EFFECTS OF ADRENORECEPTOR ACTIVATION AND AGING ON SKELETAL MUSCLE ARTERIOLES AT REST AND DURING RAPID ONSET VASODILATION

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by

ALEX W. MOORE

Dr. Steven S. Segal, Dissertation Supervisor

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

EFFECTS OF ADRENORECEPTOR ACTIVATION AND AGING ON SKELETAL MUSCLE ARTERIOLES AT REST AND DURING RAPID ONSET VASODILATION

presented by Alex W. Moore,

a candidate for the degree of doctor of philosophy,

and hereby certify that, in their opinion, it is worthy of acceptance.

Professor Steven S. Segal

Professor Harold Laughlin

Professor Frank Booth

Professor Eileen Hasser

Professor Doug Bowles

Professor Paul Fadel

To my wife

And the hope of a life together

Luke 18:28-30

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EFFECTS OF ADRENORECEPTOR ACTIVATION AND AGING ON SKELETAL MUSCLE ARTERIOLES AT REST AND DURING RAPID ONSET VASODILATION

Alex W. Moore
Dr. Steven S. Segal, Dissertation Supervisor

ABSTRACT

Sympathetic nerve activity (SNA) induces arteriolar vasoconstriction via α-adrenoreceptor (αAR) activation. Whether αAR activation affects the spread of rapid onset vasodilation (ROV) in contracting muscle is unknown. Differential αAR distribution in vascular smooth muscle has been proposed to mediate functional sympatholysis, however the αAR subtype distribution in locomotor muscle is undefined. This dissertation determined: 1) the effects of constitutive αAR activation on the spread of ROV within contracting muscle, 2) the functional αAR distribution in locomotor muscle of the mouse, and 3) the influence of αAR on ROV during aging. In arterioles of the gluteus maximus muscle (GM), I tested the hypotheses that: 1) adrenoreceptor subtype distribution is heterogeneous and 2) adrenoreceptor activation modulates the spread of ROV. The left GM of young (3-month) anesthetized C57BL/6 mice were studied using intravital microscopy. Distinct anastomotic, 1A, 2A, and 3A arterioles were studied at rest and following single muscle contraction in the presence or absence of topical αAR agonists and antagonists. Functional αAR distribution differed between proximal and distal arterioles. Constitutive αAR activation inhibited the spread of ROV between regions of the GM. It also reduced the amount of ROV seen in old (~20-month) versus young male mice. I conclude that functional αAR are heterogeneously distributed in arteriolar networks and serve to modulate regional vasodilation.

CHAPTER 1

INTRODUCTION

Role of arterioles in blood flow control

Blood flow to skeletal muscle is coupled to metabolic demand (Gorczynski *et al.*, 1978; Laughlin & Armstrong, 1982). As demonstrated in the intact circulation using micropressure measurements along vascular networks, the greatest resistance to blood flow occurs along arterioles and the feed arteries from which they originate (Fronek & Zweifach, 1975; Bohlen & Gore, 1977; Welsh & Segal, 1996). The microcirculation begins where feed arteries enter the tissue and give rise to primary (1A) arterioles. These proximal resistance vessels govern the total amount of blood flow entering the microcirculation. The daughter branches are referred to as second-order (2A) and third-order (3A) arterioles and serve to control the distribution of blood flow within the tissue and the magnitude of capillary perfusion. Thus arteriolar networks direct cardiac output to the capillary beds supplying muscle fibers according to motor unit recruitment (Fuglevand & Segal, 1997). Arterioles control the resistance to blood flow through changes in their diameter. Vasoconstriction increases vascular resistance and restricts blood flow while vasodilation decreases vascular resistance and increases blood flow. According to Poiseuille's law, resistance to flow is inversely proportional to the fourth power of vessel radius. Thus small changes in vessel diameter can produce dramatic changes in blood flow. Because individual vessels exist as part of an arteriolar network, dilation of upstream vessels must accompany local dilation in order to effect substantive

changes in blood flow. Thus, vasodilation must be coordinated among daughter and parent arterioles (Kurjiaka & Segal, 1995a) along with feed arteries (Segal & Jacobs, 2001). Such integrated responses in vascular resistance networks is recognized as "ascending vasodilation" and involves the conduction of vasodilatory signals that originate within distal branches of the microcirculation along the vessel wall to induce remote dilation in proximal vessels (Hilton *et al.*, 1970).

In skeletal muscle, it is important that blood flow matches metabolic demand in order to support contractile activity. Skeletal muscle has a wide range of metabolic rates and has a well developed network of arterioles to regulate tissue blood flow. In human quadriceps muscle, resting blood flow is as low as 0.3 L min^{-1} but can exceed 10 L min^{-1} during maximal exercise (Radegran, 1997). Rapid onset vasodilation (ROV) refers to the rapid (within 1-2 seconds) dilation of arterioles immediately following a single muscle contraction (Clifford, 2007). In humans, ROV has been shown to act independently of the muscle pump (Tschakovsky *et al.*, 1996). Direct observation of ROV in arterioles has been demonstrated using animal models (Mihok & Murrant, 2004; Armstrong *et al.*, 2004; Clifford, 2007) which corroborate ROV responses inferred in humans by measuring blood flow in the supplying artery (Tschakovsky *et al.*, 1996; Shoemaker *et al.*, 1998). As shown in hamster skeletal muscle, ROV triggered by muscle contraction can initiate ascending dilation into feed arteries and thereby enhance muscle blood flow rapidly (VanTeeffelen & Segal, 2006). While the mechanism of initiating ROV remains under investigation, the speed of this response preemptively increases blood flow to

contracting muscle before the buildup of pCO_2 or metabolites can occur. However, due to skeletal muscle representing 30-50% of total body mass together with finite pumping capacity of the heart, large increases in skeletal muscle blood flow (i.e., large decreases in total peripheral resistance) may compromise systemic arterial blood pressure (Marshall *et al.*, 1961; Rowell, 1974). Therefore a mechanism for maintaining or increasing vasoconstriction (e.g., in inactive tissues) is required to offset vasodilation in exercising skeletal muscle.

The sympathetic nervous system.

Pre-ganglionic sympathetic nerves leaving the spinal cord synapse with post-ganglionic nerves in the sympathetic chain ganglia or the adrenal glands. Norepinephrine (NE) is released by sympathetic post-ganglionic neurons and acts on vascular smooth muscle to initiate contraction, resulting in vasoconstriction. Perivascular nerves were shown to innervate blood vessels as early as 1863 (His, 1863). Detailed examination of innervation patterns along arterioles revealed all levels of the arteriolar network as well as large veins are innervated while capillaries and venules are not (Woollard, 1926; Marshall, 1982).

Late in the 19th century, Bayliss and Bradford demonstrated stimulation of sympathetic perivascular nerves causes vasoconstriction and a reduction of blood flow (Bayliss & Bradford, 1894). Doppler ultrasound in humans (Delp & Laughlin, 1998; Dinenno & Joyner, 2006) and direct observations in animal models have confirmed these findings

(Marshall, 1982; VanTeeffelen & Segal, 2003). Thus in response to exercise, the sympathetic nervous system initiates vasoconstriction to restrict blood flow to inactive (resting) tissues. This action offsets vasodilation in active muscle and helps to maintain systemic blood pressure while redirecting cardiac output to support metabolic demand. In addition, sympathetic constriction of veins reduces vascular capacitance and promotes venous return to promote cardiac output. Furthermore, in vessels supplying active muscle, the activity of sympathetic nerves can restrict muscle blood flow by opposing vasodilation and suppressing ascending vasodilation, thereby maintaining elevated resistance in feed arteries and primary arterioles (Folkow *et al.*, 1971; Segal, 2000).

Adrenoreceptors on vascular smooth muscle cells

In the vasculature, sympathetic neurotransmission targets α- and β-adrenoreceptors on vascular smooth muscle cells through the release of NE (). There are two general subtypes of α-adrenoreceptors: α_1-adrenoreceptors and α_2-adrenoreceptors. Activation of α_1-adrenoreceptors initiates a signaling cascade resulting in the activation of phospholipase C and release of inositol trisphosphate (IP_3) (Minneman, 1988). Within the smooth muscle cells, IP_3 stimulates Ca^{2+} release from the sarcoplasmic reticulum and triggers the opening of voltage-sensitive Ca^{2+} channels in the sarcolemma, resulting in increased myosin-light chain phosphorylation and contraction (Bohr, 1973); i.e., vasoconstriction. Activation of α_2-adrenoreceptors activates a G_i-protein which leads to membrane depolarization and activation of voltage-gated calcium channels. Although

α_2-adrenorecepotor activation does not rely on Ca^{2+} release from internal stores, vasoconstriction is effectively initiated through this signaling pathway as well. The other class of adrenoreceptors found predominantly in vascular smooth muscle are recognized as β_2-adrenoreceptors which promote relaxation and vasodilation. These adrenoreceptors have a lower affinity to NE compared to α_1- or α_2- adrenoreceptors. The β-adrenergic signaling pathway entails activation of a G_s-protein which increases protein kinase A and intracellular cAMP, which inhibits myosin light chain kinase, inhibiting actin-myosin cross-bridge formation and vascular tone (Bohr, 1973). Remarkably, there is a paucity of information regarding the behavior of arterioles in skeletal muscle in response to β-adrenoreceptor activation.

Aging increases sympathetic nerve activity

The rate of sympathetic discharge is not constant. In addition to spontaneous fluctuations in firing rate, acute conditions, such as dehydration, hemorrhage, or exercise can transiently increase the level of sympathetic nerve activity and adrenoreceptor activation (Malpas, 2010). However disease states such as hypertension (Malpas, 2010) and the metabolic syndrome can induce chronic elevations in sympathetic nerve activity and adrenoreceptor activation (Perin *et al.*, 2001; Grassi *et al.*, 2005). In otherwise healthy individuals, increasing age has been shown to elevate sympathetic nerve activity, thereby increasing peripheral vascular resistance and reducing muscle blood flow at rest and during exercise (Dinenno *et al.*, 1999). While the effects of aging and increased sympathetic nerve activity on exercise hyperemia have

been well-characterized in humans (Dinenno & Joyner, 2006; Proctor & Parker, 2006), direct observations of microvessels controlling blood flow to skeletal muscle of aged animals have been limited to steady state contractions (Bearden *et al.*, 2004b; Bearden *et al.*, 2007). In contrast, many of the activities encountered in daily life involve short bursts of activity and remarkably little is known of how aging may affect associated responses of ROV.

Adrenoreceptor activation in the microcirculation

While the earliest studies examining innervation of the vasculature date back almost 150 years, only recently have the effects of sympathetic nerve activity and adrenoreceptor activation been investigated in the microcirculation. Building on the original work of Mellander (Mellander, 1960), Marshall demonstrated that sympathetic vasoconstriction is more pronounced in smaller arterioles immediately upstream from capillary beds than larger feed arteries (Marshall, 1982). As inferred above, sympathetic nerve activity can also inhibit vasodilator signaling along the wall of arterioles (Kurjiaka & Segal, 1995a) and feed arteries (Haug *et al.*, 2003; Haug & Segal, 2005b) and thereby suppress ascending vasodilation to restrict muscle blood flow**.** Although sympathetic nerve activation produces constriction of arterioles within skeletal muscle, smaller arterioles undergo dilation and 'escape' the sympathetic stimulus even at rest (Marshall, 1982; Boegehold & Johnson, 1988). The ability of arterioles to escape sympathetic stimulation and dilate in response to muscle contraction has been termed 'functional

sympatholysis' (Remensnyder *et al.*, 1962). Such behavior has been shown to be more prominent in distal arterioles than in proximal feed arteries (Folkow *et al.*, 1971). As an integrated systemic vascular response, sympathetic nerve activity appears to maintain arterial blood pressure by constricting resistance vessels supplying inactive tissues and restricting the dilation of feed arteries and proximal arterioles that supply active muscles (Thomas and Segal, 2004). At the same time functional sympatholysis within active skeletal muscle promotes arteriolar dilation to maximize perfusion of capillary beds supplying contracting muscle fibers (Folkow *et al.*, 1971; Segal & Jackson, 2005).

Remarkably, there is a paucity of data concerned with the effects of constitutive adrenoreceptor activation in the microcirculation of resting skeletal muscle. Typically, vascular responses are recorded in the presence of designated levels of adrenoreceptor activation superimposed over whatever tonic activation may be present. Additional adrenoreceptor activation is accomplished by several methods. Direct electrical stimulation of specific sympathetic ganglia or of perivascular nerve fibers depolarizes synaptic nerve terminal resulting in NE release (often together with ATP and neuropeptide Y), thereby stimulating α-adrenoreceptors. However neurotransmitter release causes feedback inhibition via pre-synaptic α_2-adrenoreceptors inhibiting NE release during repeated stimulation. To avoid this complication, or in instances where perivascular nerve stimulation is not possible, many studies have delivered exogenous NE (or its analogs) to stimulate post-junctional adrenoreceptors. Consequently, the

majority of what is known about adrenoreceptor activity in the microcirculation is based on vasoconstriction mediated by heightened levels of adrenoreceptor activation.

Limitations of current understanding

While the interactions between the skeletal muscle microcirculation, adrenoreceptor activation, and exercise hyperemia have been well studied, several questions remain. Direct observation of resistance arterioles with intravital microscopy lends itself to animal models, yet few studies have been performed with muscles of locomotion. This shortcoming is attributable to the cremaster muscle being the most widely used preparation for intravital microscopy of "skeletal muscle" due to its thinness and suitability for imaging. However the primary role of the cremaster muscle is to support the testes and control their temperature. Therefore such preparations are restricted to male animals. Indeed, there is a paucity of data based upon the microcirculation of parallel-fibered locomotor muscle.

It is unknown how adrenoreceptor activation affects ROV and the spread of dilation in response to a muscle contraction. Because previous studies have typically used rhythmic contraction of entire muscles, it has not been possible to resolve how the pattern of muscle fiber recruitment may affect regional vasodilation and thereby determine the distribution of blood flow within a muscle. Nor has the functional distribution of adrenoreceptors in arteriolar networks been determined in locomotor muscle. Further, the effects of constitutive adrenoreceptor activation and aging on

rapid vascular adjustments to exercise (e.g., ROV) have yet to be investigated. As the mouse is increasingly used as a model for investigating relationships pertinent to human health and disease, the present experiments used C57BL/6 mice as it is a strain that is used widely in the scientific community. Experiments were performed using the gluteus maximus muscle as a representative locomotor muscle (Bearden *et al.*, 2004b; Lampa *et al.*, 2004).

Purpose of dissertation research

The purpose of this dissertation research was threefold: 1) Determine the functional distribution of α-adrenoreceptors in a locomotor muscle. 2) Determine the effects of constitutive α-adrenoreceptor activation on vasodilation (ROV) in response to muscle contraction. 3) Determine the effects of aging on ROV in light of increased constitutive α-adrenoreceptor activation. Chapter 2 of this dissertation investigates the role of constitutive adrenoreceptor activation on the spread of vasodilation following a single muscle contraction. Particular attention is focused on where dilation occurs with respect to active vs. inactive muscle regions. Using selective and non-selective agonists and antagonist, Chapter 3 investigates the functional adrenoreceptor distribution in arteriolar networks. Chapter 4 investigates how constitutive adrenoreceptor activation in young and old animals affects vasodilation and blood flow in response to single tetanic contractions (ROV) and during rhythmic submaximal contractions. Chapter 5 provides a summary of previous chapters in addition to presenting pilot data and a rationale for future studies.

CHAPTER 2:

REGIONAL ACTIVATION OF RAPID ONSET VASODILATION ACCORDING TO MOTOR UNIT RECRUITMENT IN MOUSE SKELETAL MUSCLE: REGULATION THROUGH α-ADRENORECEPTORS

Contributing authors

A.W. Moore, S.E Bearden, S.S. Segal

Summary

Exercise onset entails motor unit recruitment and the initiation of vasodilation. Dilation can ascend the arteriolar network to encompass proximal feed arteries but is opposed by sympathetic nerve activity (SNA), which promotes vasoconstriction and inhibits ascending vasodilation by activating α-adrenoreceptors. Whereas contractile activity can antagonize sympathetic vasoconstriction, more subtle aspects of this interaction remain to be defined. We tested the hypothesis that constitutive activation of α-adrenoreceptors governs blood flow distribution within individual muscles. The mouse gluteus maximus muscle (GM) consists of inferior and superior regions. Each muscle region is supplied by its own motor nerve and feed artery with anastomotic arterioles (AA; resting diameter ~25 μm) that span both regions. In anesthetized male C57BL/6 mice (3-5 months old), the GM was exposed and superfused with physiological saline solution (35 °C; pH 7.4). Stimulating the inferior gluteal motor nerve (0.1 ms pulse @ 100 Hz for 500 ms) evoked a brief tetanic contraction and produced rapid (< 1 s) onset vasodilation (ROV; diameter change, 10 ± 1 μm) of AA along the active (inferior) muscle

region but not along the inactive (superior) region (n=8). In contrast, microiontophoresis of acetylcholine (ACh; 1 μm micropipette tip, 1000 nA, 500 ms) initiated dilation that traveled along the AA from the inferior into the superior muscle region (diameter change, 5 ± 2 μm). Topical phentolamine (1 μM) had no effect on resting diameter but α-adrenoreceptor inhibition allowed ROV to spread along AA into the superior (inactive) muscle region (dilation, 7 ± 1μm; $P<0.05$), where remote dilation to ACh doubled ($P<0.05$). These findings indicate that constitutive activation of α-adrenoreceptors in skeletal muscle can inhibit the spread of vasodilation within arteriolar networks and thereby direct blood flow to active motor units.

Introduction

The intensity of contractile activity establishes the metabolic demand of skeletal muscle and thereby dictates the oxygen requirements of active motor units. During maximal exercise, blood flow to active skeletal muscle can increase 50- to 100-fold above rest (Saltin *et al.*, 1998) through complementary mechanisms involving integrated systemic and local cardiovascular responses. Systemically, an increase in sympathetic nerve activity (SNA) activates β_1-adrenoreceptors of the myocardium and α-adrenoreceptors in the resistance vasculature thereby increasing and redistributing cardiac output to favor active muscles (Rowell, 1974). Direct observations of skeletal muscle preparations have confirmed that SNA can produce vasoconstriction; however, arterioles in resting muscle can 'escape' from this effect (Marshall, 1982; Boegehold & Johnson, 1988). The ability of arterioles to override sympathetic vasoconstriction is enhanced in contracting

skeletal muscle with such “functional sympatholysis” (Remensnyder *et al.*, 1962) serving to increase blood flow to active muscle fibers. Overcoming the constrictor action of SNA appears to be more pronounced in smaller, distal arterioles when compared to that of larger, proximal arterioles (Anderson & Faber, 1991) and increases with intensity of muscle contraction (VanTeeffelen & Segal, 2003).

As metabolic demand increases, vasodilation ascends the arteriolar tree to encompass proximal feed arteries which supply arteriolar networks within the muscle. Such spreading of vasodilation along the vasculature is integral to increasing total blood flow into the active muscle (Hilton, 1959; Segal & Jacobs, 2001). Conversely, as SNA increases, blood flow to skeletal muscle is restricted by constricting the feed arteries (VanTeeffelen & Segal, 2003). An increase in SNA can also inhibit the ability of dilatory signals to spread along arterioles (Kurjiaka & Segal, 1995b) and feed arteries (Haug *et al.*, 2003) and thereby suppress ascending vasodilation (VanTeeffelen & Segal, 2003). Historically, providing new insight into the interplay between muscle contraction and SNA has relied upon experimental protocols using sustained bouts of rhythmic muscle contractions and have focused on the efficacy of sympathetic vasoconstriction during steady-state contractile activity (Remensnyder *et al.*, 1962; Anderson & Faber, 1991; Thomas *et al.*, 1994; Thomas *et al.*, 1998; VanTeeffelen & Segal, 2003). Further, such studies have imposed sympathetic neurotransmission via nerve stimulation or have administered exogenous norepinephrine (NE) to activate α-adrenoreceptors during a period of steady-state muscle contractions. Since most daily activities do not invoke

substantial increases in SNA, we questioned whether more subtle interactions between basal adrenoreceptor activation and locomotor muscle activity may govern muscle microvascular function than has been recognized previously.

A rapid increase in blood flow following a single brief contraction was first reported for the human forearm (Corcondilas *et al.*, 1964). Ensuing studies in humans have determined that active vasodilation can occur within a single cardiac cycle following muscle contraction (Walloe & Wesche, 1988; Tschakovsky *et al.*, 1996). Indeed, direct observation of arterioles within skeletal muscle preparations of rodents has confirmed a rapid onset vasodilation (ROV) that occurs within 1-2 seconds of muscle contraction (Marshall & Tandon, 1984; Mihok & Murrant, 2004; VanTeeffelen & Segal, 2006; Jackson *et al.*, 2010). Directly observing that ROV can encompass feed arteries external to the muscle (VanTeeffelen & Segal, 2006) indicates that a vasodilatory signal generated in response to a brief muscle contraction can travel upstream along the vessel wall. Because non-oxidative pathways of ATP production can sustain intense muscle contraction for less than a minute (Sahlin *et al.*, 1998), rapidly increasing blood flow to deliver oxygen and remove metabolic byproducts is essential to peak contractile performance. Remarkably, little is known of whether the activation of αAR may influence arteriolar responses during the first few seconds following muscle contraction. Further, whereas imposed SNA and exposure to NE can increase vasomotor tone during rhythmic muscle contractions (Remensnyder *et al.*, 1962; VanTeeffelen & Segal, 2003), it can take a minute or longer for muscle SNA to increase following the onset of

contractile activity (Mark *et al.*, 1985). Thus, it is unknown whether constitutive levels of α-adrenoreceptor activation can modulate the magnitude of ROV or the ability of the dilatory signal to travel along the resistance vasculature.

Interactions between α-adrenoreceptor activation and muscle fiber contraction have typically been studied by measuring limb blood flow in animals (Remensnyder *et al.*, 1962; Thomas *et al.*, 2001) and in human subjects (Dinenno & Joyner, 2006). Those who have focused on observing the microcirculation have stimulated the entire muscle either directly with field stimulation (Anderson & Faber, 1991) or indirectly via the motor nerve (VanTeeffelen & Segal, 2003). In such studies, it has not been possible to distinguish between active vs. inactive motor units due to simultaneous activation of all muscle fibers. As shown in the rat hindlimb using radioactive microspheres, muscle blood flow is distributed according to muscle fiber type and motor unit recruitment (Laughlin & Armstrong, 1982; Mackie & Terjung, 1983). The pattern of somatic innervation can result in distinct functional regions of a muscle that share a common vascular supply (Bearden & Segal, 2005). How an increase in blood flow may be directed to active vs. inactive muscle regions remains poorly understood, in part due to the lack of an appropriate experimental model in which such behavior can be resolved *in vivo*.

To investigate possible interactions between sympathetic and somatic neuroeffector regulation of muscle blood flow distribution at exercise onset, the goal of this study was to implement an experimental model that enabled definitive visual resolution of active

vs. inactive muscle regions in light of arteriolar network topology and vasomotor responses. Using intravital microscopy, we studied arteriolar networks within the mouse gluteus maximus muscle (GM). As illustrated in Figure 1, the GM contains distinct superior and inferior functional regions each governed by its respective motor nerve and vascular supply (Lampa *et al.*, 2004; Bearden & Segal, 2005). Arteriolar networks of respective regions are often joined by anastomotic arterioles (AA) that span respective muscle regions (Fig. 1). Using this model, we tested the hypothesis that contraction of one muscle region can initiate vasodilation that spreads into the adjacent (inactive) region along AA. Our central question focused on whether constitutive activation of α-adrenoreceptors in resting muscle influenced the spread of ROV between active and inactive muscle regions and could thereby selectively direct muscle blood flow to active motor units.

Methods

Animal care and use

All procedures were approved by the Animal Care and Use Committee of the University of Missouri and performed in accord with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Male C57BL/6 mice (3-5 months old, body mass = 25-30g) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed at ~ 22 ºC on a 12 h–12 h light–dark cycle with food and water provided *ad libitum* for at least one week before being used in an experiment. At the end of each day's

experiments, the anesthetized mouse was euthanized with an overdose of pentobarbital sodium (intraperitoneal injection) and cervical dislocation.

