [via barium chloride ($BaCl_2$) and ouabain, respectively; T2] and in the presence of combined nitric oxide synthase (L-NMMA) and cyclooxygenase inhibition (ketorolac; T3). Drugs in this protocol were not infused during exercise due to potential confounding influences on basal vascular tone. In T2, the total hyperaemic responses were significantly (P<0.05) attenuated from control at 30 seconds (-49±5%), 1 minute (-34±5%), and 2 minutes (-20±5%) of exercise and FBF was not different in the last 2 minutes of exercise. In T3, addition of L-NMMA+ketorolac reduced resting FBF but only had a further effect on exercise FBF in the first 15 sec. In Protocol 2 (n=8), all study drugs were infused prior to and during exercise to more directly assess the impact of inhibition on steady-state hyperaemia. Steady-state FBF was significantly reduced during T2 *vs* T1 (133±15 *vs* 167±17 ml min^{-1}; Δ from control: -20±3%), and further reduced during T3 (120±15 ml min^{-1}; -29±3%). The effect of inhibition on vasodilatation (forearm vascular conductance) was greater than FBF (Δ from control: T2: -28±2%; T3: -40±3%). Our data indicate vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase as a novel contributing vasodilatory pathway in the complex hyperaemic response to exercise at the onset and during steady-state exercise and reveal a role for NO and PG during steady-state hyperaemia when hyperpolarization via these means is inhibited.

Introduction

The regulation of blood flow and oxygen delivery to contracting muscle is a complex response that involves mechanical factors, the sympathetic nervous system, and local metabolic and endothelium-derived substances that can influence vascular tone (Saltin *et al.*, 1998). Regarding the latter, prior studies in animal models established that disruption of the

endothelium can attenuate the hyperaemic response to muscle contraction (Sagach *et al.*, 1992; Berdeaux *et al.*, 1994; Segal & Jacobs, 2001) and many subsequent studies have attempted to identify the endothelial-derived substances that may regulate blood flow during exercise. Additionally, local metabolic substances that elicit vasodilatation independent of the endothelium, such as K^+, have been suggested to be involved in exercise hyperaemia (Hazeyama & Sparks, 1979a; Wilson *et al.*, 1994; Juel *et al.*, 2007), particularly at the onset of muscle contractions (Mohrman & Sparks, 1974; Armstrong *et al.*, 2007). However, to date, obligatory local vasodilators for exercise hyperaemia are still questioned in humans (Joyner & Wilkins, 2007).

Endothelium-dependent vasodilatation occurs via the synthesis of nitric oxide (NO) and vasodilating prostaglandins (PGs; i.e. prostacyclin) (Feletou & Vanhoutte, 2006) or through hyperpolarization of the vascular smooth muscle (Garland *et al.*, 1995; Edwards *et al.*, 1998). In young healthy humans, combined inhibition of NO and PGs can reduce femoral blood flow during knee extension exercise (Boushel *et al.*, 2002; Mortensen *et al.*, 2007); however, no effect of prior blockade of these pathways is observed in the human forearm during mild- and moderate-intensity rhythmic handgrip exercise (Schrage *et al.*, 2004; Crecelius *et al.*, 2011b). The exact pathways by which endothelial-derived hyperpolarization occurs have not been established and thus fewer studies have attempted to address the contribution of this pathway to vascular regulation during exercise. In animals models, disruption of hyperpolarization (Milkau *et al.*, 2010) or conducted vasodilatation (Segal & Jacobs, 2001), to which vascular hyperpolarization largely contributes (Domeier & Segal, 2007), attenuates contraction-induced hyperaemia. A few studies in humans have attempted to inhibit the synthesis of candidate hyperpolarizing pathways including cytochrome p450 metabolites (Hillig *et al.*, 2003), calcium-

activated potassium (K_{Ca}) channels (Mortensen *et al.*, 2007), and ATP-sensitive potassium channels (K_{ATP}) (Schrage *et al.*, 2006). These studies observed only a modest, if any, reduction in skeletal muscle blood flow during steady-state exercise, even when combined with inhibition of NO and PGs. Combined inhibition can be an important approach given evidence these pathways may be interrelated and able to compensate for one another (Taddei *et al.*, 1999). An important point is that in these prior human studies, it is difficult to assess the efficacy of the inhibitors utilized to attenuate a hyperpolarizing stimulus. Further, hyperpolarization of vascular smooth muscle can occur independent of these means as well as be stimulated by non-endothelial-derived sources, such as increased interstitial [K^+] (Hazeyama & Sparks, 1979b; Edwards *et al.*, 1998). Thus, there are multiple lines of evidence that would suggest vascular hyperpolarization is involved in the hyperaemic response to muscle contractions and this question remains unanswered in humans.

The dynamic nature of the hyperaemic response to muscle contractions is well appreciated and while the regulation of steady-state muscle blood flow is of interest, the transition from rest to exercise presents another intriguing aspect of vascular regulation (Behnke *et al.*, 2002; Wray *et al.*, 2005; Clifford, 2007). Similar to steady-state exercise hyperaemia, inhibition of NO and PGs, independently or in combination, does not impair the *change* in blood flow observed at the onset of muscle contractions (Shoemaker *et al.*, 1996; Shoemaker *et al.*, 1997; Radegran & Saltin, 1999; Nyberg *et al.*, 2010) while only combined inhibition of NO and PGs in the leg lowers absolute leg blood flow at exercise onset (Nyberg *et al.*, 2010). Human studies investigating the role of vascular hyperpolarization in the initial hyperaemic response have not been performed.

Recently, we have shown that vascular hyperpolarization via the activation of inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+-ATPase contribute to a large portion (~50%) of the hyperaemic response following a single muscle contraction in the human forearm, and combined with NO and PGs account for nearly all (~80%) of the total vasodilatory response (Crecelius *et al.*, 2013a). In addition, activation of K_{IR} channels and Na^+/K^+-ATPase can be stimulated by both K^+ (Edwards *et al.*, 1998; Crecelius *et al.*, 2012) and adenosine triphosphate (ATP) (Crecelius *et al.*, 2012), two substances proposed to be involved in vascular regulation during exercise in humans (Gonzalez-Alonso *et al.*, 2002; Kirby *et al.*, 2012; Saltin, 2012). With this information as a background, the purpose of the present experiment was to test the hypothesis that vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase contributes to exercise hyperaemia both at the onset of muscle contractions and during steady-state levels of muscle blood flow. Further, we sought to determine whether there would be a combined role for NO and PGs after inhibiting these pathways of vascular hyperpolarization.

Methods

Subjects

With Institutional Review Board approval and after written informed consent, a total of 19 young healthy adults (Protocol 1: 7 men, 4 women; age = 23±1 years; weight = 71.6±2.6 kg; height = 172±3 cm; body mass index = 24.1±0.7 kg m^{-2}; forearm volume (FAV) = 848±41 ml; Protocol 2: 4 men, 4 women; age = 24±2 years; weight = 69.0±3.0 kg; height =173±3 cm; body mass index = 23.1±0.8 kg m^{-2}; FAV = 811±62 ml; means±S.E.M.) participated in the present study. All subjects were sedentary to moderately active, non-smokers, non-obese, normotensive (resting blood pressure <140/90 mmHg), and not taking any medications. Studies were

performed after an overnight fast and 24 hour abstention from caffeine and exercise with subjects in the supine position with the experimental arm abducted to 90° and slightly elevated above heart level upon a tilt-adjustable table. 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 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) for local administration of study drugs and blood sampling. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml hr^{-1} with heparinized saline. The two side ports were used for drug infusions (Kirby *et al.*, 2008; Crecelius *et al.*, 2010). Heart rate (HR) was determined using a 3-lead ECG (Cardiocap/5, Datex-Ohmeda, Louisville, CO, USA).

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 securely fixed to the skin 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. In Protocol 1, brachial artery diameter measurements were made in duplex mode from images recorded on a VHS tape at end-diastole and between contractions (in triplicate) during rest, and at 6, 12, 24, 60, 90, 120, 180, 240, and 300 seconds of exercise. All measurements were made by the same operator. An exponential line of best fit was generated for these data and diameters were extrapolated from this function at relevant MBV timepoints. We utilized this approach in an attempt to minimize the effect of random diameter measurement error on blood flow (Saunders *et al.*, 2005). In Protocol 2, diameter measurements were made in triplicate at rest and the end of exercise. Forearm blood flow (FBF) was then 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 was 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.

Rhythmic handgrip exercise

Maximal voluntary contraction (MVC; mean 41±2 kg, range 29 – 52 kg) was determined for the experimental arm as the average of three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3% of each other. Forearm exercise during the trials was performed with weight corresponding to 10% MVC (mean 4.1±0.2 kg, range 2.9 – 5.2 kg) attached to a pulley system and lifted 4–5 cm over the pulley at a duty cycle of 1s contraction–2 s relaxation (20 contractions per minute) using both visual and auditory feedback

to ensure the correct timing as described previously (Kirby *et al.*, 2008; Crecelius *et al.*, 2010). We chose this mild intensity rhythmic handgrip exercise to limit the contribution of systemic haemodynamics to forearm hyperaemic responses and eliminate reflex activation of the sympathetic nervous system (Seals & Victor, 1991). Thus, our experimental model aimed to isolate the local effects of muscle contractions on forearm hyperaemia without engaging potentially confounding systemic influences on vascular tone. Previous studies in our laboratory have determined that MVC is not affected by any of the vasoactive substances, particularly barium chloride and ouabain, administered in these studies (Crecelius *et al.*, 2013a)

Vasoactive drug infusion

All vasoactive drug infusions occurred via the brachial artery catheter to create a local effect in the forearm and saline was utilized as a control infusate. Specific timing and duration of infusions is provided below in the *Experimental Protocols* section.

To inhibit hyperpolarizing vasodilatory mechanisms (Crecelius *et al.*, 2012), ouabain octahydrate (Na^+/K^+-ATPase inhibitor; Sigma 03125, St. Louis, MO, USA) was infused at 2.7 nmol min^{-1} in combination with barium chloride ($BaCl_2$; K_{IR} channel inhibitor; 10% w/v BDH3238, EMD Chemicals, Gibbstown, NJ, uSA) at 0.9 μmol dl FAV^{-1} min^{-1} with a minimum dose of 8 μmol min^{-1} to a maximum dose of 10 μmol min^{-1}. This dose of $BaCl_2$ is twice that which we have previously administered in our laboratory (Crecelius *et al.*, 2012, 2013a) and which has been shown to elicit a plasma concentration of ~50 μM (Dawes *et al.*, 2002). Given that repeated muscle contractions cause a significant and prolonged elevation in blood flow, we increased the administered dose of $BaCl_2$ in an attempt to maintain a concentration *within the blood* similar to that which we have shown before to effectively inhibit both potassium-mediated

vasodilatation (Crecelius *et al.*, 2012) as well as the haemodynamic response following a single muscle contraction (Crecelius *et al.*, 2013a) while still being selective for K_{IR} channels (<100 μM) (Jackson, 2005). Pilot studies within our laboratory were performed that demonstrated this increased dose was still safe, well-tolerated by subjects and did not cause systemic haemodynamic or plasma electrolyte changes. Ouabain and $BaCl_2$ were prepared in saline and confirmed sterile and free of fungus/endotoxin and particulate matter with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS, USA) prior to use.

To block endothelium-dependent vasodilatory pathways of interest, we administered N^G-monomethyl-L-arginine [L-NMMA; NO synthase (NOS) inhibitor; Clinalfa/Bachem, Weil am Rhein, Germany] to inhibit the production of NO in combination with ketorolac [non-selective cyclooxygenase (COX) inhibitor; Hospira, Lake Forest, IL, USA] to inhibit the synthesis of PGs. The doses of L-NMMA and ketorolac were 5 mg min^{-1} and 1200 μg min^{-1} respectively. We have previously demonstrated these doses to be effective during a similar exercise protocol (Dinenno & Joyner, 2003; Schrage *et al.*, 2004; Crecelius *et al.*, 2010)Forearm volume used for normalization for specific vasoactive drugs was determined from regional analysis of whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc, Bedford, MA, USA).

Experimental Protocols

Protocol 1: Onset exercise hyperaemia

Both experimental protocols are presented in Figure 10. The main purpose of Protocol 1 was to determine the effect of inhibiting hyperpolarizing vasodilator mechanisms (via $BaCl_2$ and ouabain) alone, and in combination with NOS-COX blockade (via L-NMMA and ketorolac,

respectively) on the hyperaemia that occurs at the onset of repeated muscle contractions. In order to do this, subjects performed 10% MVC rhythmic handgrip exercise for 5 minutes in control conditions (Trial 1; saline), following combined infusion of $BaCl_2$ and ouabain (Trial 2), and following combined infusion of $BaCl_2$, ouabain, L-NMMA, and ketorolac (Trial 3). Based on our previous observations regarding acute fluctuations in resting vascular tone with $BaCl_2$ and ouabain infusion (Crecelius *et al.*, 2013a), we chose to stop the infusion of all blockers prior to the start of exercise, to best insure accurate resting blood flow measures and appropriately quantify the rapid blood flow responses compared with control conditions. This method of drug infusion was previously used by our laboratory and shown effective at significantly reducing the blood flow response to a single muscle contraction (Crecelius *et al.*, 2013a).

In trial 1, saline was infused for three minutes prior to the start of resting blood flow measures. A single contraction (10% MVC) was performed at 30, 60, and 90 seconds of this infusion in order to facilitate delivery of the infusion to the vasculature of the muscle fibres recruited for this type of contraction (Crecelius *et al.*, 2013a). Resting blood flow measures were taken for 30-60 seconds and the subject was then instructed to begin the 5 minutes of contractions. Beat-to-beat blood flow was measured throughout the entire exercise protocol. Subjects rested comfortably for 20 min before the start of the next exercise trial. Trial 2 followed a similar timeline with the difference being that ouabain was infused for 15 minutes and $BaCl_2$ for 3 minutes prior to the start of resting blood flow measures. Trial 3 was performed in a similar fashion with the exception that the pre-exercise infusions consisted of 15 minutes of ouabain, 3 minutes of $BaCl_2$, and 5 minutes each of L-NMMA and ketorolac.

Protocol 2: Steady-state exercise hyperaemia

Based on our observations in Protocol 1 (see Results), we believed it was necessary to perform a second protocol where we continued infusion of our inhibitors throughout the muscle contractions in order to determine whether we had underestimated the effect of our blockers on steady-state exercise hyperaemia in Protocol 1. The trials in Protocol 2 mimicked those of Protocol 1 (control, combined $BaCl_2$+ouabain, and combined $BaCl_2$+ouabain+L-NMMA+ketorolac) with the difference that all inhibitors were continued throughout the entire exercise duration.

Data acquisition and analysis

Data were collected and stored on a computer at 250 Hz and were analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Mean arterial pressure (MAP) was determined from the arterial pressure waveform. For Protocol 1, FBF and MAP were analyzed in 3-sec bins that corresponded to each contraction:relaxation (1:2 sec) cycle. This type of analysis was carried out for the first 3 minutes, and 30-second averages were used at minutes 4 and 5 of exercise. In the case that the MBV signal quality obtained during a 3-second cycle was altered due to operator error, a mathematical average of the preceding and subsequent bins' MBV was used. This occurred in less than 2% of all bins analyzed. HR was determined at rest, and each minute of exercise thereafter.

In Protocol 1, the total exercise-induced hyperaemic and vasodilator responses were calculated as the sum of FBF and FVC, respectively, above baseline for 30, 60, 120, 180, 240, 300 seconds of exercise [area under curve (AUC)]. To help characterize the onset of exercise hyperaemia, the mean response time was calculated as the time at which 63% of the increase in

FBF from rest to steady-state (average of min 4 and 5 FBF) was reached (Shoemaker *et al.*, 1997). In Protocol 2, the last 30 seconds of exercise was averaged to represent steady-state FBF and FVC.

To quantify the impact of vasoactive drugs in each protocol on FBF, the magnitude of inhibition of exercise hyperaemia was calculated as: (FBF inhibition - FBF control)/(FBF control) × 100. Changes in FVC, as well as AUC were calculated in a similar manner.

Statistics

Data are presented as mean±S.E.M. In Protocol 1, 15 second averages of forearm haemodynamics were calculated and used in a two-way repeated measures ANOVA (timepoint × drug condition) and *post hoc* pairwise comparisons were completed with Student-Newman-Keuls pairwise tests. In Protocol 2, paired, two-tailed Student's t-tests were used to make the three comparisons between control, $BaCl_2$+ouabain, and $BaCl_2$+ouabain+L-NMMA+ketorolac conditions for forearm haemodynamic data. In both protocols, for systemic haemodynamic variables (HR, MAP), a two-way repeated measures ANOVA [timepoint (rest vs exercise) × drug condition] was used and *post hoc* pairwise comparisons were completed with Student-Newman-Keuls tests. Significance was set *a priori* at $P<0.05$.

Results

Protocol 1: Onset exercise hyperaemia

In control (saline) conditions, FBF increased rapidly in response to muscle contractions (Figure 11A). In trial 2, prior infusion of $BaCl_2$+ouabain had no effect on resting FBF (Table 3) but significantly attenuated FBF for the first 2 minutes of exercise (Figure 11B) and there was a

significantly longer mean response time compared to control conditions (52 ± 5 *vs* 31 ± 5 sec; $P < 0.05$). The total hyperaemic response was significantly attenuated with prior $BaCl_2$+ouabain and this effect was more pronounced earlier in the exercise bout (30 sec: -49 ± 5%; 60 sec: -34 ± 5%, 120 sec: -20 ± 5%, 180 sec: -13 ± 4%, 240 sec: -10 ± 4%, 300 sec: -8±3%; Figure 12). In trial 3, infusion of L-NMMA+ketorolac in addition to $BaCl_2$+ouabain prior to exercise significantly reduced resting FBF (Table 3) as expected. Absolute FBF at the onset of exercise was further attenuated from the $BaCl_2$+ouabain condition in the first 15 seconds of exercise but not thereafter, although was significantly lower than control conditions (Figure 11B). A similar pattern was evident when the total hyperaemic response was quantified and the reduction from control conditions was calculated (30 sec: -65 ± 2%; 60 sec: -40 ± 3%, 120 sec: -23 ± 4%, 180 sec: -15 ± 4%, 240 sec: -14 ± 4%, 300 sec: -14±4%; Figure 12). The mean response time in this condition (55 ± 3 sec) was similar to following $BaCl_2$+ouabain ($P = 0.55$) and significantly lower than control ($P < 0.05$). FBF at minute 5 of exercise was slightly, but significantly lower in the $BaCl_2$+ouabain+L-NMMA+ketorolac condition (Figure 11B). In general, changes in FVC paralleled those observed in FBF (Table 3).

Systemic haemodynamics (HR and MAP) were largely unchanged throughout the trial and under all conditions. Small (2-3 mmHg) but statistically significant increases in MAP were observed in the condition of prior $BaCl_2$+ouabain+L-NMMA+ketorolac infusion versus control. Additionally, small (2-4 beats min^{-1}) but again statistically significant reductions in HR were observed in the third trial versus both control and $BaCl_2$+ouabain. There was also a slight but significant effect of exercise on HR. No significant interaction between time and drug condition was detected for MAP ($P = 0.62$) or HR ($P = 0.35$).

Protocol 2: Steady-state exercise hyperaemia

With continuous infusion of $BaCl_2$ and ouabain throughout muscle contractions (trial 2), FBF and FVC were significantly lower than in control conditions (trial 1) after 5 minutes of rhythmic handgrip exercise (ΔFBF = -20±3%; ΔFVC = -28±2%, Figure 13A and 13B, respectively). In trial 3, inhibiting NO and PG synthesis in addition to K_{IR} channels and Na^+/K^+-ATPase via $BaCl_2$ and ouabain, respectively, resulted in a further attenuation of exercise hyperaemia and vasodilatation (compared to control: ΔFBF = -29±3%; ΔFVC = -40±2%, Figure 13A and 13B, respectively). The percent reduction in FBF and FVC from control steady-state exercise levels was significantly greater for the condition of continuous $BaCl_2$+ouabain+L-NMMA+ketorolac infusion than $BaCl_2$+ouabain alone, and these changes were also greater for FVC than for FBF (Figure 14).

Systemic haemodynamics for Protocol 2 are presented in Table 4. Exercise resulted in small but significant changes in MAP and HR. Additionally, minor statistically significant increases in MAP were observed in the different drug conditions.

Discussion

The purpose of the current study was to determine the contributions of vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase to exercise hyperaemia at the onset of muscle contractions and during the steady-state portion of the blood flow response and to determine whether inhibition of these pathways revealed a compensatory role of NO and PGs. The primary novel findings of the present study are as follows. First, activation of K_{IR} channels and Na^+/K^{+-}ATPase contribute largely to the onset of exercise hyperaemia following repeated muscle contractions. Additionally, in line with prior findings in the human forearm, NO and PGs do not play a significant role in this response except for the initial 15 seconds.

Second, we demonstrate for the first time in humans a significant contribution of vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^{+-}ATPase to steady-state exercise hyperaemia. Interestingly, inhibition of these pathways reveals a significant role for NO and PGs in mediating steady-state exercise hyperaemia which has not been previously observed in this model of mild-intensity rhythmic handgrip exercise. Our collective findings indicate that vascular hyperpolarization contributes significantly to exercise hyperaemia, particularly at the onset of contractions.

Onset exercise hyperaemia: contributing signaling pathways (Protocol 1)

Beginning with the first muscle contraction of exercise, there is a rapid increase in blood flow that occurs immediately (within one cardiac cycle) (Clifford, 2007; Kirby *et al.*, 2007; Crecelius *et al.*, 2013a). With repeated muscle contractions, this increase in blood flow continues in a bi-phasic manner comprised of an initial rapid increase that plateaus by ~5-7 sec and a second, slower phase that begins ~20 sec after the onset of exercise and continues in an intensity-dependent manner until steady-state hyperaemia has been achieved (Tschakovsky & Sheriff, 2004; Saunders *et al.*, 2005). In the present study, we observe similar dynamic characteristics in the hyperaemic response to mild intensity forearm handgrip in control conditions (Figure 11*A*). Previous studies in the human forearm demonstrated that inhibition of NO or PGs does not impact the rapid onset of exercise hyperaemia (Shoemaker *et al.*, 1996; Shoemaker *et al.*, 1997) nor does antagonism of muscarinic receptors (Shoemaker *et al.*, 1997) that would be bound by acetylcholine either from an endothelial source (Martin *et al.*, 1996) or spillover from the motor neuron junction (Welsh & Segal, 1997). Studies in animals suggest that during muscle contractions, flow is controlled at the level of the feed arteries (Segal, 2005) via

conducted vasodilatation that originates in the distal resistance arterioles (Welsh & Segal, 1998). Hyperpolarization is known to contribute more robustly to distal vessel vasodilatation as opposed to vasodilator autcoids such as NO and PGs (Shimokawa *et al.*, 1996; Sandow *et al.*, 2002) and is capable of stimulating conducted vasodilatation whereas in general, NO and PGs do not (Hoepfl *et al.*, 2002; Domeier & Segal, 2007). Recently, we demonstrated that vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+ATPase largely contributes to the total vasodilatation following a single muscle contraction (Crecelius *et al.*, 2013a), the first such observation in humans. For the present study, we determined what role hyperpolarization via these pathways has in the onset of hyperaemia in response to repeated muscle contractions.

