ADVANCING AGE PRODUCES SEX DIFFERENCES IN VASOMOTOR KINETICS DURING AND AFTER SKELETAL MUSCLE CONTRACTION

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ABSTRACT

Little is known of the vasomotor responses of skeletal muscle arterioles during and following muscle contraction. We hypothesized that aging leads to impaired arteriolar responses to muscle contraction and recovery. Nitric oxide (NO) availability, which is age-dependent, has been implicated in components of these kinetics. Therefore, we also hypothesized that changes in the kinetics of vascular responses are associated with the (NO) pathway. Groups were young (3 mo), old (24 mo), endothelial (NO) synthase knockout (eNOS-/-) and Nω-nitro-L-arginine (LNA) -treated male and female C57BL6 mice. The kinetics of vasodilation during and following 1 min of contractions of the gluteus maximus muscle were recorded in second order (2A, regional distribution) and third order (3A, local control) arterioles. Baseline, peak (during contraction) and maximal diameters (pharmacologic) were not affected by age or sex. The kinetics of dilation and recovery were not different between males and females at the young age. There was a significant slowing of vasodilation at the onset of contractions (~2-fold; p<0.05) and a significant speeding of recovery (~5-fold; p<0.05) in old males vs old females and vs young. eNOS-/- and LNA did not affect the kinetics at the onset of muscle contraction. eNOS-/- mimicked the rapid recovery of old males in 2A; acute NO production (LNA) explained ~50% of this effect. These data demonstrate fundamental age-related differences between the sexes in the dynamic function of skeletal muscle arterioles. Understanding how youthful function persists in females but not males may provide therapeutic insight into clinical interventions to maintain dynamic microvascular control of nutrient supply with age.
INTRODUCTION

Microvascular blood flow control within skeletal muscle may differ between men and women and between young and old. These differences may compromise muscle function. For example, greater fatigue resistance in women vs. men (22) is abolished during ischemia (35), suggesting that blood flow control plays a central role in these differences. Moreover, blood flow control within skeletal muscle declines with age, contributing to a decline in physical activity tolerance (17, 18, 45) although the mechanisms are not well understood and differences between the sexes not known. Blood flow during steady state exercise is an important measure of vascular function. However, steady state activity is only common during deliberate repose (e.g., sleep), laboratory experiments, or during planned physical exercise. Even most sporting events contain only brief periods of steady state effort. For skeletal muscle, most of the day requires rapid adaptation to changing activity levels. The available evidence during forearm exercise (31) suggests that microvessels respond rapidly (<5 s) to skeletal muscle contraction and that the responses are similar between young male and female subjects. The role of aging on sex differences in the kinetics of microvascular dilation to contraction has not been determined.

When muscles begin to contract, they demand an increase in blood flow to match metabolic demand. Following contractions, some period of sustained dilation is necessary for nutrient supply in recovery. A slow adaptation, relative to tissue needs, at the onset of exercise compromises normal function and a rapid decrease in nutrient availability following exercise compromises recovery since many metabolic processes are still ongoing (14, 23). There is a paucity of information concerning the dynamic regulation of microvascular tone. Pharmacologic blockade of nitric oxide synthase (NOS) with L-NMMA reduces femoral artery blood flow after a bout of exhaustive knee extension without affecting the kinetics at the onset of exercise or the steady state blood flow (30). Removal of the gene for endothelial NOS (eNOS-/-) causes perfusion of working skeletal muscle to recover faster following contractions (48), reducing blood flow reserve and possibly delaying recovery. The effect of eNOS-/- on the kinetics of vasodilation at the onset of muscle contractions have not been reported. It remains unknown whether the phenotype observed in eNOS-/- is due solely to loss of NO availability or whether associated compensatory mechanisms also play a role, which would provide further
insight into mechanistic control. Only male subjects were included in these studies; whether eNOS−/− or NOS blockade similarly affect females is unknown.

The purpose of this study was to test the hypothesis that the rate at which microvessels respond to the onset and offset of muscle contraction is altered with age and that these responses differ between males and females. We further investigated the mechanistic role of eNOS vs nitric oxide per se in dynamic microvascular control during both the onset and recovery phases of muscle contraction.

