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AGE AND SEX DIFFERENCES IN LOCAL VASODILATION AND EXERCISE HYPEREMIA

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ABSTRACT

Local alterations in vasodilator responsiveness may significantly influence the leg hyperemic response to dynamic exercise in aging humans in a sex-specific manner. For example, older women exhibit lower values for peak calf vascular conductance (normalized to muscle mass) and submaximal leg blood flow during cycling relative to young women, an age difference not observed in comparable populations of men. However, the extent to which a) leg vasodilatory responsiveness is altered with age in women and b) age-associated differences in the leg hyperemic response to exercise are more substantial in women than men have not been comprehensively investigated. Accordingly, this series of five studies was designed to examine local alterations in leg vasodilatory responsiveness as a function of age in women, directly test the hypothesis that older women would exhibit greater reductions in leg exercise hyperemia than men during exercise not limited by central mechanisms, and examine the consequences of observed alterations in leg vasodilation during submaximal exercise in older women.

The purpose of the first study was to compare age differences in brachial and popliteal flow-mediated dilation (FMD) and shear rate in women. Ultrasound-derived diameters and Doppler flow velocities of the brachial and popliteal arteries were measured in 14 young ([mean ± S.E.M] 22 ± 1 yrs) and 14 older (70 ± 2 yrs) healthy women at rest and during and after 5 min of calf occlusion. Peak shear rate did not differ with age in either artery, but the normalized response of the brachial and popliteal arteries (%FMD per unit change in shear rate) was lower with age (55% and 53%, respectively) but also did not exhibit limb specificity. Endothelium-independent dilation (arterial dilation to sublingual nitroglycerin) was blunted (by 45-65%) in brachial and popliteal arteries of older women. Brachial and popliteal artery FMD were similarly reduced with age in women; this may be attributable in part to diminished smooth muscle responsiveness.

The purpose of the second study was to test the hypothesis that metabolic inhibition of a sympathetic stimulus (i.e., sympatholysis) is reduced with age in the lower extremity vasculature of women. Popliteal artery diameter and velocity (Doppler ultrasound) were assessed in 16 young (Y: 23 ± 1 yrs) and 14 older (O: 69 ± 1 yrs) women after 5 min of distal calf occlusion (FMD), 3 min of hand immersion in ice water [cold pressor test (CPT)], and 5 min of distal calf occlusion combined with hand immersion in ice water (FMD+CPT). During the combined stimulus (FMD+CPT), the reduction in peak popliteal artery conductance (5-8%) was similar in young and older women, despite reduced resting vasoconstrictor sensitivity to CPT [Y: -27.3 ± 3.8%; O: -15.8 ± 2.2%; $p < 0.05$] and blunted muscle sympathetic nerve activity response to CPT (Y: 12.7 ± 3.6 bursts min^{-1}; O: 7.8 ± 2.5 bursts min^{-1}; $p < 0.05$) in older women. In addition, peak popliteal diameter, measured during the combined stimulus (FMD+CPT), was blunted in young but not in older women (Y FMD: 5.5 ± 0.1 mm; Y FMD+CPT: 5.4 ± 0.1 mm; $p = 0.03$; O FMD: 5.8 ± 0.2 mm; O FMD+CPT: 5.8 ± 0.2 mm) as was FMD ($p < 0.01$). Older women exhibit diminished popliteal artery reactivity and reduced sympatholysis in the leg resistance vasculature.

The purpose of the third study was to test the hypothesis that exercise-induced vasodilator responses are greater in young women than men. Sixteen women (22 ± 1 yrs) and 15 men (24 ± 1 yrs) with similar fitness and activity levels performed graded quadriceps exercise (supine, single-leg knee extensions, 40 contractions/min) to maximal exertion while femoral artery diameter and velocity (Doppler ultrasound) were measured simultaneously with beat-to-beat blood pressure during each 3-min work rate (4.8 and 8.0 watts/stage for women and men, respectively). The hyperemic response to exercise (slope of femoral blood flow vs. absolute work rate) was greater ($p < 0.01$) in women as was femoral blood flow at absolute work rates >15 W. The leg vasodilatory response to exercise (slope of calculated femoral vascular conductance vs. work rate) was also greater in women than in men ($p < 0.01$) because of the sex difference in

hyperemia and the women's lower mean arterial pressure (approximately 10-15 mmHg) at all work rates ($p < 0.05$). The vasodilatory response to dynamic leg exercise is greater in young women than in young men.

The purpose of the fourth study was to test the hypothesis that sex-specific age differences in exercising leg hemodynamics persist during small muscle mass exercise that is not limited by cardiac output. Thirty-one young (15 men: 24 ± 1 yrs; 16 women: 22 ± 1 yrs) and 31 older (13 men: 71 ± 2 yrs; 18 women: 67 ± 1 yrs) adults performed graded single leg knee extensions to maximal exertion. Femoral artery blood velocity and diameter (Doppler ultrasound) and beat-to-beat arterial blood pressure were measured during each 3-min work rate (4.8 and 8.0 watts/stage for women and men, respectively). Despite lower resting leg blood flow and vascular conductance, older men exhibited relatively similar exercising leg hemodynamic responses. Older women exhibited lower hyperemic (Y: 52 ± 3 $mL \cdot min^{-1} \cdot W^{-1}$ vs O: 40 ± 4 $mL \cdot min^{-1} \cdot W^{-1}$ and $p = 0.02$) and vasodilatory responses (Y: 0.56 ± 0.06 $mL \cdot min^{-1} \cdot mmHg^{-1} \cdot W^{-1}$ vs O: 0.37 ± 0.04 $mL \cdot min^{-1} \cdot mmHg^{-1} \cdot W^{-1}$; $p < 0.01$) to exercise compared to young women. The lower vasodilator responses in older women were not abolished by consideration of age differences in hemoglobin, quadriceps muscle mass, muscle recruitment and mechanical influences on muscle perfusion. Local (non-cardiac) factors underlie the sex-specific effects of age on exercising leg hemodynamics in healthy adults.

The purpose of the fifth study was to test the hypothesis that active muscle oxygen extraction is higher in older women during a sustained bout of knee extensor exercise. In 9 young (25 ± 1 yrs) and 13 older (67 ± 1 yrs) healthy women, femoral artery hemodynamics (Doppler ultrasound) were assessed at rest and during nine minutes of ~17-18W single-knee extensor exercise. Near-infrared spectroscopy (NIRS) was used to measure changes in deoxyhemoglobin (HHb) and oxyhemoglobin (O_2Hb) relative to rest to assess oxygen extraction (Δ HHb) and

capillary blood volume (Δ HHb + O_2Hb). During exercise, femoral blood flow and vascular conductance were significantly ($p < 0.05$) lower in O compared to Y (Y: 1430 ± 101 $mL \cdot min^{-1}$ and 16.6 ± 1.2 $mL \cdot min^{-1} \cdot mmHg^{-1}$ vs O: 1127 ± 64 $mL \cdot min^{-1}$ and 10.9 ± 0.6 $mL \cdot min^{-1} \cdot mmHg^{-1}$). While oxygen extraction increased more rapidly in Y, there were no age differences ($p > 0.10$ for all comparisons) beyond the fourth minute of exercise. In addition, the relative increase in microvascular blood volume was not significantly different during exercise in Y vs. O (age effect: $p=0.87$). NIRS-derived measurements of changes in deoxygenated hemoglobin do not support the hypothesis that older women compensate for the blunted leg hyperemic response to knee extensor exercise through an augmentation of quadriceps oxygen extraction; knee extensor work may be accomplished by other metabolic or microvascular adjustments to exercise in older women.

These studies provide evidence for alterations in local vasodilator control such that older women exhibit reduced conduit artery responsiveness to increases in shear rate and sympathetic outflow and blunted metabolic inhibition of a sympathetic stimulus in the calf resistance vasculature. Moreover, while young women demonstrate augmented leg vasodilation to small muscle mass exercise relative to young men, aging is associated with a sex-specific effect on leg exercise hyperemia such that older women exhibit lower leg vasodilation and older men relatively similar leg vasodilation relative to young counterparts during leg exercise. Exercise tolerance, microvascular oxygen extraction, and capillary blood volume were all unaffected by blunted whole limb vasodilation during sustained single-knee extensor exercise. However, reduced leg hyperemic responses to exercise in older women could be functionally limiting with respect to fatigability and/or tolerance to daily physical activities and large muscle mass exercise, especially when coupled with additional cardiovascular stress (e.g., heat and dehydration, or cardiovascular pathologies).

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LIST OF ABBREVIATIONS

ABI	Ankle-brachial index
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
AUC	Area under the curve
BMI	Body mass index
cm	Centimeters
CPT	Cold pressor test
HHb	Deoxygenated hemoglobin
DHEA	Dehydroepiandrosterone
DXA	Dual energy x-ray absorpiometry
%D	Dilation
ECG	Electrocardiogram
EDHF	Endothelial-derived hyperpolarizing factor
EMG	Electromyography
ET	Endothelin
FBF	Femoral blood flow
FMD	Flow-mediated dilation
FVC	Femoral vascular conductance
g	grams
HDL	High-density lipoprotein
kg	kilograms
LDL	Low-density lipoprotein

m	meters
MAP	Mean arterial pressure
mmHg	Millimeters of mercury
mL	Milliliters
MRI	Magnetic resonance imaging
MSNA	Muscle sympathetic nerve activity
NIRS	Near infrared spectroscopy
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NTG	Nitroglycerin
O_2Hb	Oxygenated hemoglobin
PBF	Popliteal blood flow
PCO_2	Partial pressure of carbon dioxide
PG	Prostaglandins
PO_2	Partial pressure of oxygen
PVC	Popliteal vascular conductance
PWV	Pulse wave velocity
SaO_2	Arterial oxygen saturation
S.E.M.	Standard error of mean
SR	Shear rate
$\dot{V}O_{2max}$	Maximal oxygen uptake
W	Watts
WR	Work rate

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Chapter 1

INTRODUCTION

Background and Significance

During dynamic exercise in humans, oxygen delivery to the working muscle is increased to adequately meet the metabolic demand of the exercising muscle. The increased oxygen delivery is accomplished by an increase in blood flow perfusing the muscle vasculature through both central (increased cardiac output and blood pressure) and peripheral (release of local vasodilators and vasoconstrictors as well as action of the skeletal muscle pump) mechanisms. In addition, in order to maintain systemic pressure in the presence of the great vasodilatory capacity of muscle (Andersen & Saltin, 1985; Richardson *et al.*, 1993), sympathetic nerve activity is augmented such that inactive vascular beds (the splanchnic, renal, and inactive muscle beds) are constricted, thereby directing blood from the inactive to active muscle (Savard *et al.*, 1989; Rowell, 1993). The presence of locally released vasodilators from the working muscle, vascular endothelium, and red blood cells (Clifford & Hellsten, 2004) counters the sympathetic vasoconstriction in the working muscle vasculature in a process termed functional sympatholysis (Remensnyder *et al.*, 1962; Hansen *et al.*, 2000b). These acute adjustments to dynamic exercise, coupled with the increased oxygen extraction across the working muscle, serve to meet the metabolic demand of working muscle while maintaining systemic blood pressure. Therefore, the magnitude of the muscle blood flow response to dynamic exercise in humans is influenced by increases in cardiac output, the quantity of skeletal muscle dilated, and the balance between local metabolic vasodilation and sympathetic vasoconstriction in the active muscles.

Normal aging is associated with a number of alterations that could compromise muscle blood flow or its regulation during dynamic exercise; these include diminished cardiac pumping capacity, structural alterations in the vasculature, reductions in skeletal muscle mass and/or quality (e.g., increased intramuscular fat), greater muscle sympathetic neural outflow, and alterations in local vascular control mechanisms (Proctor & Parker, 2006). Indeed, evidence suggests that there are age-related alterations in active muscle blood flow and its regulation during dynamic exercise in humans (Wahren *et al.*, 1974; Lawrenson *et al.*, 2003; Poole *et al.*, 2003; Proctor *et al.*, 2003a; Proctor *et al.*, 2003b; Proctor *et al.*, 2004b). However, to date there is little evidence regarding the sex-specific influence of age on active muscle blood flow, due to the lack of studies establishing baseline sex differences in young humans as well as a dearth of studies conducted on older women. Given that the integrated response to and capacity for exercise is dependent on vasodilation of the active muscle vasculature, investigating sex-specific alterations in the control of limb blood flow during dynamic exercise is important for better understanding documented sex-differences in physical function (Merrill *et al.*, 1997; Smith & Baltes, 1998; Richardson, 2003) and dynamic exercise responses (Ogawa *et al.*, 1992; Fleg *et al.*, 1995) in older adults.

Two models for investigating the influence of age and/or sex on muscle hyperemia have traditionally been utilized in humans. The first model involves measurement of limb blood flow responses (via techniques such as dye dilution, thermodilution, plethysmography, Doppler ultrasound, and near-infrared spectroscopy) during either large (two-leg cycling or walking/running) or small (forearm or knee extensor) muscle mass exercise (Andersen *et al.*, 1985; Jasperse *et al.*, 1994; Magnusson *et al.*, 1994; Fleg *et al.*, 1995; Mortensen *et al.*, 2005). While this model provides an integrated approach to studying limb exercise hyperemia, the limitations imposed by factors such as challenging cardiac pumping capacity, evoking significant counterregulatory reflexes, and simulating multiple vasoregulatory pathways can render isolation

of specific mechanisms underlying age and sex differences difficult. Thus, an alternative approach has been to use non-exercising models (i.e., arterial infusions of vasoactive substances, reactive hyperemia, response to sympathetic stimulation and flow-mediated dilation) to isolate the influence of age and sex on local dilator and/or constrictor pathways which may then contribute to alterations in limb blood flow during exercise. While admittedly an indirect approach, the latter does provide a more simplified model for studying local vasodilation or vasoconstriction in which the stimulus for the elicited vasoreactive responses can be more carefully controlled.

With respect to potential age and sex differences in leg vasodilation observed using both of the above-described models, the following have been observed: 1) Leg blood flow and vascular conductance were reduced during submaximal cycling exercise in older women but not men relative to young controls (Proctor *et al.*, 2003a; Proctor *et al.*, 2003b), and 2) the reduction in leg vasodilator capacity with age appears to be more extensive in women than men (Martin *et al.*, 1991; Newcomer *et al.*, 2005; Proctor *et al.*, 2005; Ridout *et al.*, 2005; Wray *et al.*, 2006). These findings cumulatively imply that the local determinants of vasodilation in the exercising leg vasculature may be altered by age in women such that leg hyperemic responses to dynamic exercise are more impaired in women than men. However, extensive investigations of local dilatory alterations in the leg vasculature of older women are lacking, and comparisons of leg blood flow responses to exercise in either young or older men vs. women have not been systematically investigated. Thus, the evidence supporting the hypothesis that peripheral alterations in the control of leg blood flow during dynamic exercise with age are more substantial in women than men remains speculative.

Accordingly, this series of dissertation studies investigated local factors influencing the leg blood flow and vascular conductance responses to dynamic exercise as modified by age and sex in healthy humans. The first two studies were designed to extend findings regarding age-related changes in local vasodilatory responses in the forearm in men and women and/or leg in

men to the lower leg vasculature in women. Using a non-exercise stimulus to induce vasodilation (alone and paired with sympathetic stimulation) in the absence of the central cardiac responses evoked by dynamic exercise, these studies provided evidence of alterations in the local leg dilatory response in women. They also served as a basis for further studies investigating alterations in the control of leg blood flow during exercise. The third study utilized an exercise model in which central limitations to exercise were minimized (i.e., supine, single-knee extensor exercise) to establish data on baseline sex differences in leg vasodilatory responses to graded exercise in young humans. The fourth study then investigated the hypothesis that these leg vasodilatory responses would be altered by age in a sex-specific manner. Finally, the fifth study addressed the implications of the lower leg vasodilatory responses unique to older women by investigating microvascular responses (e.g., changes in oxygen extraction and blood volume) to a sustained bout of knee extensor exercise in young vs. older women. Collectively, these studies focused on clarifying the influence of healthy aging on leg vasodilation in both sexes with the rationale that previously documented sex differences in the aging cardiovascular system (Fleg *et al.*, 1995; Najjar *et al.*, 2004; Narkiewicz *et al.*, 2005) have not been extended to the understanding of exercising leg blood flow in healthy humans.