When $BaCl_2$ and ouabain were infused prior to contractions to inhibit Na^+/K^+-ATPase and K_{IR} channels, respectively, there was a significant attenuation of the initial hyperaemic response for the first 2 minutes of exercise (Figure 11*B*). Beyond 2 minutes, there was no longer a significant effect on the absolute level of blood flow due; however, the initial impact on the hyperaemia was of a large enough magnitude to affect the total (area under the curve) hyperaemic response through the 4th minute of exercise (Figure 12). In this protocol, due to fluctuations in resting blood flow that we have observed during infusion of $BaCl_2$ and ouabain in our laboratory, we stopped drug infusions prior to the start of muscle contractions so we could appropriately quantify resting FBF and appropriately quantify the rapid blood flow responses compared to control conditions. Given this approach, we sought to more directly address a role for vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase during steady-state hyperaemia in Protocol 2 where all inhibitors were coinfused during muscle contractions (see section below).

Several lines of evidence suggest that there is crossover between the various vasodilator pathways, and that in the presence of inhibition of one pathway, another may compensate in order to preserve blood flow and oxygen delivery, particularly during muscle activation or decreased arterial oxygen content (Taddei *et al.*, 1999; Hillig *et al.*, 2003; Markwald *et al.*, 2011). Thus, we investigated whether in the presence of inhibition of K_{IR} channels and Na^+/K^+-ATPase, there would be a role for NO and PGs, despite evidence suggesting they do not contribute to initial exercise hyperaemia in the human forearm (Shoemaker *et al.*, 1996; Shoemaker *et al.*, 1997). Combined infusion of L-NMMA and ketorolac to enzymatically inhibit the production of NO and PGs, respectively, reduced resting blood flow as we anticipated (Table 3). Absolute FBF was also inhibited beyond that which occurred with $BaCl_2$+ouabain at the start of forearm contractions. However, this further impairment diminished rapidly over time, and by 30 seconds there was no longer a significant reduction in forearm blood flow from the $BaCl_2$+ouabain condition (Figure 11*B*). Similarly, there was minimal additional effect of NO and PG inhibition on the total hyperaemic response (Figure 12). It is possible that the reduction in resting blood flow during combined NO and PG inhibition attributed to the initial and brief reduction in exercise hyperaemia, similar to prior observations of lower resting blood flow and slower mean response times when the arm was positioned above versus below heart level (Shoemaker *et al.*, 1996). In the presence of $BaCl_2$+ouabain +L-NMMA+ketorolac, muscle blood flow was still able to increase rapidly and achieve levels significantly greater than rest and near steady-state levels by three minutes of exercise, indicating redundant vasodilator signals, even from the onset of muscle contractions.

Steady-state exercise hyperaemia: contributing signaling pathways (Protocol 2)

To date, multiple studies have attempted to identify the various vasodilator pathways involved in exercise hyperaemia and most have focused on the steady-state portion of the response (Delp & Laughlin, 1998; Joyner & Wilkins, 2007; Saltin, 2007). Steady-state muscle blood flow is thought to reflect a homeostatic balance between oxygen delivery via increases in blood flow and oxygen demand of the contracting muscle (Rowell, 1993). In the human forearm, the results of previous studies have been extremely unimpressive in terms of the magnitude of reduction in muscle blood flow consequence to vasodilator inhibition. In fact, combined inhibition of NO and PGs, two important endothelium-dependent dilators does not impact the rest to steady-state muscle blood flow response (Schrage *et al.*, 2004; Crecelius *et al.*, 2011b). Given animal studies that suggested an important role for the endothelium (Sagach *et al.*, 1992; Berdeaux *et al.*, 1994) and particularly for conducted hyperpolarizing stimuli (Segal & Jacobs, 2001; Milkau *et al.*, 2010) in exercise hyperaemia and the lack of involvement of NO and PGs, we wanted to address the role of vascular hyperpolarization in muscle blood flow during exercise in humans.

One issue with attempting to address hyperpolarizing pathways in humans is a lack of specific antagonists and agonists of the proposed pathways involved in initiating and conducting hyperpolarization in the vasculature. For instance, in human studies that have attempted to inhibit potential pathways of vascular hyperpolarization [cytochrome p450 metabolites (Hillig *et al.*, 2003), K_{Ca} (Mortensen *et al.*, 2007) and K_{ATP} (Schrage *et al.*, 2006) channels], the efficacy of inhibition was not assessed, and thus caution is warranted when interpreting their negative findings. Previously, we have demonstrated that combined administration of ouabain and $BaCl_2$ can inhibit KCl-mediated vasodilatation, a stimulus for direct vascular hyperpolarization

(Crecelius *et al.*, 2012). In the present study we use this established pharmacology (Crecelius *et al.*, 2012, 2013a) to test the role of vascular hyperpolarization in muscle blood flow regulation during continued muscle contractions.

Due to observed fluctuations in resting blood flow in the present studies and in our past experience with ouabain and $BaCl_2$ (Crecelius *et al.*, 2012, 2013a), in Protocol 1 where we were focused on capturing the onset of exercise hyperaemia, we did not co-infuse these inhibitors during contractions. However, when we did not observe a significant effect during steady-state exercise (Figure 10) we questioned whether there was "wash-out" of our inhibitors as blood flow increased. Subsequently, we performed studies in Protocol 2 in which we coinfused our inhibitors throughout muscle contractions as is more typical of our approach for these types of exercise studies (Crecelius *et al.*, 2011a; Crecelius *et al.*, 2011b). Under these conditions of continuous inhibition, we do in fact observe a significant contribution of vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase to steady-state exercise hyperaemia (~20%; Figures 13 and 14). Our observed effect is more pronounced for FVC (~30%), likely due to a slightly greater pressor response during $BaCl_2$+ouabain which may reflect a slight systemic effect of our drug infusions. If this were the case, we could be potentially *underestimating* the effects of our inhibitors as the greater perfusion pressure could serve to maintain blood flow (Amann *et al.*, 2011; Richards *et al.*, 2011).

Interestingly, inhibition of NO and PGs in addition to vascular hyperpolarization resulted in a further attenuation of steady-state muscle blood flow (Figures 13 and 14). This finding is in contrast with the lack of a combined role for NO and PGs in the human forearm in previous studies (Dinenno & Joyner, 2004; Schrage *et al.*, 2004; Crecelius *et al.*, 2011b) but highlights the ability of vasodilator pathways to compensate for one another, a phenomenon that has been

observed in a variety of previous studies (Taddei *et al.*, 1999; Hillig *et al.*, 2003; Markwald *et al.*, 2011). Similar to when $BaCl_2$+ouabain were given alone, in the presence of $BaCl_2$+ouabain+L-NMMA+ketorolac, the reduction in FVC (~40%) was greater than that of FBF (~30%). It is important to emphasize the magnitude of the effect of combined inhibition of vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase and NO and PGs on FBF and FVC during steady-state exercise. A 40% reduction in forearm vasodilatation (FVC) is profound, particularly in a small muscle mass such as the forearm that typically shows resistance to decreased blood flow following pharmacological inhibition of vasodilator pathways. Further, this change approaches the observed effects of completely inhibiting conducted vasodilation on (~45% reduction in contraction-induced hyperaemia) (Segal & Jacobs, 2001).

Potential stimuli for signaling through vascular hyperpolarization and NO and PGs during exercise

As previously stated, there are a variety of substances that can stimulate vascular hyperpolarization to occur, both those that are endothelium-dependent and some that occur independently of this cell layer. Adenosine triphosphate (ATP) is a vasoactive agent released from endothelial and red blood cells in response to deoxygenation, hypercapnia, and mechanical stresses (Bodin *et al.*, 1991; Sprague *et al.*, 1996; Gonzalez-Alonso *et al.*, 2002; Kirby *et al.*, 2012; Crecelius *et al.*, 2013b) and causes profound vasodilatation that is endothelium-dependent (Duza & Sarelius, 2003). We have shown in humans that ATP-mediated vasodilatation occurs primarily through vascular hyperpolarization via activation of K_{IR} channels (Crecelius *et al.*, 2012). Additionally, we have demonstrated a modest component of ATP-mediated vasodilatation that occurs through NO and PG synthesis (Crecelius *et al.*, 2011a), and thus

particularly during steady-state exercise where a significant role for NO and PGs is revealed when hyperpolarization is inhibited, ATP may be one potential stimulus for these pathways.

Another potential stimulus for vascular hyperpolarization is increased interstitial K^+ as a result of muscle contractions (Hazeyama & Sparks, 1979b; Edwards *et al.*, 1998; Armstrong *et al.*, 2007; Kirby & Carlson, 2008) or as an endothelial-derived hyperpolarizing factor (Edwards *et al.*, 1998). Agonist binding to receptors on endothelial cells can cause intracellular calcium changes that stimulate small- and intermediate-conductance K_{Ca} channels to open and K^+ to efflux into the interstitial space or microdomain signaling complexes at the physical intersection of vascular smooth muscle and endothelial cells (Ledoux *et al.*, 2008). In animal models where these small- and intermediate-conductance K_{Ca} channels can be specifically inhibited, a significant attenuation in contraction-induced hyperaemia is observed (Milkau *et al.*, 2010). While the K^+ ion would not account for the compensatory involvement of NO and PG during steady-state exercise (Crecelius *et al.*, 2012), it could be involved in stimulating both K_{IR} channels and Na^+/K^+ATPase (Edwards *et al.*, 1998; Crecelius *et al.*, 2012) that in the present study, we show contribute both to the onset and steady-state levels of exercise hyperaemia.

Experimental Considerations

Our group has recently utilized the pharmacological approach of $BaCl_2$ and ouabain to inhibit vascular hyperpolarization via K_{IR} channel activation and Na^+/K^+-ATPase, respectively (Crecelius *et al.*, 2012, 2013a). While these inhibitors are safe for use, given that they are not used clinically, we attempt to limit the total number of subjects exposed to these agents and the total dose given to any one subject. Therefore, we have not repeated control experiments within this protocol to demonstrate effective inhibition of hyperpolarization-mediated (KCl)

vasodilatation, preserved vasodilatory capacity [to endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) agonists], and preserved maximal force production (Crecelius *et al.*, 2012, 2013a). Similarly, given our extensive use of L-NMMA and ketorolac (Crecelius *et al.*, 2010; Crecelius *et al.*, 2011a; Crecelius *et al.*, 2012) and various tests of efficacy (Dinenno & Joyner, 2003; Schrage *et al.*, 2004), we have not repeated those control studies within this experiment. Related to the effects of our inhibitors, as stated in our Methods section, we used double the dose of $BaCl_2$ from our previous studies in an attempt to maintain a concentration in the blood during exercise that was similar to at rest, in spite of the large increases in blood flow that occur with contractions. It is possible that our dose of $BaCl_2$ may be slightly beyond the range of specific inhibition of K_{IR} channels (typically <100 μM) and non-selectively inhibit other potassium channels, particularly, K_{ATP} channels (Jackson, 2005). However, given that previously there was no significant effect of inhibiting K_{ATP} channels during steady-state exercise (Schrage *et al.*, 2006), we do not feel this changes our primary conclusions.

The forearm model, while advantageous for many reasons also has some inherent limitations. We acknowledge that the muscle mass engaged in rhythmic handgrip exercise is relatively small, and extrapolating our results to more traditional modes of whole-body exercise would need to be done cautiously. It may be desirable to test ideas related to the role of hyperpolarization in regulating vascular control during larger muscle mass exercise that engage the sympathetic nervous system in order to assess the influence of these pathways on the interaction with sympathetic vasoconstriction and overall blood pressure regulation; however, safety concerns of systemic administration of $BaCl_2$ in doses suitable to appropriately inhibit K_{IR} channels preclude this at the present time.

Conclusions

Continuous skeletal muscle contractions result in a rapid and significant rise in muscle blood flow in order to deliver oxygen to meet the increased metabolic need of the tissue. The regulation of muscle blood flow during exercise is a complex response and there is great interest in understanding the various vasodilator signals that contribute to this response. Here, we show that vascular hyperpolarization via K_{IR} channel and Na^+/K^+-ATPase activation significantly contributes to the hyperemia observed at the onset and during steady-state muscle contractions. Additionally, NO and PGs have only a modest contribution to the initial (10-15 sec) rise in muscle blood flow and interestingly, in the presence of inhibition of K_{IR} channels and Na^+/K^+-ATPase, we reveal a role for these dilators during steady-state exercise hyperaemia, an observation that was not previously made in this experimental model. The primary stimuli for activation of K_{IR} channels, Na^+/K^+-ATPase, and synthesis of NO and PGs remains unknown at this time.

Table 3. Protocol 1: Forearm and Systemic Haemodynamics

* $P<0.05$ *vs* control; † $P<0.05$ *vs* $BaCl_2$+ouabain

†MAP: Main effect of condition ($BaCl_2$+ouabain+L-NMMA+ketorolac *vs* control; $P < 0.05$)

‡HR: Main effect of condition ($BaCl_2$+ouabain+L-NMMA+ketorolac *vs* control and *vs* $BaCl_2$+ouabain, $P < 0.05$); Main effect of timepoint (Min 1-5 *vs* Rest; $P < 0.05$); no significant interaction ($P = 0.35$)

FVC=forearm vascular conductance; HR=heart rate; MAP=mean arterial pressure

Timepoint	***Condition***	***FVC (ml^{-1} min^{-1} 100mmHg^{-1})***	***MAP † (mmHg)***	***HR ‡ (beats min^{-1})***
Rest	Control	36.4±3.4	93±2	56±3
	$BaCl_2$+ouabain	31.8±2.3	94±3	57±4
	$BaCl_2$+ouabain+L-NMMA+ketorolac	25.8±2.5*†	96±2	53±3
Exercise Minute 1	Control	140.2±9.8	94±3	61±4
	$BaCl_2$+ouabain	109.3±7.6*	96±3	59±4
	$BaCl_2$+ouabain+L-NMMA+ketorolac	99.5±5.7*†	98±2	57±4
Exercise Minute 2	Control	151.3±11.9	94±2	59±4
	$BaCl_2$+ouabain	134.9±9.6*	96±2	59±3
	$BaCl_2$+ouabain+L-NMMA+ketorolac	128.5±7.9*	97±2	56±4
Exercise Minute 3	Control	150.2±9.8	94±3	57±5
	$BaCl_2$+ouabain	139.6±8.3*	96±3	59±4
	$BaCl_2$+ouabain+L-NMMA+ketorolac	134.7±9.9*	97±2	57±3
Exercise Minute 4	Control	155.6±10.8	95±2	60±4
	$BaCl_2$+ouabain	147.5±8.7	95±3	59±3
	$BaCl_2$+ouabain+L-NMMA+ketorolac	142.7±10.3*	97±2	57±3
Exercise Minute 5	Control	166.0±15.3	95±2	59±4
	$BaCl_2$+ouabain	157.1±10.3	95±2	59±4
	$BaCl_2$+ouabain+L-NMMA+ketorolac	145.4±9.2*	97±2	58±4

Table 4. Protocol 2: Forearm and Systemic Haemodynamics

†MAP: Main effect of timepoint (Exercise *vs* Rest; $P < 0.05$) and condition (all conditions different; $P < 0.05$), no significant interaction ($P = 0.07$).

‡HR: Main effect of timepoint (Exercise *vs* Rest; $P < 0.01$), no significant main effect of condition ($P = 0.32$) or interaction ($P = 0.47$)

HR=heart rate; MAP=mean arterial pressure

Timepoint	***Condition***	***MAP † (mmHg)***	***HR ‡ (beats min^{-1})***
Rest	Control	84±2	56±3
	$BaCl_2$+ouabain	90±3	54±3
	$BaCl_2$+ouabain+L-NMMA+ketorolac	94±4	56±3
Steady-State Exercise (Minute 5)	Control	86±3	60±3
	$BaCl_2$+ouabain	95±2	60±3
	$BaCl_2$+ouabain+L-NMMA+ketorolac	100±3	59±3

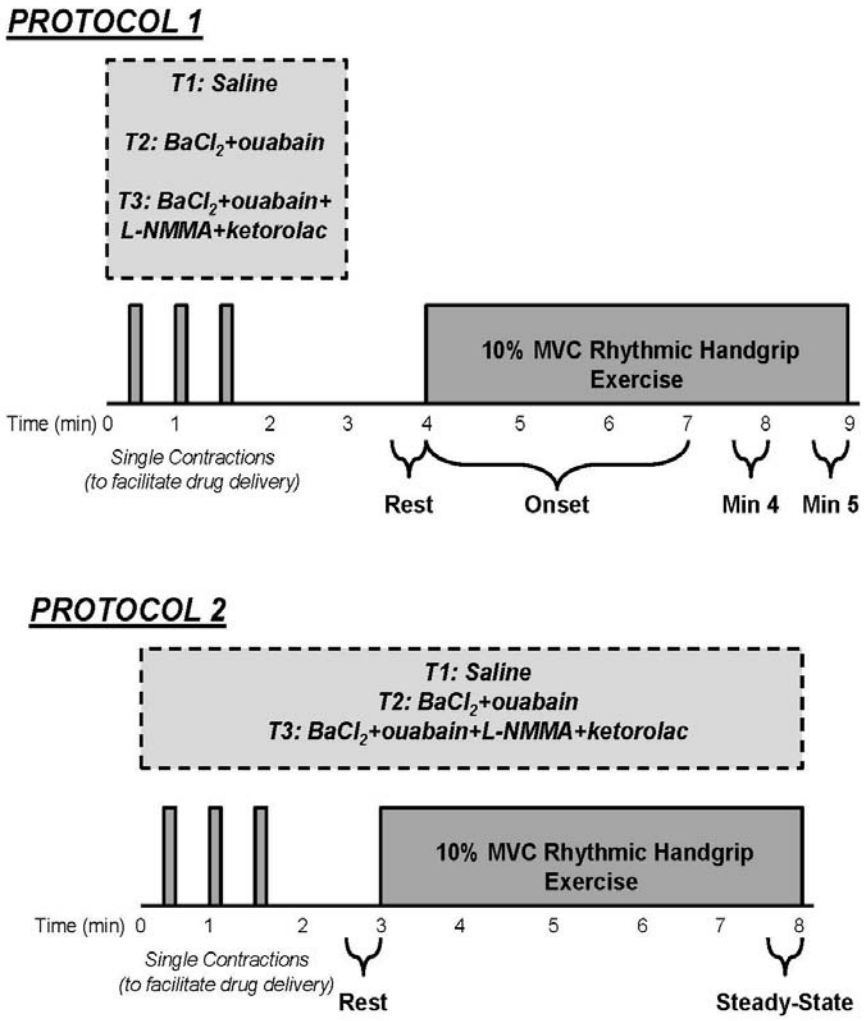

Figure 10. Experimental Timeline
In both protocols, three trials were performed. Trial 1 consisted of saline infusions as a control condition. Trial 2 consisted of the combined infusion of barium chloride ($BaCl_2$; K_{IR}-channel inhibitor) and ouabain (Na^+/K^+-ATPase inhibitor) and Trial 3 consisted of the combined infusion of $BaCl_2$, ouabain, L-NMMA (to inhibit nitric oxide synthesis) and ketorolac (to inhibit prostaglandin synthesis). During the loading period of drug infusions at rest, three single contractions were performed to facilitate drug delivery. In Protocol 1, to avoid previously observed fluctuations in baseline haemodynamics, drugs were stopped before exercise commenced, and measurements were made at rest, the onset of 10% MVC rhythmic handgrip exercise, and throughout the 5 minute bout of exercise. In Protocol 2, drugs were continuously coinfused throughout rest and the entire handgrip exercise trial.

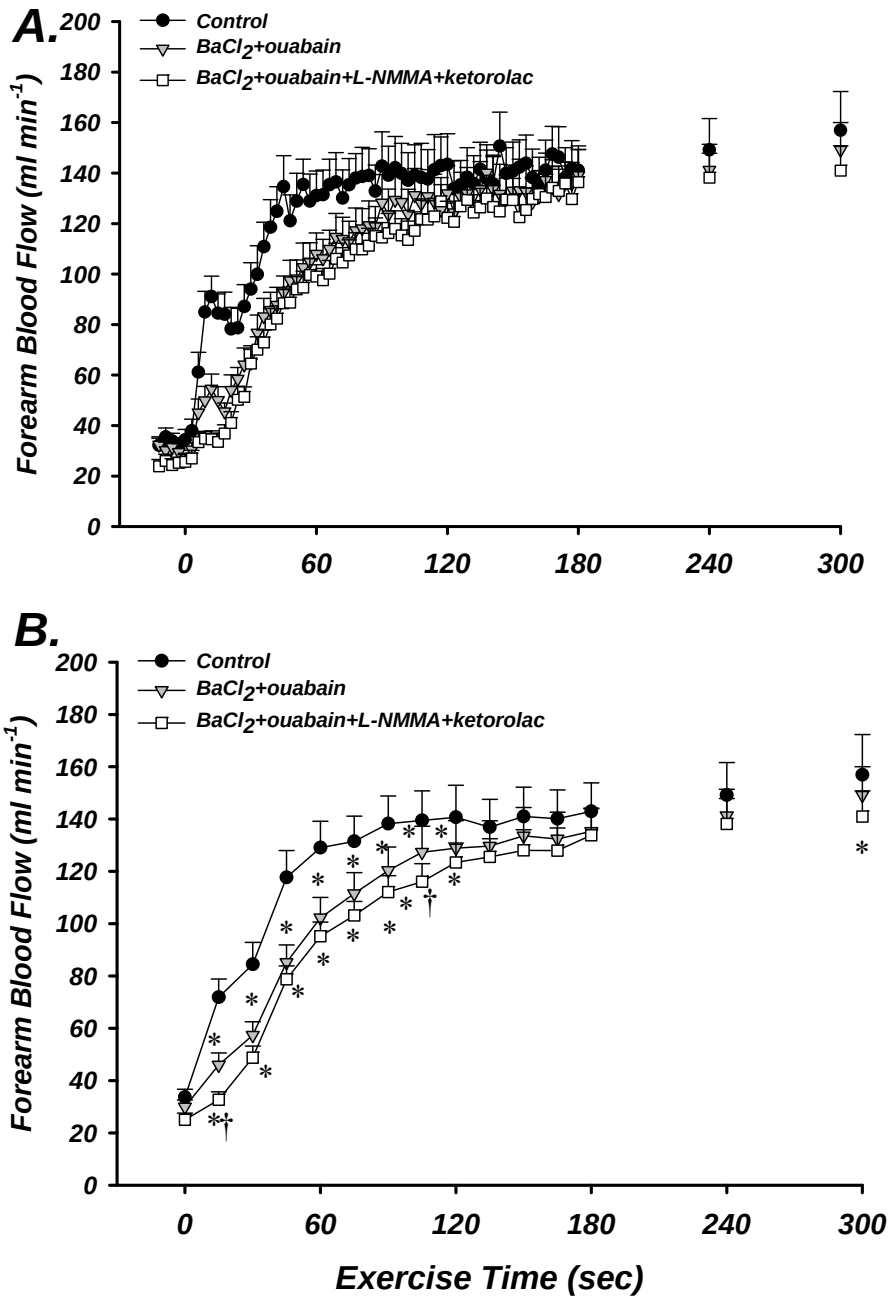

Figure 11. Protocol 1: Effect of inhibition of hyperpolarizing and endothelium-dependent vasodilator mechanisms on the onset of exercise hyperaemia
A) Forearm blood flow across the three experimental conditions are shown at rest and during 10% MVC rhythmic handgrip exercise. Data is in 3-sec bins corresponding to a contraction:relaxation cycle during exercise. *B)* Statistical analysis was performed on 15 sec averages of blood flow data (5 3-sec bins). $BaCl_2$+ouabain significantly reduced the onset of exercise hyperaemia and L-NMMA+ketorolac had an additional effect within the first 15 sec of exercise. * $P < 0.05$ *vs* control; † $P < 0.05$ *vs* $BaCl_2$+ouabain