Anesthesia and muscle preparation

A mouse was anesthetized with an initial intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1}) which was supplemented as needed. Hair was carefully shaved from the hindquarters and the mouse was placed on an acrylic platform for surgical procedures and experimental observations. Oesophageal temperature was maintained at 37-38 ºC using conducted heat from a warming plate positioned underneath the mouse. While viewing through a stereomicroscope, the left GM was exposed by removing the overlying skin and connective tissue, carefully dissecting the GM away from its origin along the spine and reflecting the muscle away from the body while preserving its insertion on the femur. The GM was spread over a transparent silicone rubber pedestal (Sylgard 184; Dow Corning, Midland, MI, USA) that was integral to the acrylic platform and the muscle edges were pinned to approximate *in situ* dimensions. Exposed tissue was superfused continuously with a physiological saline solution (35 °C, pH 7.4) containing (in mM): 131.9 NaCl, 4.7 KCl, 2 $CaCl_2$, 1.17 $MgSO_4$ and 18 $NaHCO_3$ equilibrated with 5% CO_2 / 95% N_2. Chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Intravital microscopy

Upon completion of surgical procedures, the preparation was transferred to the fixed stage (Burleigh® Gibraltar®; Mississauga, Ontario, Canada) of an intravital microscope (Nikon E600FN; Melville, NY, USA). The superfused GM was equilibrated for at least 30 min prior to experimental procedures. To allow observing different fields of view without disturbing micropipettes positioned for motor nerve stimulation or microiontophoresis (described below), the microscope was mounted on an X-Y translational platform (Burleigh® Gibraltar®). Using Köhler illumination from a long-working-distance condenser (Numerical Aperture, 0.52), images were acquired with a Nikon 20x SLWD objective (Numerical Aperture, 0.35) coupled to a charge-coupled device video camera (KP-D50; Hitachi Denshi; Japan) and viewed on a digital monitor (ViewEra V191HV; Walnut, CA, USA) at a total magnification of 1300X.

Arterioles were selected for study based upon criteria described in **Experimental Protocols**. Internal vessel diameter was measured (spatial resolution, ~ 1 μm) as the widest distance between luminal edges with a video caliper (Microcirculation Research Institute; Texas A&M University; College Station, TX, USA) that was calibrated with a stage micrometer (0.01 x 100 = 1 mm; Graticules Ltd.: Tonbridge Kent, England). The output from the caliper was sampled at 40 Hz using a Powerlab/400 system (AD Instruments; Colorado Springs, CO, USA) coupled to a personal computer.

Motor nerve stimulation

Contraction of the inferior region of the GM was evoked selectively by stimulating the inferior gluteal nerve (IGN; see Fig. 1). The nerve was cut proximally to provide a distal stump that was drawn into a suction electrode fabricated from borosilicate glass capillary tube (GC120F-10, Warner Instruments, Hamden, CT, USA) pulled to a fine tip (P-97; Sutter Instruments, Novato, CA, USA), which was broken and heat-polished to an inner diameter of ~100 µm. The electrode holder (Warner) was secured in a 3-axis micromanipulator (DT3-100; Siskiyou Corp.; Grants Pass, OR, USA) mounted on the acrylic platform. A monophasic, square-wave stimulator (S48; Grass Instruments, Quincy, MA, USA) delivered current through a stimulus isolation unit (SIU5, Grass) to elicit brief single maximal tetanic contractions. Stimulation in this manner elicited contraction only of muscle fibers in the inferior region of the GM as confirmed routinely by visual inspection; the superior region of the GM remained quiescent throughout experiments.

Microiontophoresis

To assess the ability of a vasodilatory signal to travel along the AA, a brief pulse of acetylcholine (ACh) was delivered to the inferior gluteal feed artery at its site of entry (see Fig. 1) using microiontophoresis. Borosilicate glass capillary tubes (GC120F-10) were pulled (P-97, Sutter) to an inner diameter of ~1 µm, backfilled with ACh (1 M), and secured in the 3-axis micromanipulator. A silver wire secured at the edge of the preparation served as the reference electrode. Using an iontophoresis programmer

(Model 260, World Precision Instruments (WPI); Sarasota, FL, USA), a retain current of 250 nA prevented leakage of ACh from the micropipette and the ACh stimulus was delivered using a monophasic square-wave pulse (1 µA, 500 ms). Vasomotor responses were measured at defined sites along the anastomotic arteriole based on the functional border between superior and inferior regions of the GM (see Fig. 1).

Experimental Protocols

Oxygen sensitivity

Following equilibration and prior to initiating an experimental protocol, each GM preparation was evaluated for the sensitivity of 2A to elevated oxygen as in index of tissue viability. This was done by equilibrating the superfusion solution with 21% O_2 / 5% CO_2 (balance N_2) for 5 min, determining whether 2A responded to elevated oxygen (typically 5-10 µm constriction), then re-equilibrating with 5% CO_2 / 95% N_2 for the remainder of the experiment.

Protocol 1

We first characterized ROV responses of arterioles within the inferior region of the GM to single tetanic contractions elicited via stimulation of the IGN (Fig. 2). For this purpose, second-order branches (2A) of arteriolar networks contained entirely within the inferior muscle region were selected as they serve to distribute blood flow within the GM (Bearden *et al.*, 2004b; Jackson *et al.*, 2010). One 2A was studied per mouse. With constant pulse duration (0.1 ms) and stimulation frequency (100 Hz), the IGN was

stimulated for 100 to 1000 ms (in random order across experiments) to produce single maximal tetanic contractions of varying duration. Internal vessel diameter was recorded at rest before and immediately following tissue displacement during contraction. Arterioles recovered for 3-5 min after each contraction to restore baseline diameter before a subsequent stimulus was delivered. Based upon results from these experiments, a train duration of 500 ms was used for subsequent experiments as it consistently evoked robust submaximal dilations of the 2A (Fig. 2) and thereby allowed us to determine whether such reproducible vasomotor responses could be influenced by experimental interventions described in **Protocols 2-4**.

Protocol 2

A separate group of mice was studied to investigate the effect of regional motor unit activation on the spread of ROV. Vasomotor responses were recorded at one of four locations along the AA spanning inferior (active) and superior (inactive) regions of the GM (Fig. 1). Respective observation sites were defined relative to the observed border between active and inactive muscle regions with distance from the border reference to a calibrated eyepiece reticule. Two sites were located within the active muscle region: 800 μm (a) and 500 μm (b) towards the inferior edge of the GM and two sites were located within the inactive muscle region: 500 μm (c) and at 800 μm (d) towards the superior edge of the GM. With order randomized across experiments, a separate 500-ms contraction was performed for each observation site. After a 3-5 min recovery

period to restore resting diameter, the stimulus was repeated while observing another designated site.

Additional experiments were performed in a separate group of mice to determine if vasodilation spread beyond the active muscle region and, if so, whether there was directionality to the dilatory response. Two observation sites at equal distances (~ 500 μm) from respective edges of the inferior GM were selected: the inferior gluteal feed artery (which supplies the active muscle region), and a site along the AA located within the inactive muscle region (corresponding to site "c" above). With order randomized across experiments, a separate contraction was performed for each site observed. To confirm that arterioles in the superior GM were able to undergo ROV, a third observation site was selected by locating an arteriole supplied by the superior gluteal feed artery (e.g., site "e" in Fig. 1) and not connected directly to the AA. Vasomotor responses at site "e" were recorded following a single contraction using the following stimuli: IGN stimulation, IGN stimulation in the presence of a non-selective α-adrenoreceptor blocker (phentolamine, as an additional control for **Protocol 3**) in the superfusion solution, and during field stimulation of the entire muscle.

Protocol 3

To test the hypothesis that inhibition of α-adrenoreceptors would promote the spread of ROV from active into inactive muscle regions, a separate group of mice was studied. Vessel responses to a single 500-ms tetanic contraction were recorded at locations a-d

(from **Protocol 2**) before and after equilibrating phentolamine (1 μM) in the superfusion solution for at least 15 min. In preliminary experiments (n=4), the effectiveness of α-adrenoreceptor blockade was confirmed by verifying that arteriolar constriction (typically ~ 50%) in response to NE (1 μM) in the superfusate was abolished by phentolamine.

Protocol 4

A separate group of mice was studied to determine whether vasodilation could travel along AA from the inferior region to the superior region of the GM and, if so, whether this response was affected by α-adrenoreceptors. Acetylcholine was delivered by microiontophoresis at the site where the inferior gluteal feed artery enters the GM while diameter was observed at the site of stimulation ("local") and at sites (a) and (d) as described in **Protocol 2** (i.e., designated sites along the AA located nearest and furthest from the ACh stimulus, respectively). A separate ACh stimulus was delivered for observing each site with several min of recovery between each stimulus to restore resting diameter. Because overlying skeletal muscle fibers prevented direct access (or reproducible ACh delivery) to AA embedded within the muscle, stimulating at the site of FA entry into the muscle provided direct access of the ACh micropipette to the vessel and resulted in consistent vasomotor responses. To test whether constitutive activation of α-adrenoreceptors modulates the ability of the dilatory signal to travel along arterioles, observations were made under control conditions and again in the presence of 1 μM phentolamine in the superfusion solution.

Statistics

Data were analyzed using One- and two-way Repeated Measures Analysis of Variance (Prism 5, GraphPad Software; La Jolla, CA, USA). When significant F-ratios were obtained, post hoc comparisons were made using Bonferroni or Tukey tests. Summary data are expressed as mean ± S.E. Differences were accepted as statistically significant with $P < 0.05$.

Results

A total of 38 mice were used for the present experiments. Muscle preparations were included in these experiments only if arterioles demonstrated oxygen sensitivity (typically 5-10 µm reduction in diameter) and robust spontaneous vasomotor tone, confirmed at the end of each day's experiment by adding sodium nitroprusside (SNP, 100 µM) to the superfusate and recording maximal diameter at each of the observation sites along the AA (Table 1).

Protocol 1

A representative trace of the time course of an arteriole undergoing ROV is shown in Figure 2A. Arterioles dilated within 1 second of muscle contraction; i.e., within the time required to refocus the vessel edges following tissue displacement. Increasing the duration of a tetanic contraction increased the magnitude of dilation of ROV (Figure 2B; $P < 0.05$) confirming recent findings in the GM (Jackson *et al.*, 2010).

Protocol 2

For each GM preparation, visual inspection confirmed that stimulation of the IGN produced contraction in the inferior muscle region while the superior muscle region remained quiescent. As summarized in Figure 3, robust ROV occurred along AA segments located within the active region of the GM (sites “a” and “b”). In contrast, there was little change in AA diameter within the inactive muscle region (sites “c” and “d”). Thus there was a significant difference in AA behaviour between active and inactive muscle regions ($P<0.05$). Resting and maximum diameters at sites “a-d” are given in Table 1 and demonstrate robust spontaneous vasomotor tone and thus the ability of all sites along the AA to undergo dilation.

Subsequent experiments tested for directionality to the spread of vasodilation beyond the inferior muscle region. Stimulation of the IGN initiated ROV that ascended into the inferior gluteal FA but this dilation did not spread along the AA into the inactive (superior) muscle region (Figure 4). However, as shown in Figure 5, when the entire GM contracted in response to field stimulation, ROV occurred in arterioles within the superior muscle region (e.g., site “e” in Fig. 1).

Protocol 3

To test whether the spread of ROV from active into inactive muscle regions could be affected by αAR, responses to contraction of the inferior muscle region were measured along AA at sites “a-d” before and during inhibition of α-adrenoreceptors. Remarkably,

with no effect on resting diameter, the magnitude of ROV increased ($P < 0.05$) along AA in the presence of phentolamine, particularly within the inactive muscle region (Fig. 6). In contrast, phentolamine did not enable dilation at site "e" in response to contraction of the inferior muscle region (Fig. 5). Resting and maximum diameters respective observation sites are given in Table 1.

Protocol 4

As an independent test of the ability a dilatory signal to travel along the AA, ACh was delivered from a micropipette near the proximal edge of the inferior feed artery (Fig. 1). Robust dilations were recorded at the site of stimulus delivery and at site "a" (Fig. 7). Dilation of the AA was also recorded within the superior muscle region at the site furthest from the ACh stimulus (site "d" in Fig. 1) but was diminished in amplitude relative to responses at site "a" (5 ± 2 vs. 16 ± 1 μm, respectively; $P < 0.01$). Consistent with the affect of αAR inhibition on the spread of ROV along AA, remote dilation at site (d) in the superior muscle region more than doubled during blockade of α-adrenoreceptors (Figure 7).

Discussion

The increase in blood flow to active skeletal muscle is distributed according to patterns of motor unit recruitment but how such regionality in blood flow control may be governed within the microcirculation is poorly understood. The present study has investigated this relationship by observing arterioles controlling blood flow to the

gluteus maximus muscle, a parallel-fibered muscle comprised of ~70% type IIB, 13% type IIA and 17% type I fibers (Lampa *et al.*, 2004). This powerful hip extensor consists of inferior and superior regions, each with a distinct motor nerve and vascular supply. Respective arteriolar networks are often interconnected by AA that span both muscle regions (*see Figure 1)*. Through observing ROV in response to brief tetanic contraction of the inferior GM via stimulating the IGN, a principal new finding of the present study is that dilation along AA was constrained to the inferior muscle region and its feed artery. However, following α-adrenoreceptor inhibition with phentolamine, ROV then spread along the AA from the inferior into the superior muscle region despite the latter remaining inactive. Arterioles within the superior region demonstrated ROV but only when their surrounding muscle fibers contracted. Complementary experiments using ACh microiontophoresis demonstrated that α-adrenoreceptor inhibition with phentolamine significantly enhanced the ability of a dilatory response to travel along AA from the inferior into the superior muscle region. These findings are the first to suggest that a constitutive level of α-adrenoreceptor activation can modulate the spatial domain of vasodilation and thereby direct blood flow to active regions within a skeletal muscle.

Motor unit recruitment and blood flow distribution

Previous studies of arteriolar responses to contractile activity in the GM have used field stimulation to activate the entire muscle (Bearden *et al.*, 2004b; Bearden, 2007; Jackson *et al.*, 2010). Control experiments demonstrate that under such conditions, vasodilation occurs in arterioles throughout the muscle (Figure 6). In contrast, isolation and

stimulation of the IGN enabled selective recruitment of functionally-defined motor units within the inferior muscle region, thereby enabling us to test whether vasodilation was constrained to the region of active muscle fiber and, if so, how the physiological regulation of vasodilation may occur along individual AA capable of increasing the supply of blood to both muscle regions. Our finding that arteriolar dilation occurs within the region of active muscle fiber is consistent with earlier studies in which muscle fiber bundles were activated using direct stimulation with a microelectrode positioned in the tissue (Gorczynski *et al.*, 1978; Marshall & Tandon, 1984). A limitation of such studies is that healthy motor units typically have muscle fiber dispersed through a much larger cross-sectional region of the muscle than that of their individual fibers (Burke & Tsairis, 1973; Fuglevand & Segal, 1997) in contrast to bundles of adjacent fibers. Nevertheless, as demonstrated for the mouse GM (Lampa *et al.*, 2004) and confirmed in the present study, groups of motor units can be localized to a particular muscle region or functional 'compartment'. Further, as shown in rats (Laughlin & Armstrong, 1982) and miniature swine (Armstrong *et al.*, 1987), the distribution of blood flow during treadmill running is consistent with regional differences in muscle fiber type and motor unit recruitment.

Adrenoreceptor activation and the spread of vasodilation

Functional sympatholysis describes the ability of contracting skeletal muscle to override sympathetic vasoconstriction evoked by SNA or the administration of α-adrenoreceptor agonists (Remensnyder *et al.*, 1962; Anderson & Faber, 1991; VanTeeffelen & Segal, 2003). In a reciprocal fashion, stimulating perivascular sympathetic nerves or exposure

to αAR agonists inhibits the spread of vasodilation along arterioles (Kurjiaka & Segal, 1995b) as well as feed arteries (Haug *et al.*, 2003; Haug & Segal, 2005b). In contrast to such overt activation of α-adrenoreceptors experimentally, the present experiments are the first to investigate whether the spread of vasodilation along arterioles can be affected by a constitutive, albeit subtle level of α-adrenoreceptors in the intact system. Moreover, this functional interaction was examined in light of a mechanism that may govern blood flow distribution within skeletal muscle in response to regional motor unit recruitment.

When stimulating the inferior GM through the IGN under control conditions, ROV was constrained to the active muscle region. Remarkably, inhibiting α-adrenoreceptors with phentolamine enabled ROV to spread along AA from the active muscle region into the quiescent (superior) muscle region (Figure 6). At the same time, other arterioles within the superior GM did not undergo ROV. However, when the entire muscle contracted in response to field stimulation, dilation of arterioles within the superior GM confirmed that they too were capable of ROV when their surrounding muscle fibers contracted (Figure 5). Collectively, these experiments suggest that constitutive activation of α-adrenoreceptors modulates the ability of dilation to travel along the arteriolar wall rather than the ability of arterioles to respond to muscle contraction. The lack of an effect of α-adrenoreceptor inhibition with phentolamine on resting arteriolar diameters (Fig. 6B) is consistent with previous reports (Marshall & Tandon, 1984; Jackson *et al.*,

2010). Despite no change in resting diameter, the blunted ROV seen in Old (20-month) male C57BL/6 mice was restored to levels seen in young (3-month) mice with phentolamine delivered in the same manner used in the present study (Jackson *et al.*, 2010). Moreover, exposing the GM of young mice to NE (1 nM) had no affect on resting diameter (1 nM) yet blunted ROV to the extent seen in old males (Jackson *et al.*, 2010). Thus subtle activation of α-adrenoreceptors (or inhibition thereof) can have significant effects on arteriolar reactivity without overt changes in the resting vasomotor tone. As shown in the present study, preventing the spread of dilation along the AA into inactive muscle indicates a subtle interplay between α-adrenoreceptors and motor unit recruitment that contributes to selective dilation to active muscle regions. These findings provide new insight with respect to how muscle blood flow can be preferentially distributed to active muscle regions during locomotion (Laughlin & Armstrong, 1982; Armstrong *et al.*, 1987).

We have recently reported that the inhibition of α-adrenoreceptors had no affect on ROV in male mice of similar age to those studied here (Jackson *et al.*, 2010). This finding is consistent with the present observations at site "a" (Figs. 1 and 6). Our previous study used field stimulation to contract the entire GM thus all arterioles were surrounded by active muscle fibers, as was the case for site "a". In contrast, while site "b" in the present study was also in the inferior muscle region, it was located adjacent to the inactive (superior) muscle region and may therefore not have received as great of a

dilator stimulus from active muscle fibers. Thus ROV at site "b" was enhanced during α-adrenoreceptor blockade. Nevertheless, the actions of phentolamine observed here were most apparent in the inactive region of the GM (Figures 1 and 6, sites "c" and "d"). Earlier studies in the rat spinotrapezius muscle reported that phentolamine did not enhance arteriolar dilation following tetanic contractions (Marshall & Tandon, 1984). However those experiments elicited maximal dilation which thereby precluded the possibility of enhancing vasodilation through experimental intervention. Thus the present data collectively suggest that the primary effect of α-adrenoreceptor blockade was to enable the dilatory signal to travel along the AA from its origin within the inferior muscle region.

Once initiated, the spread of vasodilation in response to muscle contraction appears to rely on cell-to-cell signaling events similar to those initiated by ACh, as disruption of the endothelium abolishes both responses (Segal & Jacobs, 2001). Perivascular sympathetic nerve stimulation or application of α-adrenoreceptor agonists inhibits the spread of vasodilation initiated by ACh (Kurjiaka & Segal, 1995b; Haug *et al.*, 2003; Haug & Segal, 2005) and the ability of vasodilation to ascend into feed arteries (VanTeeffelen & Segal, 2003). We therefore investigated whether constitutive α-adrenoreceptor activity could modulate the spread of vasodilation along AA initiated by stimuli other than muscle fiber contraction. As shown in Figure 7, the spread of vasodilation triggered by ACh microiontophoresis was also enhanced with phentolamine, thereby substantiating the

effect of phentolamine on the ability of ROV to spread from active to inactive muscle regions (Fig. 6). It should be recognized that conducted responses to ACh have typically been studied upstream from the site of stimulation but this was not possible in the GM due to anatomical constraints: Because AA are embedded in muscle fibers, reproducible responses to ACh delivery from a micropipette were only possible when the stimulus was delivered near the origin of the AA (Figure 1). Responses at remote sites to both stimuli were consistently enhanced by phentolamine. Therefore, it appears that, despite having negligible effect on vasomotor tone, constitutive levels of α-adrenoreceptor activation help to localize vasodilation to vessels supplying active muscle regions under physiological conditions. This conclusion in strengthened by observing that ROV encompassed the inferior gluteal feed artery (Fig. 4), implying that there is directionality in the spread of vasodilation so as to favor blood flow distribution to active muscle regions.

Summary

The present study demonstrates that regional vasodilation can be initiated within the mouse GM muscle through selective activation of motor units. Thus ROV is limited to arterioles within regions of contractile activity. While ROV is capable of ascending into proximal feed arteries supplying the active muscle region, it does not spread across AA into inactive muscle regions despite the ability of vasodilation to travel along the AA. Constraining dilation to the region of motor unit recruitment can be explained by subtle,

constitutive activation of α-adrenoreceptors that effectively restricts the spread of dilation. In turn, blockade of α-adrenoreceptors with phentolamine enhanced the magnitude of ROV within the active muscle region and enabled dilation to spread into inactive muscle regions. These findings support the idea that blood flow can preferentially increase to active motor units within a functionally compartmentalized muscle via complementary interaction between ROV and a constitutive level of α-adrenoreceptor activation *in vivo*.

Author Contributions

A.W. Moore, S.E. Bearden and S.S. Segal contributed to the conception, experimental design, analysis and interpretation of the experiments contained in this study. A.W. Moore performed all of the experiments. All co-authors have approved the version submitted to be considered for publication.

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	Observation Site					
Diameter (μm)	**FA**	**a**	**b**	**c**	**d**	**e**
Rest	52±3	31±2	28±2	13±1	16±1	19±2
Maximum	65±2	52±1	49±2	34±1	36±2	36±1

Table 2.1. Resting and maximum diameters at defined anatomical sites of the gluteus maximus muscle. Resting diameter measured during equilibration with 5% CO2 / 95% O2 in the superfusion solution. Maximum diameter measured during superfusion with SNP (100 μM). See Figure 1 for observation sites indicated by FA and a-e. For each site: n ≥ 7 independent observations.

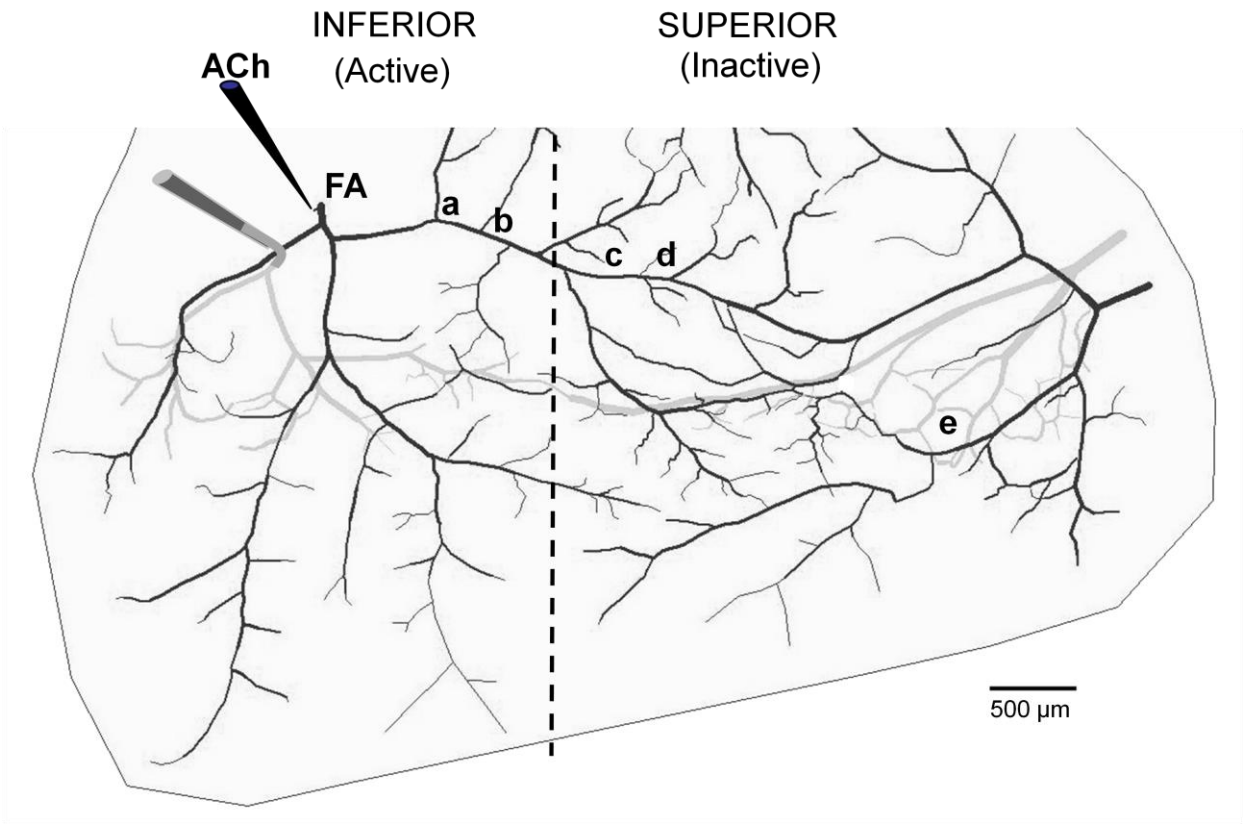

Figure 2.1. Motor innervation and arteriolar supply of the mouse gluteus maximus muscle preparation. Arterioles are labeled black and motor nerve branches are labeled gray (venules and capillaries omitted for clarity). Dashed line represents the functional border between Inferior (active) and Inferior (inactive) muscle regions of GM. Note anastomotic arteriole (AA) spanning respective regions and connecting respective arteriolar networks. Designations “a-d” represent observation sites along AA; “e” represents arteriole in superior muscle region not connected to AA. “FA" represents the feed artery external to the GM as referred to in Methods and Results. Suction microelectrode is shown holding motor nerve of inferior region. Micropipette for ACh microiontophoresis is shown with its tip positioned adjacent to site that the inferior gluteal feed artery enters the muscle.