METHODS

All procedures were approved by the Institutional Animal Care and Use Committee of Idaho State University and were performed in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. C57BL6 mice were used (n = 41; 23-36 g). Selection of the C57BL6 mouse strain is based upon its acceptance as a “general purpose strain” and commercial availability at all ages. Mice were housed under standard conditions and provided food and water ad libitum. At the end of experiments, mice were euthanized by overdose of sodium pentobarbital. Male and female mice were studied from two age groups: Young = 3 mo (range 2-4 mo; postpubescent) and Old = 24 mo (range 23-25 mo; median lifespan for this strain). Additionally, young mice with genetic deletion of the gene for endothelial nitric oxide synthase (eNOS−/−) were studied. Table 1 shows the number of animals used in each group.

Intravital Microscopy. Mice were anesthetized with pentobarbital (75 mg per kg body mass; intraperitoneal injection). For intravital microscopy, a gluteus maximus muscle was exposed by removing the overlying skin and connective tissue, cut free from its proximal attachment, reflected away from the body and positioned on a clear Sylgard pedestal at in vivo length (8). Preparations were superfused continuously with warmed physiological saline solution (PSS, 36 °C) of the composition (in mM): NaCl 131.09, KCl 4.69, CaCl2 1.8, MgSO4 1.99. Oxygen tension of the PSS was minimized by bubbling with 5% CO2 / 95% N2, maintaining pH at 7.38-7.44. PO2 of the superfusion fluid is 10-11 mm Hg in the superfusion line and 28-29 mmHg over the preparation (Strathkelvin Instruments model 781; calibrated immediately prior to measurements). For LNA
experiments, N\textsuperscript{\textomega}-nitro-l-arginine (LNA) was added to the superfusion solution to a final concentration of 10\textsuperscript{-4} and allowed to superfuse the preparation for 45-60 min prior to further measurements. The optical image was coupled to a CCD video camera through a 20X objective on a Leica DMLFS microscope and displayed on a CCTV video monitor (Tatung) to a final magnification of 1670X. Experiments were recorded on a digital video device (DVD) recorder for later analysis. This recording was necessary due to the rapid microvascular responses at the onset of contractions and the difficulty in tracking vessel diameter during muscle contraction in real-time. DVD recordings were played back at quarter-time (sometimes slower). Internal vessel diameter was measured (spatial resolution ~1 µm) from the edges of the vessel lumen using a video caliper. Diameter data are recorded at 40 Hz (160 Hz real time) and converted to real time by interpolation every 250 ms. The muscle is parallel-fibered with fibers running from the iliac crest and lumbar spine to the femur (gluteal tuberosity) and iliotibial band. The gluteus maximus is primarily a fast twitch muscle (24, 25) and is used in voluntary locomotion (8). We have also reported that the topology of the microvascular network is genetically determined and does not change throughout life (7, 8). For these reasons we are able to study the ‘same’ vessel (same branch and vessel segment within the microvascular network) in every animal.

Muscle contraction was stimulated by field current through platinum/iridium tipped wires (Grass Stimulator, 0.2 ms square-wave pulses). Minimum voltage for a single twitch was consistently 0.7-1 V. The muscle was stimulated at 10X minimal twitch voltage which drives a vigorous contraction with reproducible vascular responses but without muscle fatigue over the course of an experiment. During pilot data collection, we noted that bouts of contractions stimulated at much higher voltages, closer to maximal force production, often delayed recovery to beyond 30 minutes. Since this muscle is primarily of fast twitch (especially type 2B), it is possible that these very strong contractions resulted in tissue trauma. Based on electromyographic recordings from several quadrupeds (including rodents), published gait analyses, our own muscle frequency-force measurements, and observations of our own mice on voluntary running wheels, the following parameters were considered: 1) these muscles are active for 80-250 ms for 2-6 contractions per second depending on walking/running/sprinting (e.g., 6 contractions at 80 ms for a sprint or 2 contractions for 250 ms at a walk) and 2) force increases in the gluteus maximus muscle over a range of 10 Hz to a plateau beyond 90 Hz with 50% peak force generated at 40 Hz.
moderate work-rate was selected for these experiments in order to best approximate the mid-range of muscle function, which is comparable to daily activities: 40 Hz, 3 contractions per second, 166 ms contraction time.