Specific Aims and Hypotheses

Specific Aim 1

The purpose of Study #1, "Age and flow-mediated dilation: a comparison of dilatory responsiveness in the brachial and popliteal arteries," was to determine whether findings regarding peripheral vasodilator responsiveness in men (i.e., greater influence of age on reductions in arm vs. leg vasodilatory responsiveness; (Newcomer *et al.*, 2005; Wray *et al.*, 2006)) would be observed in women.

Hypothesis 1A**:** Vasodilation of a lower extremity artery (FMD of the popliteal artery) is blunted compared with that of the brachial artery in young and older women.

Hypothesis 1B: Popliteal artery FMD is reduced in older subjects compared with young subjects to a lesser extent than brachial artery FMD.

Specific Aim 2

The purpose of Study #2, "Evidence for reduced sympatholysis in leg resistance vasculature of healthy older women," was to determine the effect of age on lower limb sympatholysis in women in light of the evidence suggesting sympatholysis is reduced with age during two-leg cycling in men (Koch *et al.*, 2003), handgrip exercise in men (Dinenno *et al.*, 2005) and handgrip exercise in women (Fadel *et al.*, 2004).

Hypothesis 2A: Resistance vessel dilation (the hyperemic calf blood flow response to five minutes of distal cuff occlusion) is blunted to a greater extent by superimposition of sympathetic stimulation in older compared to young women.

Hypothesis 2B: Popliteal artery FMD (the conduit dilatory response to five minutes of distal cuff occlusion) is blunted to a greater extent by superimposition of sympathetic stimulation in older compared to young women.

Specific Aim 3

The purpose of Study #3, "Sex differences in leg vasodilation during graded knee extensor exercise in young adults," was to compare leg blood flow and vascular conductance responses during small muscle mass dynamic exercise in young men and women to determine whether leg vasodilator responses to exercise are sex-specific in young humans, as has been shown in exercising rats (Rogers & Sheriff, 2004) and non-exercising human forearms (Kneale *et al.*, 2000).

Hypothesis 3: Young women exhibit augmented leg vasodilator responses relative to young men during graded knee extensor exercise to maximal exertion.

Specific Aim 4

The purpose of Study #4, "Sex-specific influence of aging on exercising leg blood flow," was to determine if the lower leg vasodilatory response in women previously observed during cycling exercise persisted during graded single-knee extensor exercise, when the confounding influence of central cardiac limitations on leg hyperemia is minimized.

Hypothesis 4: There is an age by sex interaction in the leg hemodynamic responses to graded exercise, with older women exhibiting a significantly greater age-related reduction in leg blood flow and vascular conductance relative to young controls than older men.

Specific Aim 5

The purpose of Study #5, "Age and microvascular responses to sustained leg exercise in women" was to compare changes in microvascular oxygen exchange (oxygen extraction, microvascular blood volume, and blood volume heterogeneity) during a sustained bout of higher intensity (~17-18W) of knee extensor exercise.

Hypothesis 5: Older women exhibit significantly greater oxygen extraction during sustained knee extensor exercise than young women, consistent with an adaptive response (to maintain leg $\dot{V}O_2$ and exercise capacity) to the reduced leg blood flow exhibited by older women during this mode of exercise.

Chapter 2

REVIEW OF LITERATURE

This chapter will address the literature relevant to the topics comprising this dissertation by discussing 1) the control of leg blood flow by both central and peripheral mechanisms during dynamic exercise, 2) the influence of age, sex and age by sex interactions on the mechanisms controlling skeletal muscle hyperemia, and 3) the specific findings providing evidence of age and sex differences in vasodilatory responses that underlie the following five studies.

Overview of the Leg Blood Flow Response to Exercise

During dynamic, large muscle mass exercise (e.g., two-leg cycling or running), blood flow to the working muscle increases proportionally to the metabolic demand of the working muscle, as demonstrated by the tight relationship between muscle blood flow and running speed in rats (Laughlin & Armstrong, 1982) as well as oxygen uptake and work (Richardson *et al.*, 1993) or tissue perfusion (Rådegran *et al.*, 1999) in humans. The increase in muscle blood flow is accomplished by three mechanisms: 1) an increase in cardiac output through an augmentation of venous return, heart rate and cardiac contractility accomplished by neural, muscular and hormonal mechanisms, 2) vasoconstriction of the non-exercising regions (renal, splanchnic, skin, and inactive muscle vasculatures), and 3) vasodilation of the active muscle vasculature (Rowell, 1993; Laughlin & Korzick, 2001). Given that the maximal perfusion of exercising skeletal muscle (in 2-3 kg knee extensor muscles) in humans has been estimated to be anywhere from 250

to 400 mL/100 g muscle/min (Andersen & Saltin, 1985; Rowell, 1988; Richardson *et al.*, 1993), it has been suggested that the vasodilatory capacity of the skeletal muscle for outstripping the pumping capacity of the human heart will be reached if only 1/3 of the active skeletal muscle mass in the body (approximately 30kg) is engaged in intense exercise (Andersen & Saltin, 1985). Consequently, it is widely assumed that the third mechanism of increasing leg blood flow (local vasodilation of the active vasculature) tonically competes with heightened sympathetic outflow evoked by exercise such that the active vasculature is exposed to simultaneous vasodilator and vasoconstrictor influences in order to maintain systemic pressure. When exercise intensity and/or the active muscle mass engaged in exercise is low, local dilatory influences largely counter the augmented sympathetic outflow observed during exercise (Remensnyder *et al.*, 1962; Peterson *et al.*, 1988; Strange, 1999; Buckwalter & Clifford, 2001)); however, when exercise intensity with a large active muscle-mass is high, leg vascular conductance may be restrained by sympathetic vasoconstriction, particularly if cardiac output is reduced (Secher *et al.*, 1977; Savard *et al.*, 1989; Strange *et al.*, 1990; Pawelczyk *et al.*, 1992; Magnusson *et al.*, 1997).

Central Mechanisms Involved in the Control of Leg Blood Flow

As mentioned above, neural and hormonal mechanisms contribute to the centrally-mediated regulation of leg blood flow during dynamic exercise. With respect to the delivery of blood to the active muscle, increased venous return, heart rate and contractility of the cardiac muscle underlie the observed increase in cardiac output during dynamic exercise. Withdrawal of parasympathetic tone followed by sympathetic activation of the beta-1 receptors increases heart rate (Robinson *et al.*, 1966). Moreover, increases in venous return (via the muscle and respiratory pumps and venoconstriction) mediate an augmentation of end-diastolic volume (Rowell, 1993), which, when coupled with the inotropic effects of norepinephrine and epinephrine on cardiac

contractility to reduce end-systolic volume (Katz, 1992), result in an increase in stroke volume. By increasing perfusion pressure and also decreasing venous pressure (i.e., skeletal muscle pump), these mechanisms augment blood supply and oxygen delivery to the working muscle. In addition, neural control of leg hyperemia is accomplished primarily through the sympathetic nervous system, as studies in rats and hamsters demonstrate that sympathetic nerves are widely distributed throughout the systemic vasculature and the arteriolar network in skeletal muscle (Fleming *et al.*, 1989; Grasby *et al.*, 1999). Physical exercise stimulates the sympathetic nervous system in parallel with contraction intensity and the mass of involved muscle (Mark *et al.*, 1985; Savard *et al.*, 1989); it is believed that central command, the exercise pressor reflex arising from stimulation of mechanically and metabolically sensitive nerve endings in the working muscle, and consequent resetting of the arterial baroreflex (Alam & Smirk, 1937; Melcher & Donald, 1981; Raven *et al.*, 1997) cumulatively underlie the increased sympathetic outflow evoked by exercise. The sympathoexcitation during exercise is directed to all tissues, serving to redistribute cardiac output from inactive vascular regions and modulating exercising muscle blood flow (Joyner *et al.*, 1992; Keller *et al.*, 2003) in coordination with the local dilatory factors described below. Finally, other systemic vasoconstrictors—angiotensin II, vasopressin, and endothelin-I—also increase during dynamic exercise and may play a role in modulating the redistribution of blood flow to the working muscle, as evidenced by work in rats and swine (Yanagisawa *et al.*, 1988; Symons & Stebbins, 1995; Symons *et al.*, 1999).

Peripheral (Local) Mechanisms Involved in the Control of Leg Blood Flow

The delivery of the increased cardiac output during dynamic exercise to metabolically active skeletal tissue is controlled predominantly by local vasoactive factors (both vasodilators and vasoconstrictors) that affect the vascular tone of the resistance arterioles such that there is a

net increase in vasodilation (Dodd & Johnson, 1991; Rowell, 1993; Laughlin & Korzick, 2001). The contribution of vasodilation of the larger vessels in the arterial vasculature to exercise hyperemia, especially conduit arteries, is considered to be much less significant than the resistance arterioles and feed arteries, as demonstrated by work in both rats and humans (Jasperse & Laughlin, 1997; Rådegran & Saltin, 2000). In addition, vasodilators also appear to inhibit sympathetic vasoconstriction in the working muscle, producing effective functional sympatholysis (Remensnyder *et al.*, 1962; Hansen *et al.*, 2000b; Joyner & Thomas, 2003) such that blood flow to working muscle is not impacted by the sympathetic nervous system until exercise intensity with engagement of a large muscle mass is significantly high (Strange, 1999). One of the enduring mysteries of skeletal muscle hyperemia to date is identifying the local mechanisms involved in the dilatory response to exercise, as there appear to be multiple and redundant mechanisms involved in the hyperemic process. Certainly, the concept that active muscle blood flow is so closely related to the metabolic rate of the working muscle (Granger *et al.*, 1975) suggests that one or more metabolites from actively contracting muscle and/or red blood cells underlie the local vasodilatory response to exercise. For example, possible metabolic signals related to contraction include tissue and/or blood PO_2 and PCO_2, hydrogen ions, osmolarity, adenosine and adenine nucleotides, potassium, lactate, histamine, kinins, phosphates, prostaglandins, muscle-derived nitric oxide and ATP released from red blood cells (Vanhoutte & Mombouli, 1996; Stamler *et al.*, 1997; Hellsten *et al.*, 1998; Juel *et al.*, 2000; Clifford & Hellsten, 2004; Gonzalez-Alonso *et al.*, 2006)). In addition, arterial endothelial cells release vasoactive substances (nitric oxide, prostanoids, endothelium-derived hyperpolarizing factor) in response to physical forces (shear stress and vessel stretch) and chemical substances found within the blood (Bjornberg *et al.*, 1990; Davies *et al.*, 1992; Hillig *et al.*, 2003; Shipley *et al.*, 2005) which may regulate vascular tone during exercise. However, work to date to isolate the substances necessary for exercise hyperemia in humans has very rarely shown a necessary role for any metabolite or

endothelial-derived factor, as reductions in hyperemia from blocking various vasoactive substances are many times non-existent, transient, or of a limited magnitude (Klabunde *et al.*, 1988; Engelke *et al.*, 1996; Frandsen *et al.*, 2001; Joyner & Wilkins, 2007; Mortensen *et al.*, 2007). The same holds true for investigations involving the vasoactive substances relevant to metabolic inhibition of the sympathetic stimulus during exercise. Since sympatholysis is restricted to the working muscle and related to the intensity of exercise (Marshall, 1982; Thomas *et al.*, 1997; Ruble *et al.*, 2002; Wray *et al.*, 2004b), local muscle metabolic factors such as potassium, prostacyclin, ATP, and nitric oxide have been investigated with conflicting results in dogs and humans (Skinner & Costin, 1969; Hansen *et al.*, 2000a; Chavoshan *et al.*, 2002; Rosenmeier *et al.*, 2004), again raising the likelihood of redundant and multiple mechanisms involved in sympatholysis. These unresolved findings are most likely also explained in part by the degree to which muscle hyperemia and functional sympatholysis are dependent on intensity of exercise, time after onset of exercise, and active muscle fiber types used for contraction, as indicated by studies of the rat vasculature (Laughlin & Armstrong, 1982; Armstrong & Laughlin, 1983; Thomas *et al.*, 1994). Additional contributors to muscle hyperemia, again also dependent on factors such as exercise intensity, modality, and muscle fiber type, involve the skeletal muscle pump (i.e., contraction of active muscle vasculature increases venous emptying and impedes arteriolar inflow in a rhythmic fashion; (Laughlin, 1987; Tschakovsky *et al.*, 1996; Sheriff, 2003; Rothe, 2005)), myogenic control (response of the smooth muscle to increases or decreases in transmural pressure; (Johnson & Henrich, 1975; Meininger & Davis, 1992)) and conducted vasodilation (propagation of the dilatory signal upstream via gap junctions between endothelial and/or smooth muscle cells; (Segal *et al.*, 1999; Emerson & Segal, 2000)), although myogenic responsiveness appears to be more a important mediator of vascular tone at rest, at least in rats (Meininger *et al.*, 1987), and studies providing evidence of conducted vasodilation have thus far been limited to work in animal models.

Differences Between Large and Small Muscle Mass Leg Exercise

Responses to large and small muscle mass leg exercise (in particular, two- vs one-leg cycling or kicking) can be difficult to assess based on whether comparisons are made at the same absolute (i.e., systemic metabolic demand represented by total body oxygen uptake) or relative (i.e., percent of maximal oxygen uptake) work intensity. For example, at absolute work intensities, heart rate, arterial pressure, total peripheral resistance, and pulmonary ventilation are greater in small muscle mass exercise than large muscle mass exercise given that relative work intensity during these conditions is higher with a smaller muscle mass (Bevegard & Shepherd, 1967; Freyschuss & Strandell, 1968; Davies & Sargeant, 1974). Lewis et al. provided the most comprehensive evaluation of cardiovascular and ventilatory responses to one-leg and two-leg cycling by having subjects perform a maximal oxygen uptake test for large (two-leg cycling) and small (one-leg cycling and arm exercise) muscle mass exercise and then comparing responses at both the same relative and absolute work intensities. Using this model, authors concluded that while heart rate, mean arterial pressure, and total peripheral resistance were greater in small vs. large muscle mass exercise at absolute work intensities, when related to relative load, oxygen uptake, cardiac output, heart rate, catecholamine concentrations and arteriovenous oxygen difference were greater with increasing muscle mass, although mean arterial pressure was inversely related to muscle mass (Lewis *et al.*, 1983). These findings support the concept that central cardiovascular adaptations to exercise (heart rate and cardiac output) increase as a function of oxygen uptake and therefore rise to a greater extent during large muscle mass than small muscle mass exercise at comparable relative intensities; mean arterial pressure and peripheral vascular resistance are influenced by the size of the muscle mass dilated during exercise and therefore will be greater during small muscle mass exercise at any relative work rate, although differences between one- and two-leg exercise are minimal. In addition to these

differences, the factors limiting maximal exercise are different in one- vs two-leg exercise. Davies and Sargeant administered a 45% oxygen mixture to subjects performing one- and two-leg exercise and found that $\dot{V}O_{2max}$ was improved by 10% in two-leg exercise with no increase in $\dot{V}O_{2max}$ during one-leg exercise (Davies & Sargeant, 1974). This observation suggests that the factor limiting two-leg exercise involves is imposed by cardiac output whereas the limit to performance in one-leg exercise is peripheral in nature, a finding that has been supported by subsequent investigations (Secher *et al.*, 1977; Richardson *et al.*, 1995; Mortensen *et al.*, 2005). Moreover, while baroreflex modulation of leg vascular conductance remains intact during both single-knee extensor (Keller *et al.*, 2003; Wray *et al.*, 2004a) and two-leg cycling (Potts *et al.*, 1993; Raven *et al.*, 1997; Fadel *et al.*, 2001) exercise, the need for baroreflex-modulated support of mean arterial pressure is greater when a larger muscle mass is fully vasodilated; for this reason, single-knee extensor exercise is thought to reduce the potential baroreflex-mediated influence (that occurs either in synergy with or in opposition to the muscle chemoreflex) during high-intensity dynamic large muscle mass exercise (Strange *et al.*, 1990; Rowell, 1993; Secher & Volianitis, 2006). Finally, changes in sympathetic outflow (as estimated by muscle sympathetic nerve activity, or MSNA) differ between exercise modalities that utilize one or both legs. For example, while there is a decrease in MSNA during low-intensity one-leg and two-leg cycling (Saito & Mano, 1991; Saito *et al.*, 1993; Callister *et al.*, 1994)), the suppression of MSNA persists as exercise intensity increases during one-leg cycling (Saito & Mano, 1991) whereas MSNA increases linearly with increasing work rate during two-leg cycling (Saito *et al.*, 1993; Callister *et al.*, 1994). In agreement with this finding is Ray et al's observations that MSNA does not increase during graded intensity and sustained knee extensor exercise (Ray, 1993; Ray *et al.*, 1993), suggesting that the sympathetic nervous system is progressively stimulated during dynamic large muscle mass cycling exercise of increasing intensity but not similar relative intensity single leg exercise. Curiously, Savard et al. demonstrated that norepinephrine spillover

increases from rest to exercise with single-knee extensor exercise and proportionally to the active muscle mass engaged in exercise (i.e., adding additional leg and arm exercise)(Savard *et al.*, 1989); the discrepancy between the MSNA and norepinephrine responses to single-knee extensor exercise might be explained by methodological issues associated with using norepinephrine spillover to estimate muscle sympathetic nerve activity (i.e., contribution of skin sympathetic nerve activity and/or increased washout due to increased leg blood flow (Ray, 1993)). Regardless, Savard et al. did not see reductions in leg blood flow and vascular conductance during one-leg knee extensor exercise associated with the increased norepinephrine spillover, supporting the concept that sympathetic vasoconstriction does not affect leg hemodynamic responses during small muscle mass leg exercise to the extent it may during large muscle mass leg exercise (Secher *et al.*, 1977; Richardson *et al.*, 1995; Strange, 1999). Thus, the preceding studies illustrate that there are important differences in the systemic, sympathetic and regulatory responses to large and small muscle mass leg exercise (i.e, two- vs. one-leg exercise). Given the potentially greater influence of central limitations, sympathetic outflow, and counterregulatory reflexes on large muscle mass dynamic leg exercise, a small muscle mass model can be used to reduce the above-mentioned limiting factors to leg vasodilation. The differences between these two exercise models thus form the basis for studies investigating both central and peripheral alterations in the control of leg blood flow during dynamic exercise.