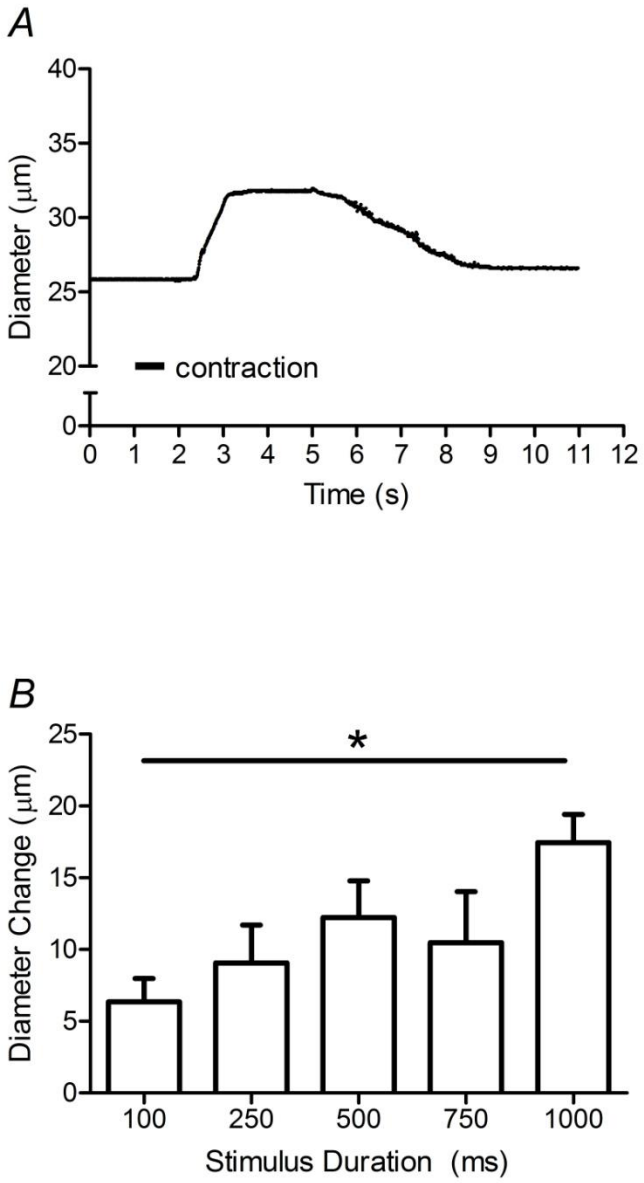

Figure 2.2. Characterization of ROV in response to inferior gluteal motor nerve stimulation. Single tetanic muscle contractions were evoked using 0.1 ms pulses delivered at 100 Hz, 30V for 100-1000 ms duration. Vessels dilated rapidly (<1 s) to a peak response within ~ 30 s. A**.** Representative tracing of arteriolar diameter response within active region to a 500 ms tetanic contraction. B: Diameter changes in second-order arterioles (2A). * Main effect of stimulus duration: $P < 0.01$. Resting diameter, 19 ± 2 μm; maximum diameter 42 ± 2 μm (n=5).

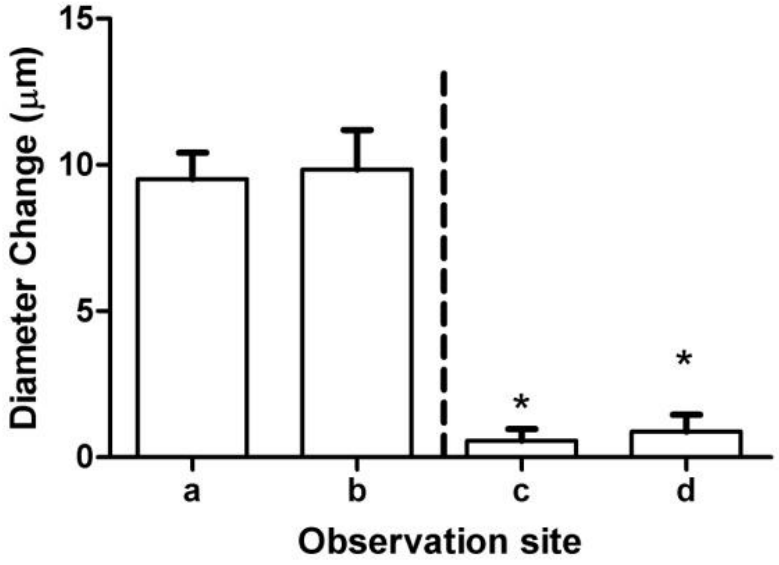

Figure 2.3. Vasodilation is confined to the active inferior muscle region. In response to a single tetanic contraction, the diameter change during ROV was pronounced along the AA within the active muscle region (sites a and b) despite negligible dilation within the inactive muscle region (sites c and d). The dashed vertical line represents the border between active and inactive regions Sites a-d are as shown in Figure 1. *Significantly different from site b, $P<0.01$ ($n=8$). See Table 1 for resting and maximum diameters.

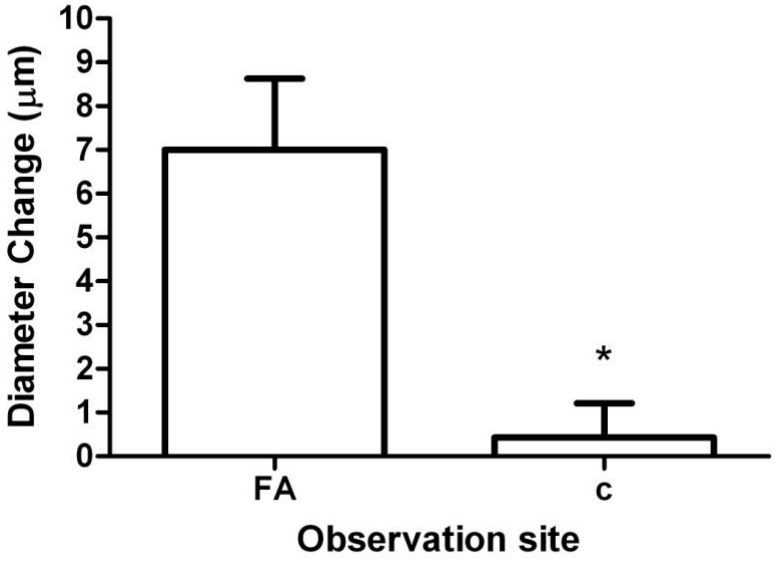

Figure 2.4. Spreading dilation external to active inferior muscle region is unidirectional. Spreading dilation was measured at equidistant sites beyond the edges of the active muscle region (~ 500 μm beyond respective muscle edges, see FA and site "c" in Fig. 1). In response to a single tetanic contraction of the inferior region of the GM, dilation ascended out of the active region into the inferior feed artery (FA), but not along the AA into the inactive (superior) muscle region. * Significantly different from upstream, $P<0.01$ (n=7).

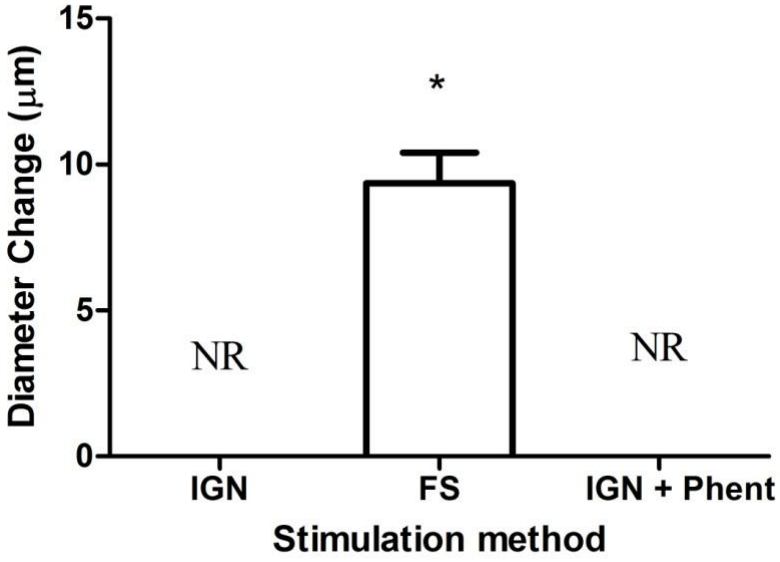

Figure 2.5. Arterioles of superior muscle region undergo ROV when their muscle fibers contract. Arterioles arising from the feed artery supplying the superior region of the GM (site "e" in Fig. 1) responded with ROV when the entire muscle was activated with field stimulation (FS) but not when the inferior muscle region was contracted selectively via stimulating the inferior gluteal nerve (IGN). Addition of phentolamine had no effect on arterioles at site "e" in response to IGN stimulation (IGN + Phent). *P<0.01, FS vs. IGN (n=5). NR, no response. Resting and maximal diameter of site "e" is given in Table 1.

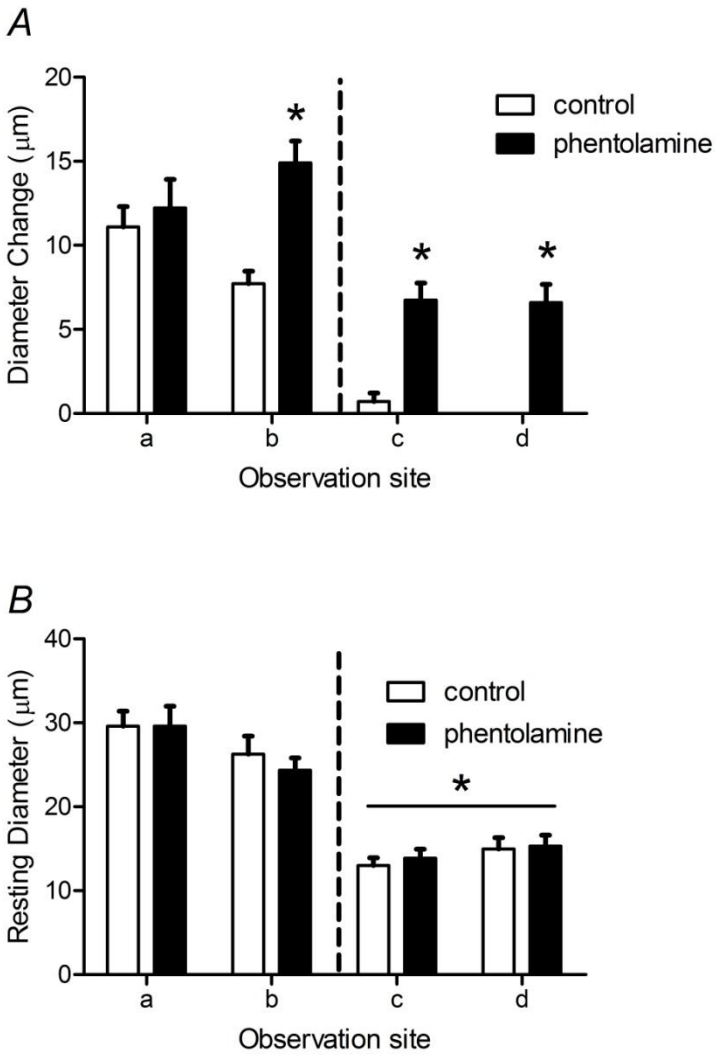

Figure 2.6. Phentolamine enhanced the spread of ROV without changing baseline diameter. The inhibition of α-adrenoreceptors promoted the spread of ROV along AA from active into inactive muscle regions. Dashed vertical line represents border between active and inactive muscle regions (see Figure 1). * P<0.05 vs. Control (n=7). **B:** Phentolamine had no significant effect on resting diameters at any of the defined sites along AA (n=7) * P<0.05 for sites c and d vs. sites a and b. See Table 1 for resting and maximal diameters.

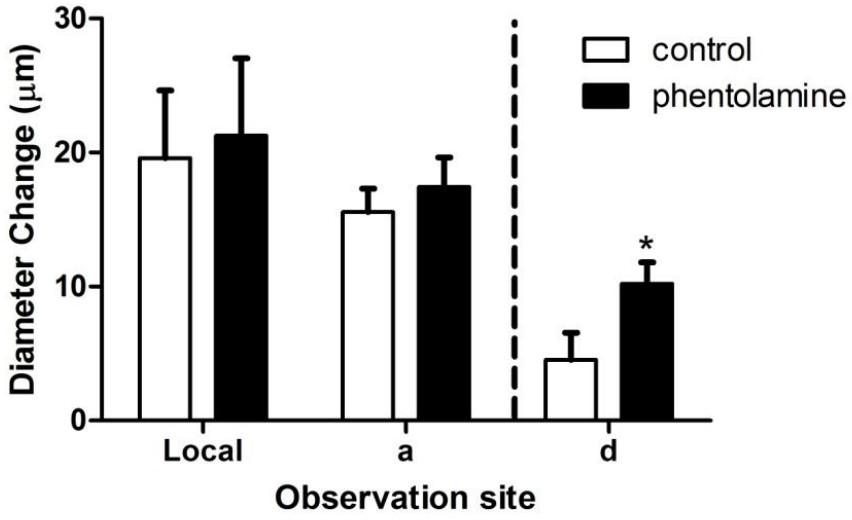

Figure 2.7. Phentolamine enhanced the spread of vasodilation along AA in response to ACh microiontophoresis. Acetylcholine was delivered as a brief pulse (500 ms, 1 μA) onto the inferior feed artery (see Fig. 1). A rapid vasodilation initiated locally traveled along the AA into the inactive (superior) muscle region. In the presence of phentolamine (1 μM), the magnitude of dilation at site "d" region increased. The dashed vertical line represents the border between inferior and superior muscle regions. *Significantly different from Control, $P < 0.01$ (n=7).

CHAPTER 3

REGIONAL HETEROGENEITY OF ADRENORECEPTOR SUBTYPES IN ARTERIOLAR NETWORKS OF SKELETAL MUSCLE

Contributing authors

A.W. Moore, W.F Jackson, S.S. Segal

Summary

Activation of vascular adrenoreceptors (ARs) governs the magnitude and distribution of muscle blood flow in accord with the distribution of AR subtypes. Functional studies in the rat cremaster muscle indicate that α_1AR predominate in proximal arterioles (first-order, 1A) while α_2AR predominate in distal arterioles (third-order, 3A). However, little is known of AR subtype distribution in arteriolar networks of locomotor skeletal muscles, particularly in the mouse. We tested the hypotheses that functional AR subtype exhibits heterogeneity among branches of arteriolar networks in a locomotor muscle and that the nature of this heterogeneity can vary between muscles having diverse functions. In anesthetized male C57BL/6J mice (3 mo), concentration-response curves (10^{-9} M – 10^{-5} M, 0.5 log increments) were evaluated in the gluteus maximus muscle superfused with physiological saline solution (35 °C, pH 7.4; n ≥ 5 per group). Norepinephrine (NE, nonselective αAR agonist) constricted 1A, 2A and 3A with similar potency and efficacy. Phenylephrine (PE; α_1 AR agonist) evoked greater ($p<0.05$) constriction in 3A (inhibited by 10^{-8} M prazosin; α_1 AR antagonist) while UK-14304 (UK; α_2AR agonist) evoked greater ($P<0.05$) constriction in 1A (inhibited by 10^{-7} M

rauwolscine; α_2AR antagonist). Isoproterenol (βAR agonist) dilated 1A, 2A, and 3A with similar potency and efficacy; these dilations were inhibited by 10^{-7} M propranolol (βAR antagonist) which otherwise had no effect on responses to NE, PE, or UK. Complementary experiments in the mouse cremaster muscle revealed a pattern of AR distribution that, while distinct from the GM, was consistent with that reported for the rat cremaster muscle We conclude that functional AR distribution in arteriolar networks of skeletal muscle varies with muscle function as well as vessel branch order.

Introduction

Adrenoreceptors (AR) on vascular smooth muscle play an important role in mediating sympathetic nervous activity through regulating arterial blood pressure and peripheral vascular resistance. Norepinephrine (NE) released from sympathetic nerves elicits vasoconstriction and restricts blood flow by activating post-junctional αAR on arteriolar vascular smooth muscle cells. The magnitude of vasoconstriction in response to sympathetic nerve stimulation varies among arterioles of differing size and branch order within the resistance network (Rosell, 1980; Marshall, 1982; Boegehold & Johnson, 1988; VanTeeffelen & Segal, 2003).

Adrenoreceptor subtypes have been demonstrated in resistance vessels of the mouse, rat, dog, and human (Medgett & Langer, 1984; Faber, 1988; Aaker & Laughlin, 2002; Wray *et al.*, 2004; Lambert & Thomas, 2005). Comprehensive studies of the functional distribution of AR subtypes in arteriolar networks (i.e., to mediate the actions of

norepinephrine and its analogs) have focused on the cremaster muscle in male rats (Faber, 1988; Anderson & Faber, 1991; McGillivray-Anderson & Faber, 1991; Ohyanagi *et al.*, 1991). For proximal (first-order, 1A) arterioles of the cremaster muscle, activating α_1AR caused relatively greater constriction compared to activating α_2AR. In contrast, for distal (third-order, 3A) arterioles, α_2AR activation caused greater constriction than did α_1AR activation (Faber, 1988).

When compared to proximal arterioles, the distal branches of arteriolar networks appear more responsive in constricting during increases in sympathetic nerve activity. Further, distal branches exhibit greater escape from sympathetic vasoconstriction in resting muscle and more readily undergo functional sympatholysis during skeletal muscle contraction (Anderson & Faber, 1991; VanTeeffelen & Segal, 2003). Such differential responses between arteriolar branch orders have been attributed to corresponding differences in the susceptibility of specific AR subtypes in the resistance vasculature to the actions of metabolic factors. Findings suggest that relative to α_1AR-induced constriction, α_2AR-induced constriction is more susceptible to being inhibited by metabolic activity associated with muscle contraction as well as during tissue hypoxia (McGillivray-Anderson & Faber, 1990; Anderson & Faber, 1991; Aaker & Laughlin, 2002; Wray *et al.*, 2004; Lambert & Thomas, 2005).

In the context of blood flow control, differential AR distribution has been used to explain why distal arterioles more readily undergo functional sympatholysis during

muscle contraction when compared to proximal arterioles. Thus dilation of distal arterioles effectively maximizes capillary perfusion and the functional surface area for exchange between microvessels and parenchymal cells while proximal arterioles and feed arteries remain constricted (and restrict muscle blood flow) to maintain systemic perfusion pressure (Segal, 2000; Joyner & Thomas, 2003; Thomas & Segal, 2004). In contrast to producing locomotion, the cremaster muscle functions to support the testes and regulate its temperature. Thus, it is not clear whether the properties of functional AR distribution determined in the rat cremaster muscle (Faber, 1988) can be accurately extrapolated to skeletal muscles that are integral to locomotion.

Whereas α_1AR and α_2AR activation elicit vasoconstriction, βAR activation elicits vasodilation (Guimaraes *et al.*, 1993). In contrast to established roles for αARs, the function of βARs during exercise is largely unknown because previous studies of αAR function have typically blocked βAR function with propranolol (Medgett & Langer, 1984; Flavahan *et al.*, 1987; Faber, 1988) and have done so without ascertaining whether βARs affected responses to the activation of αARs. Thus, the extent to which βAR activation is capable of producing arteriolar dilation is poorly understood. Moreover, it remains to be determined whether blockade of βARs enhances vasoconstriction during the activation of αARs (Morris, 1994; Torp *et al.*, 2001).

In the GM of C57BL/6 mice, recent findings illustrate that subtle activation of αAR (i.e., below that causing any change in diameter) can blunt arteriolar dilation to brief tetanic

contractions and restrict muscle blood flow at rest as well as during exercise (Jackson *et al.*, 2010). However, as holds for other locomotor muscles, the functional distribution AR subtypes in the GM is unknown. In the present study, our purpose was to determine whether AR subtypes vary with arteriolar branch order in a representative locomotor muscle in the C57BL/6 mouse. Using intravital microscopy to study arteriolar networks in the GM, we tested the null hypothesis that activation of α_1AR and α_2AR would produce similar effects in 1A, 2A and 3A. Complementary experiments determined whether the functional distribution of α_1AR and α_2AR in GM arterioles differed from those in the mouse cremaster muscle. As the effect of βAR activation in arteriolar networks of skeletal muscle has received little attention and not been investigated in the mouse, we determined whether βAR were functional in the microcirculation of the GM and whether βAR inhibition affected arteriolar responses to αAR activation.

Methods

Animal care and use

All procedures were approved by the Animal Care and Use Committee of the University of Missouri and performed in accord with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Male C57BL/6 mice (3-5 months old; 25-30g) were obtained from Jackson Laboratories (Bar Harbor, ME, USA) and housed at 20-23 °C on a 12 h - 12 h light - dark cycle with food and water provided *ad libitum*. At the end of experimental procedures, anesthetized mice were euthanized with an overdose of pentobarbital (intraperitoneal injection) and cervical dislocation.

Surgical preparation of GM and Cremaster muscles

A mouse was anesthetized with pentobarbital sodium (intraperitoneal injection, 60 mg/kg; supplemented as needed to prevent withdrawal from toe pinch). Hair was shaved from the surgical region. Esophageal temperature was maintained at 37-38 ºC by placing the mouse on an electrically-heated aluminum warming plate that was positioned on an acrylic platform. One muscle was studied per animal. Exposed muscles were superfused continuously (~3 ml/min) with a bicarbonate-buffered physiological salt solution (PSS; 34-35°C, pH 7.4) of the following composition (in mM): 131.9 NaCl, 4.7 KCl, 2 $CaCl_2$, 1.17 $MgSO_4$ and 18 $NaHCO_3$ equilibrated with 5% CO_2 / 95% N_2.

Gluteus maximus muscle. The left GM was prepared by removing overlying skin and connective tissue, separating the muscle from the spine along its origin, and reflecting it away from the body while maintaining its insertion on the femur. The muscle was spread over a transparent pedestal (Sylgard® 184; Dow Corning, Midland, MI, USA) and pinned at the edges to approximate in situ dimensions (Jackson et al 2010; Bearden et al 2004).

Cremaster muscle. The left cremaster muscle was prepared by making a midline incision ventral surface of the scrotal sac, carefully separating the muscle from surrounding connective tissue, and then opening it along the ventral axis. An orchiectomy was performed, the cremaster muscle was spread over a transparent pedestal (Sylgard® 184) and pinned at the edges to approximate in situ dimensions (Hungerford et al, 2000).

Intravital microscopy

The completed experimental preparation was transferred to the fixed stage (Burleigh® Gibraltar®; Mississauga, Ontario, Canada) of an intravital microscope (Nikon E600FN; Melville, NY, USA) and equilibrated for at least 30 min prior to beginning a protocol. The microscope was mounted on an X-Y translational platform (Burleigh® Gibraltar®) enabling to be moved to different observation sites without disturbing the preparation. Images were acquired using a 20X objective (numerical aperture, 0.35) using Köhler illumination from a long-working-distance condenser (numerical aperture, 0.52). Images were digitized with a charge-coupled device video camera (KP-D50; Hitachi Denshi; Japan) and observed on a digital monitor (ViewEra V191HV; Walnut, CA, USA) at a total magnification of 1300X. A video caliper (Microcirculation Research Institute; Texas A&M University; College Station, TX, USA) calibrated to a stage micrometer (100 X 0.01 = 1 mm: Graticules Ltd., Tonbridge, Kent, UK) was used to measure internal vessel diameter (spatial resolution ≤ 1 μm), which was defined as the widest distance between luminal edges. Output from the caliper was sampled at 40 Hz using a Powerlab/400 system (AD Instruments; Colorado Springs, CO, USA) coupled to a personal computer.