In each preparation, arteriolar responses throughout a bout of muscle contractions and recovery were measured in a second order and a third order vessel. The arteriolar trees in this muscle are typically comprised of four branch levels from the first order arterioles (1A) entering the muscle through the fourth order arterioles (4A) giving rise to capillaries (7). Thus we chose to study the distributing vessels within the muscle which are responsible for regional (2A) and local (3A) control of blood flow, respectively. Vasodilator responses were quantified as response amplitude (i.e., change in diameter), calculated as the difference between the peak response and the resting value prior to stimulation. Responses are also calculated as the percentage of maximal (obtained at the end of each experiment). At the end of each experiment, sodium nitroprusside (SNP; 10^{-5} M) or ACh (10^{-4} M) is applied globally to obtain a 'maximal' diameter; this provides an absolute reference across experiments. Baseline and maximal diameters also provide an index of vasomotor tone during each experiment.

Kinetic analyses of vessel diameter during dilation and recovery were determined using computerized non-linear curve fitting allowing for a delay component (GraphPad Software Inc, San Diego, CA). The basic formula for this is: \( D_t = B + A \cdot (1-e^{-(t-TD)/TC}) \) for the on kinetics and \( D_t = B - A \cdot (1-e^{-(t-TD)/TC}) \) for the off kinetics; where \( D_t \) = diameter at time \( t \), \( B \) = baseline diameter (on kinetics) or dilated steady state diameter (for the off kinetics), \( A \) = amplitude of the response, \( TD \) = time delay and \( TC \) = time constant.

Statistical comparisons were evaluated across all groups for each parameter by ANOVA followed by Tukey post hoc analysis to identify differing pairs. Alpha was set at 0.05 for determination of significance prior to analyses. Data are presented as means ± SEM.

RESULTS
For the young mice, there were no differences between males and females for any parameters (wild type, LNA or eNOS -/-; n= 4-5 of each sex per group). Therefore, data for young mice were pooled within each group. Baseline, maximal and peak contraction-induced diameters of 2A and 3A vessels are shown in Table 1.

At the onset of contractions vasodilation was slower in old male mice than old female mice, young mice of either sex, eNOS-/- mice of either sex, or LNA treated mice of either sex (p<0.05). At the offset of contractions (during recovery) vasoconstriction to baseline was faster in old male mice than old female mice and young mice of either sex but was not different from eNOS-/- mice; LNA treatment achieved 54% and 43% of the effect in the 2A and 3A vessels, respectively (i.e., 54% and 43% of the difference between young and old male mice). There were no differences between old male and old female mice in pre-contraction baseline diameter or in the steady state diameter achieved during muscle contraction.

**DISCUSSION**

The primary novel findings of this investigation are: 1) in male, but not female mice, aging leads to a significant reduction in the rate of vasodilation at the onset of muscle contraction and accelerates the return to baseline during recovery from a bout of muscle contractions that mimic the natural function of the muscle (figure 1), 2) this phenotype occurs without a difference in baseline, maximal or peak contraction induced diameters, 3) slow vasodilation during the on-kinetics in old male mice can not be attributed to alterations in eNOS enzyme expression or acute lack of NO production , 4) rapid recovery to baseline during the off-kinetics following contractions can be created by genetic knockout of eNOS but this effect can only partially be created by acute blockade of NO production and 5) the mechanistic roles of eNOS-/- and NOS blockade are similar in young males and females.

An important observation in the present study was that dilation often followed the first or second contraction as noted by other authors using a variety of preparations (2, 4, 20, 44). However, old males required approximately twice as long to begin dilating (figure 2). In pilot experiments designed to establish the optimal contraction paradigm, we noted that more vigorous contractions elicited with high stimulation frequencies or
voltages predictably generated a robust dilation following a single contraction, even when the contraction duration was reduced to 100 ms, while the contraction stimulus used in these experiments resulted in dilation to the first contraction in approximately 30-40% of cases. Whether the rapidity of these dilations in this preparation are due to metabolic demand, alterations in ion gradients (e.g., potassium (3)) or a mechanical response to vigorous compressive forces (13) remains to be determined.