Sex Differences in Leg Hemodynamic Responses to Exercise in Young Humans

To date, a comprehensive comparison of exercising leg hemodynamic responses between young men and women has not been published. Existing data related to this topic thus fall into three categories: 1) Comparisons of limb vascular responses to non-exercise dilator or constrictor stimuli (i.e., pharmacological infusions, reactive hyperemia, and flow-mediated dilation), 2)

findings of sex-specific systemic responses (blood pressure, cardiac output, baroreceptor responses, and sympathoexcitation) in exercising or non-exercising models that have the potential to affect leg blood flow responses during exercise in men and women, and 3) studies on limb vasodilatory responsiveness conducted using animal models. With respect to the peripheral sex differences in limb vascular responses observed through non-exercise models in humans (the first category), conclusions generally support an augmented vasodilator and blunted vasoconstrictor response in the peripheral vasculature in young women. For example, young women exhibit augmented brachial artery flow-mediated dilation (Sarabi *et al.*, 1999; Levenson *et al.*, 2001) and beta-adrenergic mediated forearm vasodilation (Kneale *et al.*, 2000) relative to young men. Moreover, the forearm vasodilatory response to acetylcholine (Dietz, 1999) as well as peak calf reactive hyperemia (Proctor *et al.*, 2005; Ridout *et al.*, 2005) tend to be higher in women, while the forearm vasoconstrictor response to norepinephrine (Kneale *et al.*, 2000; Bowyer *et al.*, 2001) and calf vasoconstrictor response to cold pressor and isometric handgrip manuevers (Hogarth *et al.*, 2007) are blunted in women relative to men.

With respect to the second category of data, there are documented sex differences in baroreflex responsiveness, with female rats demonstrating blunted baroreflex-mediated increases in sympathetic activity during phenylephrine/sodium nitroprusside infusions (Foley *et al.*, 2005) and young women exhibiting less effective baroreflex buffering of blood pressure than men (Christou *et al.*, 2005) as well as blunted sympathetic nerve activity responses to head-up tilt (Shoemaker *et al.*, 2001). In addition, Deschenes et al. noted significantly higher plasma lactate, systolic blood pressure, and plasma volume shifts in young men than women during sustained submaximal cycling exercise (Deschenes *et al.*, 2006). There are also sex-differences in cardiac function during exercise, with men exhibiting greater increases in left ventricular ejection fraction attributable to greater reductions in end-systolic volume during supine exercise (Adams *et al.*, 1987), and women (who also have lower blood volume and oxygen carrying capacity)

demonstrating reduced stroke volume and cardiac output during exercise at both relative and maximal intensity than men (Astrand & Rodahl, 1974; Wiebe *et al.*, 1998). It has also has been reported that plasma catecholamine responses to small muscle isometric exercise are greater in men (Gustafson & Kalkhoff, 1982), as are metaboreflex responses (i.e. increases in muscle sympathetic nerve activity) to handgrip exercise (Ettinger *et al.*, 1996).

Finally, regarding data assessing limb vascular responses with use of animal models, in the most revealing study, Rogers and Sheriff (Rogers & Sheriff, 2004) reported that female rats exhibit greater hindlimb vascular conductance during incremental treadmill exercise than male rats, suggesting that limb vasodilator responses to dynamic exercise are sex-specific in rats. Additional literature on sex differences in peripheral vasodilator and vasoconstrictor responses in the skeletal muscle vasculature in animal models is plentiful, although most studies have been conducted using isolated arterioles from rats, mice or pigs using non-exercise models not directly representative of exercise. For example, Huang et al. have noted reduced myogenic constriction and augmented flow-induced dilation in arterioles isolated from female vs. male rats (Huang *et al.*, 1997, 1998). Also, Laughlin et al. found that femoral arteries from female swine exhibit greater endothelium-mediated vasorelaxation (via infusions of bradykinin and acetylcholine) than do those from males (Laughlin *et al.*, 2001). In addition, acetylcholine and shear-induced vasodilation evoke only nitric-oxide mediated dilation in rat tail arteries isolated from males but both nitric oxide and EDHF-mediated dilation in similar arteries isolated from females (Pak *et al.*, 2002). Studies have also indicated that blood vessels from male species may exhibit greater vasoconstriction to substances such as endothelin (Ergul *et al.*, 1998; Tatchum-Talom *et al.*, 2000; Kellogg *et al.*, 2001) and thromboxane (Higashiura *et al.*, 1997; Sullivan & Davison, 2001), and the administration of alpha-adrenergic agonists in rat mesenteric resistance arterioles is sex-specific such that adrenergic stimulation evokes smooth muscle release of vasoconstrictor prostaglandins in males but not females (McKee *et al.*, 2003).

These findings provide evidence that the leg hemodynamic responses to exercise in young humans may be sex-specific given the existing data on limb vasodilator and vasoconstrictor responses to various stimuli in male and female animals and humans as well as the differing systemic responses to homeostatic challenges. In support of the general pattern of augmented peripheral vasodilator capacity in females, Proctor et al. recently observed evidence of greater leg blood flow responses in women compared to men at absolute work rates measured during graded leg cycling (Koch *et al.*, 2005). However, this finding has not been confirmed with a study statistically designed to test sex differences and, utilizing the two-leg cycling model, may have evoked sex-dependent central influences on leg blood flow that would confound interpretation of the results. Recently, Gonzales et al. reported the first data on sex differences in forearm blood flow during dynamic handgrip exercise to exhaustion, demonstrating that young men and women had similar forearm blood flow responses to handgrip exercise but women exhibited greater exercising forearm vascular conductance due to consistently lower mean arterial pressure (Gonzales *et al.*, 2007). While these data cumulatively support the hypothesis that females exhibit greater limb vasodilatory responsiveness to exercise, whether the peripheral hemodynamic responses to exercise in the leg are quantitatively greater in women compared to men is not currently known. *Accordingly, the third dissertation project was designed to test this hypothesis in young healthy humans*.

Potential Mechanisms Underlying the Observed Sex Differences in Vasoreactivity

Estrogenic Factors. Through a series of inhibitory blockades (blocking nitric oxide, prostaglandins, and autonomic function) and comparisons of ovariectomized and then estrogen-replaced rats, Rogers and Sheriff determined that the nature of their sex-specific findings (i.e., greater hindlimb vascular conductance during treadmill exercise in female vs. male rats) was

partially attributable to estrogenic modulation of vascular responses mediated through nitric oxide- and prostaglandin-dependent pathways (Rogers & Sheriff, 2004). In addition to effects on endothelial-derived vasodilators, estrogen can also act as a direct smooth muscle vasorelaxant (Sudhir *et al.*, 1995) and potent antioxidative agent of the reactive oxygen species generated through exercise (Brandes & Mugge, 1997; Barbacanne *et al.*, 1999; Bailey *et al.*, 2007), actions which may also serve to augment femoral vascular conductance. To this end, estrogen can blunt increases in MSNA during static handgrip exercise in young women (Ettinger *et al.*, 1998), as well as mitigate smooth muscle contraction induced by endothelin and thromboxane (Matsuda *et al.*, 1995; Kahonen *et al.*, 1998; David *et al.*, 2001) and blunt smooth muscle cell contraction through alteration of calcium handling mechanisms and the protein kinase C pathway (Crews & Khalil, 1999) in rats. Finally, Huang et al. found that pressure-induced myogenic constriction of female rat arterioles is reduced due to an estrogen-mediated enhancement of NO (Huang *et al.*, 1997). Again, however, it must be cautioned that the extent to which the acute influence of estrogen on constrictor pathways translates into chronic adaptations to exercise is not known. While there are estrogen receptors located in both the vascular endothelium and smooth muscle cells that may alter gene transcription of many vasoactive metabolites, such as prostacyclin synthase, prostacyclin cyclooxygenase, endothelin-1, and endothelial nitric oxide synthase (Mendelsohn, 2002), additional research is necessary to determine whether chronic and cyclical estrogen exposure alters dilator and constrictor pathways in humans during exercise. Finally, it should be noted that Rogers and Sheriff further concluded that the lower mean arterial pressure observed during exercise in females compared to males was independent of estrogen, autonomic function, NO synthase, or cyclooxygenase (Rogers & Sheriff, 2004). Thus, there may be non-estrogenic factors contributing to observed sex differences in vasoactive responses of males and females, including the influence of progesterone, testosterone, and other androgens (Orshal & Khalil, 2004; Hutchison *et al.*, 2005; Ross *et al.*, 2006; Seyrek *et al.*, 2007) as well as differences

in body size and composition (Raison *et al.*, 1991; Jensen *et al.*, 1998; Wascher *et al.*, 1998), vascular structure and stiffness (van der Heijden-Spek *et al.*, 2000; Debasso *et al.*, 2004)) metabolic capacity and/or response of the muscle (Simoneau *et al.*, 1985; Russ & Kent-Braun, 2003; Clark *et al.*, 2005) and adrenergic/vasodilator receptor density and downstream signaling in the vasculature (Kneale *et al.*, 2000; Keys *et al.*, 2005).

Age Differences in Exercising Leg Blood Flow

During dynamic contractions of a very small muscle mass (rhythmic hand gripping) active muscle blood flow appears well-preserved with advancing age (Jasperse *et al.*, 1994). However, during moderate intensity exercise involving one or both legs (>50% of peak work rate), active muscle blood flow is generally lower with advanced age in healthy adults; this has been reported in chronically endurance-trained men (Wahren *et al.*, 1974; Proctor *et al.*, 1998), sedentary men (Beere *et al.*, 1999; Poole *et al.*, 2003), and more recently in women (Proctor *et al.*, 2003a; Proctor *et al.*, 2004a). In moderately active older men, submaximal leg exercise may (Carlson & Pernow, 1961) or may not (Magnusson *et al.*, 1994; Proctor *et al.*, 2003b) be associated with lower leg blood flow responses compared with younger subjects. In spite of variable results between studies, perhaps reflecting the diversity of blood flow measurement techniques, exercise modalities and protocols and subject fitness levels, the overall conclusion from the literature is that human aging is associated with lower leg blood flow responses to submaximal leg exercise. In most of these studies, the older subjects also exhibited higher arterial blood pressures at a given submaximal work rate than their younger counterparts (Beere *et al.*, 1999; Poole *et al.*, 2003; Proctor *et al.*, 2003a; Proctor *et al.*, 2003b; Proctor *et al.*, 2004a), such that leg vascular conductance during leg exercise is significantly reduced with age as well. Blood flow to the legs during *peak* exercise is also reduced in older men and women, in part due

to the reduced pumping capacity of the aged heart (Beere *et al.*, 1999; Poole *et al.*, 2003; Proctor *et al.*, 2003b; Proctor *et al.*, 2004b).

Influence of Fitness on Leg Blood Flow Responses to Exercise in Aged Adults

As mentioned above, leg hemodynamic responses measured with a common measurement technique (thermodilution) during a common exercise modality (two-leg cycling exercise) are reduced with age in very sedentary (Beere *et al.*, 1999; Poole *et al.*, 2003) and very well trained (Proctor *et al.*, 1998) older men, but not in normally-active older men (Proctor *et al.*, 2003b). Koch et al. investigated these discrepant findings (Koch *et al.*, 2005) by examining the relationship between leg blood flow and leg oxygen consumption in each of the published studies. With respect to the trained older men (Proctor *et al.*, 1998), the lower leg blood flow responses were accompanied by an elevated oxygen extraction such that leg oxygen consumption was maintained similar to young trained subjects during submaximal cycling exercise. Thus, the reduced leg blood flow in older trained men may represent an adaptive response to reduced cardiac reserve. By contrast, in sedentary older men, leg oxygen consumption was reduced during submaximal cycling exercise (Beere *et al.*, 1999; Poole *et al.*, 2003), suggesting that the reduced leg blood flow exhibited by these men may represent a maladaptive aging response unaccompanied by compensation. The normally active older men with similar leg blood flow responses to cycling exercise as young men (Proctor *et al.*, 2003b) also had preserved leg oxygen consumption, further supporting the hypothesis that fitness may modulate leg hemodynamic responses to exercise in older men. Interestingly, Martin et al. found that aerobic training significantly increased peak calf blood flow and vascular conductance in older men while only marginally increasing these responses in older women, suggesting that the influence of fitness

and/or physical activity on leg blood flow in older adults may be sex-specific (Martin *et al.*, 1990).

Age Differences in Small vs Large Muscle Mass Leg Exercise Responses

The extent to which age-associated limitations in cardiac pump function are responsible for lower blood flow and/or vasodilator responses within the leg muscles of older adults will likely depend upon the particular exercise model studied. During large muscle mass exercise at a given submaximal work rate, cardiac output is either reduced (Strandell, 1976; Faulkner *et al.*, 1977; McElvaney *et al.*, 1989; Thomas *et al.*, 1993) or unchanged (Becklake *et al.*, 1965; Saltin, 1986; Kenney & Ho, 1995) in older vs. younger adults. The extent to which cardiac output limits leg perfusion (or contributes to altered leg vascular control) during submaximal exercise in older vs. younger adults is currently unknown. The few aging studies that have measured both cardiac output and leg blood flow provide evidence for an age-associated alteration in the percentage of cardiac output distributed to the exercising legs (Magnusson *et al.*, 1994; Beere *et al.*, 1999; Proctor *et al.*, 2003a; Proctor *et al.*, 2003b), at least in men. In an attempt to minimize the confounding influence of age-associated limitations in cardiac output, some investigators have examined age-related differences in leg blood flow responses to isolated knee extensor exercise. Three previous studies have reported age group comparisons of leg hemodynamics with this exercise model in healthy men (Magnusson *et al.*, 1994; Lawrenson *et al.*, 2003; Donato *et al.*, 2006). Magnusson et al (Magnusson *et al.*, 1994) observed similar leg blood flow responses during submaximal and maximal knee extensor exercise in younger and older men, while Lawrenson et al. (Lawrenson *et al.*, 2003) and Donato et al. (Donato *et al.*, 2006) reported an age-associated deficit in leg blood flow that persisted across all exercise work rates. In spite of these differences, leg vascular conductance at any given work rate was lower in the older vs. younger

men in all studies, although Lawrenson et al. reported data in leg vascular resistance units which confounds direct interpretation of the data given the nonlinear relationship between blood flow and resistance (Lautt, 1989). However, the magnitude of the age-related effect on exercising leg hemodynamic responses may be different in small muscle mass vs large muscle mass exercise. For example, the slope of the vasodilatory response in the Lawrenson study was well-preserved (estimated conductance units) or augmented (reported resistance units) with age, unlike findings from a comparable two-leg cycling study using the same population, investigators and measurement techniques (Poole *et al.*, 2003), and the general magnitude of the age difference was smaller in knee extensor exercise (i.e., 25% difference in leg vascular conductance) than that measured during two-leg exercise (i.e., 60% difference in leg vascular conductance) in the same aforementioned studies (Lawrenson *et al.*, 2003; Poole *et al.*, 2003). These findings suggest that while there may be substantial central supply or regulatory alterations associated with reductions in leg blood flow in older sedentary men, there also may be peripheral alterations in the vasculature relevant to the local control of muscle hyperemia. Comparisons of this nature cannot be extended to women or non-sedentary populations of men given the paucity of equivalent data.