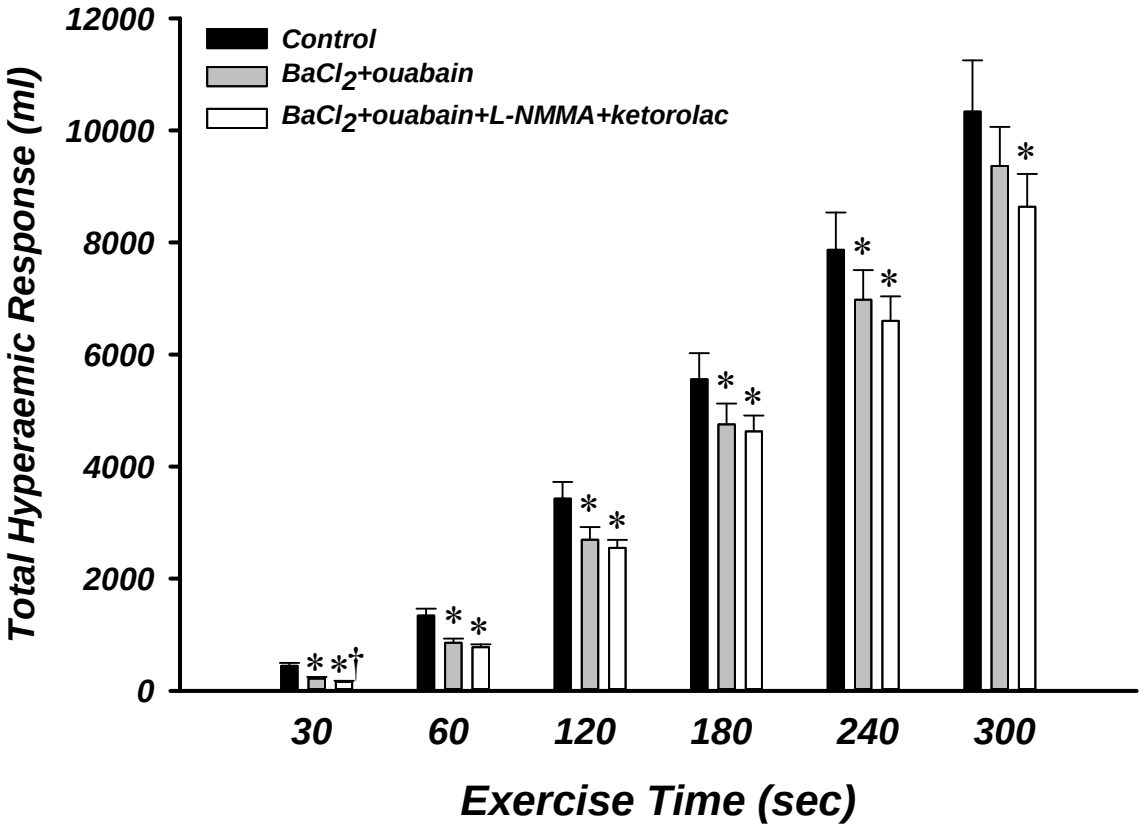

Figure 12. Protocol 1: Effect of inhibition of hyperpolarizing and endothelium-dependent vasodilator mechanisms on the total hyperaemic response to exercise
The total hyperaemic response (area under the curve) was calculated for 30 and 60-sec increments for all experimental conditions. $BaCl_2$+ouabain significantly reduced the hyperaemia until the 5th minute of exercise. The addition of L-NMMA+ketorolac to $BaCl_2$-+ouabain appeared to only have a greater effect within the first portion (30 sec) of exercise. * $P < 0.05$ *vs* control; † $P < 0.05$ *vs* $BaCl_2$+ouabain

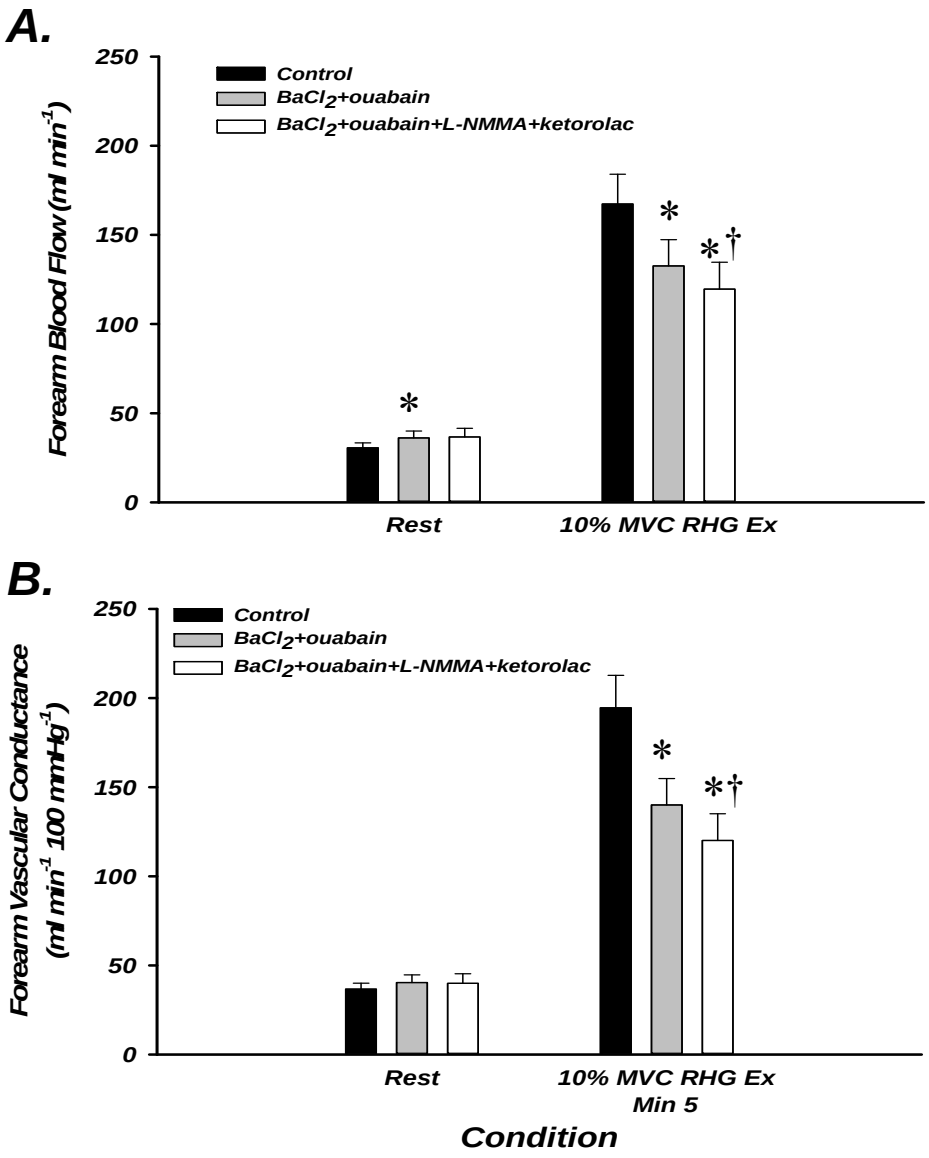

Figure 13. Protocol 2: Effect of inhibition of hyperpolarizing and endothelium-dependent vasodilator mechanisms on steady-state exercise hyperaemia
In Protocol 2, with continuous infusion of the inhibitors, a significant reduction in FBF (*A*) and FVC (*B*) was observed with combined $BaCl_2$+ouabain infusion. Here, the additional of L-NMMA+ketorolac to $BaCl_2$+ouabain further reduced both steady-state FBF and FVC.
* $P < 0.05$ *vs* control; † $P < 0.05$ *vs* $BaCl_2$+ouabain

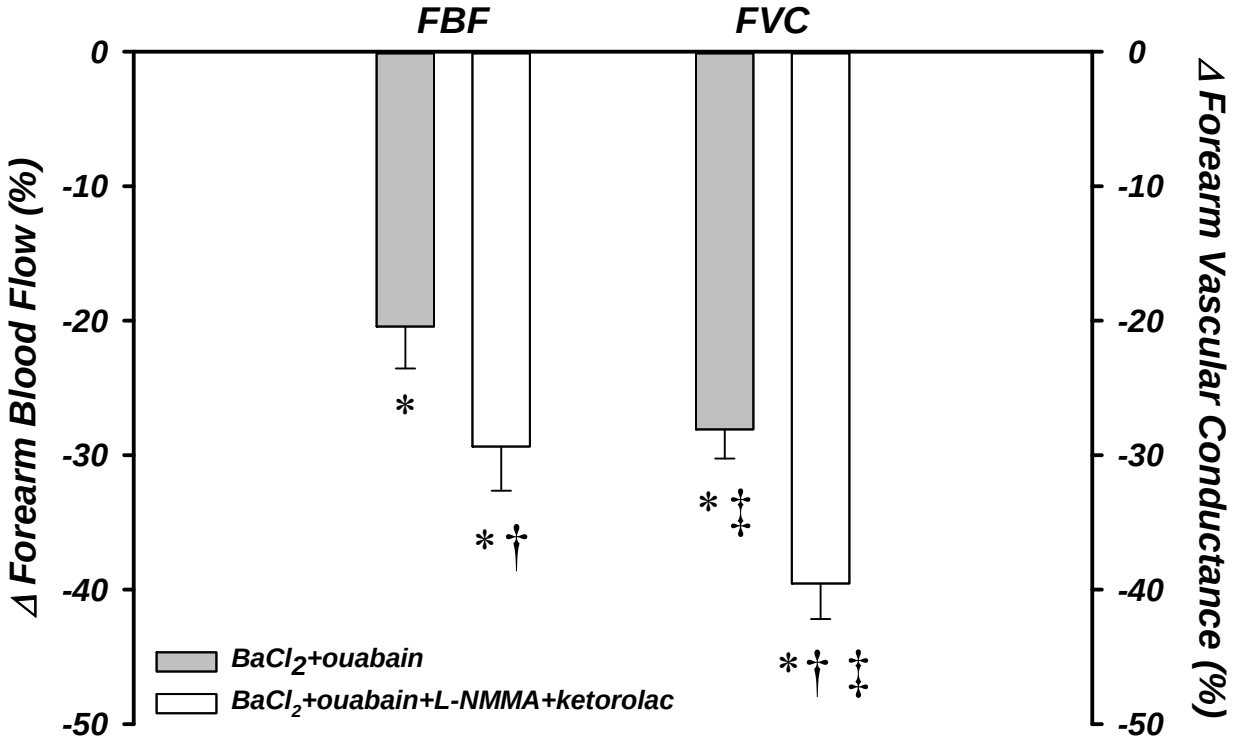

Figure 14. Protocol 2: Changes in steady-state forearm haemodynamics with inhibition of hyperpolarizing and endothelium-dependent vasodilator mechanisms
The reduction in steady-state exercise FBF and FVC was significantly greater with combined $BaCl_2$+ouabain+L-NMMA+ketorolac than $BaCl_2$+ouabain alone. Additionally, the changes in FVC were more profound than those in FBF. * $P < 0.05$ *vs* zero; † $P < 0.05$ *vs* $BaCl_2$+ouabain; ‡ $P < 0.05$ *vs* FBF.

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CHAPTER IV – MANUSCRIPT III

Preserved functional sympatholysis with combined inhibition of inwardly-rectifying channels and Na^+/K^+-ATPase in the human forearm[3]

Summary

Sympathetic vasoconstriction in contracting skeletal muscle is blunted relative to that which occurs in resting tissue. The underlying signaling of this functional sympatholysis remains a debated issue in human physiology. We recently demonstrated that activation of inwardly-rectifying (K_{IR}) potassium channels and Na^+/K^+-ATPase contributes to exercise hyperaemia, but whether these pathways are able to modulate α-adrenergic vasoconstriction during contractions has not been investigated. We tested the hypothesis that α-adrenergic vasoconstriction is augmented during exercise following inhibition of K_{IR} channel and Na^+/K^+-ATPase via barium chloride ($BaCl_2$) and ouabain respectively, and unaffected by the inhibition

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of nitric oxide (NO) and prostaglandins (PGs), two other potential vasodilators involved in vascular responses during exercise. In 11 healthy, young humans, we measured forearm blood flow (Doppler ultrasound) and calculated vascular conductance (FVC) at rest, during steady-state stimulus conditions (pre-PE), and after 2 min of phenylephrine (PE; α_1-adrenoceptor agonist) infusion via brachial artery catheter. Subjects performed either moderate (15% maximal voluntary contraction) rhythmic handgrip exercise (RHG) or received adenosine (ADO) in all conditions: control (saline), combined enzymatic inhibition of NO and PG synthesis via L-NMMA (NO synthase) and ketorolac (cyclooxygenase), and combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase (L-NMMA+ketorolac+$BaCl_2$+ouabain). All trials occurred after local β-adrenoceptor blockade (propranolol). PE-mediated vasoconstriction was calculated (%ΔFVC) in each condition. As expected, in control conditions, vasoconstriction during exercise was significantly attenuated as compared to ADO (-21±3% *vs* -46±6%; $P<0.05$). These responses were unchanged with L-NMMA+ketorolac (-24±3% *vs* -47±5%; $P<0.05$) as was steady-state exercise hyperaemia (231±21 ml min^{-1} *vs* 221±22 ml min^{-1}; $P=0.66$). Additional inhibition of K_{IR} channels and Na^+/K^+-ATPase significantly attenuated exercise hyperaemia (151±21 ml min^{-1}; $P<0.05$) but in contrast to our hypothesis, had no effect on vasoconstriction during exercise (-27±3%) despite an augmented response during adenosine (-71±3%). Thus, exercise still significantly blunted PE-mediated vasoconstriction relative to ADO during L-NMMA+ketorolac+$BaCl_2$+ouabain. Our findings confirm that vasodilatation via K_{IR} channels and Na^+/K^+-ATPase significantly contribute to exercise hyperaemia but are not likely to mediate functional sympatholysis in humans.

Introduction

Muscle contractions stimulate vasodilatation of the resistance vasculature, leading to large increases in muscle blood flow to the active tissue. Due to the profound capacity of skeletal muscle blood flow during exercise (Andersen & Saltin, 1985), some level of sympathetically mediated vasoconstriction is needed in order to maintain mean arterial blood pressure (Marshall *et al.*, 1961; Buckwalter & Clifford, 2001). It has been shown that the degree to which sympathetically mediated vasoconstriction occurs in the active muscle is blunted relative to inactive muscle (Anderson & Faber, 1991; Thomas *et al.*, 1994; Tschakovsky *et al.*, 2002; Dinenno & Joyner, 2003; Kirby *et al.*, 2008). This phenomenon, termed functional sympatholysis (Remensnyder *et al.*, 1962), is thought to allow for optimal blood flow and therefore oxygen delivery to the active tissue (Joyner & Thomas, 2003) while some vasoconstriction occurs in order to limit the overall decrease in total peripheral resistance. Various laboratories have demonstrated the existence of functional sympatholysis (Tschakovsky *et al.*, 2002; Rosenmeier *et al.*, 2003; Keller *et al.*, 2004; Rosenmeier *et al.*, 2004; Parker *et al.*, 2007; Wray *et al.*, 2007; Kirby *et al.*, 2008; Fadel *et al.*, 2012); however, to date, the signaling pathways that contribute to this response have not been well described in healthy humans.

Functional sympatholysis can occur at the post-junctional level of α-adrenoceptors, (Thomas *et al.*, 1994; Buckwalter *et al.*, 1998; Rosenmeier *et al.*, 2003) and as such, investigations have targeted a variety of vasodilator signaling pathways within the vessel itself which can contribute to exercise hyperaemia as potentially being involved in this response While some studies have suggested there may be a role for nitric oxide (NO) in blunting sympathetically-mediated vasoconstriction during muscle contractions (Thomas & Victor, 1998; Hansen *et al.*, 2000; Grange *et al.*, 2001; Chavoshan *et al.*, 2002), other investigations of this

pathway propose little involvement (Dinenno & Joyner, 2003; Buckwalter *et al.*, 2004). Further, when NO is inhibited in combination with prostaglandins (PGs), another endothelium-dependent vasodilator, there is only a modest enhancement of sympathetically-mediated vasoconstriction during muscle contractions (Dinenno & Joyner, 2004). Given that vasoconstrictor responses in a quiescent tissue were also slightly augmented in this study, it does not appear that NO and PGs are the primary mediators of functional sympatholysis in humans. Limited studies have explored other vasodilator pathways that may modulate sympathetically-mediated vasoconstriction during muscle contractions (Thomas *et al.*, 1997; Keller *et al.*, 2004) and support is growing for the role of pathways that result in vascular hyperpolarization in blood flow control during exercise (Segal & Jacobs, 2001; Milkau *et al.*, 2010; Crecelius *et al.*, 2012b; Crecelius *et al.*, 2013).

To date, few exogenous vasodilator substances are capable of blunting sympathetically-mediated vasoconstriction similar to what occurs during exercise. Specifically, in humans, exogenous ATP has directly been shown to be sympatholytic (Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008; Kirby *et al.*, 2011) and we have shown ATP-mediated vasodilatation is largely independent of NO and PG synthesis (Crecelius *et al.*, 2011a), primarily occurring through vascular hyperpolarization via inwardly-rectifying potassium (K_{IR}) channel activation (Crecelius *et al.*, 2012a). Stimulation of K_{IR} channels leads to membrane potential hyperpolarization which can also occur with activation of Na^+/K^+-ATPase (Jackson, 2005). Additionally, *in vitro* evidence suggests a prominent role for K_{IR} channels in amplifying hyperpolarization of any origin and facilitating robust cell-to-cell communication (Jantzi *et al.*, 2006). We have recently shown that activation of K_{IR} channels and Na^+/K^+-ATPase contributes to exercise hyperaemia in the forearm (Crecelius *et al.*, 2012a) and there is also limited evidence that a hyperpolarizing

stimulus may be able to override noradrenaline-induced vasoconstriction in humans (Pickkers *et al.*, 2004).

Along these lines, there appears to be important associations between sympathetically-mediated vasoconstriction, endothelial and vascular smooth muscle cell membrane potential (hyperpolarization and depolarization), and conducted vasodilatation, or dilatation that spreads electrically along a vessel length, including upstream from the site of stimulation (Segal, 1994). Specifically, it appears as though there is a reciprocal relationship between α-adrenoceptor stimulated membrane depolarization and conducted vasodilatation via hyperpolarization in that an agent that stimulates hyperpolarization and conducted vasodilatation can overcome sympathetically-induced vasoconstriction while at the same time, sympathetic stimulation attenuates conducted vasodilatation (Kurjiaka & Segal, 1995; Haug & Segal, 2005). However, whether or not vascular hyperpolarization specifically contributes to the ability of voluntarily contracting skeletal muscle to blunt direct post-junctional α-adrenergic vasoconstriction is yet to be determined. With this information as background, we tested the hypothesis that vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase plays a role in functional sympatholysis during rhythmic handgrip exercise in young healthy humans.

Methods

Subjects

With Institutional Review Board approval and after written informed consent, a total of 11 young healthy adults (8 men, 3 women; age = 23±1 years old; weight = 67.6±2.9 kg; height = 173±3 cm; body mass index = 22.4±1.1 kg m^{-2}; forearm volume (FAV) = 913±58 ml; means ± SEM) participated in the present study. All subjects were sedentary to moderately active, non-

smokers, non-obese, normotensive (resting blood pressure <140/90 mmHg), 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) for local administration of study drugs and blood sampling. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml hr^{-1} with heparinized saline. The two side ports were used for drug infusions of vasoactive drugs (Kirby *et al.*, 2008; Crecelius *et al.*, 2010). In addition, an 18 or 20 gauge (depending on visual inspection of vein size), 5.1 cm catheter was inserted in retrograde fashion into an antecubital vein of the experimental arm for deep venous blood samples. Saline was continuously infused through this catheter at a rate of approximately 3 ml min^{-1} for the duration of the study to keep it patent (Crecelius *et al.*, 2011b). Heart rate (HR) was determined using a 3-lead electrocardiogram (Cardiocap/5, Datex-Ohmeda Louisville, CO, USA).

Blood gas analysis

Brachial artery and deep venous blood samples were immediately analyzed with a clinical blood gas analyzer (Rapid Point 400 Series Automatic Blood Gas System, Siemens

Healthcare Diagnostics, Deerfield, IL, USA) for partial pressures of oxygen and carbon dioxide (pO_2 and pCO_2), fraction of oxygenated haemoglobin (FO_2Hb), oxygen content, pH, and $[K^+]$.

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 securely fixed to the skin 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, steady-state conditions pre-constriction, and end constrictor effect (see Experimental Protocol below). Forearm blood flow (FBF) was calculated as: $FBF = MBV \times \pi (\text{brachial artery diameter}/2)^2 \times 60$, 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.

Rhythmic handgrip exercise

Maximal voluntary contraction (MVC; mean 41±4 kg, range 19-67 kg) was determined for the experimental arm as the average of three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL, USA) that were within 3% of each other. Forearm exercise during the trials was performed with weight corresponding to 15% MVC (mean 6.1±0.6 kg, range 2.9 – 10.1 kg) attached to a pulley system and lifted 4–5 cm over the pulley at a duty cycle of 1s contraction–2 s relaxation (20 contractions per minute) using both visual and auditory feedback to ensure the correct timing as described previously (Kirby *et al.*, 2008; Crecelius *et al.*, 2010). We chose this mild intensity rhythmic handgrip exercise to limit the contribution of systemic haemodynamics to forearm hyperaemic responses and eliminate reflex activation of the sympathetic nervous system (Seals & Victor, 1991). Previous studies in our laboratory have determined that MVC is not affected by any of the vasoactive substances, particularly barium chloride and ouabain, administered in these studies (Crecelius et al., 2013)

Vasoactive drug infusion

All drug infusions occurred via brachial artery catheter to create a local effect in the forearm. Phenylephrine (see below) is capable of stimulating β-adrenoceptor-mediated vasodilatation (Torp *et al.*, 2001). In order to avoid this potentially confounding effect, we infused propranolol (non-selective β-adrenoceptor antagonist, West-Ward Pharmaceutical Corp., Eatontown, NJ, USA) at a loading dose of 200 μg min^{-1} for 5 min (total = 1000 μg) and continued infusions at a maintenance dose of 50 μg min^{-1} prior to any experimental trials and for the duration of the protocol. This dose of propranolol has been documented to block forearm vasodilatation to isoproterenol (Johnsson, 1967) and we have used this approach in similar

studies where phenylephrine-mediated vasoconstriction has been investigated (Dinenno *et al.*, 2002a; Dinenno *et al.*, 2002b). Regarding any potential effects of β-adrenoceptor inhibition during exercise, evidence indicates that β-mediated vasodilatation does not contribute to exercise hyperaemia in a similar experimental model (Hartling *et al.*, 1980).