To the extent possible, arteriolar networks selected for study were standardized across preparations. During the 30-min equilibration period, arteriolar branch orders were classified as follows. First-order (1A): The most proximal branch completely embedded within striated muscle fibers; second-order (2A): Originating from the 1A; Third-order

(3A): originating from the 2A. A single 1A, 2A, and 3A segment of a given arteriolar network was studied in each mouse with respective observation sites maintained throughout the experiment by reference to anatomical landmarks. Each observation site was at least 150 μm downstream from the origin of the arteriolar branch from its parent vessel. In accord with the final magnification, each branch was observed individually by shifting the field of view during diameter measurements.

Administration of agonists and antagonists

Agonists were administered topically via addition to the superfusion solution and expressed as final concentrations in the superfusate. Adrenoreceptor agonists and antagonists were prepared fresh on the morning of an experiment. The superfusion solution was delivered by gravity feed onto the preparation from a 60-ml chamber consisting of a vertically-mounted syringe secured within an aluminum heater block (SW-60, Warmer Instruments; Hamden, CT USA). Chamber volume was maintained by gravity feed from a 500-ml reservoir. For cumulative concentration-response curves to AR agonists, inflow from the reservoir was shut to fix the volume of solution within the chamber. The appropriate volume of stock concentration of agonist or antagonist was added to achieve the desired final concentration; bubbling of gas within the chamber ensured rapid mixing. Stock solutions were prepared at concentrations ensuring that the volume added was always less than 1% of the chamber volume. Arteriolar diameters were recorded and inflow from the reservoir was then restored. Once the original volume in the chamber was attained, inflow from the reservoir was shut and the next

concentration of the agonist was administered. This procedure was repeated throughout concentration-response determinations. For treatment with antagonists, the desired concentration was prepared in the 500-ml reservoir supplying the superfusion chamber to which agonists were added. When different antagonists were used for a given experiment, each was prepared in its own reservoir. Drugs and chemicals and were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Experimental Protocols

The overall goal of these experiments was to determine the functional distribution of α_1ARs, α_2ARs and βARs in first-, second- and third-order arterioles of the mouse GM.

Vessel classification and criteria for study.

During a 30-min equilibration period, arteriolar branch orders were classified as follows. First-order (1A): The most proximal branch embedded within striated muscle fibers, with the 1A origin defined as the site where its feed artery entered the muscle; second-order (2A): Originating as the first major bifurcation from the 1A; Third-order (3A): originating as the first major bifurcation from the 2A. For each branch order, the observation site was at least 150 µm downstream of its origin. Respective sites were observed individually in each muscle by shifting the field of view with reference to anatomical landmarks. After the initial 30-min equilibration, arterioles were evaluated for sensitivity to oxygen as a sensitive index of tissue integrity by equilibrating the superfusion solution with 21% O_2 / 5% CO_2 (balance N_2) for 5 min. During this period, 2A

and 3A branches typically constricted by 5-10 µm. The superfusion solution was then re-equilibrated with 5% CO_2 / 95% N_2 for the remainder of the experimental protocol. At the end of experiments, maximum diameter of each site was determined during superfusion with 10^{-4} M sodium nitroprusside (SNP) Only preparations in which arterioles exhibited spontaneous vasomotor tone, constricted to elevated oxygen, and dilated robustly to SNP were included in the study. More than 90% of muscles preparations studied during the present experiments satisfied these criteria.

Protocol 1: Functional αAR distribution in the GM

We first evaluated the stability of AR activation in GM arterioles over the time course of a typical experiment (~ 3 hours). One group of mice was used to evaluate the nonselective αAR agonist NE. Another group of mice was used to evaluate the α_1AR-selective agonist PE and a third group to evaluate the α_2AR-selective agonist UK-14304. Each agonist was studied half-log increments (10^{-9} M – 10^{-5} M unless stated otherwise) by evaluating arteriolar diameters to cumulative increases in concentration. Each concentration of agonist was equilibrated for 3 min or until vessel diameters had reached stable values and the internal diameters of 1A, 2A and 3A were recorded (order randomized). After the final agonist concentration was evaluated, superfusion with control PSS was restored for ~10 min while arterioles recovered to their initial (post 30-min equilibration) baseline diameters. This entire procedure was repeated 4 times for each agonist. One agonist was studied in each GM preparation.

Protocol 2: Functional βAR distribution in the GM

While βAR in the peripheral vasculature are reported to promote vasodilation (Vanhoutte *et al.*, 1981; Guimaraes *et al.*, 1993), little is known of how βAR activation affects arteriolar diameter *in vivo*. Nor has it been determined whether concomitant inhibition of βAR affects vasomotor responses to αAR activation. Therefore the functional distribution of βAR in arterioles of the GM was assessed by evaluating 1A, 2A and 3A diameters during cumulative addition of the βAR agonist isoproterenol (ISO, 10^{-12} M – 10^{-6} M). In one group of mice, responses to ISO were reevaluated after equilibrating with propranolol (10^{-7} M; βAR antagonist) for at least 15 min. In 3 additional groups, we determined whether βAR inhibition with propranolol affected constrictions to NE, PE or UK. Only one αAR agonist was evaluated in each muscle preparation using this approach.

Protocol 3: Selectivity of αAR agonists and antagonists

To investigate the selectivity of functional αAR subtypes in arterioles of the mouse GM, we evaluate the selectivity of αAR antagonists on responses to selective αAR agonists. One agonist was evaluated in each mouse. Antagonists were equilibrated for ~15 min before testing for their effects.

Protocol 3A: Effect of αAR subtype antagonists on responses to α_1AR activation.

Responses to PE were first evaluated under control conditions. The agonist was removed and the preparation was superfused with control PSS for ~10 min. Responses to PE were then reevaluated in the presence of rauwolscine (10^{-7} M; α_2AR antagonist),

which was then removed and the preparation was superfused with control PSS for ~10 min. Responses to PE were then evaluated a final time in the presence of prazosin (10^{-8} M, α_1AR antagonist).

Protocol 3B: Effect of αAR subtype antagonists on responses to α_2AR activation.

Responses to UK-14304 were first evaluated under control conditions. The agonist was removed and the preparation was superfused with control PSS for ~10 min . Responses to UK-14304 were then reevaluated in the presence of prazosin (10^{-8} M), which was then removed and the preparation was superfused with control PSS for ~10 min. Responses to UK-14304 were then evaluated a final time in the presence of rauwolscine (10^{-7} M).

Protocol 4: Tissue specificity of αAR subtype distribution in arteriolar networks

Experiments were performed in the cremaster muscle of additional mice to determine whether functional αAR distribution of 1A, 2A and 3A varied between muscles. Diameter responses to NE were evaluated first. Following washout and recovery, responses to PE or UK were then evaluated (order randomized across experiments). Following washout and recovery, responses to the third agonist was evaluated.

Data presentation and statistical analysis

Data are presented as the measured diameter change (in μm) from resting baseline = $[(D_{rest} – D_{treatment})]$, as % maximal constriction = $[(D_{rest} – D_{treatment})/ D_{rest}]$ (where 100% corresponds to lumen closure), or as % maximal dilation = $[(D_{treatment} – D_{rest})/(D_{max}–$

D_{rest})]. The diameter following the initial 30-min equilibration was taken as D_{rest}. The diameter recorded at a given agonist concentration is designated $D_{treatment}$. The diameter recorded in the presence of 10^{-4} M SNP is designated D_{max}.

Data were analyzed using One- and Two-way Repeated Measures Analysis of Variance (Prism 5, GraphPad Software; La Jolla, CA, USA). When significant F-ratios were obtained, post hoc comparisons were made using Bonferroni or Tukey tests. Summary data are expressed as mean ± S.E. Differences were considered statistically significant at $P < 0.05$.

Results

A total of 69 mice were used in the present experiments. Resting and maximal diameters of 1A, 2A and 3A for the GM and cremaster muscles studied are given in Table 1.

Protocol 1: Functional αAR distribution in the GM

A comparison of relative responses to selective and nonselective αAR activation is presented in Figure 1. The concentration-response curves to respective agonists were stable over time (Figs. 8-10). Therefore, data from the 4 repeated determinations were averaged for each mouse. In response to NE (Fig. 1A), respective branch orders had similar % maximal peak constrictions (1A = 88 ± 7%, 2A = 97 ± 3%, 3A = 100 ± 0%). However 3A exhibited greater ($P<0.05$) responses than 1A or 2A between $3x10^{-8}$ M and

10^{-6} M. Respective log EC_{50} values were not significantly different (1A: -5.9 ± 0.1; 2A: -5.9 ± 0.1; 3A: 6.1±0.3). In response to PE (Fig. 1B), peak constrictions of 3A (66 ± 5% of maximal) were greater ($P<0.05$) than those of 1A (32 ± 3%) or 2A (47 ± 2%). However the actual changes in diameter were similar across branch orders (*see Fig. 3B*). In response to UK-1304 (Fig. 1C), constrictions were greater ($P<0.05$) for 1A (56 ± 3%) compared to 2A (31 ± 5%) or 3A (35 ± 6%). This pattern of response indicates greater functional activity of α_1AR in 3A and of α_2AR in 1A.

Because constrictions to PE or UK-14304 did not reach an apparent plateau, EC_{50} values could not be determined for these agonists. In preliminary studies, exposure to PE at a concentration > 10^{-6} M was found to disrupt reproducible responses to consecutive concentration-response curves, thereby establishing 10^{-6} M as the upper limit for these experiments.

Protocol 2: Functional βAR distribution in the GM

Each arteriolar branch order dilated in response to βAR activation with ISO (Fig. 2). Peak absolute changes in diameter for 1A, 2A and 3A were not significantly different (18 ± 4 μm, 18 ± 3 μm and 17 ± 2 μm, respectively). Despite differences in resting and maximal diameters between branch orders (Table 1), when responses to ISO were expressed relative to respective maximal dilations (i.e., diameter changes) obtained with SNP (Table 1) there were no significant differences in the efficacy of or sensitivity between branch orders. This similarity in behavior was reflected in respective log EC_{50} values (1A:

-8.7 ± 0.2; 2A: -8.8 ± 0.2; 3A: -9.2 ± 0.2). In the presence of propranolol (10^{-7} M), responses to ISO were shifted to the right by nearly 2 log-orders ($P<0.01$), confirming its effectiveness as an inhibitor of βAR (Fig. 11). Nevertheless βAR inhibition had no affect on responses to NE, PE, and UK-14304 in 1A, 2A or 3A (Fig. 3).

Protocol 3: Selectivity of αAR agonists and antagonists

The data for these experiments are presented for 1A, 2A and 3A in Figures 4, 5 and 6, respectively. In response to PE, constriction of 1A was unaffected by rauwolscine (Fig. 4B) but was inhibited by prazosin (Fig. 4A). In response to UK-14304, 1A constriction was effectively maintained (except for an attenuated response at 10^{-5} M) in the presence of prazosin (Fig. 4C) but was inhibited by rauwolscine (Fig 4D). For 2A, constriction in response to PE was unaffected by rauwolscine (Fig. 5B) but was inhibited by prazosin (Fig. 5A). In response to UK-14304, 2A constriction was preserved in the presence of prazosin (Fig. 5C) but was inhibited by rauwolscine (Fig 5D). For 3A, constriction in response to PE was effectively maintained in the presence of rauwolscine (except for an attenuated response at 10^{-6} M Fig. 6B) but was inhibited by prazosin (Fig. 6A). In response to UK-14304, 3A constriction was preserved in the presence of prazosin (Fig. 5C) but was inhibited by rauwolscine (Fig 6D). Collectively, these data support the use of prazosin (10^{-8} M) and rauwolscine (10^{-7} M) as selective antagonists of α_1ARs and α_2ARs, respectively, in arterioles of the mouse GM.

Protocol 4: Tissue specificity of αAR subtype distribution in arteriolar networks

Comprehensive studies of arteriolar responses to adrenergic agonists and antagonists in the rat cremaster muscle indicated that α1AR predominated in 1A and α2AR predominated in 3A (Faber, 1988; Anderson & Faber, 1991; Ohyanagi *et al.*, 1991). Because our results for the mouse GM indicate a different pattern of functional AR subtype distribution in respective arteriolar branch orders, we investigated responses (% maximal constrictions) to NE, PE, and UK-14304 in arterioles of the mouse cremaster muscle (Fig. 7). For 1A, constrictions to NE were consistently less ($P<0.05$) than those of 2A or 3A (Fig. 7A). Constrictions to PE appeared greatest in 3A and were significantly different from 1A and 2A ($P<0.05$) at 10^{-6} M PE (Fig. 7B). For UK-14304, 2A and 3A constricted progressively as agonist concentration increased with 3A exhibiting greater ($P<0.05$) responses than 2A (Fig. 7C). In contrast, 1A showed little response to UK-14304. This pattern of response in the cremaster muscle indicates greater functional activity of α_1AR in 1A and of α_2AR in 3A which contrasts with that determined here for the mouse GM (Figs. 1A and 1B) while being consistent with the pattern reported for the rat cremaster muscle (Faber, 1988; Anderson & Faber, 1991; Ohyanagi *et al.*, 1991).

Discussion

This is the first study to examine the functional distribution of α_1 and α_2 adrenoreceptors in multiple branch orders of a locomotor muscle in the mouse. We demonstrate that functional AR subtype distribution in the GM varies between branch orders. Our data suggest α_2AR reactivity is most prominent in proximal (1A) arterioles

while α_1AR reactivity is most prominent in distal (3A) arterioles. This pattern of distribution contrasts with that reported for the rat cremaster muscle, where α_1AR reactivity predominates in 1A and α_2AR reactivity predominates in 3A. Importantly, our findings in the mouse cremaster muscle coincide with the pattern of αAR subtype distribution reported in the rat cremaster muscle while differing from that of the mouse GM. Based on these results, we conclude that adrenoreceptor distribution is not uniform across branch orders of the GM, and results from the cremaster muscle cannot be extended to this locomotor muscle. These findings illustrate the importance of determining the pattern of adrenoreceptor distribution in the tissue under investigation. Whereas responses to combined or selective αAR were unaffected by βAR blockade, the functional distribution of βAR in GM arterioles appears uniform across branch orders. Upon activation, signaling events initiated by βAR produce maximal dilation of arterioles that is not different from that obtained with the NO donor SNP. These are the first data to demonstrate such efficacy of βAR-mediated dilation of arterioles controlling blood flow to skeletal muscle *in vivo*.

Experimental activation of αAR

In the peripheral vasculature, sympathetic nerves release neurotransmitters which act on post-junctional AR to initiate vasoconstriction. Experimentally, several methods have been employed to effect this response including direct perivascular nerve stimulation as well as pharmacological administration of AR agonists (Haug & Segal, 2005b). Perivascular sympathetic nerve activation can be accomplished by stimulation of ganglia

in the sympathetic chain (Fleming *et al.*, 1987; Boegehold & Johnson, 1988; Thomas *et al.*, 2001) or through electrodes positioned on the feed artery (Marshall, 1982; VanTeeffelen & Segal, 2003). However, pre-junctional inhibition of neurotransmitter release or co-release of other substances (e.g., ATP and NPY) may affect the pattern of post-junctional AR activation and vasoreactivity (Vanhoutte *et al.*, 1981). In the present study, we were concerned with post-junctional AR distribution on arterioles and sought to avoid possible confounding mechanisms associated with nerve activation. Pharmacological agonists can be administered through intravascular injection (Remensnyder *et al.*, 1962; Dodd & Johnson, 1993) or topical application in the superfusion solution (Faber, 1988; Dodd & Johnson, 1993; Haug *et al.*, 2003). We used the latter approach for the present experiments to minimize possible systemic effects of intravascular delivery. Topical application of NE mimics the constriction observed with perivascular nerve stimulation (Medgett & Langer, 1984; Dodd & Johnson, 1993; Haug *et al.*, 2003). Our time controls based upon repeated concentration-response curves confirm that AR sensitivity and efficacy were well-maintained over time (Fig. 8-10). Furthermore, in contrast to earlier studies using nerve stimulation (Marshall, 1982; Boegehold & Johnson, 1988; Dodd & Johnson, 1993; VanTeeffelen & Segal, 2003), arterioles observed here did not undergo 'escape'. Lack of escape during agonist administration may well be attributable to the constant application of drug through the superfusate instead of sympathetic nerve stimulation.

Effects of combined αAR activation

Because individual vessels within a resistance network may have differential sensitivities to NE (Marshall, 1982), it is of interest to ascertain the effects of combined α-AR activation on multiple branch orders within the mouse GM. Cumulative addition of NE into the superfusate produced robust constriction through activation of α_1 and α_2 AR in 1A, 2A and 3A of the mouse GM (Figs. 1A and 3A). In the GM, each arteriolar branch order had similar maximum responses and Log EC50 values to NE (Fig. 1). This behavior contrasts with our findings in the mouse cremaster muscle (Fig.7A) and with earlier studies of the rat spinotrapezius muscle (Marshall, 1982), where distal arterioles appeared to have greater sensitivity to NE. In addition, all branch orders in the GM underwent nearly complete closure at the highest concentrations of NE (Figs 1A and 3A), which was not the case for 1A in the cremaster (Fig. 7). The large vasoconstriction observed with NE and consistent, reproducible responses suggest that branch orders within the GM are highly sensitive to αAR activation and NE produces similar relative responses.

Effects of selective α-AR activation

Concentration-response experiments to NE established that αAR were expressed in 1A, 2A and 3A of the GM (Fig. 1C). However our goal was to determine the profile of functional αAR subtypes in respective arteriolar branch orders. Remarkably, selective activation of α_1AR and α_2AR resulted in different response characteristics in 1A, 2A and 3A (Fig. 1). The α_1AR agonist PE evoked similar absolute changes in diameter changes in

respective branches (Fig. 3B). Nevertheless, when these data were normalized to account for corresponding differences in arteriolar diameter, respective constrictions were significantly different with 3A > 2A > 1A (Fig. 1B). It therefore appears that functional α_1AR are distributed unevenly between arteriolar branch orders, with 3A being the most responsive to PE. In turn, α_2AR stimulation with UK-14304 evoked constrictions in 1A which were relatively greater than evoked in 2A or 3A. Indeed, 3A arterioles showed little constriction to α_2AR stimulation (Fig. 1C) with a 10% relative response corresponding to a diameter change of ~1 μm in these branches (Fig. 3C)**.** Finding that 3A constricted to closure in response to NE and to PE but not to UK-14304 supports our contention that α_2-adrenoreceptors are heterogeneously distributed among arteriolar branch orders of the mouse GM. Furthermore, finding that either PE or UK-14304 alone were less potent than NE suggests that neither α_1AR nor α_2AR-induced constriction are not as effective as combined α_1AR + α_2AR activation in constricting arterioles.

Effects of selective αAR antagonists on selective αAR activation

Effective use of αAR antagonists has been a powerful tool in resolving which AR subtype mediates specific physiological responses (Vanhoutte *et al.*, 1981; Timmermans *et al.*, 1987). In the GM preparation, it was unknown if blockade of one αAR subtype would increase or decrease the sensitivity of arterioles to the remaining αAR subtype. Moreover, high concentrations of antagonists (especially prazosin) have an affinity for both classes of αAR subtypes thus the inhibition of α_1AR may also depress responses to

α_2AR activation. To address this concern we performed comprehensive control experiments to directly test the selectivity of αAR subtype antagonists as used in the present study. The α_1AR antagonist prazosin (10^{-8} M) effectively inhibiting responses to PE (Figs. 4A, 5A, 6A) and was most pronounced in 3A (Fig. 6A), where responses to AR stimulation were mediated primarily through α_1AR (Fig. 1). Although prazosin reduced maximal constriction to UK-14304 in 1A slightly (Fig. 4B), it had no other significant effects on responses to UK-14304 (Figs. 4B, 5B and 6B). The α_2AR antagonist rauwolscine (10^{-7} M) produced the largest inhibition to UK-14304 in 1A where the potency of UK-14304 was greatest. It also inhibited responses in 3A where the sensitivity to and potency of UK-14304 was relatively low compared to 1A (Figs. 1C and 6D). Neither prazosin nor rauwolscine had significant effects on resting arteriolar diameters (data not shown). Thus if there were constitutive activation of αAR for arterioles in the GM at rest, it was at a level below that required to enhance spontaneous vasomotor tone. This conclusion is consistent with recent findings in the GM, where the nonselective AR antagonist phentolamine (10^{-6} M) also had no effect on resting arteriolar diameter (Jackson *et al.*, 2010). We conclude that, as used in the present study, prazosin and rauwolscine were selective inhibitors of respective αAR subtypes.

Regional differences in the functional distribution of αAR subtypes

The most comprehensive examination of functional αAR distribution in the microcirculation of skeletal muscle has come from studies in the rat cremaster muscle

(Faber, 1988; Anderson & Faber, 1991; McGillivray-Anderson & Faber, 1991; Ohyanagi *et al.*, 1991; Ohyanagi *et al.*, 1992). Findings from these experiments indicate that functional α_1AR predominate in 1A while functional α_2AR predominate in 3A of the rat cremaster muscle. Because our findings in the mouse GM indicated a reciprocal pattern of αAR subtype expression, we tested whether such properties were inherent to the GM or whether they were manifest in other vascular beds of the mouse. Our experiments in the mouse cremaster illustrate that while PE produces robust constriction of each branch order, its effects tend to predominate in 1A. In contrast, UK-14304 evoked the greatest constrictions of 3A in the cremaster muscle, with little effect on the diameter of 1A (Fig. 7C). Remarkably, α_2AR activation evoked the greatest constrictions in 1A of the GM (Fig. 1C). Our data from arterioles of the mouse cremaster muscle are therefore consistent with the αAR subtype distribution described for arterioles of the rat cremaster muscle (Faber, 1988; Anderson & Faber, 1991).

The present findings collectively indicate that αAR subtype distribution in arteriolar networks varies between the GM and the cremaster muscle in the mouse. While the GM is a representative locomotor muscle, the cremaster muscle serves to support and thermoregulate the testes in males. Although adrenergic sensitivity has been shown to vary between resistance vessels of different skeletal muscles (Hilton *et al.*, 1970; Gray, 1971; Laughlin & Armstrong, 1987), the reason for such variability between vascular beds is unclear. Studies in the rat cremaster muscle have indicated that the preponderance of α_2AR on 3A contributes to the ability of these vessels to escape from

sympathetic vasoconstriction in response to acidosis or metabolic demand (Anderson & Faber, 1991; McGillivray-Anderson & Faber, 1991). However our finding that functional α_1AR predominate in 3A of the GM contrasts with such inferences. Nevertheless, in light of significant differences in the pattern of functional αAR subtype distribution between the GM and cremaster muscle found in the present study, it is clear that findings based upon the cremaster muscle need not apply to skeletal muscles involved in locomotion.

Activation and inhibition of βARs

The present findings uniquely illustrate that stimulation of βAR with isoproterenol is capable of eliciting near-maximal dilation of arterioles in the GM and that it does so with similar efficacy and potency across branch orders (Fig. 2). Given such powerful capacity to relax arteriolar smooth muscle, we tested whether constitutive activation of βAR may influence constrictor responses to αAR activation. The selectivity of propranolol's actions was demonstrated by showing that it had no effect on responses to NE, PE, or UK-14304 (Fig. 3). Finding that propranolol (10^{-7}) effectively inhibited responses to isoproterenol confirms its selectivity for βAR (Fig. 11). Because propranolol had no effect on resting arteriolar diameters (data not shown) we conclude that constitutive βAR activity was not present under the conditions of our experiments. This conclusion is supported by earlier studies illustrating that that βAR were are not active during stimulation of αAR (Marshall, 1982; Morris, 1994). Nevertheless, the release and circulation of catecholamines can activate βAR (Guimaraes *et al.*, 1993) and investigators have used propranolol proactively to block these receptors without

evaluating their role in responses to adrenergic agonists (Medgett & Langer, 1984; Flavahan *et al.*, 1987; Faber, 1988). Demonstrating such a powerful dilatory response to βAR activation in arterioles of a locomotor muscle raises the question of its physiological role in the regulation of peripheral resistance. Addressing this role is ripe for future study.

Summary and conclusions

The location of AR subtypes and the control they exert over the resistance vasculature is integral to sympathetic control of muscle blood flow concomitant with the regulation of blood pressure, particularly during muscular exercise. The present study provides evidence that αAR subtypes are not uniformly distributed in arteriolar networks of the mouse GM. Third-order arterioles exhibited relatively greater responses to PE than UK-14304, indicating that functional α_1AR activation is able to evoke relatively greater constriction in distal arterioles when compared to that evoked by α_2AR. In contrast, 1A in the GM exhibited relatively greater responses to UK-14304 than to PE, indicating that α_2AR activation is able to evoke relatively greater constriction in proximal arterioles. Prazosin (10^{-8}M) effectively inhibited α_1AR while preserving the α_2AR responses. Rauwolscine (10^{-7}M) effectively inhibited α_2AR responses while preserving α_1AR responses. This pattern of functional αAR subtype distribution in arterioles of the GM differed from that we found in the cremaster muscle, where functional α_1AR activation predominates in 1A while functional α_2AR activation predominates in 3A. The functional distribution of βAR appears to be uniform across arteriolar branch orders as 1A, 2A and

3A responded similarly to isoproterenol with near-maximal dilations. Vasoconstriction to αAR activation was not affected by βAR inhibition as propranolol had no effect on arteriolar responses to NE, PE or UK-14304 while clearly inhibiting responses to isoproterenol. The consistency observed among our results obtained with agonists and antagonists targeted to AR subtypes confirms the selectivity of our pharmacological interventions.