Peripheral Mechanisms Potentially Contributing To Altered Leg Blood Flow with Age

Attenuated muscle blood flow responses during exercise in the aged could reflect, at least in part, structural changes within the skeletal muscle microcirculation (i.e., fewer and/or smaller arterial vessels as well as stiffer vessels). Direct assessment of these structural properties in human skeletal muscle is difficult. Biopsy-based estimates of skeletal muscle capillary density, a surrogate measure of arteriolar density (Saltin & Gollnick, 1983; Delp, 1998), suggest there are age-associated losses of microvessel density in the lower leg muscles of older men and women (Coggan *et al.*, 1992), although Bearden et al. did not find evidence of changes in arteriolar

segment number, length or surface area in aged rats (Bearden *et al.*, 2004). Peak conductance following limb arterial occlusion, a broadly used index of arteriolar cross-sectional area (Rosei *et al.*, 1995; Tanaka *et al.*, 1998), declines with advancing age in the calf vasculature of older men and women (Proctor *et al.*, 2005; Ridout *et al.*, 2005), providing functional evidence for age-associated reductions in the size of the arteriolar bed in the leg. In addition, while femoral-ankle pulse wave velocity (an index of lower extremity stiffening) increases (Filipovsky *et al.*, 2005), intima-medial thickness of the femoral artery increases (Dinenno *et al.*, 2000; Moreau *et al.*, 2002) and compliance of lower extremity arteries may decrease with age in humans (Debasso *et al.*, 2004; Thijssen *et al.*, 2007a), stiffening of the microvasculature in humans has not been well documented. Muller-Delp et al. investigated this question in isolated rat soleus arterioles, finding that wall thickness of soleus muscle arterioles was increased in aged rats without an accompanying increase in calculated vascular stiffness (Muller-Delp *et al.*, 2002a). However, Bearden et al. did document increased tortuosity and branch angles in small arterioles of aging rats (Bearden *et al.*, 2004). Thus, it is not clear to what extent structural alterations and stiffening of the entire lower extremity and/or conduit arteries represent alterations in the microvasculature that would significantly affect exercise hyperemia.

Moreover, release of metabolites from working muscle, which help match muscle blood flow to metabolic demand, may also be reduced with age in the senescent muscle. Of the possible metabolic regulators of muscle blood flow (described previously), age-related differences have been reported only with respect to gene expression of nNOS in senescent animals. For example, in senescent rats, the percentage of nNOS containing fibers is significantly reduced with age in both the soleus and the extensor digitorum longus (Richmonds *et al.*, 1999). Additionally, ovariectomized rats exhibit reduced gastrocnemius nNOS and greater enhanced sympathetic vasoconstriction in the femoral artery which can be prevented by chronic treatment with 17B-estradiol (Fadel *et al.*, 2003).

Endothelium-dependent vasodilation is another potential local control mechanism that may undergo age-related impairment. Most of the available information about age-associated changes in endothelium-dependent control of muscle blood flow in humans comes from studies that infused drugs into the resting forearm via the brachial artery. These studies have generally found that acetylcholine-induced (endothelium-dependent) forearm vasodilation is blunted in sedentary older men compared to younger control subjects, whereas smooth muscle responsiveness to sodium nitroprusside is preserved (Gerhard *et al.*, 1996; DeSouza *et al.*, 2000; Taddei *et al.*, 2000; DeSouza *et al.*, 2002). With respect to exercise, Schrage et al. found that the contributions of nitric oxide and prostaglandins to forearm muscle hyperemia were 45% lower and absent, respectively, in older men compared to young men, possibly contributing to the reduced forearm blood flow observed during exercise with age (Schrage *et al.*, 2007). However, when endothelium-dependent dilators were infused into the femoral artery at rest, Newcomer et al. found that there were similar increases in acetylcholine-, substance P-, and sodium nitroprusside- induced vasodilation (% increase in blood flow above baseline) in the legs of the older compared to the young subjects. These results suggest that there are no apparent age differences in either endothelium-dependent or endothelium–independent vasodilation in the legs of sedentary men, indicative of a preserved ability of the vascular smooth muscle in the legs to respond to endothelium-derived vasodilator substances with age in men. Similarly, Wray et al. observed that flow-mediated dilation (FMD, the endothelium-dependent dilatory response of the conduit artery to an increase in fluid shear stress) was lower in the brachial but not femoral artery with age in men (Wray *et al.*, 2006); by contrast, however, Thijssen reported blunted FMD as well as endothelium-independent dilation (the response to sublingual nitroglycerin) in the femoral artery of older men (Thijssen *et al.*, 2006). Given that the relationship between conduit and resistance arteriole responsiveness with respect to age and dilation is not known (Eskurza *et al.*, 2001), and data from animal models supports a reduction in vasodilation to flow and

acetylcholine with age in rat soleus muscle arterioles (Muller-Delp *et al.*, 2002b), it is difficult to synthesize the above-mentioned findings to determine conclusively whether local leg vasodilatory responsiveness is significantly influenced with age in men. There are no data regarding this topic in women; *accordingly, the first dissertation project was designed to investigate the influence of age on leg vasodilatory responsiveness to ischemia in women*.

It is also possible that local muscle vasodilation in older adults is influenced by the elevated sympathetic outflow associated with age (Ng *et al.*, 1993), as regulation of muscle vasodilation involves a balance between vasodilator and vasoconstrictor influences. To this end, Dinenno et al. observed that 1) there was a reduction in resting leg blood flow and leg vascular conductance with age in men, 2) this reduction was associated with an age-related increase in muscle sympathetic nerve activity (Dinenno *et al.*, 1999), and 3) the lower resting leg blood flow and vascular conductance in older men relative to young men was abolished following alpha-adrenergic blockade (Dinenno *et al.*, 2001). The extent to which chronically elevated leg sympathetic tone influences exercising leg hemodynamics is not known, especially given that resting vasoconstrictor responsiveness to acute sympathetic stimulation declines with advancing age in the leg (Sugiyama *et al.*, 1996; Dinenno *et al.*, 2001; Smith *et al.*, 2007). However, metabolic inhibition of an acute sympathetic stimulus is impaired with age in the dynamically contracting forearm of women (Fadel *et al.*, 2004) and men (Dinenno *et al.*, 2005), as well as in the leg of men (Koch *et al.*, 2003). There are no data on this topic in the lower extremity vasculature in women; *accordingly, the second dissertation project was designed to test the hypothesis that metabolic inhibition of an acute sympathetic stimulus would also be reduced in the leg in older women*. Collectively, the relation between augmented chronic resting leg sympathetic tone, reduced resting adrenergic sensitivity, and heightened vasoconstrictor responsiveness during exercise in older adults with respect to local alterations in muscle hyperemia is not certain. However, given that the local vasodilatory response to exercise in

muscle is a balance between dilator and constrictor influences, it is likely that these age-related changes influence leg blood flow during exercise. Finally, it is been shown that the increased resting vascular tone associated with either aging or pathology may also be attributable to an augmented influence of endothelin (Donato *et al.*, 2005; Thijssen *et al.*, 2007b) and/or rho kinase (Kishi *et al.*, 2005; Bussemaker *et al.*, 2007); thus it is possible that exercising leg blood flow is influenced by these substances as well.

Several other mechanisms may influence leg hemodynamic responses to exercise in aged adults. These include the muscle pump, myogenic responsiveness, and conducted vasodilation. With respect to the muscle pump, there are changes in the function of the venous valves related to conditions such as varicose veins, venous hypertension, or congestive heart failure (Bevegard, 1962; Sinoway *et al.*, 1987; Shiotani *et al.*, 2002), that could reduce the effectiveness of the muscle pump in older adults. For example, Shiotani et al. found that the drop in venous pressure attributable to the muscle pump was impaired in older men with chronic heart failure (Shiotani *et al.*, 2002), while Richardson found that the speed and amplitude of exercise hyperemia was reduced in arterioles from female rats with venous congestion in heart failure (Richardson *et al.*, 2003). As advancing age is associated with increased prevalence of varicose veins, venous insufficiency and venous hypertension (Beebe-Dimmer *et al.*, 2005), and may involve cardiovascular adaptations similar to those observed in mild chronic heart failure, it is possible that the contribution of the muscle pump to exercise hyperemia declines with age in humans. Furthermore, age-related changes such as increased tortuosity and branch angles in arterioles (Bearden *et al.*, 2004) may alter the efficacy of the muscle pump.

In addition, myogenic regulation, the response of vascular smooth muscle to increases or decreases in transmural pressure (Davis *et al.*, 1984; Schubert & Mulvany, 1999), has been shown to be altered with age. Findings in animals concerning the regulation of blood flow through myogenic responses have uniformly documented reduced myogenic responsiveness in

gastrocnemius and soleus muscle arterioles with age and hindlimb unloading (Delp, 1999; Muller-Delp *et al.*, 2002a) as well as increases in myogenic set point and blunted magnitude of the myogenic response in aged mouse mesenteric arteries (Nankervis *et al.*, 2001; Gros *et al.*, 2002). Lott et al., however, who studied myogenic responsiveness to abrupt and steady-state changes in transmural pressure in the brachial artery in older and younger men and women, found that sustained increases in transmural pressure led to enhanced vasoconstriction in older subjects (Lott *et al.*, 2004). Collectively, these results do not yield a conclusive understanding of the influence of age-related changes in the regulation of exercise blood flow; the decreased myogenic responsiveness observed in animal models would not seem to explain age-related reductions in limb blood flow while the augmented responsiveness observed in the human forearm has not been extended to the human leg.

Finally, while a role for conducted vasodilation in exercising leg hyperemia has not been defined in humans, Bearden et al. recently provided the first evidence of reduced conducted vasodilation in the microvascular network of gluteus maximus muscles in old vs. adult male mice (Bearden *et al.*, 2007). While this evidence has not been extended to humans it does provide yet another mechanism potentially influencing skeletal muscle hyperemia.

Age By Sex Interactions in Leg Blood Flow Responses to Exercise and Factors Influencing These Responses

There are relatively few investigations addressing the influence of sex-specific aging on skeletal muscle vasodilation in either exercise or non-exercise models. The only exercise data to date have been provided by leg blood flow measurements collected during submaximal cycling exercise in older and younger men and women (Proctor *et al.*, 2003a; Proctor *et al.*, 2003b). While these studies were not statistically powered to detect age by sex interactions, it was found

that older, normally-active men exhibited preserved leg blood flow and leg vascular conductance responses during all submaximal work rates compared to young men, while older, normally-active women exhibited lower leg blood flow at higher work rates and lower leg vascular conductance at almost all the measured work rates compared to young women. Taking into account the approximate 10% reduction in leg muscle mass in older women by comparing leg hemodynamic responses in older and younger women matched for muscle mass did not explain the lower leg blood flow or vascular conductance observed in older women. Although cardiac output is generally lower in women than men of any age during similar intensity exercise (Astrand & Rodahl, 1974; Fleg *et al.*, 1995), both older men and older women exhibited approximately a one liter/min reduction in cardiac output during submaximal cycling exercise relative to their young counterparts. Thus, it was unclear why older men were able to direct proportionally more cardiac output to the working muscle relative to young men whereas older women did not compensate in this manner. Given that cardiac output is directed to the working muscle through vasoconstriction of inactive beds and vasodilation of active muscle vasculature, local vasoconstrictor and vasodilator mechanisms may have influenced the nature of the sex-specific findings. Evidence of sex-specific aging-related alterations in local vasodilatory responses in the lower extremity vasculature was published by Martin et al., who found that increased age was associated with reduced maximal calf conductance in women but not in men (Martin *et al.*, 1991). Additional data concerning age-related declines in peak calf conductance in men (Proctor *et al.*, 2005) and women (Ridout *et al.*, 2005) support this finding: while peak calf conductance was reduced with age in both sexes, normalizing peak calf conductance to calf muscle abolished the age association in men but not in women (Ridout *et al.*, 2005). Collectively, these two studies provided evidence of structural or vasodilatory alterations in the local hyperemic response to ischemia unique to older women which may indicate an overall age by sex interaction in vasodilatory responsiveness in humans. *Accordingly, the fourth dissertation study*

was designed to test the hypothesis that women would exhibit greater age-related alterations in vasodilatory responsiveness during dynamic leg exercise than men when central limitations to hyperemia were minimized. Using single-knee extensor exercise as an exercise model also provided data to indirectly compare (i.e., using results from the previous cycling studies vs. the fourth dissertation study) the effect of age and sex on leg hemodynamic responses to large vs. small muscle mass exercise in populations of similar fitness and health.

There are many mechanisms that could contribute to these potential age by sex interactions. Certainly, the chronic influence of female sex hormones in young women and the relatively abrupt cessation of their production at menopause have been shown to have a significant influence on both systemic and local indicators of vascular function. For example, carotid artery stiffness, brachial-ankle pulse wave velocity, blood pressure, and cholesterol increase dramatically following menopause (Peters *et al.*, 1999; Takahashi *et al.*, 2005; Izumi *et al.*, 2006). Celermajer et al. were the first to document that the reduction in brachial-artery flow-mediated dilation in men (0.21%/year) begins approximately around 40 years of age whereas women exhibit preserved FMD until the early 50s (i.e., menopause), after which the rate of decline is over twice that of men (0.49%/year)(Celermajer *et al.*, 1994). In addition, adrenal hormones (DHEA and cortisol) differ between older men and women, with age-related decreases in DHEA exhibiting sexual dimorphism such that levels of DHEA in women fall 40% during the menopausal transition (Laughlin & Barrett-Connor, 2000). Interestingly, Wu et al. found that DHEA supplementation counters the age-related attenuation of eNOS in the thoracic artery of male rats (Wu *et al.*, 2007). Moreover, male and female rats compensate for loss of nitric oxide differently with respect to responses to shear-mediated dilation in gracilis arterioles: male rats respond with an increase in prostaglandin release and female rats evoke the EDHF dilatory pathway (Huang *et al.*, 2001; Wu *et al.*, 2001). Thus, given that reductions in nitric oxide pathways are often implicated in vascular aging (Singh *et al.*, 2002; Bode-Boger *et al.*, 2003;

Woodman *et al.*, 2003; Schrage *et al.*, 2007), a sex-specific influence of aging on muscle blood flow and/or vascular dilation may also be driven in part by differing compensatory responses to the aging process in men vs. women.

Summary

This review of the relevant literature concerning leg blood flow responses to dynamic exercise reveals the following: 1) Control of exercising leg blood flow in thermoneutral conditions is a balance between central and peripheral control mechanisms aimed at meeting the metabolic demand of the working muscle while preserving systemic blood pressure, 2) The precise metabolites and pathways regulating local vasodilation and vasoconstriction in the muscle vasculature are still unknown in humans, making conclusions about the mechanisms of exercise hyperemia that are influenced by age and sex difficult, and 3) while a significant body of literature exists to support the hypothesis that both sex and age independently and interactively influence leg blood flow responses to dynamic exercise, there is little work to date specific to vasodilatory control of the leg vasculature of women and no systematic work documenting differences in peripheral leg blood flow responsiveness in either young or older males vs. females.

Chapter 3

AGE AND FLOW-MEDIATED DILATION: A COMPARISON OF DILATORY RESPONSIVENESS IN THE BRACHIAL AND POPLITEAL ARTERIES

Introduction

The vasodilator response of conduit vessels to an increase in fluid shear stress, termed flow-mediated dilation (FMD), declines with normal aging in the brachial artery (Celermajer *et al.*, 1994; Corretti *et al.*, 1995; Jensen-Urstad & Johansson, 2001). However, the influence of age on lower extremity conduit artery FMD has not been well-characterized. Lower extremity arteries are particularly susceptible to atherosclerosis, and peripheral vascular disease of the lower extremity is highly correlated with the prevalence of coronary disease (Burke *et al.*, 1995; Frangos *et al.*, 1999). To this end, it has been shown that popliteal artery FMD is reduced or abolished in subjects with hyperlipidemia and coronary disease compared to normal controls (Angerer *et al.*, 2001; Spacil *et al.*, 2002). Moreover, Sanada et al. found that 57 male and female patients with peripheral arterial disease exhibited impaired leg blood flow (but not forearm blood flow) responses to reactive hyperemia, suggesting that diminished vascular reactivity of the leg may be a better indicator of peripheral arterial disease than blunted forearm vascular reactivity (Sanada *et al.*, 2005). Unfortunately, it is not known to what extent these clinical findings reflect normal aging vs. the changes associated with the progression of vascular disease.