The kinetics adjustments in blood flow to the forearm during handgrip contractions do not differ between young males and females (age ~24 years)(31). Our data in young mice are consistent with this finding but demonstrate that the sexes adapt differently with age, implicating differences in microvascular control mechanisms that either arise de novo or are unmasked with age. Several pathways for microvascular control are known to differ between the sexes.

**Pathways of microvascular control differ between males and females.** Traditionally, the pathways involved in controlling vascular diameter have been studied as steady state responses; e.g., the final change in diameter to a drug or other stimulus. Available data from steady-state responses document key differences in vascular function between males and females. For example, numerous studies have documented sex differences in the regulation of vascular ion channels (5, 21, 33, 34, 43). Further, cyclooxygenase (COX)-derived prostaglandin H2 (PGH2) can be converted to a vasodilator (PGI2, prostacyclin) or vasoconstrictor (thromboxane A2 or by binding directly to thromboxane A2 receptors). Estrogen promotes conversion of PGH2 to prostacyclin while ovariectomy shifts the balance toward vasoconstriction (28). In some cases age appears to be a further co-factor in these sex differences. For example, prostacyclin production in men decreases with age in both human (41) and rodent (46) models. Vasomotor pathways may also adapt to age in a sex-specific manner; in situations of reduced nitric oxide bio-availability, which occur with aging and studied here, male mice respond by upregulating COX enzyme pathways while female mice respond through increases in cytochrome P-450 (42, 47), an epoxygenase enzyme that metabolizes arachidonic acid to form vasodilating epoxyeicosatrienoic acids (EETs) (16, 19) in endothelial cells (6, 10, 11).
It has been hypothesized that age-related reductions in bio-available nitric oxide (9, 26) promote endothelin-mediated constriction in males while females are less susceptible to these reductions due to a greater reliance on 'endothelium derived hyperpolarizing factors' for vasodilation (37). eNOS knockout mice exhibit the same post-contraction phenotype as our old male mice but not the slow dilation at the onset of contractions. Moreover, we show that acute blockade of NOS activity using the l-arginine mimetic L-NA explains only part of this effect. A role for NO in post-contraction hyperemia but not vasodilation at the onset of contractions is consistent with findings in humans (40). Nitric Oxide is a principle regulator of vascular homeostasis (29, 38). Deletion of the gene for eNOS caused rapid vasoconstriction toward baseline during recovery, similar to old male mice, but blockade of NO production with LNA superfusion (10^{-4}) explains only 46% and 25% of this effect in the 2A and 3A vessels, respectively. Both young male and young female mice responded similarly in the eNOS-/- and LNA conditions. Therefore, the mechanisms by which the old females persisted with a youthful phenotype are not sufficient (or are not sufficiently present) at the young age to overcome the effects of these manipulations in eNOS-/- activity or acute NO production. A partial role for nitric oxide bioavailability in our findings would be consistent with the literature, which indicates a reduction in NO bioavailability with age (27, 39). Decreased bio-availability of NO can increase sensitivity of α2-adrenoceptors on vascular smooth muscle (12). Though alpha1-adrenoceptors predominate in larger vessels, α2-adrenoceptors appear to dominate deeper in the microvascular tree (i.e., there is an increase in α2/ α1 as microvessel diameter decreases) (15). It is these α2 receptors that are most susceptible to override by muscle contraction-mediated vasodilation (1). The present data show that mechanisms sensitive to eNOS loss, aside from acute NO production per se, play a significant role during recovery but not at exercise onset. This emphasizes the multifactorial control of microvascular tone and the integrated nature of the underlying control mechanisms (32).

Dilation of 2A vessels was slower than 3A vessels at the onset of contraction in old male mice (figure 3). This may occur if the mechanisms controlling dilation of 2A vessels are relatively more impaired with age than those of 3A vessels, if vasoconstricting pathways are relatively more enhanced with age in 2A than 3A or if mechanisms coupling 3A vessels with 2A vessels are impaired. We have previously shown that communication of vasodilation along 2A vessels is impaired with age in male mice (8), though we have not examined these responses across vessel branches. During recovery from muscle contractions, 3A vessels recovered
significantly faster than 2A in young animals (of both sexes) and in old females. This was not the case in old males because 2A vessels returned toward baseline approximately 3-fold faster than their young and female counterparts. Therefore, the present data demonstrate that aging and sex may impact dynamic microvascular function differently throughout the branches of a skeletal muscle vascular network and emphasize the importance of exploring microvascular control mechanisms at all branching levels. Understanding these mechanisms and their regional differences will be important for focused therapeutic approaches.