Distal branches of the resistance network have long been recognized to more readily "escape" from sympathetic vasoconstriction (Mellander, 1960; Folkow *et al.*, 1971; Marshall, 1982). Whereas findings in the rat cremaster have associated such behavior with the prevalence of α_2AR, our present findings in the mouse GM suggest that such reasoning is not uniformly applicable across muscles and that other properties of more distal arterioles may contribute to sympathetic escape at rest or functional sympatholysis during exercise. When investigating the interaction between sympathetic neurotransmission and metabolic demand in skeletal muscle, the functional distribution of AR subtypes should be accounted for in the muscle of interest rather than drawing conclusions based upon AR subtype distribution in other preparations.

Author Contributions

A.W. Moore, W.F. Jackson and S.S. Segal contributed to the conception, experimental design, analysis and interpretation of the experiments contained in this study. A.W. Moore performed all of the experiments.. All co-authors have approved of the version submitted to be considered for publication.

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	Branch Order		
	1A	**2A**	**3A**
Gluteus maximus			
Resting (μm)	31 ± 1	20 ± 1	12 ± 1
Maximal (μm)	50 ± 1	39 ± 1	29 ± 1
Cremaster			
Resting (μm)	39 ± 1	22 ± 2	12 ± 1
Maximal (μm)	57 ± 4	39 ± 4	24 ± 3

Table 3.1. Arteriolar diameters in mouse gluteus maximus and cremaster muscles. Summary values (means ± S.E.) for internal diameters of arterioles *in vivo*. For each arteriolar branch order, resting values were recorded after the 30-min equilibration period following surgical preparation of the muscle. Maximal values recorded at the end of experiments in the presence of 10^{-4} M SNP. Data for GM arterioles are from 60 mice. Data for cremaster muscle are from 9 mice.

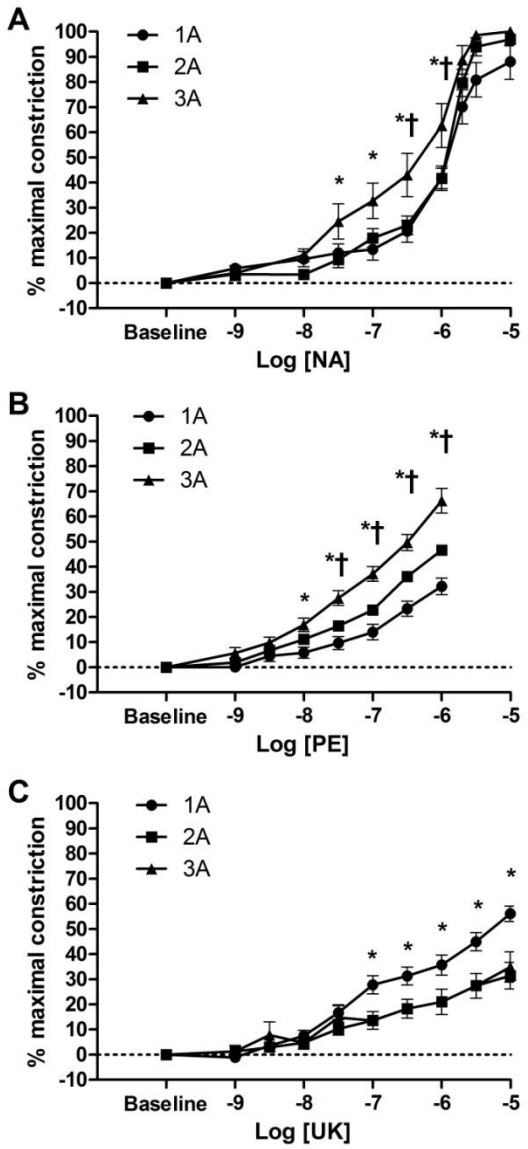

Figure 3.1. Arteriolar constriction to αAR activation in the mouse GM. A. Nonselective activation of αAR with NE produced similar % maximal constriction across arteriolar branch orders but 3A were more sensitive than 1A and 2A between $3x10^{-8}$ and 10^{-6} M. * P<0.05, 3A different from 1A. † P<0.05, 3A different from 2A (n= 11 for 1A and 2A, n= 9 for 3A). **B.** Selective activation of α_1AR with PE constricted 3A relatively more than 1A or 2A. * P<0.01, 3A different from 1A. † P<0.01, 3A different from 2A (n=21 each). **C.** Selective activation of α_2AR with UK-14304 (UK) constricted 1A relatively more than 2A or 3A. * P<0.01, 1A significantly different from 2A, and 3A (n=20 each).

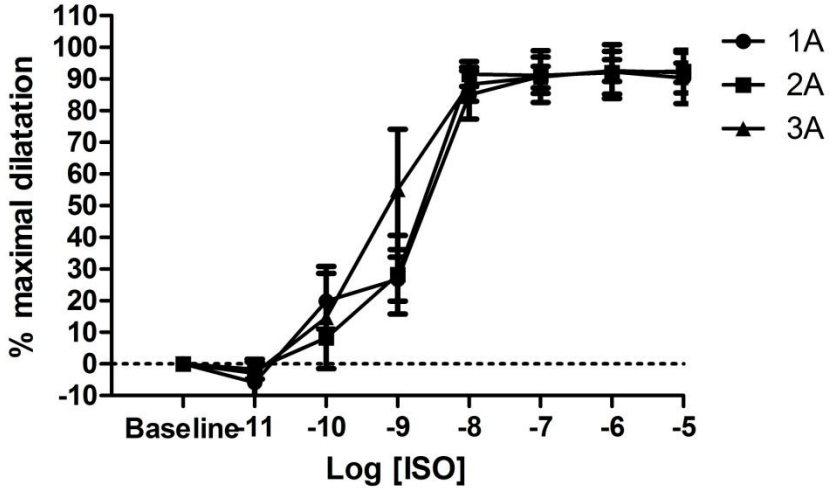

Figure 3.2. Arteriolar dilation with βAR activation in the mouse GM. Isoproterenol (ISO) dilated 1A, 2A, and 3A to 90 ± 8%, 92 ± 7%, and 92 ± 3% of the maximal dilation (diameter change in μm) obtained with 10^{-4} M SNP (refer to Table 1). There were no significant differences between arteriolar branch orders in response to ISO (n=6 arterioles for each branch order).

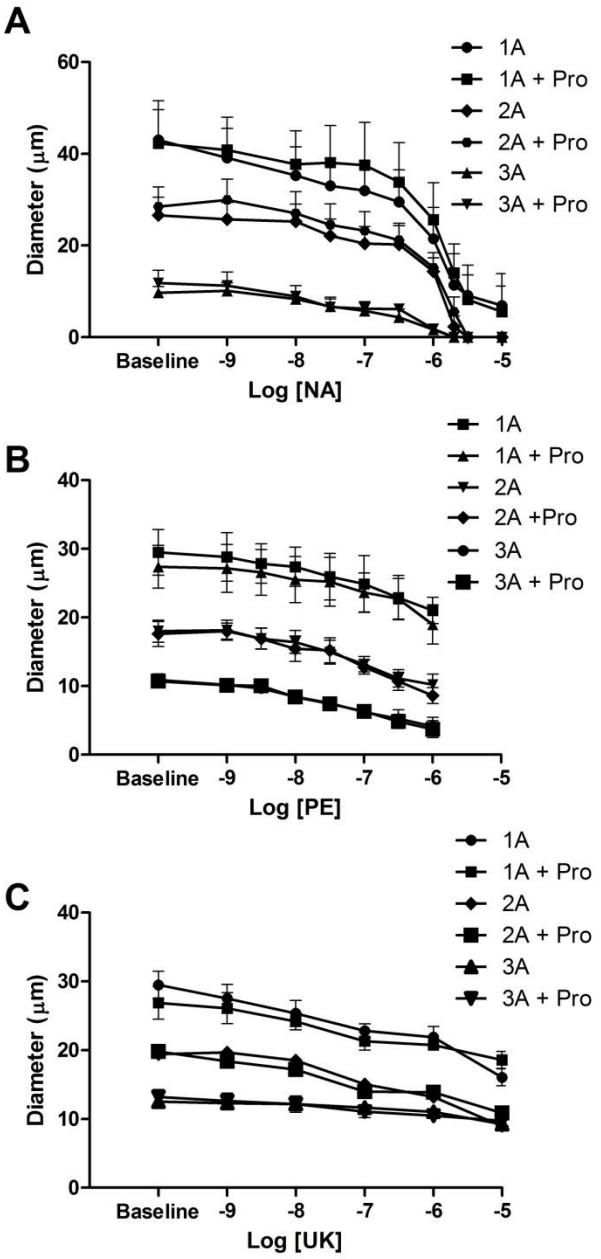

Figure 3.3. Inhibition of βAR does not alter responses to αAR agonists. Addition of propranolol (Pro, 10^{-7} M) had no significant effect on arteriolar constrictions to NE (**A**, n=4), PE (**B**, n=5), or UK-14304 (**C**, n=5).

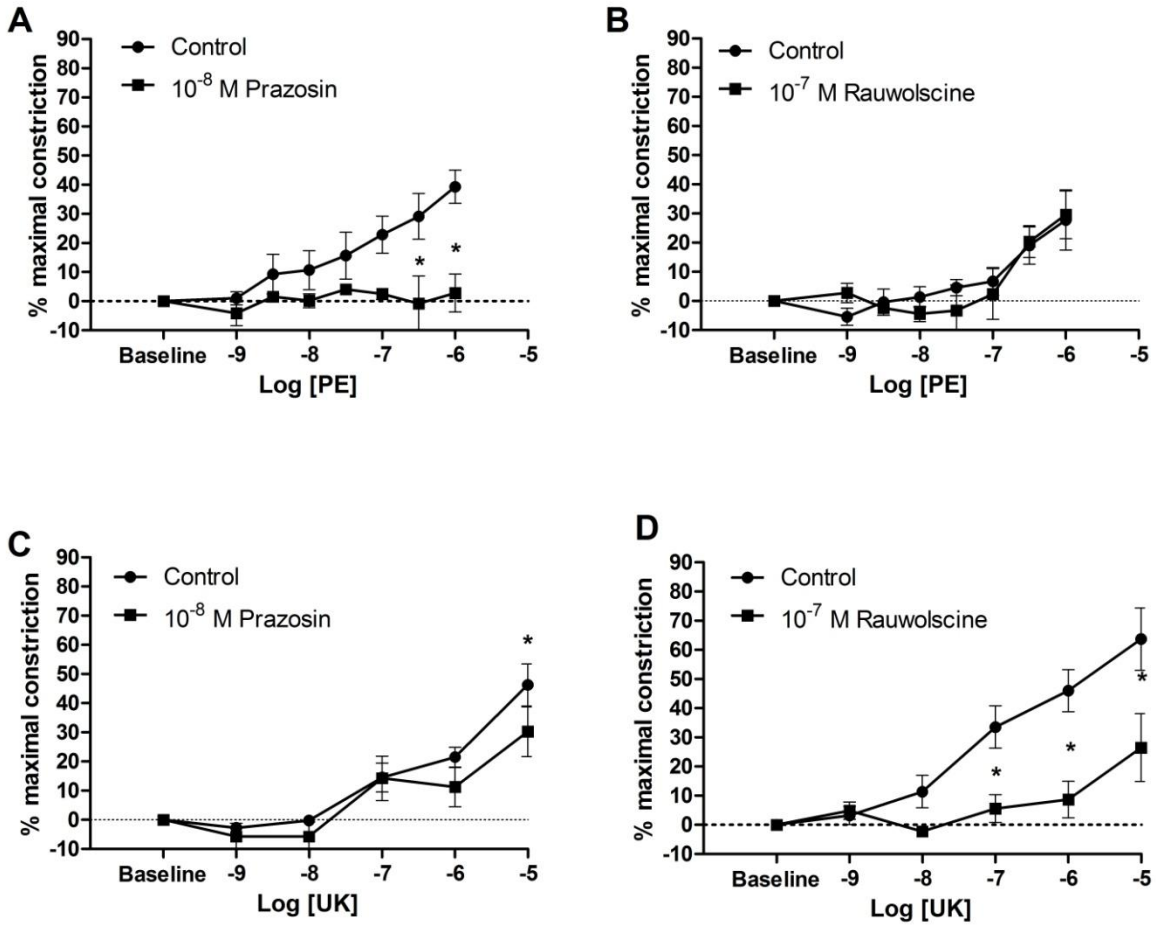

Figure 3.4. Activation and inhibition of α_1ARs and α_2ARs in 1A. A. Activating α_1AR with PE progressively increased vasoconstriction which was inhibited by prazosin (n=6). **B.** Rauwolscine had no significant affect on responses to PE (n=5). **C.** With the exception of an attenuated response at 10^{-5} M UK-14304, prazosin did not affect constrictions to α_2AR activation (n=5). **D.** Vasoconstriction to UK-14304 was inhibited by rauwolscine (n=6). *P<0.01 in **A**; P<0.05 in **C** and **D**.

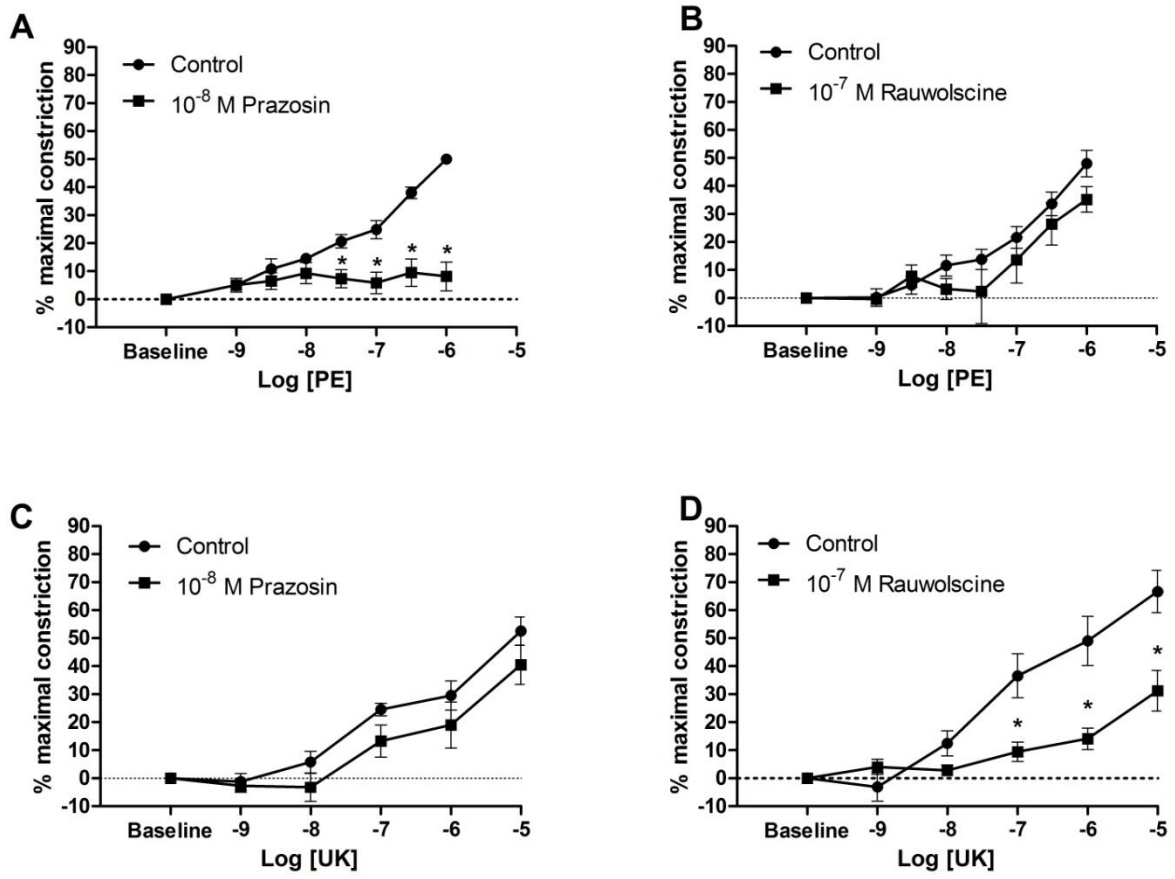

Figure 3.5. Activation and inhibition of α_1ARs and α_2ARs in 2A. A. Activating α_1AR with PE progressively increased vasoconstriction which was inhibited by prazosin (n=6). **B.** Responses to PE were preserved in the presence of rauwolscine (n=5). **C.** Responses to UK-14304 were preserved in the presence of prazosin (n=5). **D.** Vasoconstriction to UK-14304 was inhibited by rauwolscine (n=6). * P<0.01 in **A**; P<0.05 in **D**.

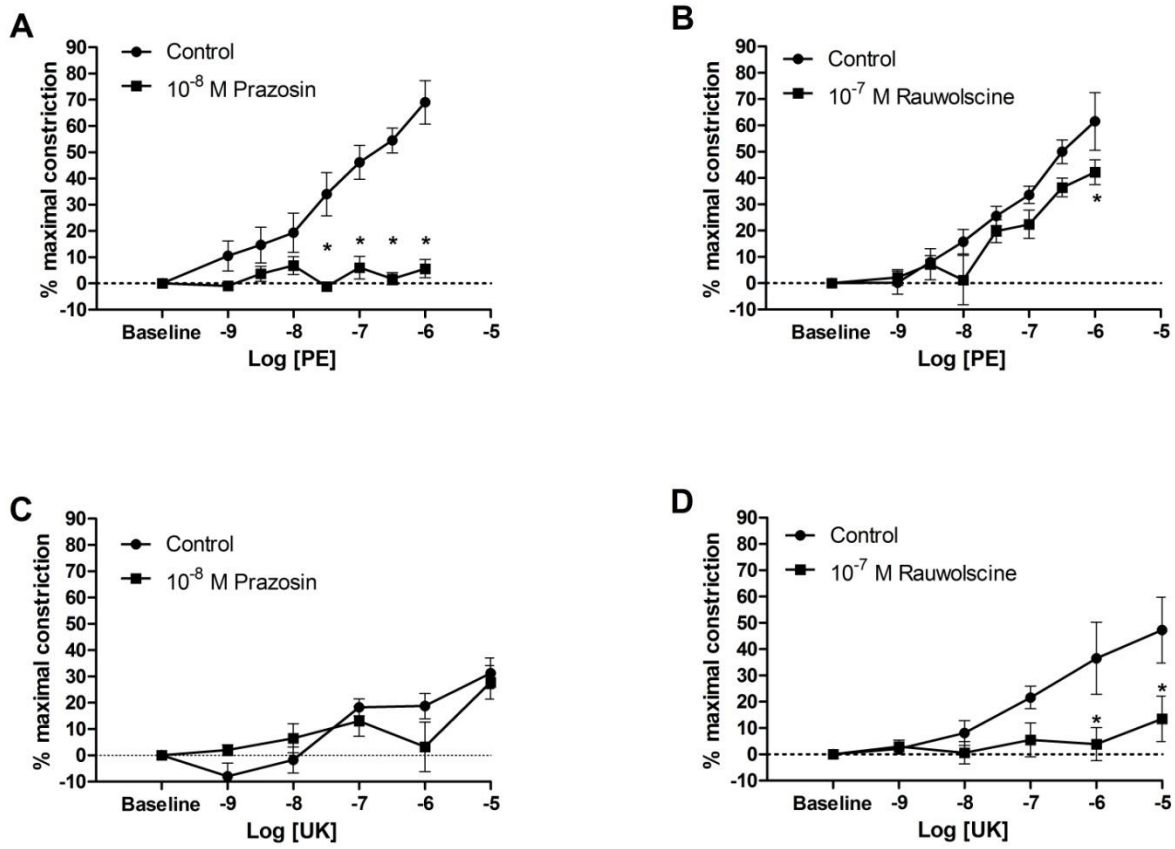

Figure 3.6. Activation and inhibition of α_1ARs and α_2ARs in 3A. **A**. Activating α_1AR with PE progressively increased vasoconstriction which was inhibited by prazosin (n=6). **B.** With the exception of an attenuated response at 10^{-6} M, responses to PE were preserved in the presence of rauwolscine (n=5). **C.** Responses to UK-14304 were preserved in the presence of prazosin (n=5). **D.** Vasoconstriction to UK-14304 was inhibited by rauwolscine (n=6). One 3A in this group constricted to closure. In all other mice, peak constriction of 3A was typically < 30% in response to UK-14304 (see Fig. 1C). * P<0.01 in **A**; P<0.05 in **B** and **D**.

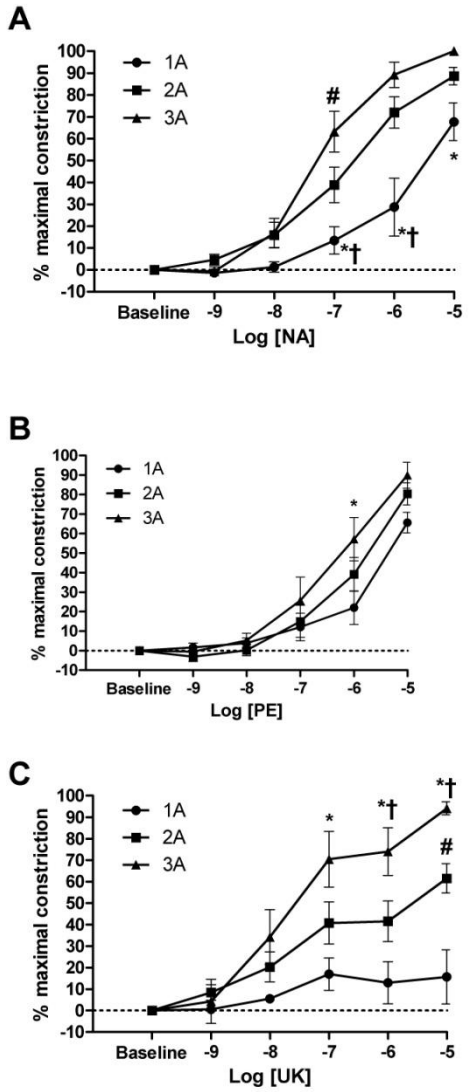

Figure 3.7: Effect of αAR agonists on arterioles in mouse cremaster muscle. A. Nonselective activation of αAR with NE produced variable levels of % maximal constriction across arteriolar branch orders with 3A more sensitive than 2A at 10^{-7} M and 1A less sensitive than 2A or 3A between 10^{-7} and 10^{-5} M. * P<0.05, 1A different from 3A. # P<0.05, 2A different from 3A. † P<0.05, 2A different from 1A. **B.** Selective activation of α_1AR with PE produced similar % maximal constriction across arteriolar branch orders but 3A were more sensitive than 1A at 10^{-6} M. * P<0.05, 3A different from 1A. **C.** Selective activation of α_2AR with UK-14304 constricted 3A relatively more than 1A or 2A. * P<0.05, 3A different from 1A. # P<0.05, 2A different than 1A. † P<0.05, 3A different from 2A. n=9 in all panels.

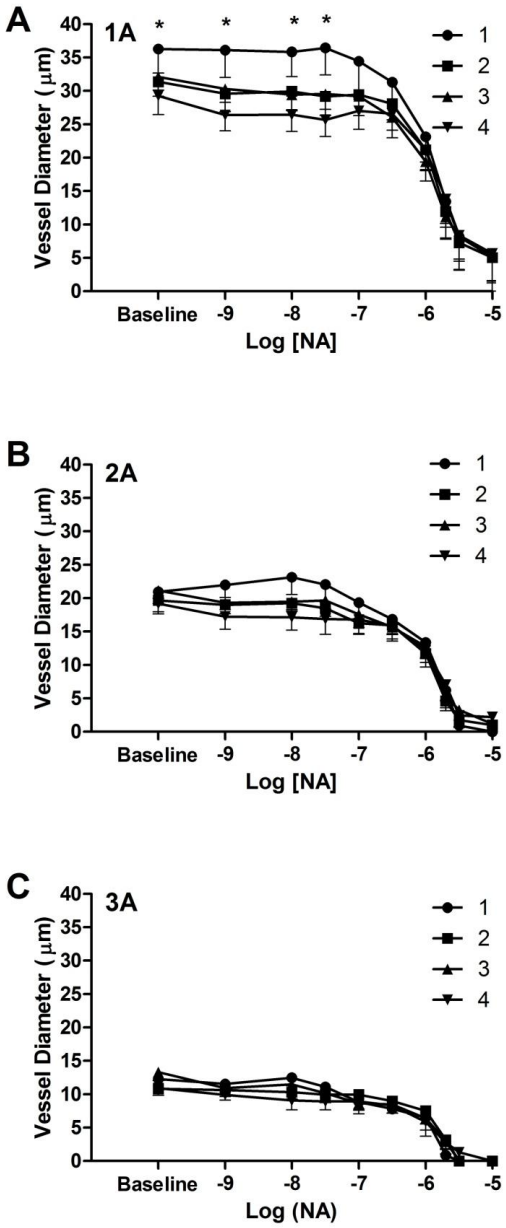

Figure 3.8. Arteriolar αAR sensitivity to NE is preserved over time. Designations 1, 2, 3 and 4 correspond to successive concentration-response evaluations as described for Protocol 1. **A.** For 1A there were no significant differences in maximal constriction or in log EC_{50} values (mean log EC_{50} = -5.9 ± 0.1). Resting tone increased slightly but significantly following the first concentration-response determination * P<0.05, 1 vs. 4. **B.** For 2A there were no significant differences in constriction to NE. **C.** For 3A there were no significant differences in constriction to NE. n = 7 in each panel.