To this end, Wray at el. recently published findings in men suggesting that a) the age-related attenuation in brachial FMD is abolished when the dilatory response is normalized to the shear stimulus, and b) femoral FMD is augmented with age (Wray *et al.*, 2006). However, as

prior research has demonstrated that there are significant sex differences in brachial FMD responses both in young (Levenson *et al.*, 2001) and older (Celermajer *et al.*, 1994) humans, it is unclear to what extent the findings of Wray et al. can be extended to women, for whom aging is accompanied by a rapid reduction in circulating sex hormones. Thus, the primary aim of the present study was to determine whether the age-related reduction in brachial artery FMD is also observed in the popliteal artery in healthy women, when baseline differences in conduit diameter are accounted for by normalizing dilation to the shear stimulus (Mitchell *et al.*, 2004; Wray *et al.*, 2005b). Additionally, we sought to compare the vasodilator responsiveness to an increase in fluid shear stress in the arm vs. leg of young and older subjects, since previous work on this topic has also been conducted solely in men (Newcomer *et al.*, 2004; Newcomer *et al.*, 2005; Wray *et al.*, 2005b, 2006). Based on the known relationship between age and brachial artery FMD as well as existing literature that has shown that popliteal artery FMD is reduced in clinical populations of patients with coronary disease (Angerer *et al.*, 2001), we hypothesized that popliteal artery FMD would be reduced in older subjects compared to young controls. Furthermore, give our previous findings concerning brachial and femoral hyperemic responses to acetylcholine (Newcomer *et al.*, 2005), we hypothesized that the vascular dilator responsiveness of the popliteal artery would be blunted compared to the brachial artery in both young and older women.

Methods

Subjects

Fourteen young (20-30 yr) and 14 older (63-79 yr) women completed the study. All subjects were nonobese (BMI $\leq$ 30), nonsmokers, had clinically normal blood chemistry (i.e. hemoglobin concentrations ranged from 11.6-14.8 $g \cdot dL^{-1}$, total cholesterol $\leq$ 240 $mg \cdot dL^{-1}$, LDL cholesterol $\leq$

150 mg·dL^{-1}), and resting supine ankle-brachial index ratings (ABI between 0.90 and 1.30). All subjects were normotensive (resting blood pressure ≤ 140/90 mmHg) and were neither extremely sedentary nor extremely fit (i.e. had cycle ergomenter $\dot{V}O_{2peak}$ values between 20 and 80% of age-predicted norms (ACSM, 2006)). Subjects were free of overt chronic diseases as evaluated by medical history questionnaire, a physical examination and resting ECG. Additionally, no subjects were taking medications having significant hemodynamic effects, including oral contraceptives (young) or hormone-replacement therapy (older) for at least the last 12 months. Younger subjects were studied in days 1-7 of their menstrual cycle to minimize the influence of cyclical changes in female hormones. On study day, subjects were asked to refrain from caffeine, aspirin, ibuprofen, or herbal supplements for at least 12 hours prior to testing. All subjects gave their written, informed consent to participate. This study was approved by the Office for Research Protections and the Institutional Review Board at The Pennsylvania State University. Subject characteristics are presented in Table 1.

Physical activity/fitness status. Subjects were interviewed in detail (type, intensity, duration, frequency) about their current and former physical activities, including occupation (current and/or former), leisure-time/recreational activities, and structured fitness activities. All younger subjects were students who did not report any physically demanding occupational or recreational tasks. The older subjects were all retired; none of the subjects reported previous physically demanding occupations. Older subjects did describe a variety of leisure-time activities (none that were more than mildly physically demanding,) such as gardening, volunteering, reading, quilting and cooking. None of the subjects participated in moderate to high intensity aerobic exercise > 3 d·wk^{-1}during the past 12 months. Additionally, all of the older subjects and a representative group of the younger subjects performed a continuous incremental leg cycle ergometer test (Lode) to maximal exertion to determine peak oxygen uptake ($\dot{V}O_{2peak}$) to

objectively quantify aerobic fitness status, as described in detail previously (Proctor *et al.*, 2004b).

Body composition. Total and regional body composition was estimated using dual-energy X-ray absorptiometery (DXA; model QDR 4500W, Hologic, Waltham, MA) with subjects in the supine position as described previously (Proctor *et al.*, 2005). Region of interest software (version 9.80 D, Hologic) was used to determine bone-free lean mass and percent fat for the forearm (from the olecranon process to distal radius or ulna) and calf (from the proximal tibia to the distal tibia or fibula) of each subject's nondominant limb (i.e., nonwriting hand and corresponding leg).

Brachial and Popliteal Artery FMD

The nondominant arm of each subject was imaged while the subject was in the supine position with the arm extended ~80° from the subject's torso at heart level. Heart rate was continuously monitored using a 3-lead electrocardiogram. A rapid inflation/deflation pneumatic cuff (D.E. Hokanson, Inc.; Bellevue, WA) was placed around the forearm, immediately distal to the olecranon process, following established guidelines for assessing FMD (Corretti *et al.*, 2002). The artery was imaged 1-3 inches proximal to the olecranon process using a 6-11MHz multifrequency linear array probe attached to a high-resolution ultrasound machine (Aspen, Acuson; Mountain View, California); once a satisfactory image using optimal B-mode imaging was obtained, the placement of the probe was marked on the upper arm to ensure that the site of measurement did not change during the trial. Ultrasound parameters were not changed during the study. Doppler velocity was also measured with the Aspen using a 68° angle of insonation held constant throughout the study. After the subject had rested in the supine position for 10 min, resting brachial artery diameter and velocity were measured for 1 min prior to inflation of the

pneumatic cuff. The cuff was then inflated to 305 mmHg for 5 min; diameter and velocity recordings resumed 1 min prior to cuff deflation and continued for 3 minutes post-inflation. Popliteal FMD was measured in a similar fashion except that the subject was lying prone with a small pillow under her non-dominant ankle, the popliteal artery was measured immediately proximal to the bifurcation (usually at or slightly above the popliteal fossa), and the pneumatic cuff was placed around the calf, 2-3 inches distal to the popliteal fossa. The order of the measurements for the popliteal and brachial arteries was randomized so that half the subjects in each age group underwent calf occlusion followed by forearm occlusion, and the other half underwent forearm occlusion followed by calf occlusion.

Brachial and Popliteal Endothelium-Independent Dilation

In 8 young and 8 older subjects, endothelium-independent dilation of the arm and leg was assessed through administration of 0.4 mg sublingual nitroglycerin (NTG), a nitric oxide donor eliciting maximal dilation representative of smooth muscle function, on two separate study visits. Diameters were measured continuously for 10 minutes following administration of NTG.

Brachial and Popliteal Artery Diameter and Velocity Analysis

For FMD measurements, brachial and popliteal artery diameters were measured for 1 min at rest, during the last minute of occlusion and as the highest diameter observed during 3 min of post-inflation imaging. Consistent with the literature, the peak diameter was observed between 50-75 seconds in most subjects (Corretti *et al.*, 2002; Betik *et al.*, 2004). Diameter measurements were sampled at end-diastole (ECG-gating was used to select images that were trigged by the R-wave of the cardiac cycle) using Brachial Imager software (Medical Imaging Applications; Iowa

City, IA). Post-test analysis of diameters was performed using edge-detection software (Brachial Analyzer Software, Medical Imaging Applications; Iowa City, IA); briefly, the technician (always the same and blind to any subject information) selected a region of interest along the arterial wall and the edge of the wall was detected by pixel density and represented by a line of best fit. Each sequence of images was reviewed by the technician and adjusted to ensure that diameter measurements were always calculated from the intima-lumen interface at both the distal and proximal vessel wall. Resting and occlusion diameters were calculated as the average of 10 images taken over the 1 min baseline and last minute of occlusion, respectively. The peak diameter was calculated by identifying the post-occlusion image with the largest diameter and averaging that image with the 5 preceding and 5 following images. FMD was then calculated as the percent change in diameter from resting baseline (% D_{rest}) or from the last minute of occlusion (% D_{occlu}). Blood flow velocity was measured at rest, during the last minute of occlusion, and as the highest velocity measured in the first 30 seconds following cuff release. A time-averaged, angle-corrected maximum velocity (highest velocity across the cardiac cycle) was calculated with a tracing of the velocity-time integral, taking the average of 3 full cardiac cycles for each measurement. For NTG measurements, the peak diameter was calculated as the post-NTG image with the largest diameter, again averaging that image with the 5 preceding and 5 following images, and calculating % dilation (% D_{NTG}) relative to the 10-second average of images pre-NTG administration.

Measurement of and Normalization to the Shear Stimulus

The shear stimulus was approximated by estimating shear rate (rest, occlusion and peak), or 4• velocity/diameter. Although blood viscosity was not measured, preventing estimation of shear stress, hematocrit was analyzed in young vs. older subjects to determine whether the

assumption that blood viscosity was not significantly different between age groups was supported. The diameter used to calculate peak shear rate was the diameter measured immediately prior to cuff release (occlusion diameter), since our pilot studies demonstrated that the diameter does not change appreciably in the first 10-12 seconds of cuff release (when peak blood velocity and shear rate are observed) and the Aspen ultrasound machine is not able to measure high-resolution diameters and velocity simultaneously. Finally, FMD was normalized to the absolute change in shear rate from either resting to peak conditions or from occlusion to peak conditions.

Statistical Analysis

Statistical analyses were performed using Minitab (Minitab, State College, PA) software. All data are reported as mean $\pm$ S.E.M. and significance was set at $p<0.05$. One-way ANOVA and Tukey post-hoc analysis were used to compare differences between young and older groups. A paired two-sample t-test was used to determine differences between resting, occlusion and peak diameters in each subject group as well as differences between FMD in the popliteal and brachial artery of each subject and an independent two-sample t-test was used to compare FMD responses between young and older subjects. Finally, the variation incorporated into the relationship between normalized brachial or popliteal FMD and age by independent variables (i.e. baseline characteristics; Table 3-1) was investigated with ANCOVA. Additional analysis of the influence of these variables was performed by comparing subsets of FMD of young and older subjects matched for each of these variables.

Results

Subject Characteristics (Table 3-1). Older subjects had significantly higher blood pressure (systolic and diastolic), total and LDL cholesterol, BMI, and body fat, as well as lower $\dot{V}O_{2peak}$.

Baseline, Occlusion and Peak Diameters and Shear Rate by Age (Table 3-2). There were no age group differences in resting, occlusion or peak shear rates or diameters (Table 3-2), although peak shear rate in the popliteal following occlusion was marginally lower ($p = 0.11$) in older subjects. There was a slight but statistically significant ($p < 0.01$) dilation observed during calf occlusion in older subjects that was present but not statistically significant in younger subjects ($p = 0.095$) and not observed in the brachial artery of either young or older subjects.

Age-associated differences in % Dilation. To investigate the effect of the slight dilation observed during calf occlusion in older subjects, we calculated % Dilation as either the dilation calculated from baseline diameter (% D_{rest}) or the dilation calculated from occlusion diameter (% D_{occlu}). Following 5 min forearm occlusion, % D_{rest} was reduced ($p = 0.03$) in older vs. young women (young = 14.6 + 1.9 %; older = 8.9 + 1.5%). Following 5 min calf occlusion, % D_{rest} was also reduced ($p = 0.04$) in older vs. young women (young = 6.7 + 0.9 %; older = 4.0 + 0.8 %). % D_{occlu} following forearm occlusion was reduced ($p < 0.01$) in older women (young = 15.8 + 0.8 %; older = 8.1 + 1.5%). % D_{occlu} following calf occlusion was also reduced ($p = 0.01$) in older women (young = 4.6 + 0.7 %; older = 1.8 + 0.4%). There was a significant difference ($p < 0.01$) between brachial and popliteal dilation (expressed relative to rest or dilation) in both young and older individuals.

Age-associated differences in brachial and popliteal normalized FMD (Figure 3-1). There was a significant difference between normalized FMD (percent change in diameter divided by the unit change in shear rate) from occlusion to peak conditions for young and older subjects in the brachial (young = 0.013 + 0.002; old = 0.006 + 0.001; $p < 0.01$) and popliteal (young = 0.011 +

0.002; old = 0.005 + 0.001; p = 0.01). However, in both young (p = 0.21) and older (p = 0.76) subjects, there was no significant difference between the normalized FMD responses in the brachial and popliteal arteries.

Endothelium-Independent Dilation (Figures 3-2 and 3-3). Following NTG administration, the dilatory response in the brachial (young = 32.1 + 1.7; old = 17.8 + 2.5; $p < 0.01$) and popliteal (young = 8.6 + 0.9; old = 3.0 + 0.7; $p < 0.01$) arteries in older women was reduced by 45% and 65%, respectively (Figure 3-2). Normalizing the FMD response to the NTG response abolished age group differences in both the brachial (young = 0.49 + 0.14; old = 0.45 + 0.18; p = 0.86) and popliteal (young = 0.53 + 0.16; old = 0.60 + 0.17; p = 0.79) (Figure 3-3).

Influence of independent variables. Due to previous work demonstrating the inverse relationship between individual characteristics such as blood pressure (Yan *et al.*, 2005), cholesterol (total and LDL) (Corrado *et al.*, 2005), and BMI (Benjamin *et al.*, 2004) on brachial artery FMD, we sought to determine whether our observations of age-related changes in FMD were mediated by independent variables. Investigating the relationship between normalized brachial or popliteal FMD, age and independent variables with ANCOVA yielded no significant results ($p > 0.05$) for the influence of total cholesterol, LDL cholesterol, percent body fat, BMI, systolic blood pressure, diastolic blood pressure, hematocrit, forearm muscle mass, or calf muscle mass. Matching a subset of 6-8 young and older subjects for each of these variables did not change the magnitude or significance of the age-associated attenuation in FMD except in the case of the blood pressure-matched subjects. In older subjects matched to younger subjects for systolic pressure, there was only a 30% attenuation in brachial FMD and a 25% attenuation in popliteal FMD. Similarly, in subjects matched for diastolic pressure, there was only a 35% reduction in brachial FMD and a 45% reduction in popliteal FMD with age.

Discussion

The major new finding of this research is that FMD is diminished to a similar extent (by 50-60%) in the brachial and popliteal arteries of healthy older women. Additionally, the relationship between the increase in shear rate due to 5 minutes of distal cuff occlusion and conduit artery dilation appears to be attenuated in older women but, contrary to our hypothesis, is not significantly different between the brachial and popliteal artery in either young or older women.

Reductions in Conduit Artery FMD with Age

In the present study, when we measured brachial artery FMD as the conventional percentage increase in diameter above resting baseline (Corretti *et al.*, 2002), we found that the dilatory response to 5 minutes of distal occlusion was reduced by approximately 40% in the older women. This reduction was comparable to that which has been reported previously in the literature (Celermajer *et al.*, 1994; Jensen-Urstad & Johansson, 2001; Ryliskyte *et al.*, 2004). Popliteal artery FMD, measured in the same manner, was reduced in older women by approximately 30%. The magnitude of this reduction was less than has been reported in the literature comparing patients with hyperlipidemia (Spacil *et al.*, 2002) and coronary disease (Angerer *et al.*, 2001) to healthy controls, although these studies evoked FMD through 5 minutes of proximal occlusion.

During the course of the 5 minute calf occlusion, we observed a small but statistically significant dilation in the popliteal artery in the older subjects. Popliteal artery diameter also increased in the younger women, but this was not statistically significant. Because the shear stimulus is directly related to the diameter measured immediately prior to cuff release (Furchgott

& Zawadzki, 1980; Palmer *et al.*, 1987; Koller & Bagi, 2002; Shipley *et al.*, 2005), we also expressed FMD as the percentage increase in diameter above occlusion diameter. % D_{occlu} tended to magnify the effect of age in popliteal artery FMD due to the greater dilatory response during occlusion of older subjects. While our only explanation for the differential popliteal dilation to date comes from the observation that myogenic dilation to sustained decreases in transumural pressure is augmented with age in the brachial artery (Lott *et al.*, 2004), we do believe that it is worthwhile to present FMD as a function of occlusion diameter since using resting diameter would encompass a dilatory change occurring during occlusion that was not caused by the peak shear stimulus. That is, inasmuch as FMD represents the dilatory response to the increase in fluid shear stress post-occlusion, the expression of dilation should only represent changes in vessel diameter occurring in direct relation to the shear stimulus, particularly when FMD is being used to detect small diameter changes (0.1-0.3 mm) in a large vessel with only 8% dilation. Thus, our subsequent calculations (normalized FMD, limb comparisons, ratio to endothelium-independent dilation) used % D_{occlu} as the representation of FMD. However, it should be noted that using % D_{rest} to represent FMD yielded the same, significant results.