Given the large increase in blood flow that occurs as a result of muscle contractions, we infused adenosine (Akorn, Lake Forest, IL, USA) at variable doses to elevate forearm blood flow of a quiescent tissue to similar levels observed during exercise and this served as a 'high flow control' in each experimental condition. We have previously demonstrated that contracting muscle blunts direct α_1- and α_2-adrenoceptor-mediated vasoconstriction, whereas vasoconstrictor responses are preserved during adenosine infusion (Tschakovsky *et al.*, 2002; Kirby *et al.*, 2008; Kirby *et al.*, 2011).

In order to stimulate sympathetically-mediated vasoconstriction, the selective α_1-adrenoceptor agonist phenylephrine (PE; Sandoz, Princeton, NJ, USA) was infused at 0.125 μg dl FAV^{-1} min^{-1}. The absolute dose of PE was adjusted to the appropriate hyperaemic condition as previously described by our laboratory (Kirby *et al.*, 2008; Kirby *et al.*, 2011) in order to normalize concentrations to this relative dose based on the observed level of forearm blood flow during adenosine infusion and exercise (see below). This relative dose of PE is twice that which we have previously utilized in male subjects (Kirby *et al.*, 2009; Kirby *et al.*, 2011) and this was done in order insure that all subjects would demonstrate vasoconstriction during exercise in control conditions, and thus provide an appropriate control for any potential augmentation of this response due to our pharmacological inhibitors.

N^G-monomethyl-L-arginine (L-NMMA; Bachem, Germany) was administered to inhibit nitric oxide synthase-mediated production of NO and ketorolac (Hospira, Lake Forest, IL, USA)

was administered to inhibit cyclooxygenase-mediated synthesis of PGs. Loading doses of L-NMMA and ketorolac were 25 mg (5 mg min^{-1} for 5 minutes) and 6 mg (600 μg min^{-1} for 10 min), respectively (Crecelius *et al.*, 2011b; Crecelius *et al.*, 2012a). In subsequent trials, L-NMMA and ketorolac were infused at maintenance doses of 1.25 mg min^{-1} and 150 μg min^{-1}, respectively.

To inhibit vascular hyperpolarization via Na^+/K^+-ATPase and K_{IR} channel activation, ouabain octahydrate (Na^+/K^+-ATPase inhibitor; Sigma 03125, St. Louis, MO, USA) was infused at 2.7 nmol min^{-1} in combination with barium chloride ($BaCl_2$; K_{IR} channel inhibitor; 10% w/v BDH3238, EMD Chemicals, Gibbstown, NJ, USA) at 0.9 μmol dl FAV^{-1} min^{-1} with a minimum absolute dose of 8 μmol min^{-1} to a maximum dose of 10 μmol min^{-1} (Crecelius *et al.*, 2012a; Crecelius *et al.*, 2012b). Ouabain and $BaCl_2$ loaded for 15 and 3 minutes, respectively prior to the first trial (exercise or adenosine) of the inhibited condition and were continued at these same doses for the duration of the trial. In the second trial of the blocked condition, infusion of all inhibitors began 3 min prior to the start of muscle contractions or adenosine infusion. Ouabain and $BaCl_2$ were prepared in saline and confirmed sterile and free of fungus/endotoxin and particulate matter with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS, USA) prior to use. Forearm volume used for normalization for specific vasoactive drugs was determined from regional analysis of whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc, Bedford, MA, USA).

Experimental protocols

Figure 15 presents the overall experimental timeline (*A*) as well as the protocol for each individual trial (*B*). After instrumentation and β-adrenoceptor inhibition, subjects performed a

bout of handgrip exercise or received intra-arterial adenosine infusion (final average dose: 46.2±5.6 μg dl FAV^{-1} min^{-1}) matched to the predicted/observed hyperaemic response to exercise. The total length of each trial was 9 minutes consisting of 3 minutes of baseline conditions and 6 minutes total of exercise or adenosine infusion. Steady-state conditions were achieved after 3 minutes of hyperaemia (min 6 of Figure 15) and the dose of PE was calculated on the basis of FAV and FBF. Vasoconstrictor infusion then began after this (4 minutes into the stimulus) and lasted for 2 minutes. Venous blood samples were taken at rest, in steady-state hyperaemic conditions (Pre-PE) and at end-PE. Arterial blood samples were only taken at baseline as these samples interfere with the ability to measure FBF and we have shown arterial blood gases to be unchanged with mild-moderate intensity rhythmic handgrip exercise (Crecelius *et al.*, 2011b). In the first exercise and adenosine trials, saline was used as a control infusate.

Given evidence for interactions of hyperpolarization and endothelium-dependent vasodilators, particularly NO, (Bauersachs *et al.*, 1996; Taddei *et al.*, 1999) prior to investigating the role of vascular hyperpolarization via K_{IR} and Na^+/K^+-ATPase activation, we inhibited the production of NO via L-NMMA. Redundancy and compensation has also been observed between NO and PG during physiological stressors (Schrage *et al.*, 2004; Markwald *et al.*, 2011) and thus to eliminate this, we inhibited PG synthesis via ketorolac. Combined inhibition of NO and PGs also served to confirm the previous findings that these pathways do not contribute to functional sympatholysis (Dinenno & Joyner, 2004). Following control trials, L-NMMA and ketorolac were administered in loading doses and exercise and adenosine (final average dose: 59.5±6.9 μg dl FAV^{-1} min^{-1}) trials were repeated in this condition of combined inhibition of NO and PGs. After these third and fourth trials, $BaCl_2$ and ouabain were infused and exercise and adenosine (final average dose: 45.2±7.5 μg dl FAV^{-1} min^{-1}) trials were performed with combined

inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase. Exercise and adenosine trials were counterbalanced in each condition between subjects. In cases where the adenosine trial preceded exercise, for the purposes of adjusting the adenosine dose, we predicted the level of hyperaemia based on our experience with exercise responses in control and inhibited conditions (Dinenno & Joyner, 2004; Schrage *et al.*, 2004; Crecelius *et al.*, 2011b; Crecelius *et al.*, 2012b). All trials were separated by 15 minutes of rest.

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). FBF, FVC, MAP, and HR represent an average of the last 30 seconds of the corresponding timepoint within a trial (Figure 15; baseline, pre-PE, PE). To quantify the vasoconstrictor effect of PE, the per cent change (% Δ) was calculated as: $(FVC_{PE} - FVC_{Pre\text{-}PE})/(FBF_{Pre\text{-}PE}) \times 100$. We use %Δ in FVC as our standard index to compare vasoconstrictor responses as this appears to be the most appropriate way to compare vasoconstrictor responsiveness under conditions where there might be differences in vascular tone and blood flow, rather than pressure, is the main variable changing (Lautt, 1989; Buckwalter & Clifford, 2001). Forearm oxygen consumption (VO_2) was calculated as: $FBF \times (O_2\ content_{arterial} - O_2\ content_{venous})$ and presented in ml min^{-1}.

Statistics

All values are reported as means ± SEM. Given the complexity of our experimental design, many comparisons are possible. We have utilized a statistical approach that focuses on the comparisons that are most relevant to our experimental questions. Absolute hypereaemic

forearm haemodynamics were assessed by two-way [timepoint (pre-PE or PE) × condition (control, L-NMMA+ketorolac, or L-NMMA+ketorolac+$BaCl_2$+ouabain)] repeated measures (RM) ANOVA inclusive of both adenosine and exercise trials. Due to the predictably large change from rest to exercise/adenosine, resting forearm haemodynamics were analyzed separately by one-way RM ANOVA. Systemic haemodynamics were evaluated by two-way RM ANOVA (timepoint × condition) with all timepoints (rest, pre-PE, PE) included. Vasoconstrictor responses (%Δ) were compared with a two-way [stimulus (exercise or adenosine) × condition (control, L-NMMA+ketorolac, or L-NMMA+ketorolac+$BaCl_2$+ouabain)] RM ANOVA. Blood gas variables for the Pre-PE and PE timepoints of exercise trials were analyzed with two-way ANOVA while a one-way ANOVA was used for baseline values. Student-Newman-Keuls *post hoc* testing was performed to make pairwise comparisons. Changes in the haemodynamic response to exercise were compared with paired Student's *t* tests. Significance was set at $P < 0.05$.

Results

Systemic haemodynamics in all experimental conditions are presented in Table 5. Small increases in MAP and HR occurred with exercise and throughout the course of the experiment and these are detailed in Table 5.

As intended by experimental design, FBF during adenosine infusion and exercise was matched prior to PE infusion in all experimental conditions (Figure 16) as was FVC (Table 5). In all trials, PE reduced FBF from these matched pre-PE levels. FBF at the end of PE infusion was always significantly greater in exercise conditions, as compared to control.

Infusion of L-NMMA+ketorolac reduced baseline FBF in both the adenosine and exercise trial but had no effect on steady-state FBF pre-PE (Figure 16). When quantified as a %Δ from control, there was no significant change in FBF and a minor decrease in FVC (Figure 17). For both adenosine and exercise, there was no impact of L-NMMA+ketorolac infusion on the vasoconstrictor responses (%Δ FVC) to PE (Figure 18).

With infusion of L-NMMA+ketorolac+$BaCl_2$+ouabain, baseline FBF was still significantly reduced from control in the exercise trial however this was not significant in the adenosine trial (P=0.27). Steady-state pre-PE FBF was significantly lower during exercise and by design in the adenosine trial (Figure 16). As shown in Figure 17, the magnitude of the effect on the exercise response was rather profound (FBF: ~40% reduction; FVC: ~45% reduction). Inhibition of NO, PG, K_{IR} channels and Na+/K+-ATPase significantly augmented PE-mediated vasoconstriction during adenosine in contrast to exercise where the vasoconstrictor response was unchanged (Figure 18). Accordingly, FBF with PE was still greater during the exercise trial as compared to during adenosine (Figure 16).

Blood gases from the arterial and deep venous samples obtained prior to and during the exercise trials are presented in Table 6. For simplicity, we have omitted presenting samples obtained during adenosine trials, as any observed differences were predictable based on changes in blood flow to a quiescent tissue and we were most interested in the exercise responses. A venous catheter was unsuccessful in one subject and due to equipment malfunction, some variables were not analyzed for all samples resulting in a range of N values (7-10) for these data. The significant changes observed were largely predictable based on the vasoconstriction induced by PE in each condition. Of note, oxygen extraction increased (increased a-vO_2, decreased pO_2 and FO_2Hb) pre-PE with infusion of L-NMMA+ketorolac+$BaCl_2$+ouabain as compared to

control and L-NMMA+ketorolac conditions. This coincided with decreased blood flow and resulted in VO_2 being maintained near prior levels. In control and L-NMMA+ketorolac conditions, a-vO_2 significantly increased with PE infusion. In contrast, this did not occur with L-NMMA+ketorolac+$BaCl_2$+ouabain and combined with attenuated FBF resulted in a significantly lower VO_2 as compared to control levels. Reduced VO_2 was paralleled with lower pH and greater pCO_2 and [K+] in this condition of combined inhibition of NO, PG, and vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase.

Discussion

The purpose of the current study was to determine whether vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase mediates functional sympatholysis in healthy humans. We specifically hypothesized that inhibition of K_{IR} channels and Na^+/K^+-ATPase would significantly impair the ability of contracting skeletal muscle to blunt α-adrenergic vasoconstriction, as compared to the vasoconstrictor response observed in resting muscle. The primary novel finding of the present study is that sympathetically-mediated vasoconstriction persists in contracting skeletal muscle during combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase, despite augmented constriction during a control vasodilator condition (Figure 18). The present results support our previous findings that activation of K_{IR} channels and Na^+/K^+-ATPase contribute to exercise hyperaemia and vasodilatation in the human forearm (Figure 17) (Crecelius *et al.*, 2012b) and that there is a minimal role for NO and PGs in mediating exercise hyperaemia (Schrage *et al.*, 2004) and sympatholysis (Dinenno & Joyner, 2003, 2004) in this model. Collectively, these results suggest that vasodilator action beyond K_{IR}

channels and Na^+/K^+-ATPase are requisite for the modulation of sympathetic vasoconstriction observed during muscle contractions in humans.

Vascular signaling pathways underlying functional sympatholysis

Identifying the sympatholytic 'factor' during muscle contractions has been the aim of a number of different investigators and findings across a range of models and utilizing various approaches have yielding conflicting results. Previous studies in the rat hindlimb clearly demonstrated a role for NO in functional sympatholysis (Thomas *et al.*, 1998; Thomas & Victor, 1998) and these findings have been indirectly substantiated in more recent human studies (Fadel *et al.*, 2004; Fadel *et al.*, 2012). However, in the human forearm, direct α_1- and α_2-adrenoceptor responsiveness during exercise is unchanged by the inhibition of NO synthase and a NO donor is incapable of modulating sympathetic vasoconstriction similar to what occurs in the vasculature of contracting skeletal muscle (Dinenno & Joyner, 2003). These findings are similar to those in experimental dogs with respect to inhibition of NO synthase not affecting direct α_2-mediated vasoconstriction during exercise (Buckwalter *et al.*, 2004). Prostaglandins, another endothelium-derived vasodilator have also been shown to be capable of blunting sympathetic vasoconstriction in certain conditions (Lippton *et al.*, 1981; Faber *et al.*, 1982); however, when synthesis of PGs was inhibited in the human forearm, similar to NO, vasoconstrictor responsiveness during exercise was unaltered (Dinenno & Joyner, 2004). In this same study, synthesis of NO and PGs was inhibited in combination, given the potential compensatory crossover of these pathways (Barker *et al.*, 1996; Osanai *et al.*, 2000). The observed sympathetic vasoconstrictor responses during exercise were slightly augmented compared to control conditions; however, they were substantially blunted compared with those at rest (Dinenno & Joyner, 2004). Given this range of

findings, as well as evidence for interaction between NO and vascular hyperpolarization (Bauersachs *et al.*, 1996; Taddei *et al.*, 1999), before addressing a potential role of hyperpolarization via K_{IR} channel and Na^+/K^+-ATPase activation, we inhibited these pathways to eliminate any potential compensatory effects and also confirm a minimal role of NO and PGs in sympatholysis.

The present findings support the previous work (Dinenno & Joyner, 2004) that α-adrenergic vasoconstriction is significantly blunted during muscle contractions as compared to a resting condition under combined NO and PG synthesis (Figure 18). Our current findings differ slightly from the previous work in that we did not see a significant augmentation of vasoconstriction during both rest and exercise conditions. This may be due to our utilization of propranolol in the current study to inhibit any potential PE-induced β-mediated vasodilatation which likely would have a NO-dependent component (Dawes *et al.*, 1997). The current study is also in agreement with previous reports that combined inhibition of NO and PGs does not impair the magnitude of steady-state exercise hyperaemia attained during mild to moderate rhythmic handgrip exercise (Figure 17) (Dinenno & Joyner, 2004; Schrage *et al.*, 2004; Crecelius *et al.*, 2011b). Taken together, the collective human data suggests that vascular control during exercise and particularly the interaction with sympathetic vasoconstriction extends beyond NO and PGs.

The majority of non-NO and PG mechanisms of vasomotion, particularly those that are endothelium-dependent, are thought to elicit hyperpolarization of vascular smooth muscle cells and there is significant interest in the role of this electrical communication within and between both the endothelial and smooth muscle cells (Emerson & Segal, 2000). Limited animal studies have pursued whether or not hyperpolarizing mechanisms can modulate sympathetic vasoconstriction and may be involved in functional sympatholysis. Investigations from Segal

and colleagues have utilized hamster preparations and determined there is a reciprocal relationship between α-adrenoceptor-mediated depolarization and pharmacologically-induced hyperpolarization; specifically, hyperpolarization that stimulates a conducted vasomotor response, or vasodilatation upstream from the site of agonist application. A reciprocal relationship is evident in that sympathetic stimulation attenuates conducted vasodilatation while conducted vasodilatation can also overcome sympathetically-induced vasoconstriction (Kurjiaka & Segal, 1995; Haug & Segal, 2005). Taken together, these data suggest that hyperpolarization and the resultant conducted vasodilatation may be the underlying pathway of functional sympatholysis.

Regarding specific mechanisms of hyperpolarization, a variety of events can stimulate this negative change in membrane potential, with a prime candidate being K^+ ion efflux through small- and intermediate-conductance calcium-activated potassium (K_{Ca}) channels (Jackson, 2005). Along these lines, non-selective inhibition K_{Ca} channels can augment PE-induced increases in perfusion pressure in an isolated rat kidney (Moreno *et al.*, 2003). More directly related to functional sympatholysis, Thomas and colleagues tested whether or not ATP-sensitive potassium (K_{ATP}) channels may mediate this response as they are metabolically sensitive and thus could be stimulated by a variety of factors within the milieu of exercising tissue (1997). In the rat hindlimb, pharmacological activation of K_{ATP} channels was able to inhibit sympathetically-stimulated vasoconstriction in the rat hindlimb and inhibition of K_{ATP} channels during muscle contractions augmented sympathetic vasoconstriction (Thomas *et al.*, 1997). Similarly, in humans, inhibition of K_{ATP} channels augmented carotid baroreflex induced sympathetically-mediated vasoconstriction during leg exercise (Keller *et al.*, 2004); however this also occurred at rest, and thus functional sympatholysis *per se* may have been unaltered. At rest,

activation of K_{ATP} channels via diazoxide infusion can limit norepinephrine-induced vasoconstriction in the human forearm (Pickkers *et al.*, 2004). These are the only studies to date we are aware of that have attempted to address the role of vascular hyperpolarization, via any pathway, in modulating sympathetic vasoconstriction during exercise and overall suggest this electrical signaling may have some role in regulating functional sympathoylsis.

We have recently demonstrated that inhibition of K_{IR} channels and Na^+/K^+-ATPase via $BaCl_2$ and ouabain, respectively, abolishes a mild hyperpolarizing stimulus (low dose KCl infusion) in the human forearm (Crecelius *et al.*, 2012a). Stimulation of either K_{IR} channels or Na^+/K^+-ATPase leads to vascular hyperpolarization and evidence suggests K_{IR} activation is important in "amplifying" the spread of hyperpolarization through the vasculature (Jantzi *et al.*, 2006). Thus, given the previous evidence to suggest that hyperpolarization may be involved in functional sympatholysis and our own data that showed activation of K_{IR} channels and Na^+/K^+-ATPase contributes to exercise hyperaemia (Crecelius *et al.*, 2012b), we utilized our established pharmacological approach to test whether these pathways contributed to the ability of muscle contractions to blunt sympathetically-mediated vasoconstriction. Contrary to our hypothesis, in the presence of inhibition of K_{IR} channels and Na^+/K^+-ATPase, vasoconstrictor responses were maintained compared to control conditions and still significantly attenuated compared to resting (adenosine) conditions (Figure 18). Interestingly, vasoconstriction during adenosine infusion, which served as a high-flow control, was significantly augmented with combined infusion of L-NMMA+ketorolac +$BaCl_2$+ouabain as compared to control and L-NMMA+ketorolac conditions. We are not certain as to why this occurred, particularly to the large magnitude that we observed. The fact that vasoconstrictor responsiveness in resting tissue was increased but unaltered during

exercise highlights the unique signaling that occurs during exercise versus other hyperaemic conditions.

Effects of inhibition of K_{IR} channel and Na^+/K^+-ATPase activity on blood parameters

In the present study, we sampled blood from a deep antecubital vein and the brachial artery catheter in order to measure a variety of potential muscle metabolites that may be contributing to our observed responses, as well as determine the oxygen consumption of the forearm tissue during exercise. These data (Table 6) strengthen the findings of our haemodynamic measures and add additional insight to the metabolic consequences of our inhibitors. Exercise stimulated predictable changes reflective of increased metabolism and as would be anticipated, in control and L-NMMA+ketorolac conditions, PE-mediated vasoconstriction and reduced forearm blood flow was coupled with increased extraction of oxygen, and thus preserved oxygen consumption.

Of interest, given the reduction in exercise hyperaemia that occurred during L-NMMA+ketorolac+$BaCl_2$+ouabain, extraction during exercise was significantly increased from the prior experimental conditions (control and L-NMMA+ketorolac). It appears this was near maximal levels of extraction as there was no significant increase with PE infusion despite a reduction in blood flow. Thus, oxygen consumption in the condition of combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase was attenuated during sympathetic stimulation. This observation may have important relevance for populations that demonstrate impaired exercise hyperaemia and sympatholysis, such as older healthy adults and hypertensive humans (Dinenno *et al.*, 2005; Kirby *et al.*, 2009; Vongpatanasin *et al.*, 2011). Given that all subjects were able to continue contractions, and thus muscle work still occurred, it is likely that non-oxidative

pathways compensated for the loss in ATP production and this is reflected in significantly lower pH and greater pCO_2 values. Whether this shift in metabolism would impair exercise tolerance over prolonged periods of time and concomitant sympathetic activation remains unknown.

Experimental considerations

Given that our primary findings regarding the role of K_{IR} channels and Na^+/K^+-ATPase in sympatholysis were negative and contrary to our hypothesis, the efficacy of our pharmacological inhibition of these pathways could be questioned. We do not believe this is likely the case as $BaCl_2$+ouabain significantly augmented vasoconstriction during adenosine infusion and significantly attenuated the hyperaemic and vasodilatory response to muscle contractions. It is possible that we have not reached *complete* inhibition of these pathways as this is difficult to test given the limitations in the magnitude of direct hyperpolarization that can be stimulated in the human forearm due to subject discomfort with high levels of KCl infusion. In this context, it is important to acknowledge that our inhibition of K_{IR} channels and Na^+/K^+-ATPase blocks only K^+ stimulated hyperpolarization and the amplification of a hyperpolarizing stimulus. We are not presently able to inhibit hyperpolarization directly, nor the ability of electrical signals to be communicated through the vessel layers.

Further related to the efficacy of our pharmacological inhibition, we observed no effect of L-NMMA+ketorolac on vasoconstrictor responses during exercise and adenosine infusion. We did however, observe a significant reduction in resting blood flow as would be expected with sufficient inhibition of NO and PGs and utilized standard doses of L-NMMA and ketorolac which have previously been shown to be effective (Dinenno & Joyner, 2003; Schrage *et al.*, 2004; Crecelius *et al.*, 2011a).

We think it is important to adjust the absolute dose of PE administered in these types of studies to the observed level of forearm blood flow in order to approximately match a similar relative concentration within the blood in all conditions (Dinenno & Joyner, 2003, 2004; Kirby *et al.*, 2008; Kirby *et al.*, 2011). It could be argued that as a result of the attenuated hyperaemic response to exercise during L-NMMA+ketorolac+$BaCl_2$+ouabain, the flow-adjusted absolute dose of PE was lower, and this explains why the vasoconstrictor response was not augmented. We do not think this is the case as the response during adenosine was augmented and this was a flow- and absolute PE dose-matched condition. Thus, our experimental approach of adjusting our PE dose does not explain the lack of effect of combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase on functional sympatholysis.

Perspectives

Activation of K_{IR} channels and Na^+/K^+-ATPase are only two of many ways in which vascular cells can hyperpolarize. In human *in vivo* studies, we are limited in the specificity and extent to which we can inhibit mediators of this important change in membrane potential. One of the motivations to investigate the role of K_{IR} channels and Na^+/K^+-ATPase in functional sympatholysis was our previous findings regarding the contributions of this pathway to ATP-mediated vasodilatation (~50%) (Crecelius *et al.*, 2012a) and the unique ability of ATP to blunt sympathetic vasoconstriction similar to what occurs during exercise (Rosenmeier *et al.*, 2004; Kirby *et al.*, 2008).