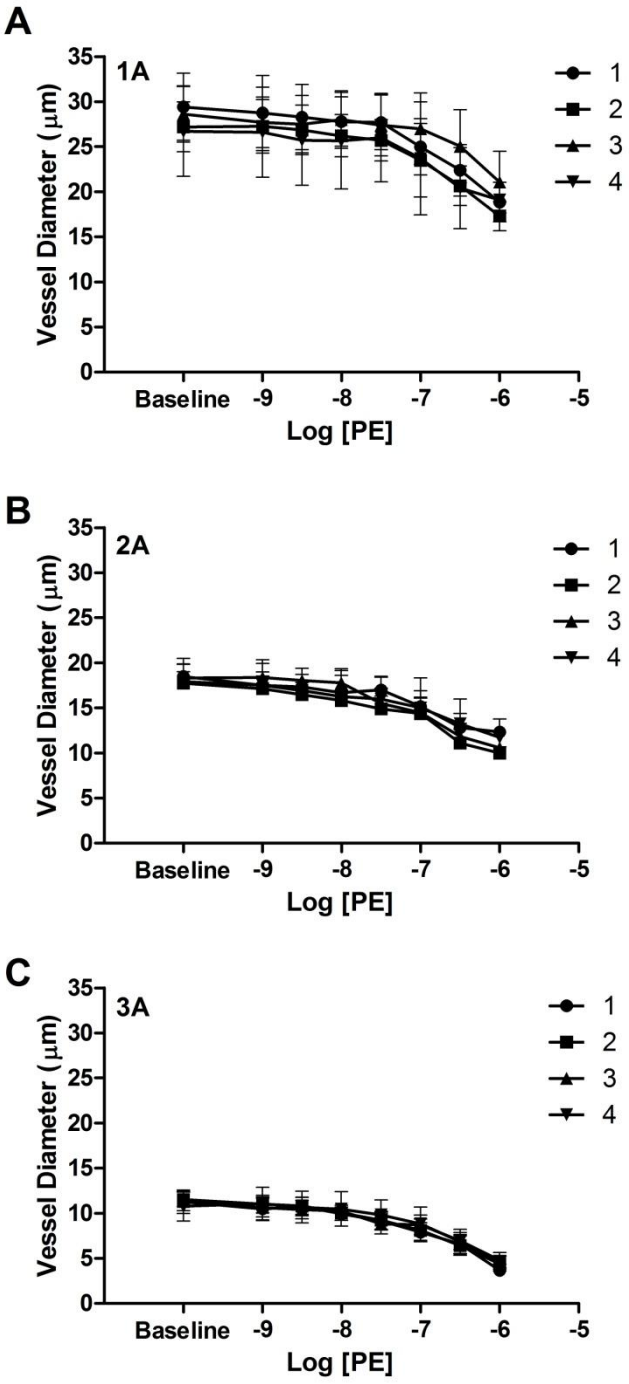

Figure 3.9. Arteriolar α_1AR sensitivity to PE is preserved over time. There were no significant differences in % maximal constriction between concentration-response evaluations in any arteriolar branch. **A**, 1A. **B**, 2A. **C**, 3A. n =5 in each panel.

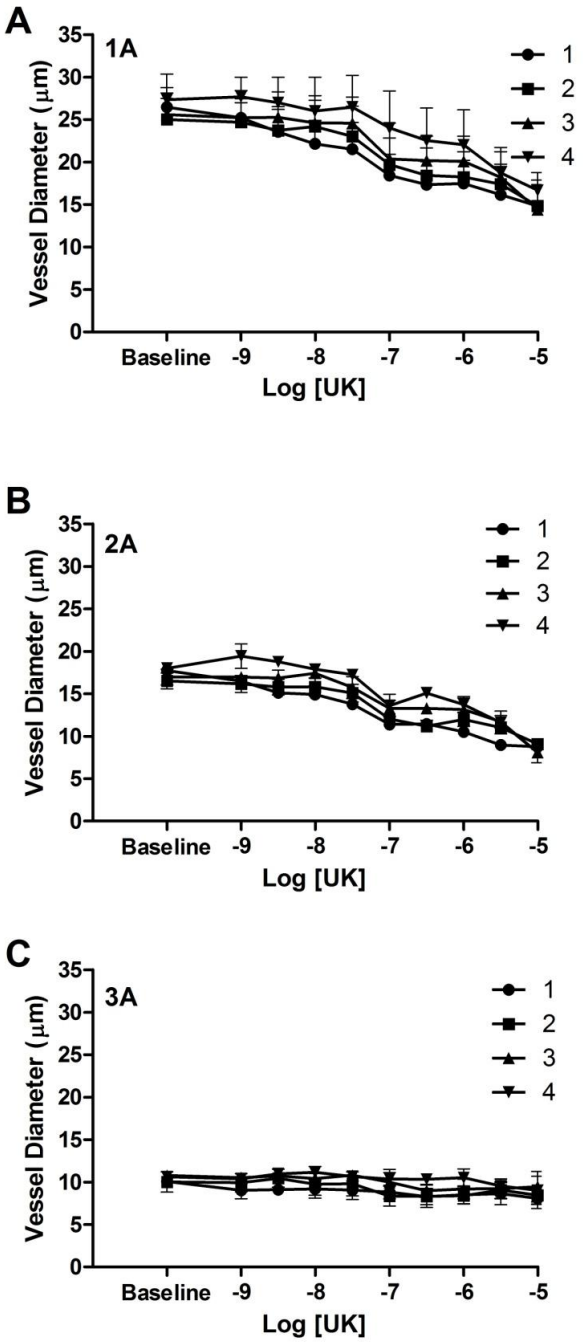

Figure 3.10. Arteriolar α_2AR sensitivity to UK-14304 is preserved over time. There were no significant differences in % maximal constriction between concentration-response evaluations in any arteriolar branch. **A**, 1A. **B**, 2A. **C**, 3A. n =5 in each panel. Note lack of constriction to UK-14304 in 3A (compare to panel C in Figs. 8 and 9).

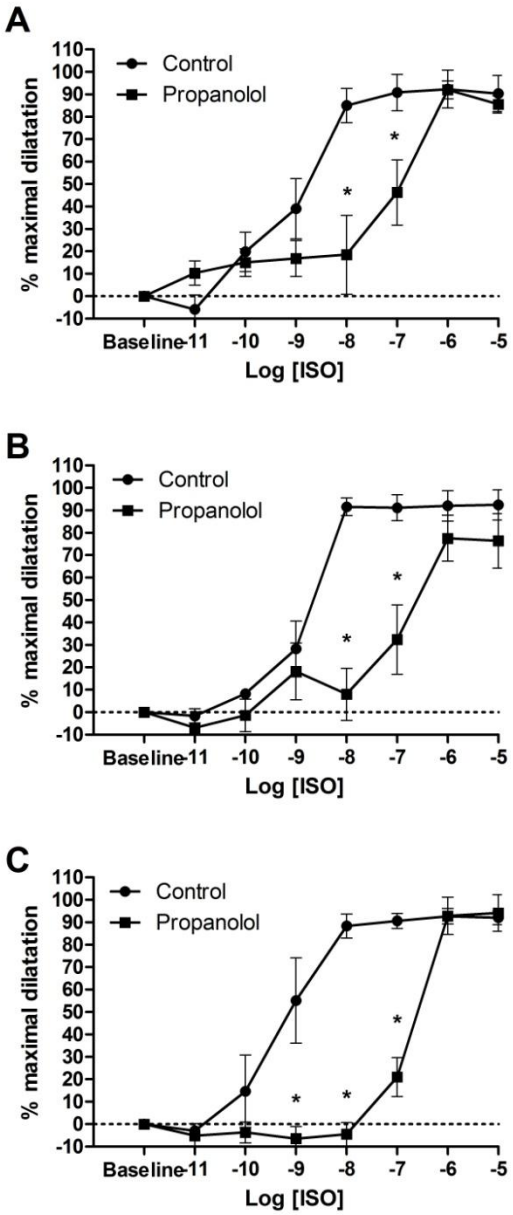

Figure 3.11: βAR activation produces dilation inhibited by propranolol. In each panel, 10^{-7} M propranolol shifted concentration-responses to isoproterenol rightward by ~2 log orders. * P<0.01, Control different from Propranolol. **A.** Responses in 1A. Log EC50 values: Control, -9.0±0.2; Propranolol, -7.0±0.2. **B.** Responses in 2A. Log EC50 values: Control, -8.8±0.2; Propranolol, -6.9±0.3 **C.** Responses in 3A. Log EC50 values: Control, -9.2±0.2; Propranolol, -6.8±0.3. n = 6 in each panels.

CHAPTER 4

CONCLUSIONS

This dissertation investigated the functional distribution of adrenoreceptor subtypes in arteriolar networks and the effects of adrenoreceptor activation on the spread of dilation in the microcirculation of skeletal muscle. The research findings encompassed three manuscripts based upon intravital studies of the microcirculation in the gluteus maximus muscle of anesthetized mice. The first manuscript encompassed the effects of constitutive α-adrenoreceptor activation on the spread of ROV within contracting muscle. The second manuscript encompassed the functional distribution of adrenoreceptors in locomotor muscle of the mouse. The third manuscript (included as the Appendix) focused on resolving the effects of α-adrenoreceptor activity on ROV in old mice. A key feature of these experiments is that they are based upon the gluteus maximus muscle and thereby provide novel insight with respect to the physiology and pharmacology of arteriolar networks in a locomotor muscle of the mouse.

Chapter 2 demonstrates that regional vasodilation can be initiated within the mouse GM muscle through selective activation of motor units. Thus ROV is limited to arterioles within regions of contractile activity. While ROV is capable of ascending into proximal feed arteries supplying the active muscle region, it does not spread across anastomotic arterioles into inactive muscle regions despite the ability of vasodilation to travel along

the anastomotic arteriole. Constraining dilation to the region of motor unit recruitment can be explained by subtle, constitutive activation of α-adrenoreceptors that effectively restricts the spread of dilation. In turn, blockade of α-adrenoreceptors with phentolamine enhanced the magnitude of ROV within the active muscle region and enabled dilation to spread into inactive muscle regions. Remarkably, this effect of α-adrenoreceptor inhibition occurred with no change in resting diameter, thereby indicating subtlety in adrenergic regulation of functional vasodilation that has not previously been recognized. These findings support the idea that blood flow can preferentially increase to active motor units within a functionally compartmentalized muscle via complementary interaction between ROV and a constitutive level of α-adrenoreceptor activation *in vivo*. Moreover, they illustrate the insight that can be gained by developing novel approaches to study the microcirculation in a limb muscle directly involved in locomotion.

Chapter 3 presents evidence that α-adrenoreceptors are distributed heterogeneously in the resistance vasculature of the mouse GM. Remarkably, this functional distribution differs from other preparations investigating adrenoreceptor distribution in the microcirculation, particularly the widely accepted findings based upon the rat cremaster muscle (Faber, 1988; Anderson & Faber, 1991; Ohyanagi *et al.*, 1991). Comprehensive control experiments illustrate the selectivity α1- and α2- adrenoreceptor activation and inhibition using pharmacological tools. Thus a low concentration of prazosin (10^{-8} M)

effectively inhibits α_1-adrenoreceptor to PE while preserving the α_2-adrenoreceptor responses to UK-14304. Rauwolscine (10^{-7} M) inhibited α_2-adrenoreceptor responses to UK-14304 while maintaining α_1-adrenoreceptor responses to PE. Finding that 3A exhibited greater responses to PE than to UK-14304 indicates that α_1-adrenoreceptors exert greater control in distal arterioles. Finding that 1A exhibited greater responses to UK14304 than PE indicates that α_2-adrenoreceptors exert greater control in proximal arterioles. These findings contrast with the findings based upon the rat cremaster muscle (Faber, 1988; Anderson & Faber, 1991; Ohyanagi *et al.*, 1991) which indicate that α_1-adrenoreceptor function predominates in 1A while α_2-adrenoreceptor function predominates in 3A. In turn, our findings from the mouse cremaster muscle confirm the adrenoreceptor distribution found in the rat cremaster muscle and thereby suggest that there are differences in functional adrenoreceptor distribution between muscles. These findings imply that studies investigating sympathetic nervous activity and functional sympatholysis should account for adrenoreceptor subtype distribution in the muscle of interest.

The functional distribution of β-adrenoreceptors appears to be uniform across arteriolar branch orders as all vessels studied responded equally to isoproterenol. Remarkably, the activation of β-adrenoreceptors with isoproterenol consistently produced maximal dilations that were not different from the actions of SNP. Vasoconstriction to α-adrenoreceptor activation does not appear to be affected by constitutive β-adrenoreceptor activity, as propranolol had no effect on responses to α-adrenoreceptor agonists though it effectively inhibited vasodilation to isoproterenol. Given the gaps that

remain in current understanding of signaling events underlying functional vasodilation, these new findings suggest that greater attention should be given to the role of β-adrenoreceptors.

To provide insight with respect to how the relationships investigated in Chapters 2 and 3 are affected by aging, the appendix provides evidence that aging is associated with microvascular dysfunction that is selective for male mice (as demonstrated in the C57BL/6 strain). Deleterious effects of aging on dynamic blood flow control in old males were highlighted by consistently blunted ROV in response to single tetanic contractions when compared to old females, young males, or young females. As blockade of α-adrenoreceptors with phentolamine restored ROV in old males and topical NE attenuated ROV in young males and females (all at concentrations that had no effect on resting diameter), we suggest that aging is associated with constitutively elevated levels of α-adrenoreceptor activation in resistance vessels of old males. Furthermore, arteriolar blood flow in old males was attenuated relative to young males at rest, during ROV, during steady state dilations to rhythmic twitch contractions and during maximal dilation of arterioles with topical SNP. As these effects were manifest despite no significant differences in systemic arterial blood pressure, resting or maximal arteriolar diameters between experimental groups, we propose that even without overt vasoconstriction or vascular remodeling, subtle levels of α-adrenoreceptor activation can effectively compromise rapid adjustments in tissue blood flow and could thereby

impair daily activities. Moreover, blunted red blood cell velocity with no difference in arteriolar diameters implicates proximal segments of the resistance network as key sites for restricting muscle blood flow with aging. In light of comprehensive human studies implicating a role for enhanced sympathetic vasoconstriction in limiting muscle blood flow, the present findings support the C57BL/6 mouse as a model system for studying how aging affects the microcirculation in mammalian skeletal muscle.

Future directions

In light of evidence for the role of the sympathetic nervous system in limiting physical performance with aging in humans, it is essential to understand how such actions are mediated in the microcirculation of skeletal muscle. Increased levels of constitutive α-adrenoreceptor activation during aging may be due to post-junctional changes in functional adrenoreceptor distribution or sensitivity. It is unknown if α-adrenoreceptor sensitivity of arterioles differs between young and old male C57BL/6 mice. Thus future studies should determine whether α-adrenoreceptor sensitivity is altered with aging and whether such changes are subtype-specific. A related question concerns why the effects of aging seen in male mice are not as apparent in female mice. Because α-adrenoreceptor activity modulates ROV, and because α-adrenoreceptor distribution is heterogeneous in the GM, the contribution of α_1- and α_2-adrenoreceptors to inhibition of ROV during aging is ripe for investigation. Based upon the present findings, appropriate combinations of α-adrenoreceptor agonists and antagonists can be applied

to determine the effects of selective adrenoreceptor blockade and activation in arteriolar branch orders of young and old male mice to provide such insight.

Final comments

At the onset of exercise, rapid onset vasodilation occurs to effectively match blood flow with increased metabolic demand. The primary effect of sympathetic nervous activity and adrenoreceptor activation during exercise is to promote vasoconstriction in inactive tissues and maintain systemic blood pressure. The present studies have demonstrated that subtle levels of α-adrenoreceptor activation play an additional, previously unrecognized role in contracting skeletal muscle: By limiting the spread of vasodilation to regions of active motor unit recruitment, constitutive activation of α-adrenoreceptors helps direct blood flow to active muscle fibers. In old males, constitutive α-adrenoreceptor activation appears to be detrimental, as it limits the amount of ROV and blood flow available to contracting muscle. Finding that the functional distribution of α-adrenoreceptors differs between muscles implies that studies of the interaction between skeletal muscle contraction and sympathetic control of the vasculature need to account for regional heterogeneity in distribution of α-adrenoreceptor subtypes. This conclusion applies to respective branches of resistance networks as well as to different muscles. Given the profound ability of β-adrenoreceptor activation to produce arteriolar dilation in vivo, this signaling pathway my warrant greater consideration in therapeutic strategies designed to improve tissue blood flow.

APPENDIX:

BLUNTING OF RAPID ONSEST VASODILATION AND BLOOD FLOW RESTRICTION IN ARTERIOLES OF EXERCISING SKELETAL MUSCLE WITH AGING IN MALE MICE

Preface

The work presented in the Appendix was initially designed and performed by Dr. Dwayne Jackson under the supervision of Dr. Steven Segal while at Yale University. This manuscript was submitted after Dr. Segal moved to the University of Missouri. Following peer-review, it became apparent that additional experiments were required to address the concerns of the reviewers and improve the overall impact of this study. Being familiar with the intricacies of the GM preparation, Alex Moore designed and performed the requisite control experiments, which supported and extended the initial findings. Alex Moore's contributions to this manuscript are found in Figure A.4 and the corresponding text. The data presented in the appendix serve as a backdrop for the experiments and data presented in Chapter 2 and 3.

Contributing authors

D.N. Jackson, A.W. Moore, S.S. Segal

Summary

Exercise capacity and skeletal muscle blood flow are diminished with aging but little is known of underlying changes in microvascular hemodynamics. Further, it is not clear how the sympathetic nervous system affects the microcirculation of skeletal muscle with aging or whether sex differences prevail in the regulation of arteriolar diameter in response to muscle contractions. In the gluteus maximus muscle of C57BL/6 mice, we tested the hypothesis that aging would impair "Rapid Onset Vasodilation" (ROV) in distributing arterioles (second-order, 2A) of Old (20-month) male (OM) and female (OF) relative to Young (3-month) males (YM) and females (YF). Neither resting (~18 μm) nor maximum (~30 μm) 2A diameters differed among groups. In response to single tetanic contractions at 100 Hz (duration, 100-1000 ms), ROV responses were blunted by half in OM relative to OF, YM or YF. With no effect in YM, blockade of α-adrenoreceptors with phentolamine (1 μM) restored ROV in OM. Topical norepinephrine (1 nM) blunted ROV in YM and YF to levels seen in OM and further suppressed ROV in OM ($P<0.05$). To evaluate arteriolar blood flow, red blood cell velocity was measured in 2A of OM and YM; respective heart rates (353±22 vs. 378±15 beats min^{-1}) and carotid arterial blood pressures (76±3 vs. 76±1 mmHg) were not different. Blood flows at rest (0.6 ± 0.1 vs. 1.6 ± 0.2 nl s^{-1}) and during maximum dilation (2.0 ± 0.8 vs. 5.4 ± 0.8 nl s^{-1}) with sodium nitroprusside (10 μM) were attenuated > 60% ($P<0.05$) in OM. Blood flow at peak ROV was blunted by 75-80% in OM vs. YM ($P<0.05$). In response to 30-s rhythmic contractions at 2, 4, and 8 Hz, progressive dilations did not differ with age or gender. Nevertheless, resting and peak blood flows in YM were 2- to 3-fold greater ($P<0.05$) than

OM. We suggest that aging blunts ROV and restricts blood flow to skeletal muscle of OM through subtle activation of α-adrenoreceptors in resistance networks.

Introduction

A reduction in physical work capacity with aging has long been attributed to the loss of muscle mass and function (Lexell, 1995). At the same time, physical activity requires adequate blood flow to support the contractile activity of muscle fibers. Human studies have demonstrated that the ability to increase blood flow to exercising skeletal muscle is impaired with aging even when accounting for lean body mass (Proctor & Joyner, 1997; Proctor *et al.*, 1998; Dinenno *et al.*, 1999; Lawrenson *et al.*, 2003). As this perfusion deficit is more pronounced in the legs than in the arms (Donato *et al.*, 2006), the effect of aging on blood flow appears to be greatest for muscles used for locomotion. In response to submaximal bicycling (Proctor & Joyner, 1997; Proctor *et al.*, 1998) or single-leg rhythmic knee extension (Lawrenson *et al.*, 2003; Parker *et al.*, 2008), a restricted increase in vascular conductance to active muscles of 'older' (typically ~60-70 years) vs. 'younger' (typically ~20-30 years) subjects reflects an increase in the resistance to blood flow, thereby implicating changes in the microcirculation. Studies of limb blood flow in humans are based primarily upon methods employing limb plethysmography, thermal dilution in venous effluent or Doppler ultrasound in proximal conduit arteries. While these data reflect regulatory events originating within the microcirculation, measurements performed for entire limbs and conduit vessels cannot ascertain where vasomotor responses occur or how they are regulated within

microvascular resistance networks. In contrast, animal models have enabled more invasive approaches to investigate the effects of aging on the microcirculation and the isolation of individual resistance vessels for *in vitro* studies has provided valuable insight into how key signaling events that underlie blood flow control are affected (Csiszar *et al.*, 2002; Muller-Delp *et al.*, 2002b; Woodman *et al.*, 2002). However, surgical isolation for *in vitro* study eliminates key physiological interactions between microvessels, muscle fibers and the autonomic nervous system that are manifest in the intact organism (Thomas & Segal, 2004).

A characteristic response to the stress of physical exertion is an increase in muscle sympathetic nerve activity (SNA) (Seals, 1989), which is integral to the redistribution and augmentation of cardiac output to support the metabolic demands of active musculature (Rowell, 1974). An increase in SNA can impose restrictions on muscle blood flow (Thomas & Segal, 2004) and this effect appears to be augmented with aging (Dinenno & Joyner, 2006; Proctor & Parker, 2006), even under resting conditions (Dinenno *et al.*, 1999). Moreover, sex differences in how SNA changes with aging are apparent. For example, although male subjects exhibited heightened SNA at an earlier age than did females, the increase in SNA with age increased more rapidly in females (Matsukawa *et al.*, 1998; Narkiewicz *et al.*, 2005). How the sympathetic nervous system affects the microcirculation of skeletal muscle with aging, and whether sex differences prevail in the regulation of arteriolar diameter in response to muscle contractions, remain inadequately defined. Indeed, more subtle effects of sympathetic neuroeffector

signaling may be manifest than previously recognized. Further insight into these relationships requires an experimental model that allows direct observation of arterioles in response to the contractile activity of skeletal muscle involved in locomotion.

Studies concerned with how the regulation of muscle blood flow is affected by aging have focused primarily on cardiovascular responses to well-defined submaximal levels of rhythmic contractions of leg or forearm musculature. Thus our current understanding of how aging impacts muscle perfusion reflects regulatory events occurring during steady-state levels of activity. In contrast, daily activities are more typically characterized by short bursts of activity with frequent transitions between different levels of energy expenditure. As reported in the human forearm (Corcondilas *et al.*, 1964) and supported by ensuing studies (Shoemaker *et al.*, 1998; Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007) muscle blood flow can increase within the first cardiac cycle following the onset of muscle contraction. Direct observations of arterioles in the hamster cremaster (Mihok & Murrant, 2004) and cheek pouch retractor (VanTeeffelen & Segal, 2006) muscles confirm that dilation can begin within the first 1-2 seconds of muscle contraction. Recent evidence from the human forearm indicates that such rapid vasodilatory responses to muscle contraction can be attenuated with aging (Carlson *et al.*, 2008). In turn, complementary observations of vasomotor kinetics in arterioles of the mouse gluteus maximus muscle (GM) have illustrated that both the initial increase in hyperemia and the ability to sustain hyperemia upon cessation of activity are impaired with aging (Bearden *et al.*, 2004; Bearden, 2007) with such effects

predominating in males. Nevertheless, how aging impacts both rapid and steady-state control of muscle blood flow within the microcirculation remains poorly understood, particularly in light of sympathetic regulation and the role of sex.