The Conduit Artery Dilator Response in Older Women is Reduced Despite Similar Increases in Shear Rate

Although there were no age differences in shear rate during rest, occlusion, or peak measurements (Table 3-2), the dilatory response in both the popliteal and brachial artery of older women was reduced compared to younger women. Moreover, when the diameter changes in either artery were normalized to the shear stimulus, FMD was still attenuated, compared to young women, by 55% and 53% in the brachial and popliteal artery, respectively (Figure 3-1). This analysis suggests that the observed age-associated reductions in conduit artery FMD are a

function of differences in the dilatory response rather than a diminished shear stimulus in older limbs. These results differ from the recently published data of Wray et al., who found there were preserved (brachial) and augmented (femoral) FMD with age (Wray *et al.*, 2005b); collectively, these findings suggest that the influence of age on FMD of both upper and lower extremity arteries may be sex-specific.

The Relationship Between Shear Rate and Diameter Increases in the Brachial and Popliteal Artery Does Not Appear to be Limb-Specific

The dilatory response of the popliteal artery to 5 minutes of distal occlusion was significantly less than that of the brachial artery (when measured as % dilation). However, the lower % dilation in the popliteal artery could reflect a reduced peak shear stimulus (i.e. ~400-500 s^{-1}) compared to that measured after 5 minutes of brachial artery occlusion (~1300-1400 s^{-1}) (Table 3-2). Given that the calf comprised a much larger lean tissue mass than the forearm in these women (DXA results from Table 1), the resistance arteriole dilation induced by 5 minutes of occlusion may simply not have been as great in the forearm as it was in the calf. However, when the dilatory response to occlusion was examined with respect to the increased shear rate following cuff release (Figure 3-1), normalized FMD did not differ between the arm and the leg in either young or older individuals. This suggests that conduit artery responsiveness to an increase in shear stress evoked by 5 min of distal cuff occlusion is not limb-specific.

What Underlies the Attenuated Arm and Leg Dilator Response but Preserved Shear Stimulus in Older Women?

Following both forearm and calf occlusion, the dilatory response of the upstream conduit artery was blunted in older women, despite increases in shear rate that were similar to those

observed in young women. Interestingly, endothelium-independent dilation was also blunted similarly in older women (Figure 3-2) such that accounting for the deficit in smooth muscle function (ratio of FMD:NTG dilation; Figure 3-3) abolished age-related dilatory differences in both the brachial and popliteal arteries. Thus, it is certainly possible that alterations in the smooth muscle response to endothelium-derived dilators evoked following 5 minutes of occlusion underlie blunted FMD. However, given that the signaling mechanisms behind FMD have not been systematically investigated in women, the lower limb vasculature, or older humans, we cannot conclude whether the observed attenuation in FMD is attributable solely to a deficit in smooth muscle signaling/responsiveness or to other age-related alterations (i.e., diminished NO production and/or bioavailability, a reduction in synthesis or release of alternative and/or additional endothelium-derived dilators, elevation in sympathetic activity (Dinenno *et al.*, 2000; Hijmering *et al.*, 2002; Dyson *et al.*, 2005) or shear-induced release of endothelin (Yanagisawa *et al.*, 1988; Yoshizumi *et al.*, 1989; Berger *et al.*, 2001)).

Why Do Our Findings Differ from the Existing Literature Concerning Limb Vascular Heterogeneity?

Previously, limb-specific differences in the dilator response to intra-arterial infusions of endothelium-dependent vasodilators have been documented such that brachial artery blood flow is increased to a greater extent than femoral artery blood flow following an infusion of acetylcholine, an NO-dependent dilator (Newcomer *et al.*, 2005). Recently, Wray et al. found that dilation of the femoral artery (per unit increase in shear rate) during knee extensor exercise was less than dilation of the brachial artery during handgrip exercise (Wray *et al.*, 2005b). Our report of similar vascular responses between the popliteal and brachial arteries may be attributable to a sex difference (men vs women), differences in vessel size (the femoral artery is

almost twice as large as the popliteal artery and as such may be less responsive to increases in shear stress or NO-dependent agonists) and/or differences in the stimuli used to assess limb-specific responses (i.e. infusion of acetylcholine vs exercise hyperemia vs tissue ischemia).

Does the Peak Shear Rate Accurately Portray Vascular Responsiveness in Conduit Arteries?

It has recently been noted that the peak shear rate, a parameter commonly used to normalize FMD to the stimulus (de Groot *et al.*, 2004; Rakobowchuk *et al.*, 2005; McGowan *et al.*, 2006) may not comprise the true nature of the shear stimulus (Pyke *et al.*, 2004; Pyke & Tschakovsky, 2005). Rather, the full one-minute post-occlusion shear rate (assessed as area under the curve, or AUC) may more closely estimate the stimulus for FMD. To this end, we compared the effects of normalizing FMD to the one-minute AUC shear rate vs peak shear rate in 8 young and older subjects and found no difference in our results and conclusions when using either normalization technique (Figure 3-4).

To What Extent Do Our Findings Reflect Primary Aging?

As noted in the results, assessing the influence of independent variables on FMD with ANCOVA did not yield significant results for any variable, possibly because the relation between FMD and cardiovascular risk factors is only apparent in younger adults with few risk factors (Witte *et al.*, 2005). However, we were able to match 6-8 young subjects to 6-8 older subjects for each characteristic to examine further whether reduced FMD was modulated by elevated baseline variables such as blood pressure, cholesterol, etc. in older subjects. In older adults matched to younger adults for both systolic and diastolic blood pressure, we found that the reduction in arm and leg FMD was blunted. This finding suggests that the influence of age on FMD may be

mediated in part through structural alterations of the peripheral vasculature that partially underlie increased blood pressure, such as intimal thickening, increased collagen content, and decreased elastin (Lakatta, 2002), as older subjects demonstrated significantly higher peripheral pulse wave velocity measurements (data not shown). Furthermore, the close relationship between age, vascular stiffness, regulation of smooth muscle tone and atherosclerosis makes it impossible to determine in this study whether vascular aging, atherosclerosis, or a combination of the two result in blunted FMD (Lakatta & Levy, 2003).

Finally, given that our study population was restricted to women, it is certainly possible that our results are indicative of a primary or additional effect of reduced sex hormones on FMD in postmenopausal women. It is beyond the scope of this paper to discuss the manner in which age and sex hormones may independently and jointly effect endothelial and smooth muscle function. However, we feel that our results are not marginalized by the sex of our study population, as the loss of sex hormones is an inherent aspect of the aging process in women.

Experimental Considerations

A current limitation of the study is that our Doppler ultrasound machine samples the peak, or center, blood velocity envelope rather than an intensity-weighted mean blood velocity that reflects the differing blood velocities across the parabolic curve comprising fluid movement in an artery. In young subjects, the correlation between peak velocity, spatially-averaged mean blood velocity and shear rate is supported by research (Silber *et al.*, 2001). However, it is not known whether in an older subject, with a stiffer vessel, the relationship between peak blood velocity and mean blood velocity is altered. To this end, Silber et al. have studied femoral and brachial artery blood velocities in young and older men and have found that in all subjects, the ratio of the center velocity to the spatially averaged mean velocity is smaller than for a fully

developed parabola and does not differ among subject groups (HA Silber, unpublished observation). Although using center velocity to estimate mean blood velocity and shear rate will result in a greater underestimation of either parameter in a large vessel, such as the femoral artery, given that larger arteries have a larger "blunt zone" in the center (Tangelder *et al.*, 1986), vessels of more similar size, such as the popliteal and brachial, will not be influenced significantly by this discrepancy (HA Silber, personal communication).

In addition, due to the difficulty subjects experienced keeping their leg in a position in which the Doppler probe could be maintained at an angle of insonation < 60° (the standard range for blood velocity measurements), with diameter still imaged accurately, the angle was standardized to 68° for all subjects for both arm and leg measurements. As intrinsic spectral broadening with an increasing angle of insonation leads to consistent overestimation of the true flow velocity (Eicke *et al.*, 1995; Hoskins, 1999), it is likely that our velocity measurements overestimate true blood velocity. However, given that our values would be systematically overestimated for the entire subject population, we are not attempting to quantify volumetric blood flow, and the standard angle of insonation of 60° also overestimates peak velocity (Hoskins, 1999), we do not feel that this issue significantly influences the results of the study.

Conclusions

The present study demonstrates that the well-documented age-associated reduction in brachial artery FMD is also observed in the popliteal artery in women. However, the age-related reduction in normalized FMD is not limb-specific; that is, while a given increase in shear rate evokes a smaller percent diameter increase in older women, this relationship is similar in both the brachial and popliteal arteries. Finally, attenuated FMD in both the arm and leg in older women

may be attributable, in part, to altered smooth muscle responsiveness to endothelium-derived substances.

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Table **3-1**: Subject Characteristics. Values are means ± S.E.M. * indicates significant ($p < 0.05$) difference between young and older subjects. [1] Data only collected in six of the fourteen young subjects due to necessity of completing testing prior to graduation.

	Young n = 14	Older n =14
Age	22 ± 1	70 ± 2
LDL Cholesterol ($mg \cdot dL^{-1}$)	77.5 ± 5.9	117.5 ± 6.9*
Total Cholesterol ($mg \cdot dL^{-1}$)	147.5 ± 6.3	204.7 ± 6.5*
Hematocrit (%)	39.1 ± 0.6	40.8 ± 0.8
Systolic Blood Pressure (mmHg)	109.3 ± 4.3	130.6 ± 3.1*
Diastolic Blood Pressure (mmHg)	68.4 ± 2.1	78.4 ± 2.7*
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	36.4 ± 2.6[1]	19.6 ± 0.7*
Body Fat (%)	27.4 ± 1.2	33.8 ± 1.3*
BMI ($kg \cdot m^{-2}$)	21.9 ± 0.7	24.5 ± 0.8*
Forearm Muscle (g)	603.1 ± 36.9	579.7 ± 18.8
Calf Muscle (g)	1615.8 ± 59.5	1446.1 ± 91.3

Table **3-2**: Brachial and Popliteal Diameters and Shear Rates. Resting, occlusion and peak diameters and shear rates are shown for young (n = 14) vs. older (n = 14) subjects. Values are means ± S.E.M. * indicates significant ($p < 0.05$) difference between peak and baseline/occlusion conditions. † indicates significant ($p < 0.05$) difference between baseline and occlusion conditions. There were no significant age group differences for any of the conditions. Peak shear rates were obtained, on average, in the first 10-15 seconds following occlusion, although velocity was monitored for 30 seconds after cuff release. Peak diameters were obtained, on average, 60-75 seconds following occlusion, although diameter was monitored for 3 minutes after cuff release.

	Diameter (mm)		Shear Rate (s^{-1})	
	Young	Older	Young	Older
Brachial				
Rest	2.96 ± 0.14	3.11 ± 0.11	212.9 ± 32.0	227.3 ± 31.6
Occlusion	2.91 ± 0.13	3.13 ± 0.11	40.5 ± 7.9†	48.0 ± 7.1†
Peak	3.37 ± 0.14*	3.37 ± 0.93*	1400.0 ± 130.2*	1330.8 ±120.2*
Popliteal				
Rest	5.55 ± 0.18	5.69 ± 0.21	63.3 ± 8.9	61.2 ± 9.5
Occlusion	5.66 ± 0.18	5.80 ± 0.20†	19.2 ± 3.8†	21.6± 4.6†
Peak	5.92 ± 0.18*	5.90 ± 0.20*	500.1 ± 48.6*	400.7 ± 35.4*

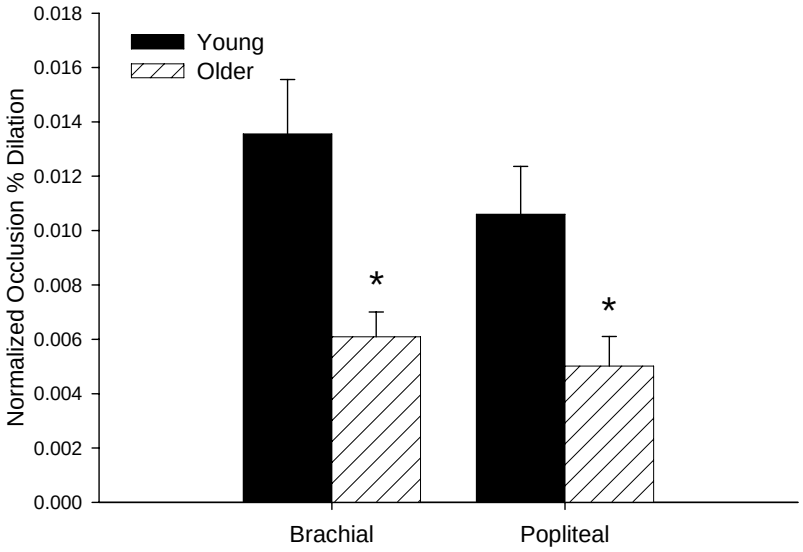

Figure **3-1**: Comparison of average normalized FMD responses (mean ± S.E.M.) following forearm or calf occlusion in young and older subjects. Dilation was calculated as the percentage increase above occlusion diameter divided by the absolute change in shear rate from occlusion to peak. * indicates significant ($p < 0.05$) difference between young and older subjects. There were no significant differences between normalized brachial and popliteal FMD in either young or older subjects.

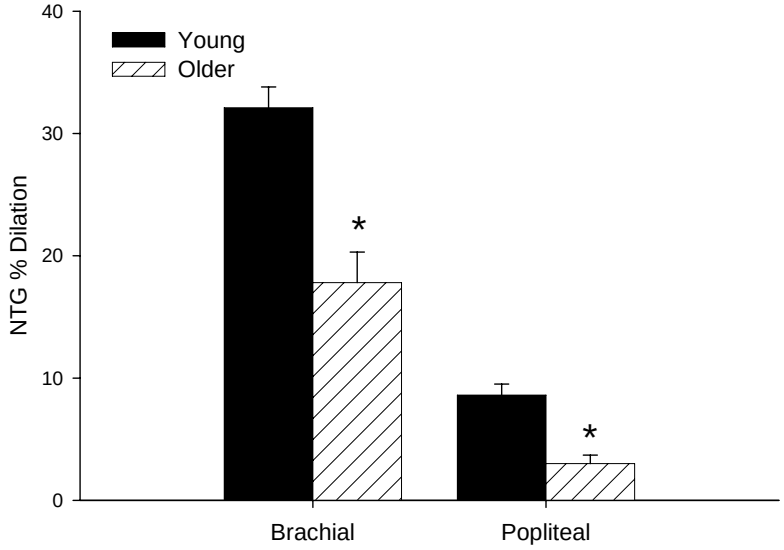

Figure **3-2**: Comparison of average brachial and popliteal responses (mean + S.E.M.) to nitroglycerin (NTG) in young and older subjects. Dilation was calculated as the percentage change from pre-NTG diameter to the maximum diameter measured during the 10 minutes following NTG administration. * indicates significant ($p < 0.05$) difference between young and older subjects.

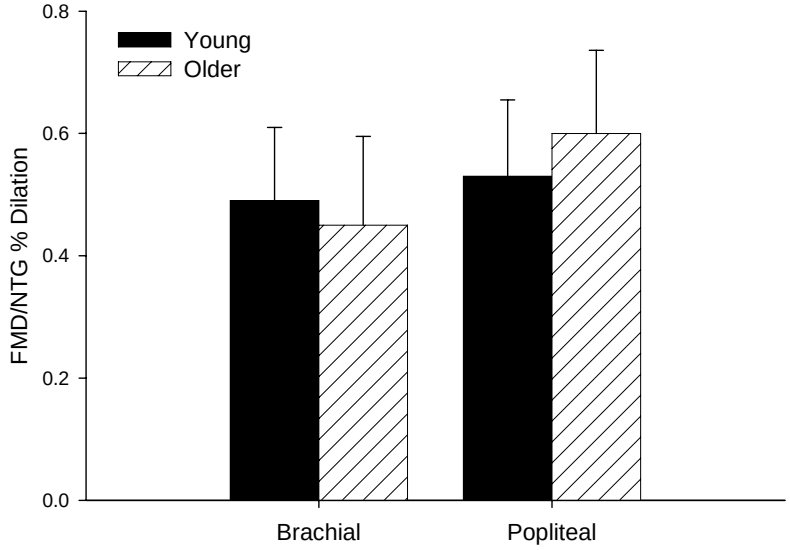

Figure **3-3**: Ratio of average endothelium-dependent (FMD; calculated in Figure 2) to endothelium-independent (NTG; calculated in Figure 4) dilation (mean $\pm$ S.E.M.) in the brachial and popliteal arteries of 8 young and 8 older subjects.