Interest in ATP and the potential role for this molecule in vascular control during exercise is significant and remains an unanswered question given the limitations in specifically inhibiting purinergic receptors ATP binds to, even in animal models. Data from our laboratory

demonstrate the ability of exogenous ATP to cause vasodilatation and modulate sympathetic vasoconstriction (Kirby *et al.*, 2008). We have also shown that NO and PGs have only a modest role in ATP-mediated vasodilatation (Crecelius *et al.*, 2011a; Crecelius *et al.*, 2012a), and thus if ATP does contribute to functional sympatholysis, a minimal role for NO and PGs as observed previously (Dinenno & Joyner, 2004) and in the current study is in line with this hypothesis. Combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase reduces ATP-mediated vasodilatation by over 50%; however, it is possible that the vascular signaling that contributes to the remaining portion of vasodilatation is the same as what allows exercise to maintain the ability to blunt sympathetic vasoconstriction following inhibition of these same pathways as observed in the present study.

What then are these remaining signaling pathways that could explain our observed responses? We propose that electrical communication, likely through intermediate- and small-conductance K_{Ca} channels and direct transfer of electrical charge through the physical connections of endothelial and vascular smooth muscle cells occurs, and is able to still stimulate hyperpolarization and elicit a conducted response that is capable of profound vasodilatation and opposition to sympathetically-induced depolarization. We have recently shown that slight augmentations in endothelial signaling, via infusion of acetylcholine can augment the ability of mild handgrip exercise to blunt sympathetic vasoconstriction and this occurs independent of NO and PG signaling (Kirby *et al.*, 2013). Thus, electrical communication and a balance between hyperpolarization and depolarization of vascular smooth muscle cells remains our proposed underlying mechanism explaining functional sympatholysis. Further development of specific pharmacological inhibitors that can attenuate cell-to-cell communication and are safe for human administration is necessary before this proposition will be able to be directly addressed.

Conclusions

Muscle contractions stimulate a large increase in blood flow and have the ability to blunt sympathetically-mediated vasoconstriction relative to what occurs in resting tissue. This functional sympatholysis is thought to preserve oxygen delivery to active tissue in the face of increased sympathetic activity that is necessary for appropriate blood pressure regulation. Many studies have investigated the contributing signaling pathways to this vascular response and the current data provides novel insight into this phenomenon. We show that acute pharmacological inhibition of K_{IR} channels and Na^+/K^+-ATPase does not impact direct α-adrenergic vasoconstriction during muscle contractions. Further, we support previous findings that these pathways contribute to exercise hyperaemia whereas NO and PGs do not contribute to exercise hyperaemia nor sympatholysis in this model. The impact of combined attenuated hyperaemia and preserved vasoconstrictor responses during exercise with combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase is metabolically significant and results in attenuated oxygen consumption and increased non-oxidative metabolism in the contracting tissue. The collective data from the present study and preceding investigations fail to explain the majority of the physiological basis of functional sympatholysis and future work should examine electrical communication within and between vascular cell layers in an attempt to better understand this important basic vascular regulatory scheme.

Table 5. Systemic haemodynamics and forearm vascular conductance in all conditions
PE, phenylephrine
* *P*<0.05 *vs* adenosine (within condition); †*P*<0.05 *vs* control; ‡*P*<0.05 *vs* +L-NMMA+ketorolac
§ *P<0.05* Main effects of Timepoint and Condition; *P<0.05* Timepoint × Condition
◊ *Timepoints*: within all adenosine trials (*P* = NS); within all exercise trials, *P*<0.05 Baseline *vs* Pre-Phenylephrine; *P* = NS Pre-Phenylephrine *vs* Phenylephrine; *Condition: P<0.05* Main effect

Condition	*Timepoint*	*Trial*	Mean Arterial Pressure (mmHg) §	Heart Rate (beats min^{-1}) ◊	Forearm Vascular Conductance (ml min^{-1} 100 $mmHg^{-1}$)
Control	**Baseline**	**Adenosine**	94 ± 2	57 ± 2	31.3 ± 2.5
		Exercise	92 ± 2	60 ± 2	35.0 ± 2.1
	Pre-PE	**Adenosine**	97 ± 2	59 ± 2	232.0 ± 20.8
		Exercise	96 ± 2	66 ±2	238.6 ± 18.5
	PE	**Adenosine**	101 ± 3	59 ± 2	116.7 ± 11.0
		Exercise	99 ± 3	64 ± 1	186.9 ± 12.3*
+L-NMMA +ketorolac	**Baseline**	**Adenosine**	96 ± 2	54 ± 2	25.3 ± 2.7†
		Exercise	98 ± 2	57 ± 2	25.3 ± 2.2†
	Pre-PE	**Adenosine**	98 ± 3	56 ± 2	204.3 ± 10.8
		Exercise	100 ± 2	62 ± 2	218.8 ± 18.9
	PE	**Adenosine**	103 ± 2	57 ± 2	111.1 ± 13.8
		Exercise	104 ± 3	61 ± 2	164.1 ± 13.2*
+L-NMMA +ketorolac +$BaCl_2$ +ouabain	**Baseline**	**Adenosine**	100 ± 3	56 ± 2	28.0 ± 2.7
		Exercise	101 ± 2	58 ± 2	26.4 ± 2.9†
	Pre-PE	**Adenosine**	106 ± 2	57 ± 2	141.7 ± 18.0†‡
		Exercise	110 ± 3	63 ± 3	136.6 ± 18.2†‡
	PE	**Adenosine**	109 ± 3	55 ± 2	38.8 ± 5.3†‡
		Exercise	113 ± 4	62 ± 2	97.3 ± 11.9*†‡

Table 6. Deep venous blood gases in exercise trials
a-v, arterial-venous; PE, phenylephrine
Pre-PE and PE were statistically analyzed separately from baseline given the predictable large changes due to exercise.
Due to technical error, n=7-10. *vs Pre-PE (within condition) †$P<0.05$ *vs* control; ‡$P<0.05$ *vs* +L-NMMA+ketorolac

Condition		pO2 (mmHg)	FO_2Hb (%)	pH	pCO2 (mmHg)	[K+] (mmol L^{-1})	a-vO_2 (ml dl^{-1})	VO_2 (ml min^{-1})
Control	**Arterial**	82.0±1.0	94.3±0.1	7.402±0.007	37.4±1.1	3.86±0.03		
	Baseline	27.9±2.1	46.2±4.9	7.347±0.008	47.5±1.1	4.04±0.09	11.1±1.1	3.6±0.4
	Pre-PE	23.9±0.9	33.9±1.6	7.271±0.011	59.8±1.9	4.75±0.07	13.6±0.7	32.6±2.9
	PE	20.4±1.6†	24.5±3.1*	7.249±0.010	62.6±1.5	4.55±0.06	15.5±0.8*	30.0±3.0
+L-NMMA +ketorolac	**Arterial**	84.1±1.6	94.7±0.2	7.408±0.009	34.8±1.1	3.90±0.05		
	Baseline	23.1±1.7	34.2±4.0†	7.321±0.009	49.2±2.0	3.93±0.12	13.5±1.0	3.5±0.3
	Pre-PE	22.7±1.0	31.3±1.8	7.278±0.014	56.0±1.9	4.68±0.13	14.1±0.6	32.3±4.1
	PE	19.7±1.3	22.9±2.7*	7.243±0.013*	61.6±3.2	4.62±0.09	16.1±0.7*	26.8±3.7
+L-NMMA +ketorolac +$BaCl_2$ +ouabain	**Arterial**	81.9±1.8	94.1±0.3	7.396±0.003	36.4±0.5	3.94±0.09		
	Baseline	26.1±2.7	44.1±5.7‡	7.328±0.009	44.7±1.4	3.30±0.11†‡	12.3±1.1	3.5±0.3
	Pre-PE	18.3±1.3†‡	19.9±1.6†‡	7.239±0.016	61.4±3.2	5.10±0.15†‡	16.4±0.5†‡	25.4±3.7
	PE	17.3±2.0	15.8±3.6*†	7.196±0.019*†‡	68.4±3.7*	5.00±0.16†‡	17.1±0.7	19.0±2.8†

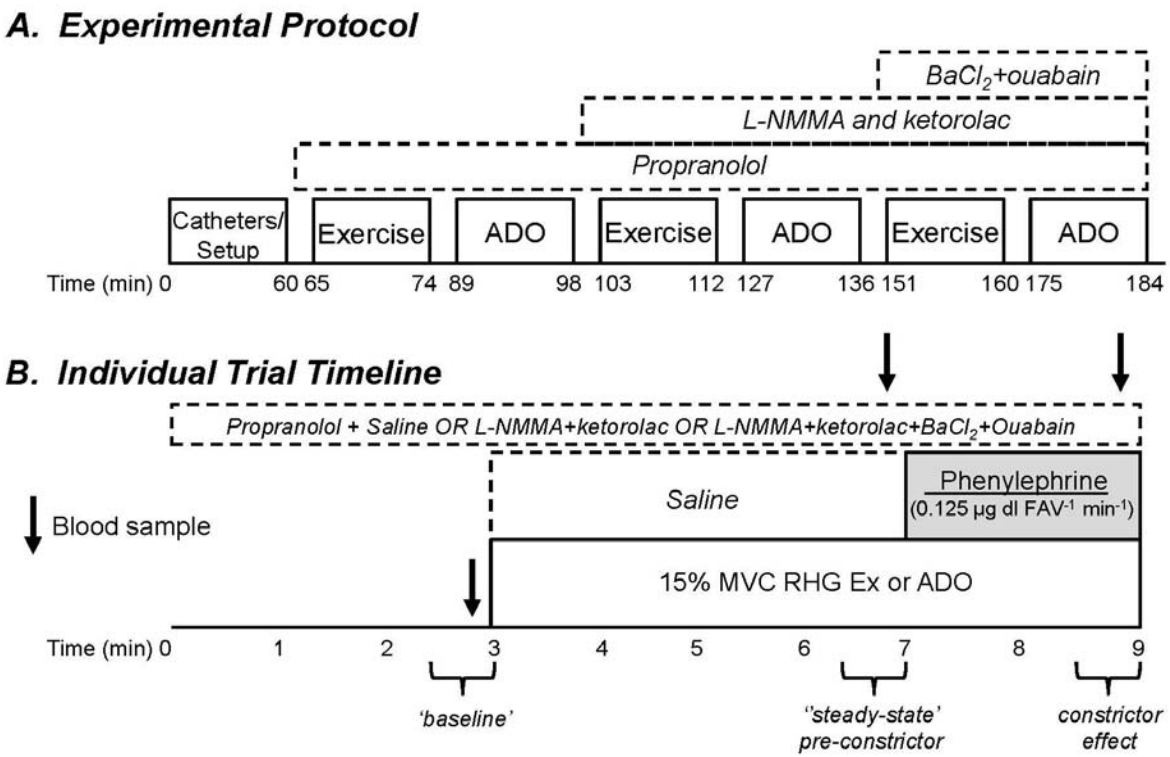

Figure 15. Experimental timeline

A. After instrumentation of brachial artery catheter, subjects received propranolol to inhibit β-adrenoceptors and then underwent exercise and adenosine (ADO) trials (see Panel *B*) in control (saline) conditions. L-NMMA and ketorolac were then administered in order in inhibit the synthesis of nitric oxide (NO) and prostaglandins (PGs), respectively and exercise and ADO trials were repeated. Finally, barium chloride ($BaCl_2$) and ouabain were infused in order to inhibit vascular hyperpolarization via activation of inwardly-rectifying potassium channels and Na^+/K^+-ATPase, respectively, in combination with NO and PGs. In each individual trial (*B*), forearm blood flow (FBF) was measured for 3 minutes of baseline. Subjects then performed exercise [15% maximal voluntary contraction (MVC) rhythmic handgrip exercise (RHG)] or received variable-dose ADO infusion to match hyperaemia observed with exercise. After four minutes of the stimulus (adenosine or exercise) "steady-state" FBF was calculated and a dose of phenylephrine (PE; α_1-adrenoceptor agonist) based on this FBF was then infused for 2 min and vasoconstrictor responses were determined. Arterial blood samples were taken at rest and venous (retrograde antecubital catheter) samples were taken at baseline, steady-state pre-PE, and end PE.

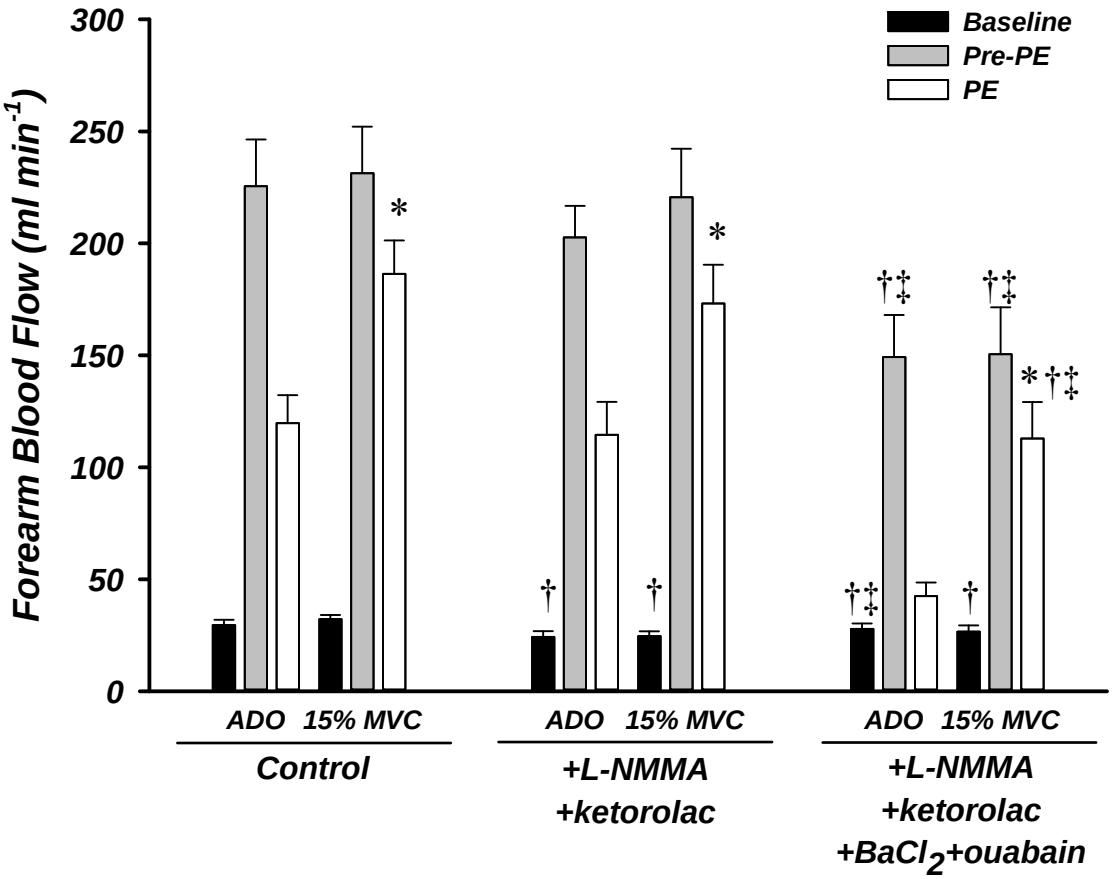

Figure 16. Absolute forearm blood flow responses across all experimental conditions

Forearm blood flow (FBF) responses are presented at rest (black bars), pre-phenylephrine (PE) vasoconstrictor stimulus (grey bars), and end-PE (open bars) in all experimental conditions. As intended, FBF was matched between adenosine (ADO) infusion and 15% maximal voluntary contraction (MVC) rhythmic handgrip exercise. PE elicited a significant reduction in FBF in all conditions. In control (saline), FBF after PE was significantly greater during exercise than ADO infusion. L-NMMA and ketorolac to inhibit nitric oxide (NO) and prostaglandin (PG) synthesis, respectively, reduced baseline FBF but had no effect on pre-PE or PE FBF for either ADO or exercise. The addition of barium chloride ($BaCl_2$) and ouabain to inhibit vascular hyperpolarization via inwardly-rectifying potassium channels and Na^+/K^+-ATPase significantly attenuated exercise hyperaemia, and FBF during PE was lower than in control and +L-NMMA +ketorolac conditions, but still greater than during the ADO trial within this condition. **P*<0.05 *vs* ADO (within condition); †*P*<0.05 *vs* control; ‡*P*<0.05 *vs* +L-NMMA+ketorolac

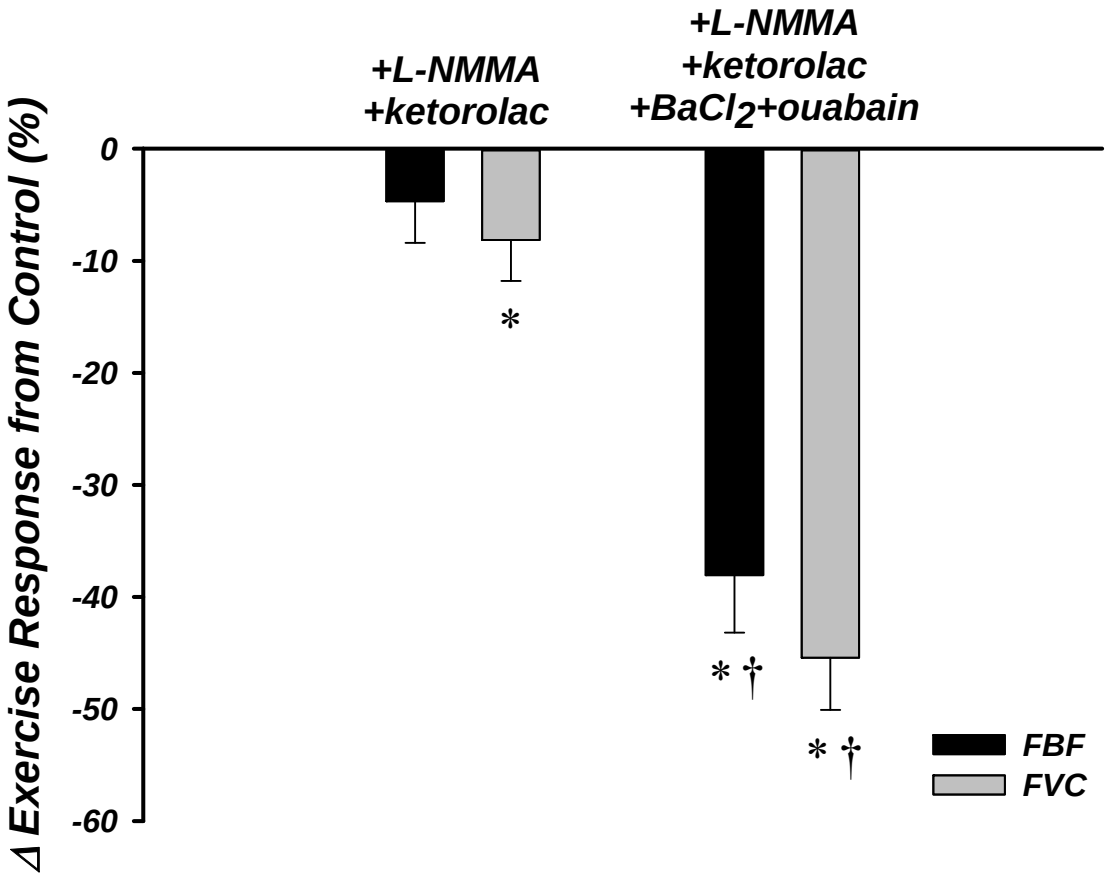

Figure 17. Impact of experimental inhibition on steady-state forearm haemodynamics during exercise

The relative change in steady-state forearm blood flow (FBF; black bars) and forearm vascular conductance (FVC; grey bars) due to pharmacological inhibition compared to the control (saline) condition is shown. Inhibition of the synthesis of nitric oxide (NO) and prostaglandins (PGs) via L-NMMA and ketorolac, respectively had no impact on FBF and reduced FVC. The additional inhibition of vascular hyperpolarization via inwardly-rectifying potassium (K_{IR}) channels (barium chloride, $BaCl_2$) and Na^+/K^+-ATPase (ouabain) had a substantial effect on both FBF and FVC, attenuating exercise hyperaemia and vasodilatation ~40% from control. *P<0.05 *vs* zero; †P<0.05 *vs* +L-NMMA+ketorolac

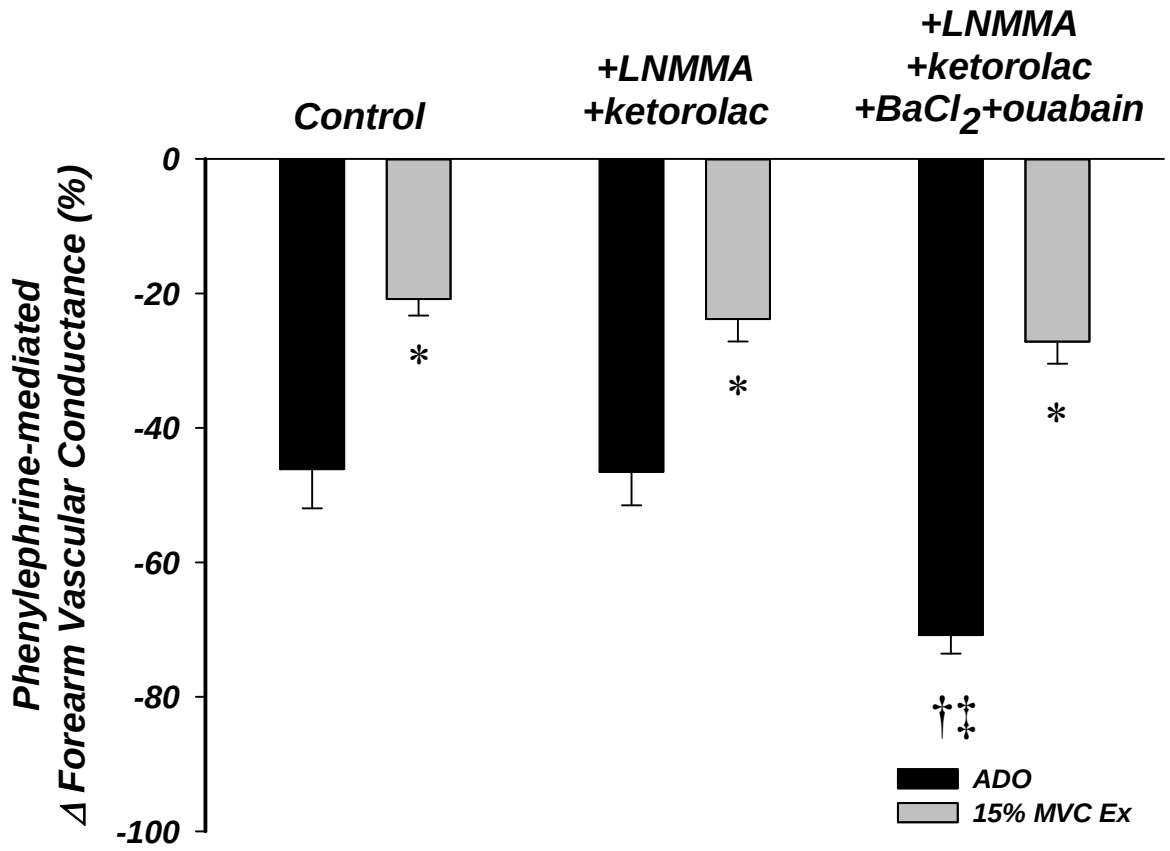

Figure 18. Phenylephrine-mediated vasoconstriction in all experimental trials

The vasoconstriction elicited by phenylephrine (PE) infusion is presented for adenosine (ADO; black bars) and 15% maximal voluntary contraction rhythmic handgrip exercise (15% MVC Ex; grey bars) trials in the following conditions: control (saline), combined inhibition of nitric oxide (NO) and prostaglandins (PGs) via L-NMMA and ketorolac, respectively, and combined inhibition of NO, PGs and vascular hyperpolarization via activation of inwardly-rectifying potassium (K_{IR}) channels (barium chloride, $BaCl_2$) and Na^+/K^+-ATPase (ouabain). In all conditions, exercise blunted PE-mediated vasoconstriction. Combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase augmented PE-mediated vasoconstriction during ADO infusions but did not alter the vasoconstrictor response during exercise. *P<0.05 *vs* ADO (within condition); †P<0.05 *vs* control; ‡P<0.05 *vs* +L-NMMA+keorolac

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CHAPTER V – MANUSCRIPT IV

Inwardly-rectifying potassium channels and Na^+/K^+-ATPase mediate reactive hyperaemia in healthy humans[4]

Summary

Rationale: Reactive hyperaemia (RH) in the forearm circulation is an important marker of cardiovascular health yet the underlying vasodilator signaling pathways are controversial and thus remain unclear. We hypothesized that vascular hyperpolarization via inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+-ATPase contributes to RH in young, healthy humans, whereas nitric oxide (NO) and prostaglandins (PGs) do not.