Limitations. Microvessels in skeletal muscle of old male mice were slow to dilate and fast to recover with muscle contraction. These data do not identify the site of dysfunction. The vascular wall may be resistant to dilation or the kinetics of release of vasodilators from skeletal muscle fibers may be impaired. For example, during sustained isometric contraction, male subjects rely on glycolysis to a greater extent than females despite similar oxidative capacity of the muscle (36). In our study, the steady state diameters at baseline and during contractions were not different across groups, suggesting that the demand for blood flow was not different. This is consistent with our previous findings that age does not impair force production or the work done during contractions by this muscle (8). Findings in humans have demonstrated that differences in muscle fatigability between the sexes (males more fatigable than females) may depend on blood flow control since ischemia abolishes the difference (35).

Conclusion. Our data are the first to investigate the interactive roles of age and sex on dynamic microvascular responses to muscle contraction in vivo. These data uniquely demonstrate age-dependent declines in vasodilation in male, but not female, skeletal muscle during onset and recovery despite the same steady state vasomotor response. eNOS-/- had a greater impact on recovery kinetics than acute NO production though neither condition affected vasodilatory kinetics at the onset of muscle contractions. These findings call specific attention to the importance of understanding the dynamic control of muscle perfusion and present an opportunity to understand how females persist with a youthful kinetic phenotype. Elucidating the mechanisms by which female mice persist with a youthful phenotype, and males do not, will shed light on potential therapeutic targets for age-related dysfunction of resistance vessels.
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REFERENCES


FIGURE LEGENDS

**Figure 1.** Representative response traces of 2A vessels in the young, old female and old male groups.

**Figure 2.** Delay prior to vasodilation at the onset of contractions (A, C) and prior to initiation of recovery of vessel diameter at the end of contractions (B, D) in 2A and 3A vessels (A/B and C/D, respectively). eNOS/- group is genetic ‘knockout’ of endothelial nitric oxide synthase, LNA is following 45 min-1 hr superfusion with $10^{-4}$ Nω-nitro-l-arginine to block endogenous NOS activity. A) Second order arterioles (2A) in old male mice are late to begin dilating compared with young, old female and eNOS/- (brackets, p<0.05). B) Second order arterioles (2A) in old male mice have a shorter delay than all other groups except eNOS/- (#, p<0.05). C) Third order arterioles (3A) in old male mice are late to begin dilating compared with old female mice (brackets, p<0.05). D) Delays in recovery in third order arterioles (3A) were not different among the groups.

**Figure 3.** Time constant for vasodilation (A, C) and recovery (B, D) of diameter in 2A and 3A vessels (A/B and C/D, respectively). Time constant is the amount of time, after the delay, required to achieve 63% of the overall change. eNOS/- group is genetic ‘knockout’ of endothelial nitric oxide synthase, LNA is following 45 min-1 hr superfusion with $10^{-4}$ Nω-nitro-l-arginine to block endogenous NOS activity. A) Second order arterioles (2A) in old male mice are slower to dilate than all other groups (asterisk, p<0.05). B) Second order arterioles (2A) in old male mice and eNOS/- are faster to recover than other groups but not different from each other (#); LNA is significantly different from all other groups (▲); (p<0.05). C) Third order arterioles (3A) in old male mice are slower to dilate than all other groups (asterisk); † indicates significantly smaller than same group for 2A; (p<0.05). D) Third order arterioles (3A) in old male mice are faster to recover than all other groups except eNOS/- (#); † indicates significantly smaller than same group for 2A; (p<0.05).
Table 1. Diameters of second and third order arterioles in mouse gluteus maximus muscle. Values are given for baseline, peak steady-state values during muscle contractions and maximal diameter to acetylcholine or sodium nitroprusside. Young groups are pooled male and female data (4-5/sex/group), which were not different (p>0.05). *significantly smaller than Young and Old Female groups.

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