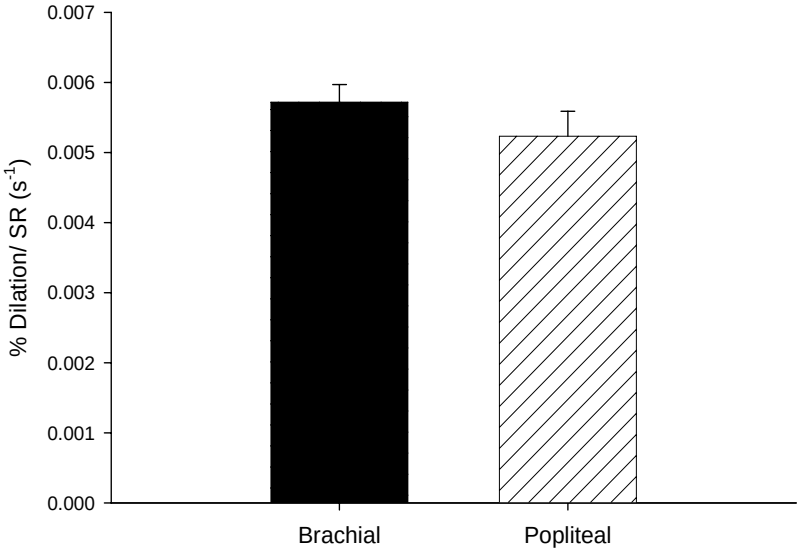

Figure **3-4**: Comparison of brachial and popliteal dilation (mean ± S.E.M.) normalized to 1 minute AUC shear rate in 8 young and older subjects. Data from young and older subjects was pooled as there were no age group differences. Dilation was calculated as the percentage increase above occlusion diameter divided by the AUC for shear rate following cuff release after 5 minutes of distal occlusion.

Chapter 4

EVIDENCE FOR REDUCED SYMPATHOLYSIS IN THE LEG RESISTANCE VASCULATURE OF HEALTHY OLDER WOMEN

Introduction

Dynamic, large muscle mass exercise evokes two competing influences: vasodilation in the working muscle vasculature, the purpose of which is to meet metabolic demand, and sympathetic vasoconstriction in blood vessels of both inactive and active circulations to maintain systemic blood pressure (Shepherd, 1987; Rowell, 1993; Saltin *et al.*, 1998). A characteristic of dynamic exercise, therefore, is that vascular conductance in the exercising muscle will be determined in part by the balance between vasoconstriction and vasodilation, such that metabolic vasodilators inhibit the vascular effects of augmented sympathetic outflow (commonly referred to as functional sympatholysis (Remensnyder *et al.*, 1962; Saltin *et al.*, 1998; Hansen *et al.*, 2000b)).

There is accumulating evidence to suggest that sympatholysis is reduced with age during two-leg cycling in men (Koch *et al.*, 2003), handgrip exercise in men (Dinenno *et al.*, 2005), and handgrip exercise in women (Fadel *et al.*, 2004); this could be one mechanism underlying age-related alterations in muscle blood flow and vascular control during exercise (Proctor & Parker, 2006). An alternative approach to studying the balance between vasodilation and augmented sympathetic tone that has been used in young adults involves the application of an acute sympathetic stimulus during brachial artery FMD, or flow-mediated dilation (Sinoway *et al.*, 1988; Hijmering *et al.*, 2002; Lind *et al.*, 2002; Spieker *et al.*, 2002; Dyson *et al.*, 2005). Doppler ultrasound-derived measurements of FMD, the dilatory response to local ischemia, offer the advantage of investigating vasodilation through both the resistance arteriole response to occlusion

(the immediate hyperemic response upon release of occlusion, as quantified by changes in flow and vascular conductance) as well as the conduit artery response to increased fluid shear stress (the percent dilation of the conduit, normalized to the shear stimulus). In addition, a carefully timed sympathetic stimulus can be applied such that the peak constrictor stimulus coincides reproducibly with peak dilatory responses. Finally, FMD can be studied in the lower extremity vasculature, a vascular bed that is more relevant to the control of blood flow and systemic pressure during large muscle mass exercise, without being confounded by age-related alterations in exercise tolerance, baroreceptor responsiveness, or central limitations (e.g. cardiac output, systemic pressure) to leg vasodilation.

Accordingly, we sought to determine the effect of an acute elevation in sympathetic tone (cold pressor test, or CPT) on both the ischemia-induced dilation of resistance vessels and the conduit artery response in the popliteal artery of young vs older women, as the effect of age on lower limb sympatholysis has not been characterized in women. Based on the above-mentioned studies (Fadel *et al.*, 2004; Dyson *et al.*, 2005), we hypothesized that both resistance vessel dilation and popliteal artery FMD would be blunted to a greater extent in older compared to young women, suggestive of reduced sympatholysis with age.

Methods

Subject Characteristics

Sixteen young (20-30 yr) and fourteen older (62-74 yr) women completed the study. All subjects were non-obese (BMI $\leq$ 30), nonsmokers, had clinically normal blood chemistry (i.e. hemoglobin concentrations ranged from 11.6-14.8 $g \cdot dL^{-1}$, total cholesterol $\leq$ 240 $mg \cdot dL^{-1}$, LDL cholesterol $\leq$ 150 $mg \cdot dL^{-1}$), and resting supine ankle-brachial index ratings (ABI between 0.90

and 1.30; VP2000, Colin Medical). All subjects were normotensive (resting blood pressure ≤ 140/90 mmHg) and were neither extremely sedentary nor extremely fit (i.e. had cycle ergomenter $\dot{V}O_{2peak}$ values between 20 and 80% of age-predicted norms (ACSM, 2006)). Subjects were free of overt chronic diseases as evaluated by medical history questionnaire, a physical examination and resting ECG. Percent body fat, estimated using dual-energy X-ray absorptiometery (DXA; model QDR 4500W, Hologic, Waltham, MA), did not differ between groups. Additionally, no subjects were taking medications having significant hemodynamic effects, including oral contraceptives (young) or hormone-replacement therapy (older) for at least the last 12 months. Younger subjects were studied in days 1-7 of their menstrual cycle to minimize the influence of female hormones. On study day, subjects were asked to refrain from caffeine, aspirin, ibuprofen, or herbal supplements for at least 12 hours prior to testing. All subjects gave their written, informed consent to participate. This study was approved by the Office for Research Protections and the Institutional Review Board at The Pennsylvania State University. Subject characteristics are presented in Table 1.

Subjects also completed a physical activity questionnaire to assess routine physical activity (Yale Physical Activity Questionnaire (Dipietro *et al.*, 1993) for older and Baecke Questionnaire of Habitual Physical Activity (Baecke *et al.*, 1982) for young). None of the subjects participated in moderate to high intensity aerobic exercise > 3 $d \cdot wk^{-1}$ or regular lower body resistance training > 2 $d \cdot wk^{-1}$during the past 12 months. To objectively quantify aerobic fitness status, all of the subjects performed a continuous incremental leg cycle ergometer test (Lode) to maximal exertion to determine peak oxygen uptake ($\dot{V}O_{2peak}$), as described in detail previously (Proctor *et al.*, 2004b).

Experimental Design

All subjects participated in Parts 1-4 (see Figure 4-1) on a single study visit in which Doppler ultrasound was used to assess popliteal diameters and velocities during FMD, CPT and CPT superimposed on FMD (FMD+CPT). The room was thermoneutral with temperature consistently maintained at 23.5±0.1°C. Blood pressure was measured at heart level in the dominant arm with an automatic blood pressure monitor (Omron HEM-705CP; Vernon Hills, IL) and heart rate was continuously monitored using a 3-lead electrocardiogram. Each part of the experiment was separated by a 15 minute rest period. A subset of young and older subjects allowed us to obtain direct recordings of muscle sympathetic nerve activity (MSNA) on a separate day (see Part 5 below).

Part 1 (FMD alone). Popliteal FMD was measured with the subject lying prone with a small pillow under the non-dominant ankle. A rapid inflation/deflation pneumatic cuff (D.E. Hokanson, Inc.; Bellevue, WA) was placed around the calf, 2-3 inches distal to the popliteal fossa. The artery was imaged immediately proximal to the bifurcation (usually at or slightly above the popliteal fossa) using a 6-11MHz multifrequency linear array probe attached to the Acuson 128XP duplex ultrasound imaging system (Siemens, New York, NY, USA); once a satisfactory image using optimal B-mode imaging was obtained, the placement of the probe was marked on the leg to ensure that the site of measurement did not change during the trial. Ultrasound parameters were not changed during the study. Doppler velocity was also measured with the Aspen using a 60° angle of insonation held constant throughout the study. After the subject had rested in the prone position for 10 min, resting popliteal artery diameter and velocity were measured for 1 min prior to inflation of the pneumatic cuff. The cuff was then inflated to ~300 mmHg for 5 min; diameter and velocity recordings resumed 1 min prior to cuff deflation and continued for 3 minutes post-inflation.

Part 2 (Cold pressor stimulation alone). Baseline Doppler measurements (diameter and velocity) were taken for 1 minute. The subject was then instructed to immerse her dominant hand (up to the wrist) in a 0-1 °C mixture of ice and water for 3 minutes while maintaining a steady, relaxed breathing pattern, consistent with previous studies using this sympathetic stimulus (Victor *et al.*, 1987; Seals, 1990). Diameters and velocities were measured throughout the ice water immersion and were continued for 3 minutes of recovery after the subject removed her hand from the ice/water mixture.

Part 3 (FMD + cold pressor stimulation). Baseline Doppler measurements (diameter and velocity) were taken for 1 minute. The FMD protocol was repeated in an identical fashion except that CPT (as described above) was superimposed on the protocol such that ice water immersion started at minute 3.5 of the 5 minute occlusion and continued for 3 minutes until 1.5 minutes post-cuff release. This timing ensured that a sufficient sympathetic stimulus would occur both during resistance arteriole dilation (i.e. occlusion) as well as during the ensuing conduit artery dilation so that both components of FMD could be examined with respect to CPT (Victor *et al.*, 1987; Seals, 1990). Recovery data were collected for 3 minutes following the end of CPT.

Part 4 (Sublingual nitroglycerin). Endothelium-independent dilation of the leg was assessed through administration of 0.4 mg sublingual nitroglycerin (NTG), a nitric oxide donor eliciting maximal dilation representative of smooth muscle function. After 1 minute of baseline diameter measurements, diameters were measured continuously for 10 minutes following administration of NTG.

Part 5 (MSNA responses to cold pressor stimulation). On a separate visit, a subset of subjects (7 young and 7 older) participated in Part 5. To assess neural sympathetic outflow, peroneal nerve MSNA was measured continuously before, during and after CPT. CPT was repeated as described above, with the that resting measurements were obtained for 3 rather than 1 minute to quantify MSNA. Heart rate, blood pressure, and MSNA measurements were taken for

3 minutes before (rest) and 3 minutes following (recovery) the 3 minute 0-1°C ice-water immersion of the dominant hand (CPT).

Measurements and Calculations

Measurement of popliteal artery diameter and velocity. For FMD measurements, popliteal artery diameters were measured for 1 min at rest, during the last minute of occlusion and as the highest diameter observed during 3 min of post-inflation imaging. Consistent with the literature, the peak diameter was observed between 50-75 seconds in most subjects (Corretti *et al.*, 2002; Betik *et al.*, 2004). Diameter measurements were sampled at end-diastole (ECG-gating was used to select images that were trigged by the R-wave of the cardiac cycle) using Brachial Imager software (Medical Imaging Applications; Iowa City, IA). Post-test analysis of diameters was performed using edge-detection software (Brachial Analyzer Software, Medical Imaging Applications; Iowa City, IA); briefly, the technician (always the same and blind to any subject information) selected a region of interest along the arterial wall and the edge of the wall was detected by pixel density and represented by a line of best fit. Each sequence of images was reviewed by the technician and adjusted to ensure that diameter measurements were always calculated from the intima-lumen interface at both the distal and proximal vessel wall. Resting and occlusion diameters were calculated as a 10 second average of images taken over the 1 min baseline and last minute of occlusion, respectively. The peak diameter was calculated by identifying the post-occlusion image with the largest diameter and averaging that image with the 5 preceding and 5 following images. Blood flow velocity was measured as the average velocity during 10 seconds of rest, the average velocity during 10 seconds of the last minute of occlusion, and the average (peak) velocity measured over the first 10 seconds following cuff release. Velocity measurements continued until 45 seconds post-cuff release, at which time 2D imaging

was optimized for diameter measurements. Again, imaging software (Brachial Analyzer Software, Medical Imaging Applications; Iowa City, IA) was used to automatically trace the velocity-time integral of time-averaged, angle-corrected maximum velocities (highest velocity across the cardiac cycle). For NTG measurements, the resting diameter was calculated as a 10 second average of images taken over the 1 min baseline and the peak diameter was calculated as the post-NTG image with the largest diameter, again averaging that image with the 5 preceding and 5 following images.

Assessment of dilation. FMD was calculated as the absolute and percent change in diameter from either rest or the last minute of occlusion to peak. We calculated post-occlusion area under the curve (AUC) shear rates using lower-resolution diameter imaging simultaneously with velocity measurements for 45 seconds following cuff release (until it was necessary to switch into high-resolution 2D mode to image peak diameters), as recent evidence suggests that AUC measurements may more closely approximate the shear stimulus than peak measurements (Pyke *et al.*, 2004; Pyke & Tschakovsky, 2005). FMD was then normalized to the 45-second AUC shear rate (4*velocity/diameter). NTG dilation was calculated as the percent change in dilation relative to baseline.

Calculation of popliteal blood flow and vascular conductance. Resting, occlusion and peak popliteal blood flows were derived from the formula:

Popliteal blood flow (PBF): Blood velocity(1000) $\cdot \pi \cdot$ (popliteal diameter/2)$^2 \cdot 60$

where the PBF is in ml·min^{-1}, the blood velocity is in m·s^{-1}, the brachial diameter is in mm, and 60 is used to convert from ml·s^{-1} to ml·min^{-1}. Resting, occlusion, and peak popliteal vascular conductance (PVC) were calculated as PBF/MAP.

Assessment of blunted vasodilatory responses during cold pressor stimulation. To examine the influence of CPT on peak reactive hyperemia, we calculated the percent reduction in hyperemic PVC during sympathetic stimulation as:

$$(\text{Peak } PVC_{FMD+CPT} - \text{Peak } PVC_{FMD}) / \text{Peak } PVC_{FMD} * 100$$

where the difference between PVC measured following 5 minutes calf occlusion and PVC measured when CPT was superimposed on occlusion is divided by PVC measured following 5 minutes calf occlusion. Additionally, we compared this value to the PVC percent reduction relative to rest observed during CPT alone (PVC_{rest}-PVC_{CPT}/ PVC_{rest} * 100) to account for any age-related differences in baseline adrenergic sensitivity. This approach has been used previously to compare vasoconstrictor responsiveness in young vs older subjects (Dinenno *et al.*, 2005).

To examine the influence of CPT on FMD, we examined all components of the dilatory response--absolute diameter changes, percent diameter changes relative to rest or occlusion, 45 second AUC SR, and percent diameter changes normalized to the AUC SR-- between FMD and FMD + CPT. This approach has been used previously to assess the influence of a sympathetic stimulus on FMD (Lind *et al.*, 2002; Dyson *et al.*, 2005).