Methods and Results: In 24 (23±1 years) subjects, we performed RH trials by measuring forearm blood flow (FBF; venous occlusion plethysmography) following 5 minutes of arterial occlusion. In Protocol 1, we studied 2 groups of 8 subjects and assessed RH in the following conditions; Group 1: control (saline), inhibition of K_{IR} channels (barium chloride; $BaCl_2$), combined inhibition of K_{IR} channels and Na^+/K^+-ATPase ($BaCl_2$+ouabain, respectively), and combined inhibition of K_{IR} channels, Na^+/K^+-ATPase, NO and PGs ($BaCl_2$+ouabain+L-NMMA+ketorolac, respectively). Group 2 received ouabain rather than $BaCl_2$ in the 2nd trial. In

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Protocol 2 (n=8), 3 RH trials were performed: control, L-NMMA+ketorolac, and L-NMMA+ketorolac +$BaCl_2$+ouabain. All infusions occurred via brachial artery catheter. Compared to control, $BaCl_2$ significantly reduced peak FBF (-50±6%; $P<0.05$) whereas ouabain and L-NMMA+ketorolac did not. Total FBF (area under the curve) was attenuated by $BaCl_2$ (-61±3%) and ouabain (-44±12%) alone and this effect was enhanced when combined (-87±4%), nearly abolishing RH. L-NMMA+ketorolac did not impact total RH FBF prior to or after administration of $BaCl_2$ and ouabain.

Conclusions: Vascular hyperpolarization via K_{IR} channels is a primary determinant of peak RH, whereas activation of both K_{IR} channels and Na^+/K^+-ATPase explains the majority of RH in humans.

Introduction

Following ischaemia caused by temporary arterial occlusion, there is significant vasodilatation and a rapid marked increase in blood flow in most tissues, including the human forearm (Patterson & Whelan, 1955; Gentry & Johnson, 1972). This phenomenon of reactive hyperaemia is independent from neural vasodilatation (Duff & Shepherd, 1953) and is thought to occur as a result of myogenic and local metabolic factors within the resistance vasculature and thus can be used as a test of microvascular function (Flammer *et al.*, 2012). Attenuated reactive hyperaemic responses have been documented in populations demonstrating a variety of risk factors that increase cardiovascular morbidity and mortality (Go *et al.*, 2013) including hypertension (Conway, 1963; Takeshita & Mark, 1980; Iwatsubo *et al.*, 1997; Higashi *et al.*, 1999), atherosclerosis (Dakak *et al.*, 1998), peripheral artery disease (Fronek *et al.*, 1973), congestive heart failure (Zelis *et al.*, 1968), and aging (Proctor *et al.*, 2005; Ridout *et al.*, 2005).

Recently, peak reactive hyperaemic flow was determined to be predictive of future cardiovascular events in a healthy population (Anderson *et al.*, 2011) as well as in at-risk patient populations (Huang *et al.*, 2007) and this relationship may be stronger than that of commonly assessed macrovascular function via flow-mediated brachial vasodilatation (Philpott *et al.*, 2009; Anderson *et al.*, 2011). Further, interventions that reduce the burden of cardiovascular disease (Go *et al.*, 2013) such as exercise (Higashi *et al.*, 1999) and angiotensin-converting enzyme inhibition (Iwatsubo *et al.*, 1997) improve reactive hyperaemia in hypertensive patients. Despite the utility of the reactive hyperaemia test as a measure of vascular health, the underlying local vasodilator signaling that contributes to this response in humans is largely unknown.

Given the strong associations between impaired reactive hyperaemia, cardiovascular disease risk, and attenuated endothelial-dependent and metabolic vasodilatation (Taddei *et al.*, 1997; Kirby *et al.*, 2012), a variety of previous investigations in humans have attempted to determine the role of numerous endothelial-derived or -dependent vasoactive and metabolically-dependent substances in mediating the response including nitric oxide (NO) (Tagawa *et al.*, 1994; Engelke *et al.*, 1996; Dakak *et al.*, 1998; Nugent *et al.*, 1999; Bank *et al.*, 2000; Raff *et al.*, 2010), prostaglandins (PGs)(Kilbom & Wennmalm, 1976; Carlsson *et al.*, 1987; Engelke *et al.*, 1996; Addor *et al.*, 2008), ATP-dependent potassium (K_{ATP}) channels (Banitt *et al.*, 1996; Bank *et al.*, 2000) and adenosine (Carlsson *et al.*, 1987; Costa *et al.*, 1999; Meijer *et al.*, 2008). The results of these studies are largely equivocal and to date, even when the production or action of these substances are inhibited in combination (Carlsson *et al.*, 1987; Engelke *et al.*, 1996; Bank *et al.*, 2000), a significant portion of both the peak and total reactive hyperaemia remains unexplained. There is growing interest in vasodilatation that occurs via non-NO and –PG mechanisms due to hyperpolarization of endothelial and vascular smooth muscle cells (Cohen &

Vanhoutte, 1995; Emerson & Segal, 2000). In this context, we recently demonstrated the ability to block a direct hyperpolarizing stimulus (KCl) via inhibition of inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+-ATPase in the human forearm and show these pathways can contribute to vasodilator responses to pharmacological stimulation of the endothelium (Crecelius *et al.*, 2012a) as well as increased metabolic demand (Crecelius *et al.*, 2012b). Whether or not vascular hyperpolarization via K_{IR} channel and Na^+/K^+-ATPase activation contributes to reactive hyperaemia in humans has never been tested.

With this information as background, we directly tested the hypothesis that vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase contributes to forearm reactive hyperaemia following temporary arterial occlusion and that NO and PGs have a minimal role in this response in healthy humans.

Methods

Subjects

With Institutional Review Board approval and after written informed consent, a total of 24 young healthy adults (18 men, 6 women; age=23±1 years (range: 18-34 years); weight=73.1±1.5 kg; height=175±1 cm; body mass index=23.9±0.5 kg m^{-2}; forearm volume (FAV)=945±39 ml; means±S.E.M.) participated in the present study. All subjects were sedentary to moderately active, non-smokers, non-obese, normotensive (resting blood pressure <140/90 mmHg), and not taking any medications. Studies were performed after an overnight fast and 24 hour abstention from caffeine and exercise with subjects in the supine position with the experimental arm abducted to 90° and slightly elevated above heart level upon a tilt-adjustable table in a cool environment (20-22°C). 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 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) for local administration of study drugs and blood sampling. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously flushed at 3 ml hr^{-1} with heparinized saline. The two side ports were used for drug infusions (Dinenno *et al.*, 2002; Crecelius *et al.*, 2012a). Heart rate (HR) was determined using a 3-lead electrocardiogram (Cardiocap/5, Datex-Ohmeda Louisville, CO, USA).

Forearm blood flow and vascular conductance

Forearm blood flow (FBF) was measured via venous occlusion plethysmography using mercury-in-salistic strain gauges and techniques as previously described (Crecelius *et al.*, 2012a). FBF was expressed as milliliters per deciliter of tissue per minute (ml dl FAV^{-1} min^{-1}. As an index of forearm vasodilatation and to account for individual differences in baseline vascular tone, forearm vascular conductance (FVC) was calculated as (FBF/MAP) × 100 expressed as (ml dl FAV^{-1} min^{-1}) $100mmHg^{-1}$. Immediately following the release of the occlusion cuff for the reactive hyperaemia (see below), the venous occlusion cuff cycled between inflation (4 seconds) and deflation (3 seconds) yielding one blood flow measurement every 7 seconds for the first 56 seconds (8 flow measures). Pilot studies in the laboratory demonstrated that 3 seconds was sufficient time for venous emptying. Given the predicted rapid fall in FBF,

we felt it was prudent to capture the maximum possible number of flow measures in the initial portion of the hyperaemic response. After 8 flow measures, the inflation:deflation cycle was changed back to 7:8 sec, as was used at rest.

Reactive hyperaemia protocol

The same cuff that was placed around the upper portion of the arm and used for venous occlusion for the measurement of FBF was used to cause arterial occlusion for each reactive hyperaemia trial. After measurement of baseline FBF, the occlusion cuff was rapidly inflated to 200 mmHg for 5 minutes of ischaemia. This location and duration of ischaemia was chosen to mimic the reactive hyperaemia protocol utilized in investigations of the contributions of various endothelial-derived vasodilator pathways to the reactive hyperaemic response (Kilbom & Wennmalm, 1976; Carlsson *et al.*, 1987; Engelke *et al.*, 1996; Nugent *et al.*, 1999) and importantly, that has recently demonstrated peak reactive hyperaemic flow to be more strongly associated with cardiovascular disease risk than measures of flow-mediated vasodilatation (Anderson *et al.*, 2011). After 5 minutes, the cuff was rapidly deflated and flow measures commenced. Forearm blood flow was measured and collected for 5 minutes post-cuff deflation; however, FBF in control conditions returned to baseline within 2.5 minutes (150 seconds) and thus we only present and statistically analyzed the data for this duration.

Vasoactive drug infusion

All drug infusions were through the brachial artery catheter to create a local effect in the forearm and saline was utilized as a control infusate. All infusions were completed during

baseline measures, prior to the 5 minutes of arterial occlusion and reactive hyperaemia. Specific timing and duration of infusions is provided below in the *Experimental Protocols* section.

To inhibit K_{IR} channels, barium chloride ($BaCl_2$; K_{IR} channel inhibitor; 10% w/v BDH3238, EMD Chemicals, Gibbstown, NJ, USA) was infused at 0.9 μmol dl FAV^{-1} min^{-1} with a range of a minimum dose of 8 μmol min^{-1} to a maximum dose of 10 μmol min^{-1} for five minutes prior to each arterial occlusion. We doubled the dose of $BaCl_2$ from that which we have previously utilized (Crecelius *et al.*, 2012a) in an attempt to maximize our blockade of K_{IR} channels while still remaining in an estimated venous plasma concentration range that is relatively selective for K_{IR} channels (Dawes *et al.*, 2002). Additionally, given we did not want to continue infusions throughout the arterial occlusion period and risk extravasation of drug, this higher concentration allowed for a greater amount of drug to be delivered during the loading period alone. To inhibit Na^+/K^+-ATPase, ouabain octahydrate (Na^+/K^+-ATPase inhibitor; Sigma 03125, St. Louis, MO, USA) was infused at 2.7 nmol min^{-1} for 15 minutes prior to arterial occlusion (Crecelius *et al.*, 2012a). On subsequent reactive hyperaemia trials, ouabain was reinfused for 5 minutes prior to arterial occlusion to provide continuous inhibition. Ouabain and $BaCl_2$ were prepared in saline and confirmed sterile and free of fungus/endotoxin and particulate matter with a standard microbiology report (JCB-Analytical Research Labs, Wichita, KS, USA) prior to use. We administered N^G-monomethyl-L-arginine (L-NMMA; NOS inhibitor; Clinalfa/Bachem, Weil am Rhein, Germany) to inhibit the production of NO in combination with ketorolac (non-selective cyclooxygenase inhibitor; Hospira, Lake Forest, IL, USA) to inhibit the synthesis of PGs. The doses of L-NMMA and ketorolac were 5 mg min^{-1} and 1200 μg min^{-1} respectively and given for 5 minutes prior to arterial occlusion. We have previously demonstrated these doses to be effective during hyperaemic stimuli (Dinenno *et al.*, 2002) and in

the current protocol, observe significant reductions in resting blood flow (see Results) consistent with effective inhibition of these pathways (Lauer *et al.*, 2001). Forearm volume used for normalization of specific vasoactive drugs was determined from regional analysis of whole-body dual-energy X-ray absorptiometry scans (QDR series software, Hologic, Inc, Bedford, MA, USA).

Experimental Protocols

In all experimental protocols, subjects rested quietly for 30 minutes after insertion of the catheter before the first experimental trial and for 20 minutes between each trial.

Protocol 1: Independent and combined effects of K_{IR} channel and Na^+/K^+-ATPase inhibition

This protocol was designed to primarily address the role of K_{IR} channels and Na^+/K^+-ATPase in the reactive hyperaemia response. In total, 16 subjects participated in this protocol. Eight of these subjects (Group 1) underwent reactive hyperaemia trials in the following conditions: (1) control (saline), (2) independent K_{IR} channel inhibition ($BaCl_2$), (3) combined K_{IR} channel and Na^+/K^+-ATPase inhibition ($BaCl_2$+ouabain), and (4) inhibition of K_{IR} channels, Na^+/K^+-ATPase, as well as the production of NO and PGs ($BaCl_2$+ ouabain+L-NMMA+ketorolac). In the other eight subjects (Group 2), the protocol was the same except that the second trial consisted of independent inhibition of Na^+/K^+-ATPase via ouabain versus $BaCl_2$ infusion.

Protocol 2: Effects of combined inhibition of NO and PGs

To address the combined role of NO and PGs in reactive hyperaemia, we performed a second protocol (n=8) that consisted of reactive hyperaemia trials in the following conditions: (1) control (saline), (2) combined NO and PG inhibition (L-NMMA+ketorolac), and (3) inhibition of the production of NO and PGs as well as K_{IR} channels and Na^+/K^+-ATPase (L-NMMA+ketorolac +$BaCl_2$+ouabain).

Protocol 3: Control vasodilator stimulus

In a subset of subjects (n=6), sodium nitroprusside (SNP; Nitropress, Hospira Inc., Lake Forest, IL) was infused at 2 µg dl FAV^{-1} min^{-1} for 5 minutes (Kirby *et al.*, 2010) in control (saline) conditions and after prior administration of all four antagonists ($BaCl_2$, ouabain, L-NMMA and ketorolac) as a negative control to confirm intact capacity of the forearm resistance vasculature to vasodilate.

Data acquisition and analysis

Data were collected and stored on a computer at 250 Hz and were analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Mean arterial pressure (MAP) was determined from the arterial pressure waveform. FBF was determined from the derivative of the forearm plethysmogram signal. For resting hemodynamic measures, the average of the last minute of baseline was used. One primary outcome variable of interest was the absolute peak hyperaemic FBF. To quantify the reactive hyperaemia response, we averaged and plotted values from each subject at all FBF timepoints (7, 14, 21, 28, 35, 42, 49, 56, 60, 75, 90, 105, 120, 135, 150 sec post cessation of arterial occlusion) and the total reactive hyperaemic

FBF [area under the curve (AUC)] was determined as the sum of FBF above baseline at each timepoint. The peak reactive hyperaemic FBF and vasodilatation (FVC) was determined for each subject individually and these values were also averaged. In all subjects, these individual peaks occurred at either the first, second, or third flow measurements. When FBF/FVC measurements for all subjects were averaged at each timepoint, the peak nearly always occurred at the first flow measurement (see Results). To quantify the impact of the vasoactive inhibitors, the magnitude of inhibition (%Δ) was calculated as: ($FBF_{peak/total}$ inhibition – $FBF_{peak/total}$ control)/($FBF_{peak/total}$ control)×100 and always quantified from the control condition. For the SNP control trials, FBF was averaged across the last minute of baseline and SNP infusion. Data for pharmacological dilator infusion was presented in as both absolute and percent changes in FBF and FVC.

Statistics

Data are presented as mean±S.E.M. Dynamic post-occlusion FBF values were analyzed via a two-way repeated measures ANOVA (time × condition). To make comparisons of peak and total reactive hyperaemic FBF and baseline hemodynamics between each of the experimental conditions within a given protocol, we used a one-way repeated measures ANOVA. We were most interested in making comparisons to control and the prior trial in order to determine whether an *additional* impact of the added inhibitors occurred. For comparisons between protocols, a one-way ANOVA was utilized. In all cases, Student-Newman-Keuls *post hoc* pairwise comparisons were made when a significant *F* was observed. Significance was set *a priori* at $P<0.05$.

Results

No significant differences in subject characteristics were detected between the 3 experimental groups. Baseline systemic hemodynamics (HR, MAP) and FBF for all experimental protocols are presented in Table 7 and baseline FVC values are presented in Table 8. For all protocols, there were no significant changes in HR or MAP during or following the 5 minutes of arterial occlusion (data not shown).

Protocol 1: Independent and combined effects of K_{IR} channel and Na^+/K^+-ATPase inhibition

In Group 1 of Protocol 2, subjects received $BaCl_2$ alone following the control trial in order to assess the independent role of K_{IR} channels in reactive hyperaemia (Figure 19). Baseline FBF and FVC was lower with $BaCl_2$ but was not significantly different from control levels in all subsequent trials of this protocol (Tables 7 and 8). $BaCl_2$ significantly reduced the peak reactive hyperaemia response (-50±6%; Figure 19A and B) and impaired FBF for the first 75 sec (Figure 19A). Taken together, the total reactive hyperaemic FBF was also significantly reduced from control levels (-62±3%; Figure 19C). The addition of ouabain did not further impact peak reactive hyperaemic FBF (-60±7%; $BaCl_2$ *vs* $BaCl_2$+ouabain; P=0.25) but approached having a significant additional effect on total reactive hyperaemic FBF (-82±4%; P=0.07). The addition of L-NMMA+ketorolac did not have a further impact (Peak: -68±7%; Total: -88±3%). Changes in peak vasodilatation (FVC) paralleled those of FBF (Table 8).

In Group 2 of Protocol 2, subjects received ouabain alone following the control trial in order to assess the independent role of Na^+/K^+-ATPase in reactive hyperaemia (Figure 20). Ouabain had no effect on peak reactive hyperaemic FBF (2±6%; Figure 20A and B) but did significantly reduce FBF during 14-90 sec of hyperaemia, resulting in a significant attenuation of

the total reactive hyperaemic FBF (-44 ±12%; Figure 20C). The addition of $BaCl_2$ significantly reduced peak hyperaemic FBF (-62±8%) as well as further reduced total hyperaemic FBF (-92±8%) whereas there was no additional effect of L-NMMA+ketorolac on either peak (-63±7%) or total hyperaemic FBF (-94±8%). Changes in peak vasodilatation (FVC) paralleled those for FBF (Table 8).

Protocol 2: Effects of combined inhibition of NO and PGs

In Protocol 3, we assessed the combined contribution of NO and PGs to reactive hyperaemia and subsequently inhibited K_{IR} channels and Na^+/K^+-ATPase (Figure 21). As would be expected with effective inhibition, L-NMMA+ketorolac significantly reduced baseline FBF and FVC (Tables 7-9). The FBF response upon cessation of arterial occlusion is shown in Figure 21. The mean of the first FBF measures was augmented with L-NMMA+ketorolac (Figure 21A); however, when each individual subjects' peak response was averaged, this comparison only approached being significant (+18±8%; P=0.069; Figure 21B). FBF was attenuated with L-NMMA+ketorolac 30-60 sec following the end of arterial occlusion (Figure 21A), yet the total reactive hyperaemic FBF remained similar to control (-10±12%; P=0.24; Figure 21C). The additional inhibition of K_{IR} channels and Na^+/K^+-ATPase via $BaCl_2$ and ouabain, respectively, significantly attenuated both peak (-61±8%; Figures 21A and B) and total (-69±6%; Figures 21C) reactive hyperaemic FBF.

Comparison of reactive hyperaemia protocols

A summary of the relative (%Δ) effects of independent and combined roles of K_{IR} channels and Na^+/K^+-ATPase, as well as combined NO and PGs as compared to control

conditions is presented in Figure 22. In these pooled comparisons, there was not a significantly greater effect on the reduction of peak FBF from $BaCl_2$ alone to combined $BaCl_2$+ouabain. Total reactive hyperaemic FBF was attenuated by $BaCl_2$ alone and ouabain alone and this effect was enhanced when these inhibitors were combined. L-NMMA+ketorolac had no independent effects on peak or total reactive hyperaemic FBF nor did they enhance any inhibition beyond that which occurred with $BaCl_2$+ouabain infusion.

Protocol 3: Control vasodilator stimulus

In order to confirm preserved vasodilator capacity after administration of $BaCl_2$+ouabain+L-NMMA+ketorolac, SNP was administered in control (saline) conditions and at the end of the experimental protocol in a subgroup of 6 subjects. Baseline FBF and FVC were reduced following infusion of $BaCl_2$+ouabain+L-NMMA+ketorolac, however there was no significant reduction in the absolute level, absolute change, or relative change in FBF and FVC during SNP infusion (Table 9).

Discussion

The primary novel finding from the current study is that vascular hyperpolarization via activation of K_{IR} channels and Na^+/K^+-ATPase explains the majority of the reactive hyperaemic response to temporary ischaemia, whereas NO and PGs have little combined role in this response (Figure 22). K_{IR} channels appear to be involved in both the peak and total FBF response; however, Na^+/K^+-ATPase only contributes to the total reactive hyperaemic FBF and not the peak FBF. The present findings lend novel and significant insight into this basic microvascular

response that has been shown to have clinical relevance in a variety of conditions that increase cardiovascular disease morbidity and mortality.