The goal of the present study was to provide new insight with respect to how aging and sex interact to affect the ability of the microcirculation to increase muscle blood flow in response to contractile activity. Experiments were performed using intravital microscopy to study the mouse GM and thereby investigate arteriolar responses to contractile activity within a skeletal muscle that is actively recruited during locomotion, adapts to exercise conditioning (Bearden *et al.*, 2004) and is common to both sexes. Moreover, activation of a single muscle minimizes requirements for adjustments in cardiac output, the demand for systemic cardiovascular regulation, or the possibility of activating baroreflexes and thereby emphasizes behavior intrinsic to the peripheral circulation. We focused on second-order (2A) arterioles based upon their strategic location between proximal feed arteries and terminal arterioles as well as their essential role in controlling blood flow distribution within the muscle (Bearden *et al.*, 2004). We tested the hypothesis that aging would impair rapid onset vasodilation (ROV) and the magnitude of arteriolar blood flow. Complementary experiments determined whether vasomotor responses were sex-specific, were manifest for distinct forms of contractile activity, and whether an underlying role for sympathetic regulation could be resolved for any differences that emerged between groups according to age or sex.

Methods

Animal care and use

Experimental procedures were approved by the Animal Care and Use Committees of The John B. Pierce Laboratory (New Haven, CT, USA) and of The University of Missouri (Columbia, MO, USA) and were performed in accord with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Experiments were performed using C57BL/6 mice with 2 age-groups studied for each gender. The total number and body mass of mice studied in each group were: old (20-month) males (OM: n=18, 35 ± 3 g) and females (OF: n=6, 35 ± 5 g) and young (3-month) males (YM: n=23, 27 ± 2 g) and females (YF: n=9, 23 ± 2 g). Mice were housed in animal care facilities of the John B. Pierce Laboratory or the University of Missouri, respectively, at ~24 °C on a 12 h/12 h light/dark cycle with food and water *ad libitum*. Mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA) or Charles River Laboratories (Wilmington, MA, USA) and housed on site for at least 1 week before being studied. Body mass was measured prior to each experiment. Upon completion of experimental procedures each day, the anesthetized mouse was euthanized by an overdose of sodium pentobarbital (intraperitoneal injection) and cervical dislocation.

Anesthesia and muscle preparation

A mouse was anaesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg kg^{-1}) which was supplemented as needed. During experiments, the depth of anesthesia was maintained at a level that preserved sympathetic modulation of

arterioles as confirmed by transient vasoconstriction in response to a firm toe pinch. The mouse was positioned in the prone position on a heating plate to maintain esophageal temperature at 37–38 °C and viewed through a stereomicroscope to prepare the left GM for intravital microscopy. After carefully shaving the surgical area, the skin overlying the muscle was removed and exposed tissue was superfused continuously (3-5 ml min^{-1}) with bicarbonate-buffered physiological salt solution (PSS; 35 °C, pH 7.4) of the following composition (mM): NaCl 137, KCl 4.7, $MgSO_4$ 1.2, $CaCl_2$ 2 and $NaHCO_3$ 18 and equilibrated with 5% CO_2–95% N_2. The origin of the GM was cut free from its insertion along the spine with great care taken to preserve the integrity of its neurovascular supply. Free edges of the muscle were gently reflected, spread evenly onto a transparent silicone rubber pedestal (Sylgard® 184; Dow Corning, Midland, MI, USA) and pinned to approximate *in situ* dimensions. Insertion of the GM onto the femur remained intact.

Intravital microscopy

Upon completion of surgery, the mouse preparation was transferred to the stage of an intravital microscope (Zeiss ACM or Nikon E600FN). The superfused GM was equilibrated for at least 30 minutes before experimental observations. Using Köhler illumination, arterioles were observed through a Zeiss UD40 objective (NA=0.41 or a Nikon 20X SLWD objective (NA=0.35), respectively, coupled to a video camera (C2400; Hamamatsu, Japan). Final magnification on the video monitor was ~1000X. Internal vessel diameter was determined as the distance between luminal edges using a video

caliper with spatial resolution ≤ 1 μm calibrated with a stage micrometer (100 X 0.01 = 1mm: Graticules Ltd., Tonbridge, Kent, UK).

Second-order arterioles (2A) were chosen for study for two reasons. First, these resistance microvessels are positioned anatomically to control the distribution of blood flow within the GM (Bearden *et al.*, 2004). Second, the consistency of arteriolar network architecture across young and old animals enables data acquisition from approximately the same region of each muscle (Bearden *et al.*, 2004). One arteriole was studied in each mouse. Following equilibration, the resting (baseline) diameter was recorded and arterioles were tested for oxygen sensitivity as follows: Superfusate O_2 was elevated from 0 to 21% (with 5% CO_2, balance N_2) for 10 minutes, arteriolar diameter was recorded, and equilibration with 5% CO_2–95% N_2 was restored for the duration of experimental procedures. Changes in arteriolar diameter (and blood flow in a subset of experiments; see *Hemodynamic measurements*, below) were evaluated in response to brief maximal tetanic contractions at 100 Hz as well as 30-s of rhythmic muscle contractions at 2, 4, or 8 Hz (see: *Skeletal muscle contractions,* below). For these experiments, each muscle preparation underwent both contraction protocols with order randomized across experiments. At the end of each day's procedures, maximum arteriolar diameter was recorded by adding sodium nitroprusside (SNP, 10 μM) to the superfusate (Bearden *et al.*, 2004; VanTeeffelen & Segal, 2006).

Skeletal muscle contractions

Contractions of the GM were evoked using electrical field stimulation (EFS). For this purpose, wire electrodes (90% Pt–10% Ir; diameter, 250 µm) were positioned in the superfusion solution on either side of the exposed muscle. Monophasic pulses (0.1 ms) were delivered at 10 V through a stimulus isolation unit (SIU5; Grass Technologies; Quincy, MA, USA) driven by a square wave stimulator (S48, Grass). Preliminary experiments confirmed that this voltage elicited reproducible contractions of the GM and of arteriolar responses for the duration of an experiment. Control experiments (n=3) confirmed that muscle fiber contractions resulted from acetylcholine release from motor nerve terminals as the addition of a nicotinic receptor antagonist (d-tubocurarine, 10 µM) reversibly abolished muscle contractions in response to EFS (Jacobs & Segal, 2000). In the presence of d-tubocurarine, the absence of arteriolar constriction with EFS further serves to confirm that EFS did not activate perivascular sympathetic nerves.

Hemodynamic Measurements

Centerline red blood cell velocity (V_{rbc}) was monitored concomitant with diameter in 2A using an Optical Doppler Velocimeter as described (Jacobs & Segal, 2000; VanTeeffelen & Segal, 2006). To ensure fidelity of these recordings, we confirmed that the V_{rbc} signal was pulsatile with the cardiac cycle. Mean red blood cell velocity (V_m) was calculated as $V_m=V_{rbc}/1.6$. Accurate velocimeter readings were not possible during tissue movement. Thus, for ROV experiments, peak V_{rbc} was recorded (for 1.5 s) during the peak of

vasodilation following a brief tetanic contraction. For rhythmic contractions, peak V_{rbc} was taken as that recorded for a similar interval immediately upon cessation of contractile activity (Welsh & Segal, 1997; Jacobs & Segal, 2000; VanTeeffelen & Segal, 2006). Blood flow was calculated as = $\pi(D/2)^2 V_m$, where *D* is diameter (Welsh & Segal, 1997; Jacobs & Segal, 2000; VanTeeffelen & Segal, 2006). Wall shear rate (WSR) during this period was calculated: WSR = 8Vm/*D*.

Experimental protocols

Rapid onset vasodilation (ROV)

Arteriolar responses to muscle contraction were first evaluated for ROV. Based upon preliminary experiments (data not shown) performed in light of earlier findings (Bearden *et al.*, 2004; VanTeeffelen & Segal, 2006), a brief maximal tetanic contraction at 100 Hz was used to evoke ROV in each experimental group. To characterize arteriolar dilations across a range of tetanic contraction durations, stimulus train durations were 100, 200, 400, 600, 800 and 1000 ms with the order randomized across experiments. The arteriole consistently returned to the initial resting baseline with 2-3 minutes of recovery between contractions. As tissue displacement occurred during contraction, diameter was recorded preceding each stimulus (resting baseline) and immediately following contraction with a delay of 1-2 s (Figure 1) that reflected the time required to refocus and reposition the video caliper.

The ROV responses were first evaluated under control conditions. Steady-state vasodilations to rhythmic contractions were then assessed as described in the next paragraph. In light of differences observed in ROV for OM compared to other groups (see Results for details), one of 2 pharmacological agents to inhibit [phentolamine (1 μM)] or activate [norepinephrine (NE, 1 nM)] α-adrenoreceptors (αAR) were investigated for an effect on ROV. Following addition to the superfusion solution, each agent was first equilibrated for 15 minutes with no measureable effect on resting arteriolar diameter. It should be recognized that a 10-fold range of tetanic contractions plus a 4-fold range of rhythmic contractions were used to define 2A responses to exercise. Therefore only one pharmacological agent could be investigated reliably in a given preparation. To match these treatments by age, phentolamine was tested in OM and OF while NE was tested in YM and YF.

Steady-state vasodilation to rhythmic contractions

As the nature of vasodilation can vary with the pattern of muscle fiber activation (VanTeeffelen & Segal, 2000; Murrant, 2005), vasomotor responses to 30-s rhythmic contractions at 2, 4 and 8 Hz (in randomized order) were also evaluated in each experimental group. Stimulation at these frequencies evokes un-fused twitch contractions that correspond to ~40% of peak tetanic tension (Bearden *et al.*, 2004). Following each 30-s period of rhythmic twitch contractions, resting baseline was reestablished consistently within 5 minutes. Arteriolar diameter was recorded preceding

contractile activity, during rhythmic contractions, and following contractions throughout recovery.

Experimental emphasis on male mice

Throughout our initial experiments, the order in which age and sex were studied was varied across respective groups. However, based upon the clear distinction of ROV responses between YM and OM (*see Figure 2*) and greater morbidity encountered for OF compared to OM, our experimental design was adjusted accordingly. To directly compare the effects of both activation and inhibition of αAR on ROV responses in the same GM preparations, a subset of experiments was performed on YM and OM (n=5 per group). To preserve the integrity of preparations for these experiments, fewer tetanic contractions were performed (400, 600, 1000 ms; randomized) under each experimental condition. Thus, following equilibration, responses to each contraction were recorded under control conditions. The preparation was equilibrated with NE (1 nM) for 5 minutes and ROV responses were revaluated. After recovering from the final contraction, the preparation was equilibrated with phentolamine for 15 minutes and ROV responses were evaluated a final time.

To provide further insight with respect to the impact of aging on skeletal muscle perfusion, arteriolar blood flow was evaluated in subsets of OM and YM. In these animals, arterial blood pressure was measured by cannulating a carotid artery with polyethylene tubing (PE-10) connected to a pressure transducer (CDX III; COBE; Arvada,

CO, USA) coupled to a Transbridge amplifier (World Precision Instruments, Sarasota, FL, USA). Blood pressures were averaged over 10 cardiac cycles to obtain a mean value for each mouse.

Chemicals and solutions

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) and dissolved in purified deionized water (dH_2O, 18.2 MΩ), with the exception of phentolamine, which was first dissolved in 100% EtOH. Stock solutions were prepared weekly, stored at 4 °C and diluted at least 100-fold in PSS to final working concentrations on the day of an experiment. Based upon preliminary experiments, NE (1 nM) was added to the superfusion solution at to subtly activate αAR without producing arteriolar constriction. In accord with our earlier studies of αAR inhibition in microvessels (Haug & Segal, 2005), the concentration of phentolamine in the superfusion solution was 1 μM (with 0.1% EtOH). Vehicle controls confirmed that 0.1% EtOH in PSS was without affect on baseline diameter or vasomotor responses.

Data acquisition, statistical analyses and presentation

Data were collected at 100 Hz using a PowerLab system (AD Instruments; Colorado Springs, CO, USA) coupled to a personal computer. Data were analyzed using SigmaStat software (version 3.11; Systat Software Inc., Point Richmond, CA, USA) and differences were accepted as statistically significant with $P<0.05$. For ROV data, one- and two-way (age, sex) Repeated Measures Analysis of Variance (RMANOVA) was used to test for the

main effect of tetanic contraction duration across the 4 experimental groups. Linear regression was performed to evaluate the correlation between the duration of contraction and the magnitude of arteriolar dilation for each animal. Slopes of these responses were compared across groups using ANOVA. The main effect of stimulus frequency during rhythmic contractions was analyzed using RMANOVA. Where treatment conditions differed between Young (NA exposure) and Old (phentolamine exposure), pair-wise comparisons were performed using multiple t-tests with a Bonferroni correction to maintain total $P<0.05$. To compare OM and YM with respect to the effect of NE and phentolamine on ROV in the same preparations, a 3-way (age, treatment, contraction duration) RMANOVA was used. When significant F-ratios were obtained with ANOVA or RMANOVA, Tukey tests were performed for post-hoc comparisons. Summary data are presented as mean values ± Standard Error (S.E.).

Results

Arteriolar responses to O_2 and hemodynamic characteristics in young and old mice

Neither baseline nor maximum arteriolar diameters differed significantly with age or gender (Table 1). However, arteriolar constriction in response to elevating superfusate O_2 from 0 to 21% was significantly greater ($P<0.05$) in YM compared to other groups (Table 1), consistent with earlier observations (Bearden *et al.*, 2004). There were no differences in respective heart rates (378 ± 15 vs. 353 ± 22 beats min^{-1}) or mean arterial blood pressures (76 ± 1 vs. 76 ± 3 mm Hg) between YM and OM.

Rapid onset vasodilation and blood flow

Vasomotor responses. The diameter change with ROV increased consistently with the duration of tetanic contraction ($P < 0.01$; Figures 2-4). Across the entire range of single tetanic contractions, there were no significant differences in ROV responses between YM, YF or OF (Figure 2A), though a subtle effect was apparent for OF at shorter durations. In striking contrast, ROV responses of OM were attenuated by nearly half ($P<0.05$) across the entire range of contraction durations (Figure 2A). The slope of the regression line determined for mean responses across the range of contractions was taken as an index of the sensitivity of ROV responses for each experimental group. As illustrated in Figure 2B, there was a strong positive correlation between increasing contraction duration and the change in arteriolar diameter. Nevertheless, the sensitivity of ROV to single tetanic contractions was reduced significantly ($P<0.05$) in OM as compared to YM, YF and OF, which were not different from each other.

Given that SNA can inhibit vasodilation (Thomas & Segal, 2004), we tested the hypothesis that inhibition of αAR would restore the magnitude of ROV responses in OM. Remarkably, with no change in resting diameter, superfusion with phentolamine (1 μM) increased ROV of OM such that these responses were no longer different from those of YM (Figure 3A). For age-matched controls, phentolamine had no effect on ROV in OF (Figure 3B). In complementary experiments, superfusion with NE (1 nM) consistently depressed ROV in YM to levels observed in OM (Figure 3A) and did so without changing

resting diameter. Though less pronounced, the effect of NE in YF was similar to that observed in YM (Figure 3B).

Adrenoreceptor treatments compared between OM and YM

Our initial experiments investigated the effect of αAR inhibition or activation by testing phentolamine in Old mice and NE in Young mice. These data are limited as they did not determine whether inhibition of α-adrenoreceptors could affect ROV in Young mice or whether stimulation of α-adrenoreceptors could affect ROV in Old mice. Therefore a subset of experiments was performed to compare the effect of both α-adrenoreceptor inhibition and activation on ROV in the same preparations of OM and YM (Figure 4). Consistent with findings in Figure 2, control ROV responses of OM were blunted consistently relative to those of YM. Despite having no effect in YM, phentolamine again increased ROV in OM ($P<0.05$) to levels not different from responses recorded in YM. In turn, NE blunted ROV in YM to levels not different from OM under control conditions and attenuated ROV even further in OM ($P<0.01$) with responses to 400 and 600 ms contractions nearly abolished.

Hemodynamic responses

At rest, 2A blood flow was blunted by > 50% in OM vs. YM (Figure 5A), as was maximum blood flow during topical exposure to SNP (2.0 ± 0.8 vs. 5.4 ± 0.8 nl s^{-1} respectively, $P < 0.05$; n=6 per group). As shown in Figure 5A, baseline blood flow recovered consistently between each tetanic contraction. Blood flow increased slightly but significantly with

the duration of contraction in both OM and YM. However, this hyperemic response to tetanic contraction was also blunted by > 50% in OM vs. YM ($P<0.05$; Figure 5A).

With no difference in resting diameter (Table 1), the reduction in 2A blood flow in OM vs. YM is explained by lower velocity of red blood cells. Baseline V_m remained stable during experiments but was blunted by more than half ($P < 0.05$) in OM vs. YM (Figure 5B) Recorded during the peak of ROV diameter responses, there was no significant effect of contractile activity on V_m in either YM or OM. Thus, V_m was consistently blunted in OM and is consistent with flow restriction by vessels located upstream from the exposed muscle [e.g., proximal feed arteries (VanTeeffelen & Segal, 2003)]. Nearly identical relationships were apparent for WSR (Figure 5C), with OM having consistently blunted values relative to YM ($P < 0.05$) at rest and in response to tetanic contraction.

Steady-state vasodilation and blood flow with rhythmic contractions

From similar baseline diameters (Table 1), arteriolar dilations consistently reached a stable plateau during 30 s of rhythmic contractions at 2, 4 and 8 Hz. The magnitude of dilation increased with stimulation frequency for all experimental groups ($P < 0.05$). In marked contrast to ROV responses (Figures 2 and 3), neither age nor sex had a significant effect on the magnitude of diameter changes to rhythmic contractions (Figure 6A) as arterioles of YM, YF, OM and OF all exhibited similar dilations under these conditions.

Arteriolar blood flow increased with stimulation frequency ($P < 0.05$) for both YM and OM. Peak responses in YM (2.3 ± 0.6 to 3.5 ± 0.8 nl s^{-1}) were 2- to 3-fold greater ($P < 0.05$) than those recorded in OM (Figure 6B) despite similar increases in arteriolar diameter, providing additional evidence for an upstream limitation to blood flow in OM compared to YM.

Discussion

The present study was undertaken using the C57BL/6 mouse as a model for investigating how aging influences the arteriolar control of blood flow within a mammalian hindlimb locomotor muscle and to determine whether such effects differ between sexes. In second-order arterioles (2A) of the gluteus maximus muscle, we demonstrate that ROV in response to single tetanic contractions was blunted consistently and selectively in 20-month old males relative to age-matched females and to 3-month old mice of both sexes. Remarkably, the inhibition of α-adrenoreceptors with phentolamine restored ROV in OM to levels seen in the other 3 groups. In turn, the blunted ROV observed in OM was reproduced in YM and YF by exposure to a concentration of NE (1 nM) that had no effect on resting diameter. Thus the blunting of ROV of 2A of OM can be explained by a subtle elevation in the resting level of arteriolar smooth muscle cell activation via α-adrenoreceptors. In distinct contrast, there were no differences between respective groups for 2A dilations during rhythmic submaximal contractions. Nevertheless, arteriolar blood flows were attenuated significantly under

all conditions for OM as compared to YM, which can be explained by a blunted ability to evoke dilation in proximal segments of the resistance network.

Rapid onset of vasodilation and its impairment with aging

The present data are the first to demonstrate ROV in the mouse microcirculation and do so in a hindlimb muscle that is active during locomotion and adapts to physical training (Bearden *et al.*, 2004). Elucidating ROV in response to a brief muscular contraction in humans (Corcondilas *et al.*, 1964) has stimulated ongoing interest in understanding such rapid responsiveness of the peripheral resistance vasculature. Direct observations of the microcirculation in hamster cremaster (Mihok & Murrant, 2004; Armstrong *et al.*, 2007b) and retractor (VanTeeffelen & Segal, 2006) muscles have resolved rapid dilation of individual arterioles in response to single contractions, providing a direct link between events described for intact limbs in humans and the ability to resolve underlying mechanisms of blood flow control in the microcirculation.

That ROV is impaired with aging in human subjects (Carlson *et al.*, 2008) underscores the relevance of the present findings in the mouse GM as a model that enables direct insight into signaling events that modulate such phenotypic changes in vasomotor control. In accord with studies of the human forearm, where blood flow increased linearly with the intensity of single contractions (Tschakovsky *et al.*, 2004), we observed ROV in the mouse GM to increase in direct proportion to the duration of single tetanic contractions (Figure 2). Moreover, these dilatory responses were blunted selectively in

OM across a 10-fold range of contraction duration (Figure 2A). Attenuation of this relationship in OM relative to OF, YM or YF (Figure 2B) indicates a reduction in sensitivity of ROV in OM. In turn, the difference between sexes that emerged here with aging suggests that the determinants of ROV are similar in male and female mice until key events associated with aging occur in OM. As active tension produced by the GM in male mice is maintained with aging throughout the frequency-force relationship (Bearden *et al.*, 2004a), the blunting of ROV is not likely to be explained by a loss of contractile function. Instead, our findings suggest a subtle effect of α-adrenoreceptor activation in OM that was not present in other experimental groups. Additional support for sex differences in the kinetics of vasomotor responses with aging comes from demonstrating the slowing of vasodilation onset (and acceleration of recovery) in arterioles of OM relative to other groups (Bearden, 2007). Such phenotypic changes may contribute to the lag in oxygen uptake at the onset of moderate cycling in older vs. younger subjects (DeLorey *et al.*, 2004).

Role for α-adrenoreceptor activation in blunting ROV and arteriolar blood flow

A unique finding of this study is restoration of ROV in OM when α-adrenoreceptors were inhibited with phentolamine (Figures 3 and 4). This gain of function led us to test whether the sympathetic neurotransmitter NE could mediate such an effect and was tested in young animals of both sexes. As illustrated in Figure 3, ROV responses were attenuated by NE in YM and YF without altering resting arteriolar diameter. While internal diameter was not changed measurably by NE, arteriolar smooth muscle cells

adopted a 'crinkly' appearance similar to that observed for arterioles in OM. The present findings thereby indicate a subtle yet constitutively higher level of arteriolar smooth muscle cell activation via α-adrenoreceptors in OM as compared to the other groups studied here. In turn, exposing OM to NE resulted in even greater attenuation of ROV (Figure 4) despite no effect on resting diameter.

The use of phentolamine as a nonselective α-adrenoreceptor antagonist precludes the ability to resolve specific roles for α_1- vs. α_2-adrenoreceptors in modulating ROV. Nevertheless, as shown in feed arteries of hamster skeletal muscle, respective adrenoreceptor subtypes can interact additively to suppress rapid dilations. For example, acetylcholine initiates hyperpolarization and rapid relaxation of microvascular smooth muscle cells (Emerson & Segal, 2000) through activating calcium-sensitive K^+ channels (Domeier & Segal, 2007). Indeed, early studies in the dog hindlimb (Mohrman *et al.*, 1973; Mohrman & Sparks, 1974) implicated a key role for K^+ in rapid hyperemic responses to single brief tetanic contractions. This conclusion is supported by recent findings in the hamster cremaster muscle illustrating the role of K^+ in mediating rapid arteriolar dilation in response to single contractions of skeletal muscle fibers (Armstrong *et al.*, 2004).

Vasodilation can ascend the resistance network into proximal feed arteries in response to muscle contraction (Hilton, 1959) and thereby increase muscle blood flow by reducing vascular resistance located upstream from intramuscular arterioles (Folkow *et*

al., 1971; Segal & Jacobs, 2001). In older humans, blood flow to exercising skeletal muscle is attenuated (Dinenno & Joyner, 2006; Proctor & Parker, 2006), though the site(s) of flow restriction were not defined. As feed arteries supplying the GM were not exposed in our surgical preparations, the elevated activation of α-adrenoreceptor that was apparent in arterioles was also likely to also be manifested in proximal branches of the vascular supply. We therefore propose that the blunting of arteriolar perfusion in OM reflects the impairment of ascending vasodilation by α-adrenoreceptor activation, such that feed artery tone remained high and thereby restricted blood flow into arterioles downstream. The blunted V_m (Figure 5B) and WSR (Figure 5C) recorded at rest and during arteriolar responses in OM are consistent with such an effect are supported by earlier findings. First, sympathetic nerve stimulation reduced blood flow into contracting skeletal muscle even during robust arteriolar dilation and was associated with inhibition of feed artery dilation (VanTeeffelen & Segal, 2003). Second, activation of α-adrenoreceptors inhibited the ability of vasodilation to conduct along feed arteries (Haug & Segal, 2005). Third, conducted vasodilation is impaired in OM relative to YM (Bearden *et al.*, 2004; Bearden *et al.*, 2007). Though the mechanism underlying the inhibition of vasodilation in proximal segments of the resistance vasculature remains to be defined, we suggest that it too may be associated with higher levels of α-adrenoreceptor activation in vascular smooth muscle cells.

Rhythmic twitch contractions dilated arterioles to similar steady-state levels irrespective of age or sex (Figure 6A). This similarity across experimental groups contrasts with the

selective blunting of ROV for OM in response to single tetanic contractions. Thus the effect of aging on arteriolar dilation depends upon the nature of contractile activity. This conclusion is consistent with the interpretation that signaling events mediating sustained vasodilation differ from those mediating rapid vasodilation (Haddy & Scott, 1975; Morganroth *et al.*, 1975). Even with similar arteriolar dilations across experimental groups, and consistent with attenuated blood flow during ROV, peak blood flow responses were consistently 2- to 3-fold greater in YM versus OM (Figure 5B). In accord with flow limitations apparent during ROV, such behavior during rhythmic contractions (Figure 6B) and during maximum dilation with topical SNP (*see RESULTS: Hemodynamic Responses*) further implies that total blood flow into the arteriolar network was restricted by proximal segments of the resistance network (Jacobs & Segal, 2000; VanTeeffelen & Segal, 2003). As these conclusions are based entirely upon hemodynamic measurements performed in male animals, future experiments are required to ascertain the nature of microvascular blood flow in female animals and how it may be affected by aging.

Experiments performed on the cremaster muscle of rats have shown that differences in the ability to evoke dilation in proximal vs. distal resistance microvessels may also reflect corresponding differences in the regional distribution of α-adrenoreceptor subtypes (Anderson & Faber, 1991). Whether regional distribution of α-adrenoreceptor subtypes is manifest in the mouse GM remains to be investigated. Nevertheless, the present findings help to explain how augmented α-adrenoreceptor activation with aging

effectively blunts resting blood flow as well as exercise hyperemia in humans (Dinenno & Joyner, 2006; Proctor & Parker, 2006). Indeed, a similar mechanism may well contribute to delaying the attainment of functional vasodilation as well as the impaired ability to sustain vasodilation into the recovery period (Bearden *et al.*, 2004; Bearden, 2007).

Sympathetic neuroeffector signaling, muscle blood flow and the role of sex

While there is general agreement that enhanced actions of the sympathetic nervous system contribute to attenuated blood flow responses with aging (Dinenno & Joyner, 2006; Proctor & Parker, 2006), it remains controversial as to whether vasomotor responsiveness to neuroeffector signaling is altered. For example, when acute sympathetic vasoconstriction (via cold-pressor test) was evaluated during bicycle ergometry, older men demonstrated greater reduction in leg vascular conductance as compared to their younger counterparts (Koch *et al.*, 2003). In contrast, when postjunctional α-adrenoreceptors in the leg of male subjects at rest were stimulated with tyramine to release NE, aging was associated with reduced responsiveness of both α_1 and α_2 AR subtypes (Smith *et al.*, 2007). These recent findings support earlier observations showing attenuated forearm blood flow reductions of older vs. young subjects (both male and female) at rest during infusions of NE into the brachial artery (Hogikyan & Supiano, 1994). In accord with the present data, lower basal vascular conductance in the legs of older vs. younger males (Dinenno *et al.*, 1999; Smith *et al.*, 2007) reflected greater tonic vasoconstriction mediated by enhanced SNA recorded

from the peroneal nerve (Dinenno *et al.*, 1999). However, when the responsiveness of isolated skeletal muscle arterioles to adrenergic receptor agonists has been evaluated directly *in vitro*, vasoconstriction to NE was similar between vessels isolated from young (4-month) and aged (24-month) rats (Muller-Delp *et al.*, 2002a). Similarly, in the mouse GM, constriction to phenylephrine (a selective α_1-adrenoreceptor agonist) was not different between YM and OM (Bearden *et al.*, 2004). Thus age-related differences in vasomotor responsiveness that have been identified in human subjects at rest or during exercise may involve cardiovascular reflexes that are avoided in isolated vessels or individual muscles of anesthetized animals as studied here.

Consistent with the present findings, recent observations in older humans indicate that ROV in the forearm was blunted relative to younger subjects (Carlson *et al.*, 2008). However, unlike the present study, this effect of aging was apparent for both sexes. Leg blood flow during moderate cycling has been reported to be reduced in both older male subjects (Proctor *et al.*, 1998) and female subjects (Proctor *et al.*, 2003a) even when accounting for active muscle mass. Nevertheless, impaired blood flow in older men relative to their younger counterparts has not been consistently observed (Proctor *et al.*, 2003b) and more recent findings emphasize that attenuated vasodilation during rhythmic single-leg knee extensions (which minimize central limitations of cardiac output) is greater in older women than in older men (Parker *et al.*, 2008). Thus apparent differences between sexes may also depend upon the nature of exercise used for evaluating muscle blood flow and whether or not systemic cardiovascular reflexes

are invoked. As estrogen therapy was found to reverse augmented sympathetic vasoconstriction in postmenopausal women (Fadel *et al.*, 2004), the role of estrogen in maintaining arteriolar perfusion (and ROV) with aging also remains to be ascertained.

The present data showing selective blunting of arteriolar blood flow in the GM of OM (Figures 5A and 6B) are supported by earlier studies in the hindlimb of Fischer 344 rats, where the ability to increase blood flow during muscle contractions as well as during vasodilator infusion was depressed in 24-month vs. 12-month males (Irion *et al.*, 1987) but not in females (Irion *et al.*, 1988). In these studies, greater muscle fatigue was associated with the reduction in muscle blood flow. As neuropeptide Y (NPY) is a co-transmitter released with NE from sympathetic nerve terminals, evidence from adult Sprague-Dawley rats suggests that such sex differences in muscle blood flow may be related to a greater role for NPY in mediating sympathetic actions on the vasculature for males as compared to females (Jackson *et al.*, 2005b, a). However, the effects of aging on the contributions of NPY to noradrenergic vasoreactivity also remain to be elucidated.

Summary

The present study provides evidence that aging is associated with microvascular dysfunction in a locomotor muscle that is selective for male C57BL/6 mice. Deleterious effects of aging on dynamic blood flow control in OM were highlighted by blunted ROV in response to single tetanic contractions when compared to OF, YM or YF. As blockade

of α-adrenoreceptors with phentolamine restored ROV in OM and topical NE attenuated ROV in YM and YF, we suggest that aging is associated with constitutively elevated levels of α-adrenoreceptor activation in resistance micro-vessels of OM. Furthermore, arteriolar blood flow in OM was attenuated relative to YM at rest, during ROV, during steady state dilations to rhythmic twitch contractions and during maximal dilation with topical SNP. As these effects were manifest despite no significant differences in systemic arterial blood pressure, resting or maximal arteriolar diameters between experimental groups, we propose that even without overt vasoconstriction or vascular remodeling, subtle levels of α-adrenoreceptor activation can effectively compromise rapid adjustments in tissue blood flow and could thereby impair daily activities. Moreover, blunted V_{rbc} with no difference in arteriolar diameters implicates proximal segments of the resistance network as key sites for restricting muscle blood flow with aging. In light of comprehensive human studies implicating a role for enhanced sympathetic vasoconstriction in limiting muscle blood flow, the present findings support the C57BL/6 mouse as a model system for studying how aging affects the microcirculation in mammalian skeletal muscle.

Author Contributions

D.N. Jackson A.W. Moore and S.S. Segal contributed to the conception, experimental design and execution, and the analysis and interpretation of data contained in this manuscript. D.N. Jackson and S.S. Segal prepared the original submission. A.W. Moore contributed additional text and all co-authors have approved the version submitted herein to be published.

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Age-group	Baseline diameter (μm)		Maximum diameter (μm)		O_2 response (μm)	
	♂	♀	♂	♀	♂	♀
Young	17 ± 1	17 ± 1	31 ± 1	29 ± 1	-10 ± 1*	-5 ± 1
Old	17 ± 1	17 ± 1	31 ± 1	30 ± 1	-6 ± 1	-4 ± 1

Table A.1. Diameters and oxygen response of 2A arterioles in mouse gluteus maximus muscle. Baseline values were recorded under control conditions (superfusate equilibrated with 5% CO_2/95%N_2). Maximum values were recorded during superfusion with SNP (10 μM). O_2 response indicates the change in diameter in response to elevating superfusate O_2 from 0 to 21%. *Arterioles of YM constricted more than other groups, $P < 0.05$ (n = 6-22 per cell).

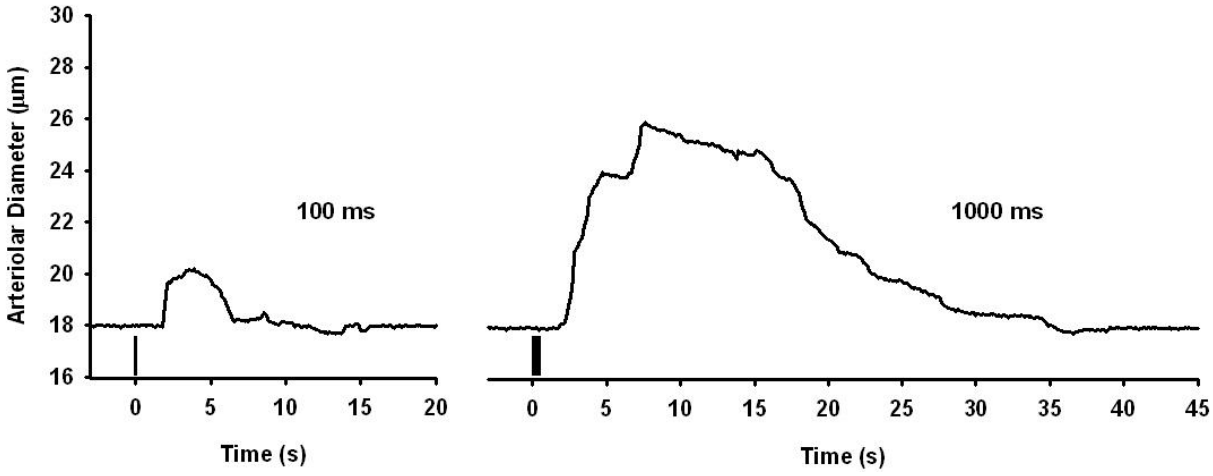

Figure A.1. Rapid Onset Vasodilation in response to brief tetanic contraction. Representative traces of diameter responses to single tetanic contractions of 100 ms (left) and 1000 ms (right) duration from a young male mouse. Vertical bars indicate stimulation at 100 Hz. As ROV was nearly instantaneous, delays in diameter responses reflect time to refocus the microscope after contraction.

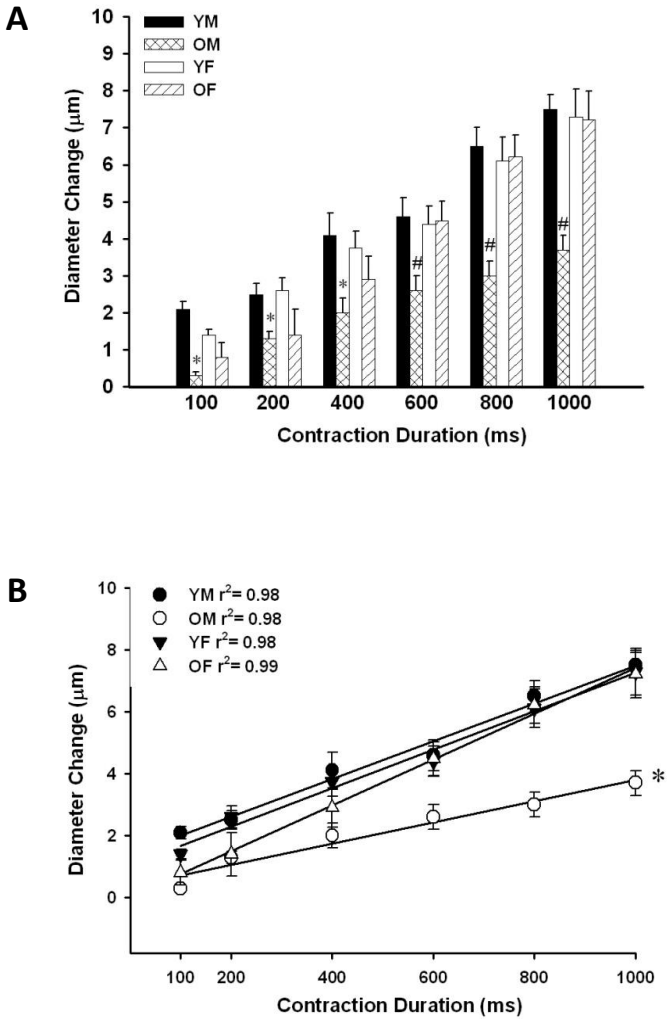

Figure A.2. Blunting of ROV in old male mice. A) Across the range of contraction durations, diameter change in response to single tetanic contractions was attenuated by nearly half in OM as compared to YM, YF or OF, (n = 6-18 per group). * P<0.05, OM vs. YM and YF; # P<0.05, OM vs. other groups. **B)** In each experimental group, the duration of muscle contraction correlated well with the change in arteriolar diameter (n = 6-18 per group). However the slope of ROV responses to increasing stimulus duration was blunted in OM. * P<0.05, OM vs. other groups.

Abbreviations: YM, young male; YF: young female; OM: old male; OF: old female.

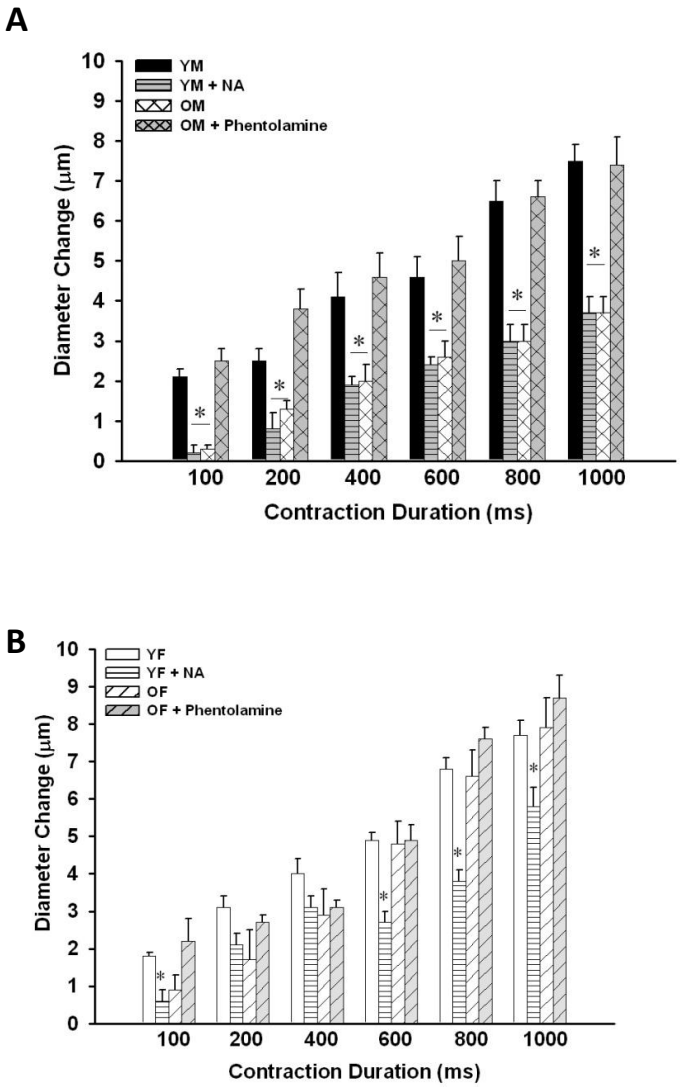

Figure A.3. Roles of α-adrenoreceptor inhibition and activation in ROV. A) Superfusion of phentolamine (1 μM) increased ROV in OM to levels not different from those recorded in YM. Conversely, superfusion of NE (1 nM) blunted ROV in YM to levels not different from those recorded in OM . Resting diameters (Table 1) were not different between conditions. * P<0.05 for OM and YM + NE vs. YM and OM + phentolamine (n = 6-8 per group). **B)** Superfusion of NE (1 nM) blunted ROV in YF (consistent with its effect in YM, Panel A), particularly at longer durations of contraction. However, superfusion of phentolamine (1 μM) had no effect on ROV responses in OF, which were not different from those in YF. Resting diameters (Table 1) were not different between conditions. * P<0.05, YF + NE vs. other groups, n = 4-6 per group). Abbreviations are defined in Figure 2.

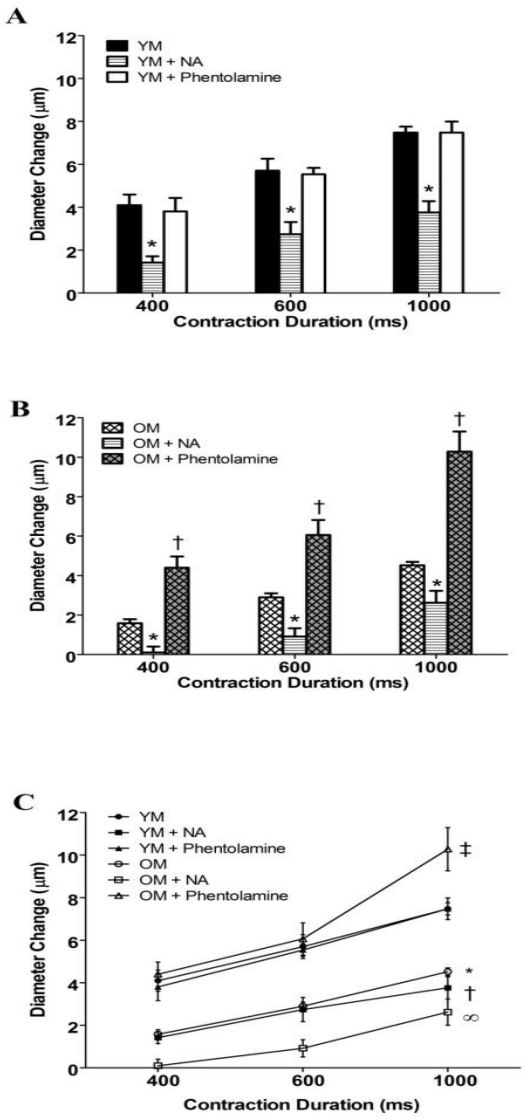

Figure A.4. Effects of NE and phentolamine on ROV in Young and Old male mice. A) *Young Males:* Topical NE blunted ROV significantly (* P<0.01). Phentolamine had no significant effect on ROV (n=5). **B)** *Old Males:* Topical NE blunted ROV significantly (* P<0.01). Phentolamine enhanced ROV significantly († P<0.01; n=5). **C)** *Comparisons of YM vs. OM (data in A and B re-plotted):* Under control conditions, ROV was blunted in OM vs. YM (* P<0.01). For YM + NE, ROV was blunted significantly below control (†P<0.01) and these levels were not significantly different from OM. For OM + NE, ROV was further depressed for each contraction (∞ P<0.01). Phentolamine had no effect in YM yet increased ROV significantly in OM (‡ P<0.01). Note that 1 OM had a particularly large response to 1000 ms contraction that elevated the mean ± SE for this data point.

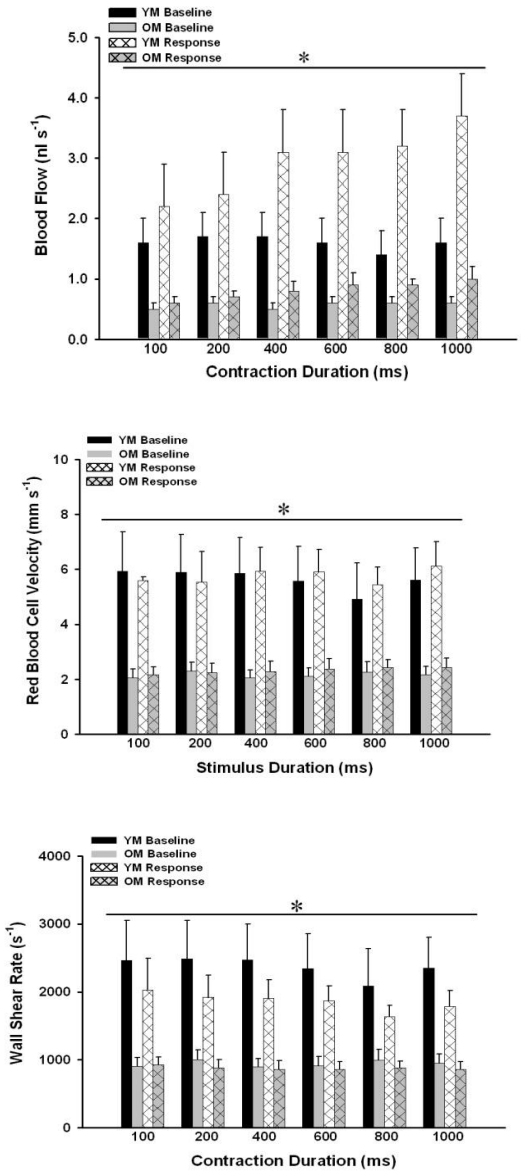

Figure A.5. Arteriolar hemodynamic responses during ROV were blunted in OM vs. YM. A) At rest and during peak dilation in response to single tetanic contractions, blood flow was greatly attenuated in OM vs. YM. **B)** Mean red blood cell velocity during peak dilation did not change with increasing stimulus duration in either YM or OM. However, baseline and response values were blunted consistently in OM vs. YM. **C)** Wall shear rate during peak dilation did not change with increasing stimulus duration in either YM or OM. However, WSR was blunted consistently in OM vs. YM. * $P<0.05$, OM vs. YM. For all panels, n=6 per group. Abbreviations are defined in Figure 2.

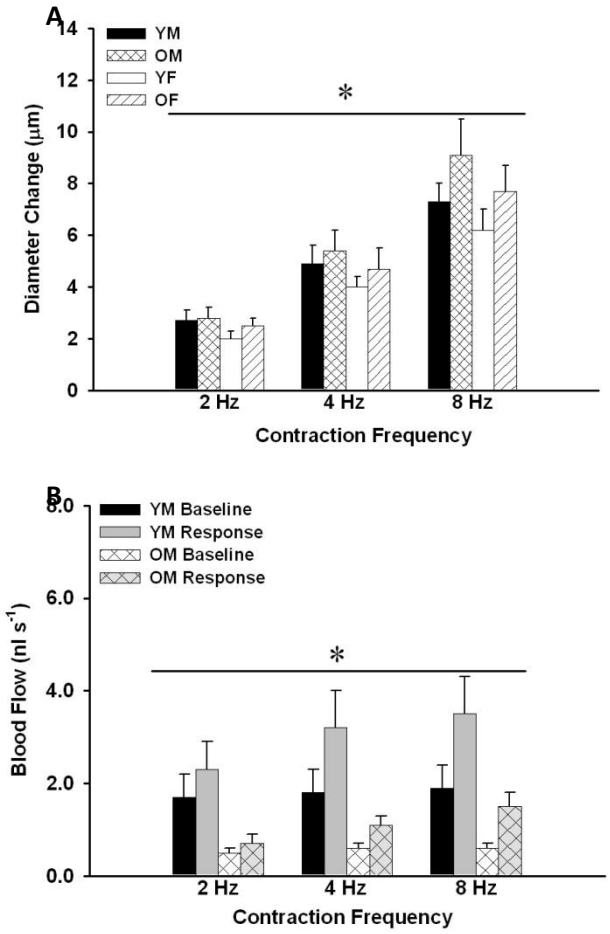

Figure A.6. With rhythmic contractions, arteriolar dilations are independent of age or sex yet blood flow is blunted in OM. A) With no difference in resting or maximal diameters (Table 1), the magnitude of arteriolar dilation increased with contraction frequency in a similar manner for each experimental group. * Main effect of stimulus frequency, P<0.05 (n = 6-18 per group). **B)** Blood flows at rest (baseline) and in response to 30 seconds of rhythmic contractions were blunted by more than half in OM compared to YM. *P<0.05, OM vs. YM (n=6 per group). Abbreviations are defined in Figure 2.

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VITA

Alex Moore is originally from St. Louis, MO. He attended Wheaton College (IL) where he majored in Kinesiology and was part of the track and cross country teams. After graduation, he attended the University of North Carolina – Chapel Hill, where he earned his master's degree in applied physiology under A.C. Hackney. He taught in the biology and kinesiology departments at Westmont College for two years before coming to the University of Missouri to work in Steve Segal's lab. While at Missouri, he was supported by a Life Sciences fellowship and a Phi Zeta grant.