Overview of classic vasodilator signaling pathways involved in reactive hyperaemia

Beginning with the initial observation of a rapid and profound hyperaemia in response to a period of ischaemia, there was interest in determining the underlying signals for this response (Duff & Shepherd, 1953; Patterson & Whelan, 1955; Kilbom & Wennmalm, 1976; Takeshita & Mark, 1980; Tagawa *et al.*, 1994). Early experiments determined that an intact nervous system was not requisite to observe this response (Duff & Shepherd, 1953; Zelis *et al.*, 1968) and subsequent studies pursued investigating local mechanisms of vascular control that might be involved (Carlsson *et al.*, 1987; Tagawa *et al.*, 1994; Banitt *et al.*, 1996; Engelke *et al.*, 1996; Dakak *et al.*, 1998; Nugent *et al.*, 1999; Bank *et al.*, 2000). Alongside these studies aimed to determine the physiological basis of reactive hyperaemia, the test itself began to be used as a measure of vascular health in a variety of at-risk populations (Conway, 1963; Zelis *et al.*, 1968; Fronek *et al.*, 1973; Takeshita & Mark, 1980; Iwatsubo *et al.*, 1997; Higashi *et al.*, 1999). Different groups of subjects that demonstrated "endothelial dysfunction" as commonly assessed by intra-arterial infusion of endothelium-dependent vasodilators (e.g. acetylcholine) or flow-mediated vasodilatation of the brachial artery were shown to have attenuated reactive hyperaemia responses (Conway, 1963; Zelis *et al.*, 1968; Fronek *et al.*, 1973; Takeshita & Mark, 1980; Iwatsubo *et al.*, 1997; Higashi *et al.*, 1999). This relationship of cardiovascular disease, endothelial health, and impaired reactive hyperaemia has further stimulated an interest in the potential mediators of this response. Importantly, recent evidence indicates that peak reactive hyperaemic flow in response to five minutes of ischaemia (via upper arm cuff inflation) may in

fact be a better predictor of cardiovascular events than the more commonly-assessed brachial flow-mediated vasodilatation (Anderson *et al.*, 2011).

It is well known that NO contributes to cardiovascular health in humans due to its multifaceted cardioprotectve properties (Cohen & Vanhoutte, 1995). Whether NO mediates reactive hyperaemia was a logical proposition and has been investigated in a variety of existing studies (Tagawa *et al.*, 1994; Engelke *et al.*, 1996; Meredith *et al.*, 1996; Dakak *et al.*, 1998; Nugent *et al.*, 1999; Bank *et al.*, 2000; Raff *et al.*, 2010). There is discrepancy within the literature, and our present finding that NO (in combination with PGs) does not contribute to peak reactive hyperaemic FBF fits with the results of most (Tagawa *et al.*, 1994; Engelke *et al.*, 1996; Nugent *et al.*, 1999; Bank *et al.*, 2000; Raff *et al.*, 2010) but not all (Meredith *et al.*, 1996; Dakak *et al.*, 1998) of these studies. Some of the previous work has shown a modest role for NO in the total hyperaemic response (Tagawa *et al.*, 1994; Engelke *et al.*, 1996; Bank *et al.*, 2000). Additionally, previous studies demonstrated only a minimal contribution of PGs to peak and/or total reactive hyperaemia (Carlsson *et al.*, 1987; Engelke *et al.*, 1996; Addor *et al.*, 2008). Even when NO and PGs were inhibited in combination, there was no impact on the peak change in FBF in response to ischaemia, whereas there was some reduction (~35%) in the prolonged hyperaemic response(Engelke *et al.*, 1996). Our current findings agree with these observations that NO and PGs do not contribute to peak reactive hyperaemic FBF, and while we observed a reduction in absolute FBF in the latter portion of reactive hyperaemia, this was not of sufficient magnitude to impair the total reactive hyperaemic FBF (Figure 21). An interesting observation in the present study was that peak reactive hyperaemia was somewhat augmented after combined NO and PG inhibition, and this could also reflect a critical role for vascular hyperpolarization in the response (Bauersachs *et al.*, 1996).

Other potential metabolic candidates for regulating reactive hyperaemia include adenosine or K_{ATP} channel activation. Augmenting adenosine signaling through caffeine (adenosine receptor antagonist) withdrawal or dipyridamole (inhibitor of cellular uptake of adenosine) does improve reactive hyperaemia; however, direct inhibition of adenosine receptors (via theophylline or caffeine) does not impair peak reactive hyperaemic FBF (Carlsson *et al.*, 1987; Meijer *et al.*, 2008) and has a minimal effect on total FBF (Carlsson *et al.*, 1987). Similarly, results from inhibition of K_{ATP} channels have been equally as unsuccessful in explaining reactive hyperaemia. Inhibition of K_{ATP} channels via sulfonylureas such as tolbutamide or glibenclamide has been shown to modestly reduce total reactive hyperaemic FBF but have no impact on the peak response in some studies (Banitt *et al.*, 1996; Bank *et al.*, 2000), whereas other investigators have demonstrated no effect of K_{ATP} channel inhibition on either peak or total reactive hyperaemia (Farouque & Meredith, 2003) . In the present study, we did not investigate these pathways based on these previous observations.

Endothelium-dependent vasodilatation that occurs independently of NO and PGs causes hyperpolarization of endothelial cells and vascular smooth muscle cells (Cohen & Vanhoutte, 1995). Smooth muscle cell hyperpolarization can also be stimulated from non-endothelial sources (Emerson & Segal, 2000). Beyond K_{ATP} channels, to the best of our knowledge, hyperpolarization has not previously been studied in regards to a potential role in reactive hyperaemia. Activation of both K_{IR} channels and Na^+/K^+-ATPase leads to hyperpolarization of vascular smooth muscle cells and recently K_{IR} channels have been shown to be particularly important for the amplification of hyperpolarizing stimuli as they are directly responsive to changes in membrane potential (Jantzi *et al.*, 2006).

Critical role for K_{IR} channels and Na+/K+-ATPase in mediating reactive hyperaemia in humans

Based on our comprehensive assessment in the present study, there is a significant role for K_{IR} channels and Na^+/K^+-ATPase in reactive hyperaemia in humans (Figure 22). Interestingly, only K_{IR} channel activation, but not Na^+/K^+-ATPase contributed to the peak reactive hyperaemic FBF. Selective inhibition of K_{IR} channels reduced the peak hyperaemic response ~50%, and a total of ~60% was observed when inhibition of Na^+/K^+-ATPase was performed simultaneously. Inhibition of K_{IR} channels and Na^+/K^+-ATPase independently reduced the total reactive hyperaemic FBF by ~60% and ~40% respectively, and this effect is enhanced when these are inhibited in combination. In this context, there is a remarkable reduction in the total response (-87±4%) from control, nearly abolishing reactive hyperaemia. Consistent with our findings of a lack of a combined role for NO and PGs when inhibited first, there was also not a consistent and significant additional effect of NO and PG blockade beyond that observed with combined K_{IR} channel and Na^+/K^+-ATPase inhibition. Collectively, the magnitude of the observed attenuation due to K_{IR} channel inhibition on peak hyperaemia, and combined K_{IR} and Na^+/K^+-ATPase on the total hyperaemic response, is by far the greatest in the known studies to date on this topic.

Experimental considerations

All of the inhibitors utilized were administered prior to arterial occlusion and reactive hyperaemia. This may lead one to question the efficacy of our inhibitors after the 5 minutes of occlusion and subsequent large increases in blood flow. While not directly assessed, given the magnitude of the effects on peak and total reactive hyperaemic FBF we observed, we do not

think this alters our primary conclusions. If anything, we may be potentially *underestimating* a role for K_{IR} channels and Na^+/K^+-ATPase in reactive hyperaemia. Further, the reactive hyperaemic responses to an identical stimulus as used in the present study have been shown to be repeatable over time (Engelke *et al.*, 1996), thus the marked impact of our inhibitors on reactive hyperaemia cannot be attributed to reduced responses with repeated trials.

$BaCl_2$ has been demonstrated to be primarily selective for K_{IR} channels up to a concentration of 100 μmol L^{-1} (Quayle *et al.*, 1997). Dawes and colleagues demonstrated that a dose at half of what we used increased antecubital venous plasma concentrations in the infused forearm to 50 μmol L^{-1} (Dawes *et al.*, 2002) and thus, it can be assumed that our dose would result in concentrations within the selective range for K_{IR} channels. At greater concentrations, $BaCl_2$ has been shown to inhibit other potassium channels, most prominently K_{ATP} channels. While we believe that $BaCl_2$ in the dose we administered is selective for K_{IR} channels, if we are in fact inhibiting K_{ATP} channels, this likely does not provide an alternate explanation for our findings as the majority of existing data shows little-to-no impact of inhibiting K_{ATP} channels on peak and/or total reactive hyperaemia response (Banitt *et al.*, 1996; Bank *et al.*, 2000; Farouque & Meredith, 2003).

Presently, the exact stimulus for vascular hyperpolarization in response to local ischaemia is unknown. Potential candidates include substances that have been shown to cause vasodilatation through K_{IR} channels and Na^+/K^+-ATPase such as K^+ (Edwards *et al.*, 1998; Crecelius *et al.*, 2012a), ATP (Crecelius *et al.*, 2012a), and bradykinin (Dwivedi *et al.*, 2005). Alternatively, direct anion influx or cation efflux might also stimulate vascular smooth muscle cell hyperpolarization, relaxation, and subsequent vasodilatation. Along these lines, evidence suggests that mechanosensitive mechanisms such as the myogenic response and stretch of

endothelial cells contribute to the earliest portion of reactive hyperaemia (Koller & Bagi, 2002). Identifying the stimulus for hyperpolarization via K_{IR} channel and Na^+/K^+-ATPase activation that occurs during reactive hyperaemia represents an intriguing future area of research and potentially would provide valuable insight into explaining impaired reactive hyperaemia responses in clinical populations.

Perspectives

The role of vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase in mediating vascular responses is a relatively new area of study in humans, and thus the direct impact of cardiovascular disease risk factors on these pathways has not been well studied *in vivo*. There is limited evidence that suggests disruptions in hyperpolarizing pathways may be both consequence (de Kreutzenberg *et al.*, 2003) and cause (Brahler *et al.*, 2009) of cardiovascular disease-related conditions such as elevated free-fatty acid levels and hypertension. Given the strong relation between attenuated reactive hyperaemia responses and cardiovascular disease morbidity and mortality (Anderson *et al.*, 2011) and as a result of this study, reactive hyperaemia and vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase, these vasodilator pathways present an exciting future direction for studies in patient populations. Moreover, these findings could be particularly important for populations that exhibit microvascular dysfunction and may serve as a target for specific therapies to improve microvascular blood flow control in humans.

Conclusions

Following temporary arterial occlusion, there is a significant increase in blood flow in the forearm vasculature of humans and the magnitude of this response reflects microvascular

function and is an important marker of overall vascular health and future cardiovascular disease risk. Here, we show that the majority of this response, in terms of both the initial peak hyperaemia as well as the total hyperaemia above baseline that occurs throughout the duration of the response depends on activation of K_{IR} channels and Na^+/K^+-ATPase. Additionally, our findings support the previous investigations that showed very little role for NO and PGs in reactive hyperaemia in humans. We propose that impaired vascular hyperpolarization via of K_{IR} channels and Na^+/K^+-ATPase likely contributes to many of the observed microvascular deficits in various patient populations.

Table 7. Baseline forearm and systemic hemodynamics for all protocols

n=8 in all groups; **P*<0.05 *vs* 1st Trial (i.e. control); †*P*<0.05 *vs* 2nd Trial (i.e. ouabain); HR=heart rate (beats min^{-1}); MAP=mean arterial pressure (mmHg); FBF=forearm blood flow (ml dl forearm $volume^{-1}$ min^{-1})

Protocol 1 – Group 1	**Control**	**$BaCl_2$**	**$BaCl_2$+ouabain**	**$BaCl_2$+ouabain+ L-NMMA+ketorolac**
HR	56±3	58±3	58±4	56±3
MAP	86±3	87±3	87±2	91±4
FBF	2.3±0.5	1.5±0.2*	2.3±0.3	1.9±0.2
Protocol 1 – Group 2	**Control**	**Ouabain**	**Ouabain+$BaCl_2$**	**Ouabain+$BaCl_2$+ L-NMMA+ketorolac**
HR	57±4	57±4	58±4	58±5
MAP	83±1	84±2	89±2*†	91±3*†
FBF	2.5±0.3	2.3±0.4	2.2±0.2	1.8±0.1*
Protocol 2	**Control**	**L-NMMA +ketorolac**	**L-NMMA+ketorolac +$BaCl_2$+ouabain**	
HR	58±4	54±3	55±4	
MAP	90±3	89±4	92±4	
FBF	2.1±0.3	1.5±0.2*	1.5±0.1*†	

Table 8. Resting and peak reactive vasodilatation in all protocols

n=8 in all groups; *P<0.05 *vs* 1st Trial (i.e. control); †P<0.05 *vs* 2nd Trial (i.e. $BaCl_2$)

	Forearm Vascular Conductance (ml (dl FAV min^{-1}) 100 $mmHg^{-1}$)			
Protocol 1 – Group 1	**Control**	**$BaCl_2$**	**$BaCl_2$+ouabain**	**$BaCl_2$+ouabain+ L-NMMA+ketorolac**
Rest	2.8±0.6	1.8±0.3*	2.6±0.4	2.1±0.3
Peak	36.3±3.4	18.0±3.2*	13.8±3.0*	10.4±2.8*†
Protocol 1 – Group 2	**Control**	**Ouabain**	**Ouabain+$BaCl_2$**	**Ouabain+$BaCl_2$+ L-NMMA+ketorolac**
Rest	3.0±0.4	2.7±0.4	2.5±0.2	2.0±0.3*
Peak	28.5±3.5	26.5±3.1	9.8±2.7*†	8.8±1.7*†
Protocol 2	**Control**	**L-NMMA +ketorolac**	**L-NMMA+ketorolac +$BaCl_2$+ouabain**	
Rest	2.3±0.3	1.6±0.2*	1.6±0.1*	
Peak	34.5±4.1	39.0±5.3	14.5±3.9*†	

Table 9. Protocol 3: Control vasodilator stimulus

n=6; **P*<0.05 *vs* control; FAV=forearm volume; FVC=forearm vascular conductance (ml (dl FAV min^{-1}) 100 $mmHg^{-1}$); SNP=sodium nitroprusside

	Baseline	**SNP 2 µg dl FAV^{-1} min^{-1}**	**Absolute Δ**	**%Δ**
Control				
FBF	2.2±0.3	12.4±1.5	10.2±1.4	499±66
FVC	2.5±0.4	15.3±2.1	12.7±1.8	546±72
$BaCl_2$+ouabain +L-NMMA+ketorolac				
FBF	1.5±0.3*	10.1±2.0	8.6±1.7	611±107
FVC	1.6±0.2*	12.1±2.6	10.6±2.4	672±100

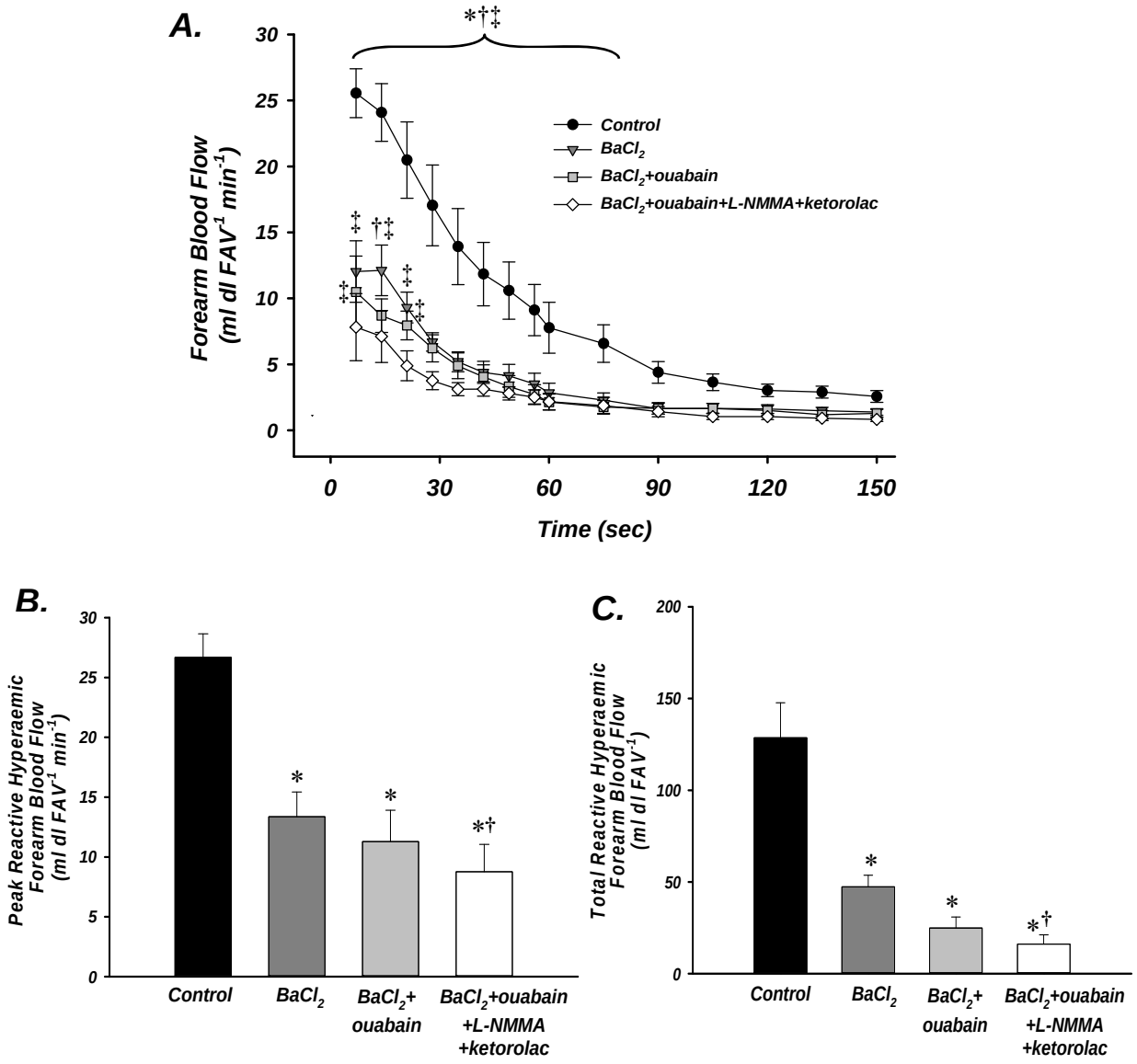

Figure 19. Protocol 1: Independent effects of K_{IR} channel inhibition (Group 1)
A. Dynamic forearm blood flow (FBF) response following 5 min of arterial occlusion in the following conditions: control (black circles), independent K_{IR} channel inhibition ($BaCl_2$; dark grey triangles), combined K_{IR} channel and Na^+/K^+-ATPase inhibition ($BaCl_2$+ouabain; light grey squares), and combined inhibition of K_{IR} channels, Na^+/K^+-ATPase, NO and PGs ($BaCl_2$+ouabain+L-NMMA+ketorolac; white diamonds). $BaCl_2$ significantly inhibited the response for the first 75 sec and there was little additional effect of ouabain, or L-NMMA+ketorolac. **P*<0.05 *vs* $BaCl_2$; †*P*<0.05 *vs* $BaCl_2$+ouabain; ‡ *P*<0.05 *vs* $BaCl_2$+ouabain+L-NMMA+ketorolac. B. Peak reactive hyperaemic FBF was significantly attenuated from control by $BaCl_2$ and ouabain had no additional effect whereas there was a slightly greater reduction with the addition of L-NMMA+ketorolac. **P*<0.05 *vs* Control; †*P*<0.05 *vs* $BaCl_2$. C. Similarly, total reactive hyperaemic FBF (area under the curve) was significantly reduced from control by $BaCl_2$ and ouabain had no additional effect whereas L-NMMA+ketorolac further reduced this response. **P*<0.05 *vs* Control; †*P*<0.05 *vs* $BaCl_2$.

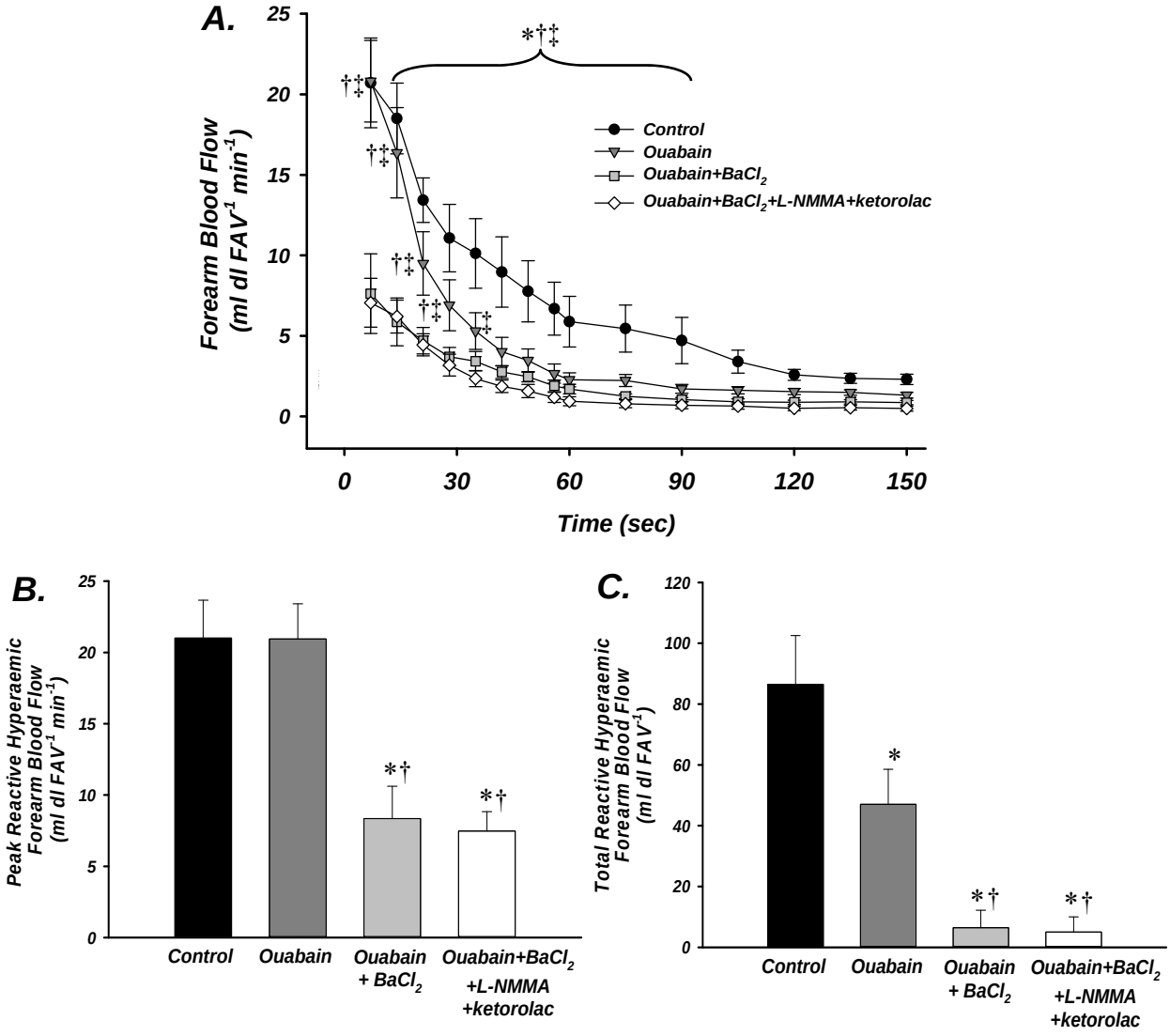

Figure 20. Protocol 1: Independent effects of Na^+/K^+-ATPase inhibition (Group 2) A. Dynamic forearm blood flow (FBF) response following 5 min of arterial occlusion in the following conditions: control (black circles), independent Na^+/K^+-ATPase inhibition (Ouabain; dark grey triangles), combined Na^+/K^+-ATPase and K_{IR} channel inhibition (Ouabain+$BaCl_2$; light grey squares), and combined inhibition of Na^+/K^+-ATPase, K_{IR} channels, NO and PGs (Ouabain+$BaCl_2$+L-NMMA+ketorolac; white diamonds). Ouabain did not affect the initial FBF, but thereafter reduced the FBF from control until 90 sec post-cuff deflation. The addition of $BaCl_2$ further attenuated FBF for the first 30 seconds, whereas addition of L-NMMA+ketorolac had no further effect. **P*<0.05 *vs* Ouabain; †*P*<0.05 *vs* Ouabain+$BaCl_2$; ‡ *P*<0.05 *vs* Ouabain+$BaCl_2$+L-NMMA+ketorolac. B. Peak reactive hyperaemic FBF was not affected by ouabain. Infusion of $BaCl_2$ significantly reduced peak FBF from control, and L-NMMA+ketorolac had no further impact. **P*<0.05 *vs* Control; †*P*<0.05 *vs* Ouabain. C. Total reactive hyperaemic FBF (area under the curve) was significantly reduced from control by ouabain and $BaCl_2$ had an additional effect whereas L-NMMA+ketorolac did not. **P*<0.05 *vs* Control; †*P*<0.05 *vs* Ouabain.

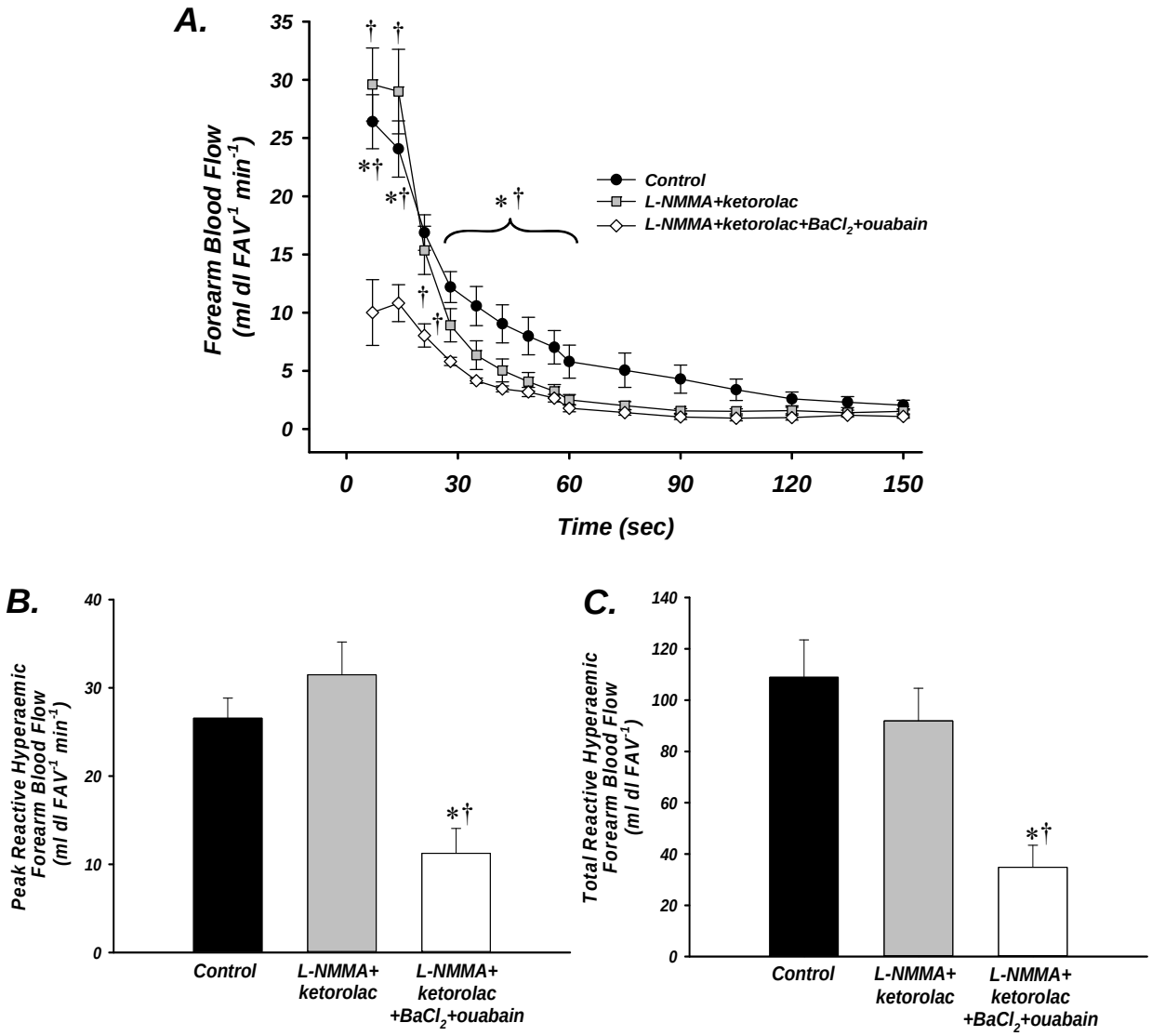

Figure 21. Protocol 2: Effects of combined inhibition of nitric oxide and prostaglandins A. Dynamic forearm blood flow (FBF) response following 5 min of arterial occlusion in the following conditions: control (black circles), combined inhibition of the synthesis of NO and PGs (L-NMMA+ketorolac; light grey squares), and combined inhibition of NO, PGs, K_{IR} channels and Na^+/K^+-ATPase (L-NMMA+ketorolac+$BaCl_2$+ouabain; white diamonds). L-NMMA+ketorolac attenuated the response from control only from 30-60 sec post-cuff deflation. The addition of $BaCl_2$+ouabain significantly reduced the initial FBF response for the first 30 sec and there was no further effect thereafter. **P*<0.05 *vs* L-NMMA+ketorolac; †*P*<0.05 *vs* L-NMMA+ketorolac+$BaCl_2$+ouabain. B. Peak reactive hyperaemic FBF was not affected by L-NMMA+ketorolac and was significantly attenuated by L-NMMA+ketorolac+$BaCl_2$+ouabain. **P*<0.05 *vs* Control; †*P*<0.05 *vs* L-NMMA+ketorolac. C. Similar to peak, total reactive hyperaemic FBF (area under the curve) was not affected by L-NMMA+ketorolac and was significantly attenuated by L-NMMA+ketorolac+$BaCl_2$+ouabain. **P*<0.05 *vs* Control; †*P*<0.05 *vs* L-NMMA+ketorolac.

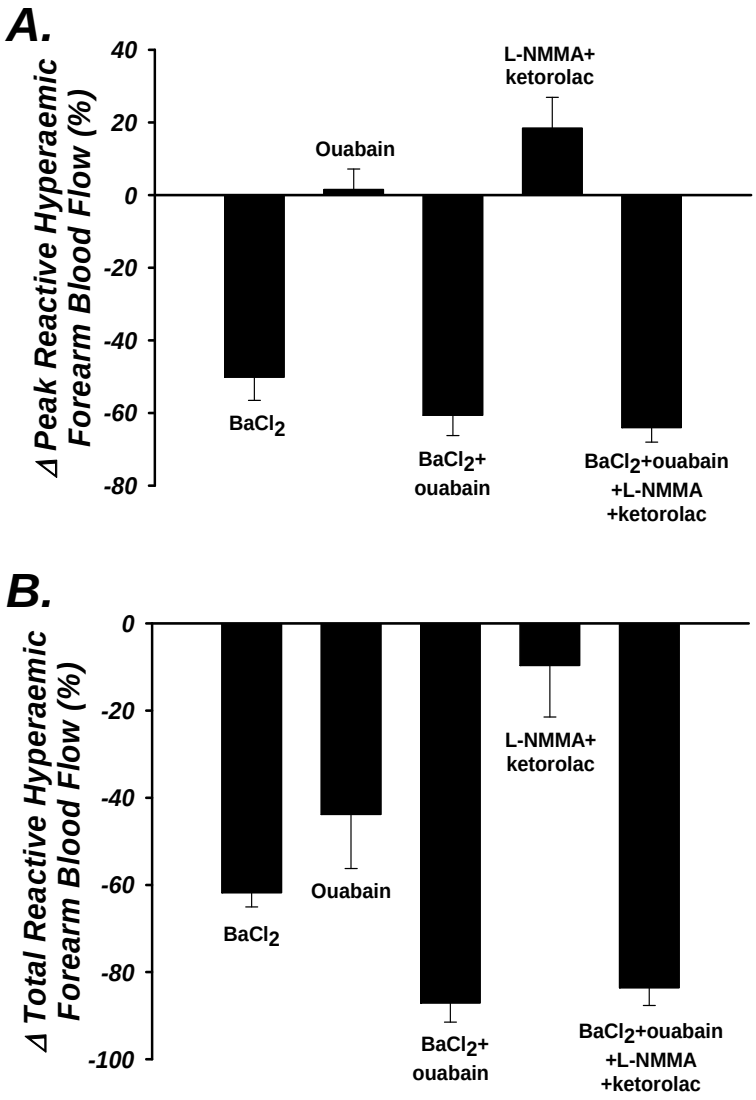

Figure 22. Summary: Effects of inhibition of K_{IR} channels, Na^+/K^+-ATPase, nitric oxide and prostaglandins on peak and total reactive hyperaemia

The combined results from the three experimental protocols are presented for both the relative impact (%Δ) on peak (A) and total (B) reactive hyperaemic forearm blood flow (FBF) in each experimental condition ($BaCl_2$: n=8; Ouabain: n=8; $BaCl_2$+ouabain: n=16; L-NMMA+ketorolac: n=8; $BaCl_2$+ouabain+L-NMMA+ketorolac: n=24). $BaCl_2$ reduced peak FBF and this attenuation was unchanged with the addition of ouabain or L-NMMA+ketorolac. Neither ouabain alone nor L-NMMA+ketorolac attenuated peak FBF. $BaCl_2$ and ouabain both independently reduced total FBF and in combination ($BaCl_2$+ouabain), the reduction was enhanced. There was no additional reduction by L-NMMA+ketorolac, nor did L-NMMA+ketorolac independently reduce total FBF. Comparisons made by 1-way ANOVA (P<0.05).

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CHAPTER VI – OVERALL CONCLUSIONS

Vasoactive signaling pathways in humans have been identified over time, and the relative contributions of these pathways in response to a variety of stimuli are of interest to our pursuit of understanding the integrative regulation of tissue blood flow and oxygen delivery. A variety of factors have been proposed to be involved in the hyperemic response to muscle contractions and arterial occlusion, as well as the modulation of sympathetic vasoconstriction during exercise. Primarily, to date, two of the most well-studied vascular signaling pathways in humans have been nitric oxide (NO) and prostaglandins (PGs). The propensity of work in the literature on these pathways is likely due to their endothelium-dependency and the relationship of endothelial health and cardiovascular disease as well as the ability to enzymatically inhibit their synthesis *in vivo*. However, recent evidence, mostly in experimental animals suggests that signaling beyond NO and PGs, through vascular hyperpolarization, may be involved in a variety of conditions. Thus, we have focused our experiments on determining the role of vascular hyperpolarization in vasomotor control in humans.

Specifically, vascular hyperpolarization can occur by a variety of means and this is one of the difficulties in establishing the role of this pathway, particularly in human studies where it is impossible to directly measure membrane potential. Activation of inwardly-rectifying potassium (K_{IR}) channels and Na^+/K^+-ATPase leads to vascular hyperpolarization and we have recently demonstrated our ability to safely inhibit these pathways in the human forearm. Thus, the preceding collection of experiments provides some of the first direct evidence establishing the significant role of vascular hyperpolarization in mediating vasodilator responses to muscle contraction and temporary arterial blood flow occlusion.

The key novel findings from present body of work are as follows: 1) vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase significantly contributes to rapid

vasodilation following a single muscle contraction and combined inhibition of these pathways along with NO and PGs nearly abolishes this response; 2) vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase mediates a portion of the vasodilatory and hyperemic response to repeated muscle contraction, both at the onset and when steady-state levels are obtained, whereas NO and PGs, do not largely contribute to this response; 3) contrary to our hypothesis, vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase appears to not be involved in functional sympatholysis; and 4) reactive hyperemia in response to temporary arterial occlusion is primarily mediated by vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase, and NO and PGs do not have an independent role in this response. Taken together, we believe that vascular hyperpolarization via K_{IR} channels and Na^+/K^+-ATPase represents a novel vascular signaling pathway in humans in response to muscle contractions and arterial occlusion. Based on the collective literature on this topic and the results from the present investigations, it is likely that this pathway may be involved in some of the age- and disease-related impairments in the responses we have examined. Thus, these findings may provide insight into understanding impaired vascular function in patient populations and as such, could represent a novel therapeutic target for reversing microvascular dysfunction.

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:	November 16, 2012	
TO:	Dinenno, Frank, Health & Exercise Science	
	Israel, Richard, Health & Exercise Science, 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:	NONE	
PROTOCOL NUMBER:	09-1186H	
APPROVAL PERIOD:	Approval Date: November 18, 2012	Expiration Date: November 17, 2013

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

Barker, Janell

Approval is to continue to recruit the remaining 216 participants with the approved recruitment and consent material. This approval also reflects an approved amendment reviewed with this continuing review to add 200 participants to the 16 remaining, to add Hannah Scott as administrative contact, use of a revised flyer and recruitment method (to recruit the younger population through RamLink using the approved flyer). 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, 2012 through November 17, 2013
Review Type:	FULLBOARD
IRB Number:	00000202
Funding:	National Institute on Aging : 40808 National Institute of Health :
	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

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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: Effects of Aging and Regular Physical Activity

PRINCIPAL INVESTIGATOR: *Frank A. Dinenno, Ph.D. 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. Trained graduate students, undergraduate students, research assistants, or research associates are assisting with the research. These studies are paid for by the National Institute on Aging, a part of the US Government.*

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

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

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

_____ 2) several visits over a few to several weeks. _____ ***(your initials)***

WHAT WILL I BE ASKED TO DO? *This consent form applies to a large research project. You are only being asked to participate in one part of the total project. Depending on the part of the research project that you are involved in, you will be asked to participate in some of the following procedures. Many potential procedures are described in the section below. However, the procedures that you will be asked to do for this part of the study have a check mark next to them. The check marks were put there by one of the researchers. The time associated with each procedure reflects the amount of time you will spend performing or undergoing the procedure, not the total time of the study. A member of the research team will fully explain each checked procedure that applies to your participation and specifically how long each session (total time) in the laboratory will be.*

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

_____ ***(your initials)***

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

_____ ***(your initials)***

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

_____ **(*your initials)***

_____ ***Graded Exercise Test.*** *If you are in the 55-90 yr-old age group, you will be asked to perform a maximal exercise test on a treadmill under the supervision of a physician. This test will occur in the Human Performance Clinical/Research Laboratory in the Department of Health and Exercise Science on the CSU campus. Sticky electrodes will be placed on your chest, and you will walk briskly or jog while the steepness of the treadmill is increased. Your blood pressure and heart beat will be closely measured during and immediately after the test. (~1 hour)*

_____ ***(your initials)***

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

_____ ***(your initials)***

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

_____ ***(your initials)***

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

_____ ***(your initials***

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

_____ ***(your initials)***

_____ ***Calf Exercise.*** *You will sit in a special chair and squeeze your calf muscles (similar to standing on your toes) while your arms, hips, and shoulders are comfortably secured. The intensity of the exercise will range from very easy to moderately difficult, and you will be asked to perform this exercise for ~10 minutes several different times throughout the study with plenty of rest in between exercise trials.*
(1 – 2 hours)

_____ ***(your initials)***

_____ ***Knee Extensor Exercise.*** *You will sit in a special chair and squeeze your thigh muscles while your hips and shoulders are comfortably secured. Your feet will be secured in specially designed boots and you will be asked to extend your leg against resistance until your ankle is about at the height of your knee, relax back to a regular seated position, and then repeat. The intensity of the exercise will range from very easy to moderately difficult, and you will be asked to perform this exercise for ~10 minutes several different times throughout the study with plenty of rest in between exercise trials. (1 – 2 hours)*

_____ ***(your initials)***

__X___ ***Maximum Voluntary Contraction.*** *This will consist of 3-4 trials where you will squeeze your muscles (either forearm, calf, or thigh) and generate as much force as you can. You will be asked to generate as much force over the course of ~3 seconds and hold this force another 5 seconds. After a 2-3 minute rest period, you will be asked to do this again. This is typically used to determine how heavy of exercise you perform so everybody is exercising at similar percentages of their maximum. (~ 20 minutes)*

_____ ***(your initials)***

_____ ***Exercise Training.***

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

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

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

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

____ ***Ischemic Exercise.*** *You will exercise your calf or forearm with a blood pressure cuff on your thigh or upper arm that is inflated very tightly to temporarily block the blood flowing to your muscle. You will be asked to perform this exercise for ~10 minutes several different times throughout the study with plenty of rest in between exercise trials. (20 – 30 minutes)*
_____ ***(your initials)***

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

____ ***Lower Body Negative Pressure.*** *You will be placed in a sealed wooden chamber while you are laying flat on a bed. The chamber is sealed at your waist. Using a standard vacuum that is attached to the chamber, suction will be applied to mimic what happens when you go from laying to standing up. This will occur several times throughout the study for about 15 minutes at a time. (~ 1 hour)* _____ ***(your initials)***

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

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

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

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

_____ ***(your initials)***

__X__ ***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)***

__X__ ***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)***

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

_____ ***(your initials)***

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

_____ ***(your initials)***

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

____ ***Blood Sample.*** *Up to 100 ml (approximately 7 tablespoons) of your blood will be drawn from a vein on the front of your elbow or artery in a standard fashion using a sterilized hypodermic needle. (~15 minutes)*

_____ ***(your initials)***

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

__***X***__ ***Venous Catheterization.*** *Your skin will be cleaned and a catheter (plastic needle) will then be inserted on the front side of your elbow and secured to the skin. In some cases, a local anesthetic might be used to reduce any discomfort. (~2-4 hours)*

_____ ***(your initials)***

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

_____ ***(your initials)***

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

_____ ***(your initials)***

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

Vasoconstrictors – cause temporary narrowing of the blood vessels (minutes)
___ Tyramine
___ Norepinephrine
_X__ Phenylephrine
___ Clonidine
___ Dexmedetomidine
___ L-NAME
_X__ L-NMMA
___ Aspirin
_X__ Ketorolac
X___ Barium Chloride
_X__ Oubain

Vasodilators – temporarily relax the blood vessels (minutes)
_X__ Acetylcholine
_X__ Adenosine
X___ Sodium Nitroprusside
___ L-Arginine
_X__ Phentolamine
X___ Adenosine Triphosphate (ATP)
___ Potassium Chloride (K^+)
___ Isoproteronol

No major effects
___ Ascorbic Acid (Vitamin C)
_X__ Propranolol
___ Aminophylline
___ Pyridoxine _____ ***(your initials)***

Oral Supplement Administration

_____ ***Ascorbic Acid (Vitamin C)***

_____ ***(your initials)***

_____ ***Muscle Biopsy.*** *A sample of muscle will be taken from a muscle on the outside of your thigh. This will take place under the supervision of a medical doctor in the Hartshorn Health Center on the CSU campus. Your skin will be temporarily numb using lidocaine, a medicine similar to novacaine. After deadening the skin, a ¼ inch incision, or cut, is made in the skin over the muscle using a sterilized scalpel. The sample is obtained using a sterilized sampling needle. The muscle sample obtained is usually about ½ the size of the eraser on the end of a pencil. You will not have to reduce your activity afterwards, but should not perform any unusual or extremely vigorous activity for a few days. You will receive written instructions regarding care of the incision, and a telephone number to contact if you have any questions. (30 - 45 minutes)*

_____ ***(your initials)***

FUTURE USE OF BLOOD OR MUSCLE SAMPLES

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

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

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

ARE THERE REASONS WHY I SHOULD NOT TAKE PART IN THIS STUDY?

If you are not 18-35 or 55-90 years of age, are pregnant, are a regular smoker, or have any diseases that would affect our measurements or significantly increase the risks associated with this study, we will not be able to include you in the research.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

(The procedures that apply to your proposed participation are checked)

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

  _____ ***(your initials)***

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

  _____ ***(your initials)***

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

  _____ ***(your initials)***

- _X____ *Body composition (DEXA) scan – the risks associated with the DEXA are very low. The radiation you will receive is less than 1/3000th of the Food and Drug Administration (FDA) limit for annual exposure. The FDA is a government organization responsible for medical safety. In other words, you could receive 3000 DEXA scans in a single year and still not meet the FDA limit for radiation exposure. In this study you will receive one scan. The more radiation you receive over the course of your life, the greater the risk of having cancerous tumors or of inducing changes in genes. The radiation in this study is not expected to greatly increase these risks, but the exact increase in such risks is not known. Women who are pregnant or could be pregnant should receive no unnecessary radiation and should not participate in this study.* _____ ***(your initials)***

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

- _____ *Exercise training – There is a slight risk of muscle strain and muscle soreness resulting from brief strong muscle contractions. Soreness should not last more than two days or affect your normal function and should get progressively less as training continues.*

  _____ ***(your initials)***

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

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

  _____ ***(your initials)***

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

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

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

- _____ *Brachial Artery Compression – There is a risk of slight discomfort at the site of compression (elbow). There is also a risk of slight discomfort or cramping if performing forearm exercise at the same time.* _____ ***(your initials)***

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

- _X____ *Venous Occlusion Plethysmography- There is a risk of temporary discomfort of the hand or foot when the blood pressure cuffs are inflated.*
  _____ ***(your initials)***

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

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

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

- _X____ *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)***

- __X___ *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)***

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

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

  _____ ***(your initials)***

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

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

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

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

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

We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. For example, your name will be kept separate from your research records and these two things will be stored in different places under lock and key. You should know, however, that there are some circumstances in which we may have to show your information to other people. For example, the law may require us to show your information to a court or to the Human Research Committee at CSU.

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

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? *For experiments that involve the blood sample, muscle sample, fine wire electrodes, and arterial or venous catheterization, you will be paid $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? *Please be aware that for this study the University has made special arrangements to provide initial medical coverage for any injuries that are **directly related** to your participation in this research project.*

The research project will provide for the coverage of reasonable expenses for emergency medical care related to the treatment of research-related injuries, if necessary.

LIABILITY:
Because Colorado State University is a publicly-funded, state institution, it may have only limited legal responsibility for injuries incurred as a result of participation in this study under a Colorado law known as the Colorado Governmental Immunity Act (Colorado Revised Statutes, Section 24-10-101, et seq.). In addition, under Colorado law, you must file any claims against the University within 180 days after the date of the injury.

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

WHAT IF I HAVE QUESTIONS? *Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the principal investigator, Frank Dinenno, Ph.D., at (970)491-3203, or via email at fdinenno@cahs.colostate.edu. If you would like to ask a medical doctor about your participation in the study, you may contact 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 Janell Barker, Human Research Administrator, at 970-491-1655. We will give you a copy of this consent form to take with you.*

This consent form was approved by the CSU Institutional Review Board for the protection of human subjects in research on November 18, 2010.

Your signature acknowledges that you have read the information stated and willingly sign this consent form. Your signature also acknowledges that you have received, on the date signed, a copy of this document containing 9 pages.

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

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

_____ _____
Name of person providing information to participant Date

_____
Signature of Research Staff

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

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