Muscle Sympathetic Nerve Activity (MSNA). Multiunit postganglionic recordings of MSNA were made using sterile 200-µm-diameter Tungsten microelectrodes of 2-3 MΩ impedance inserted percutaneously into muscle nerve fascicles of the peroneal nerve of the dominant leg, as described previously (Pawelczyk *et al.*, 2001). Briefly, a recording electrode was placed in the peroneal nerve at the fibular head or the popliteal fossa, and a reference electrode was placed subcutaneously 2-3 cm from the recording electrode, with the subject in the supine position. The nerve signal was amplified (40,000-70,000), band-pass filtered (0.7 kHz high pass; 2-3 kHz low pass), and then full-wave rectified and smoothed with a resistance-capacitance circuit (time constant, 0.1 s) to produce a recording of "integrated" MSNA. The recording electrode was adjusted until clear sympathetic bursts were recorded and the signal-to-noise ratio of the bandpass-filtered neurogram (peak-to-peak burst amplitude relative to baseline noise) exceeded two. The microneurographer evaluated the quality of MSNA recordings based on accepted criteria, including: *1*) pulse synchrony, *2*) facilitation during the hypotensive phase of

the Valsalva maneuver, and suppression during the hypertensive overshoot after release, *3*) increases in response to breath holding, and *4*) insensitivity to emotional stimuli (loud noises or stressful mental arithmetic) and stroking the skin. ECG (78534A; Hewlett Packard), beat-to-beat blood pressure (radial tonometry of the nondominant hand; Colin, Medical Instruments Corporation), and neural recordings were stored on a chart recorder (MT95K2; Astromed) and VCR tape (Vetter) for later analysis. The amplitude of each mass sympathetic discharge was quantified by digitization of the resistance-capacitance-filtered neurogram (SigmaScan, version 2.1, Jandel Scientific). Sympathetic activity was expressed as the frequency of discharges (bursts/min).

Assessment of the sympathetic stimulus. The quotient of the percent change in peak popliteal conductance from FMD to FMD + CPT conditions and the absolute change in MSNA was calculated to determine the relationship between reductions in peak vascular conductance and the stimulus for vasoconstriction.

*Reproducibility***.** We determined between-condition Doppler ultrasound measurement reproducibility as well as between-condition and between-visit CPT reproducibility (estimated by peak MAP) according to the following formula (Abbink *et al.*, 2001; de Groot *et al.*, 2004):

$(\sum (\text{first test-second test})^2)/2*(\text{number of paired observations})/(.5(\text{average of first test} + \text{average of second test}))$.

Baseline popliteal measurement reproducibility (n=30) was 0.17% for resting diameters and 5.18% for resting shear rate. FMD popliteal measurement reproducibility (n=10; subjects studied on a different visit) was 9.54% for peak diameters, 16.05% for peak shear rate, and 19.7% for FMD. Peak MAP reproducibility was 0.48% between conditions for Study Visit 1 and 0.86% between Study Visit 1 and Study Visit 2.

Statistical Analysis

Statistical analyses were performed using Minitab (Minitab, State College, PA) and SPSS (SPSS 13.0, Chicago, IL) software. All data are reported as mean + S.E.M. and significance was set at $p<0.05$. A Student's t-test for independent groups and Tukey post-hoc analysis were used to compare baseline differences between young and older groups. Repeated-measures ANOVA models were used to assess the effects of within-group differences (i.e. values at different timepoints or conditions) and between-group differences (i.e. influence of age) on different response variables such as PBF, PVC, MAP, MSNA, and normalized FMD.

Results

Influence of age on peak reactive hyperemia and vascular conductance. Following cuff release, peak blood flow (Y: 850 ± 85 $mL \cdot min^{-1}$; O: 848 ± 69 $mL \cdot min^{-1}$) and peak vascular conductance (Y: 10.0 ± 0.8 $mL \cdot min^{-1} \cdot mmHg^{-1}$; O: 9.5 ± 0.8 8 $mL \cdot min^{-1} \cdot mmHg^{-1}$) were similar between young and older women ($p > 0.40$ for both comparisons).

Effects of cold pressor stimulation of resistance vessels of young vs older women (Figure 4-2). Young subjects demonstrated a trend towards a greater percent reduction in resting blood flow (Y: -7.0 ± 3.7%; O: -3.3 ± 4.5%; p= 0.09) and a significantly greater percent reduction in PVC during CPT (p=0.03). Following superimposition of CPT on FMD, peak blood flow was significantly augmented relative to FMD alone (Y: $p<0.01$; O: $p=0.04$) in both subject groups (Y: 1016 ± 80 $mL \cdot min^{-1}$; O: 1039 ± 109 $mL \cdot min^{-1}$); however, there was no difference in peak blood flow between subject groups ($p = 0.86$). PVC was still similar in both age groups (Y: 9.2 ± 0.6 8 $mL \cdot min^{-1} \cdot mmHg^{-1}$; O: 9.0 ± 0.9 8 $mL \cdot min^{-1} \cdot mmHg^{-1}$; $p=0.58$), and was reduced similarly ($p=0.79$) respective to FMD alone. In a subset of subjects matched for baseline adrenergic responsiveness

to CPT, older subjects demonstrated a significantly greater (p=0.04) reduction in peak popliteal conductance during FMD + CPT.

Influence of age on FMD responses (Figures 4-3 and 4-4). Absolute diameter responses are shown in Figure 3. Based on the significant dilation observed during occlusion in older women we estimated FMD as both the % dilation from rest to peak as well as occlusion to peak conditions (% D from rest: Y: 8.4 ± 0.9 %; O: 3.2 ± 0.4 %; % D from occlusion: Y: 8.1 ± 0.9 %; O: 2.3 ± 0.5 %; both $p < 0.01$). The 45-sec AUC shear rate was attenuated ($p < 0.01$) in older subjects (Y AUC: 15930 ± 877 s^{-1}; O AUC: 13351 ± 1088 s^{-1}). Normalization of resting or occlusion FMD to the 45 sec AUC SR, which is believed to most closely represent the shear stimulus (Pyke & Tschakovsky, 2005), still yielded an age-associated decrease in the dilatory response to 5 minutes distal occlusion (Figure 4-4).

Effects of cold pressor stimulation on the conduit artery responses of young vs older women. (Figures 4-5 and 4-6). There were no changes in popliteal diameter during or after CPT alone in either young or older subjects. When CPT was superimposed on FMD, the dilatory response measured relative to baseline was not different from the FMD condition in young (p=0.31) and older (p=0.20) subjects (% D: Y: 7.5 ± 1.0%; O: 2.0 ± 0.6 %). However, the dilatory response measured relative to occlusion was blunted in young (p=0.05) but not older (p=0.36) subjects (% D: Y: 6.3 ± 0.8 %; O: 1.6 ± 0.8 %), as young subjects displayed a significant popliteal dilation during occlusion and a reduced peak diameter (Figure 4-3). AUC SR was unchanged (p=0.37 and 0.38, respectively) between conditions in young and older women (Y FMD+CPT: 15070 ± 867 s^{-1}; O FMD+CPT: 12123 ± 800 s^{-1}); however, in the young subjects peak SR was significantly higher and the decay curve was significantly steeper when CPT was superimposed on FMD (Figure 4-5), leading to a similar estimate of AUC SR despite different response curve characteristics. These SR changes were not observed in older subjects. Finally, the resting dilatory response normalized to the AUC SR was not significantly reduced in either subject

population (p=0.60 and 0.26 for young and older, respectively) whereas the occlusion dilatory response normalized to the AUC SR was significantly reduced in young but not older subjects (Figure 4-6). In a subset of subjects (n=7/group) matched for resting FMD (Y %D/s^{-1}: 0.00035 ± 0.00003; O: 0.00033 ± 0.00004), peak diameter was still unchanged in older women (FMD peak diameter: 5.96 ± 0.21 mm; FMD+CPT peak diameter: 5.90 ± 0.23 mm; p=0.47) such that normalized FMD estimated relative to occlusion was attenuated in young but not older women (Y FMD: 0.00065 ± 0.0002 %D/s^{-1}, Y FMD + CPT: 0.00050 ± 0.0001 %D/s^{-1}; O FMD: 0.00010 ± 0.0001 %D/s^{-1}; O FMD + CPT: 0.00010 ± 0.0001 %D/s^{-1}).

Influence of age on sublingual nitroglycerin responses (Figure 4-7). Endothelium-independent dilation was reduced in older subjects (Figure 4-7) such that normalization of FMD (calculated relative to rest or occlusion diameter) to NTG-induced dilation abolished age-group differences (Resting FMD%/NTG%: Y: 1.1 ± 0.2%; O: 1.0 ± 0.3%; p=0.79; Occlusion %/NTG%: Y: 1.1 ± 0.2%; O: 0.7 ± 0.3%; p=0.30).

Influence of age on blood pressure and MSNA (n=7/group) responses to cold pressor stimulation. While MAP was significantly higher in older subjects at most time points before, during, or after CPT, the change from rest to peak measured during CPT was similar in young vs older subjects (YΔMAP: 22.5±2.3 mmHg; OΔMAP: 24.9±2.6 mmHg; p=0.50). In the subset of subjects in whom MSNA was measured, young and older subjects exhibited similar peak MSNA during CPT (Y: 26.6±5.7 bursts·min^{-1}; O: 36.3±2.3 bursts·min^{-1}; p>0.60), but young subjects demonstrated a greater change in MSNA from rest to peak measured during CPT (YΔ MSNA: 12.7±3.6 bursts·min^{-1}; OΔ MSNA: 7.8±2.5 bursts·min^{-1}; p<0.05) as a result of lower resting MSNA (Y: 13.8±2.9 bursts·min^{-1}; O: 28.5±2.9 bursts·min^{-1}; p < 0.01). In this subset of women, the reduction in peak popliteal conductance following CPT (Y: -5.9 ± 5.2% ; O: -8.4 ± 13.6%) divided by the absolute change in MSNA was greater in older women (Y: -0.7 ± 0.8 %/ bursts·min^{-1}; O: -5.6 ±

4.8 %/ bursts·min^{-1}), although this age difference was not statistically significant (p=0.21) due to the limited sample size.

Discussion

Recent evidence suggests that the blunting of sympathetic vasoconstriction in resistance vessels (sympatholysis) may be reduced in exercising limbs of older adults. This age-related alteration has not been characterized in the lower extremity vasculature of women, and the potential for blunting of the conduit artery dilatory response to a sudden increase in shear stress (i.e., FMD) has not been examined in older adults of either sex. The present study utilized a standardized hyperemic stimulus (5 min of distal calf ischemia) and application of robust sympathetic vasoconstrictor stimulus (3 min of cold pressor stimulation spanning the last 1.5 min of ischemia and the first 1.5 min following cuff release) in healthy younger vs. older women to test the hypothesis that aging reduces lower limb sympatholysis in women. Popliteal artery FMD responses to sublingual nitroglycerin (NTG) were also studied to determine whether there may be a smooth muscle contribution to any age-dependent dilatory responses. The primary new findings were that 1) lower limb resistance vessel sympatholysis appears to be less effective in healthy older vs. younger women when the vasculature is vasodilated in response to 5 minutes of ischemia and 2) FMD of the popliteal artery is altered during acute sympathetic stimulation in young, but not older women.

Is the Vasodilatory Response to 5 Minutes of Ischemia Preserved in the Lower Legs of Older Women?

Following 5 minutes of distal cuff occlusion, there was no age difference in reactive hyperemia (expressed as either peak popliteal artery blood flow or conductance). These findings

differ from previous findings of blunted calf hyperemia with age following exhaustive ischemic exercise (Martin *et al.*, 1991) and 10 minutes of passive, proximal cuff occlusion (Ridout *et al.*, 2005) in women. These disparities are likely attributable to a) the different dilator stimulus used in the present study, as both ischemic calf exercise and 10 minutes of proximal occlusion evoke significantly greater and more sustained hyperemia than 5 minutes of distal occlusion (Martin *et al.*, 1991), and b) differences in blood flow measurement techniques between the current study (Doppler ultrasound) and the aforementioned studies (plethysmography).

Is There Evidence of Reduced Sympatholysis in Lower Leg Resistance Vessels of Older Women?

In agreement with the well-documented age-related decrease in resting adrenergic responsiveness (Seals & Esler, 2000; Dinenno *et al.*, 2005), CPT reduced resting popliteal conductance by ~15% compared to the ~28% reduction observed in young women. Following superimposition of CPT on FMD, the percent reduction in peak popliteal conductance relative to FMD alone was similar in young vs older women (~5-8%) (Figure 4-2), which is notable given that the increase in MSNA (i.e. efferent sympathetic stimulus) was blunted in older women. Furthermore, in a subgroup comparison of 9 young and 9 older women who had similar reductions (20 ± 3%) in popliteal conductance to CPT at rest, older women had approximately twice the reduction in peak popliteal conductance compared to young women when CPT was superimposed on FMD (~12 vs 6%) (Figure 4-2). In addition, superimposing CPT on FMD resulted in a significant augmentation of the peak shear rate in young (Figure 4-5) but not older women. Since CPT raises systemic pressure, and calculations of shear rate do not account for perfusion pressure, a plausible explanation for this finding is that CPT augmented peak shear rate in young women due to an intact sympatholysis combined with increased perfusion pressure. By

contrast, the increased perfusion pressure in older women did not translate into an increase in peak shear rate, even though increases in MAP during CPT were identical in both subject groups. Taken collectively, these results provide evidence to suggest that sympatholysis is blunted with age in the lower extremity resistance vasculature in women.

The metabolic milieu (i.e. release of vasoactive substances such as nitric oxide (NO), prostaglandins (PG), endothelium-derived hyperpolarizing factor (EDHF), and endothelin (ET)) of the calf resistance vasculature immediately prior to and following cuff release is not known, especially since the release of dilators and constrictors is transient and may change with age, sex, oxidative stress, and metabolite bioavailability (Lembo *et al.*, 2000; Huang *et al.*, 2001; Taddei *et al.*, 2001; Sun *et al.*, 2004). Certainly, one attractive hypothesis is that release of NO, which may underlie peak reactive hyperemia (Engelke *et al.*, 1996; Koller & Bagi, 2002), attenuates adrenergic vasoconstriction (Costa *et al.*, 2001; Chavoshan *et al.*, 2002) such that there is an age-related decrease in the NO-mediated modulation of CPT in the calf resistance vasculature. However, other interactions in the resistance vasculature between dilators, constrictors, and/or ion channels (Hansen *et al.*, 2000b) may also underlie metabolic inhibition of an acute sympathetic stimulus, rendering specific conclusions difficult without further research.

Effect of Age on Popliteal FMD

Given the significant popliteal dilation observed in older women during cuff occlusion in this study (Figure 4-3) as well as our previous study parker (Parker *et al.*, 2006), attributable perhaps to enhanced myogenic responsiveness with age (Lott *et al.*, 2004), we estimated peak dilation relative to occlusion as well as baseline. As stated previously, our rationale for this approach is that inasmuch as FMD represents the dilatory response to the increase in fluid shear stress post-occlusion, the expression of dilation should only represent changes in vessel diameter

occurring in direct relation to the shear stimulus rather than those evoked through occlusion, such as changes in transmural pressure (Parker *et al.*, 2006). Relative to occlusion diameter, older women exhibited an approximately 70% lower popliteal FMD than their younger counterparts. Calculating FMD relative to resting diameter resulted in a 60% reduction in conduit dilation in older vs young women. Interestingly, the shear stimulus, estimated as the 45 second post-cuff release AUC to reflect current thought concerning the true shear stimulus for FMD (Pyke & Tschakovsky, 2005) was significantly lower in older women due to similar blood velocity responses to ischemia yet larger overall diameters. Taking into account the reduced shear stimulus by normalizing the FMD response to these estimations of the shear stimulus (Figure 4-4) did not influence the magnitude of the age-associated reduction in FMD, in agreement with our recently published findings (Parker *et al.*, 2006). Cumulatively, these data suggest that conduit artery dilation is influenced by age in women similar to what has been observed in the forearm (Celermajer *et al.*, 1994; Jensen-Urstad & Johansson, 2001), resulting in a net dilatory deficit.

The blunted conduit artery dilation in older women could represent increased popliteal stiffening (Tai *et al.*, 1999; Debasso *et al.*, 2004), an altered balance of dilator (reduced) and constrictor (increased) release (Taddei *et al.*, 2001; Singh *et al.*, 2002; Mather *et al.*, 2004), or diminished smooth muscle responsiveness (Adams *et al.*, 1998; Newcomer *et al.*, 2005; Saka *et al.*, 2005). The latter is supported by the reduced popliteal dilation we observed in older women (Figure 4-4) in response to NTG administration, as NTG is a nitric oxide (NO) donor which evokes endothelium-independent dilation, assessing smooth muscle function. However, while the similar percent reduction in FMD and NTG responses makes it tempting to attribute blunted FMD in older women entirely to smooth muscle dysfunction, we would caution that this line of reasoning may be an oversimplification as the pathways underlying FMD in the leg, with age, and in women have not been elucidated. For example, it is possible that FMD is not nitric oxide dependent in the lower extremity vasculature of women and thus blunted FMD encompasses

alterations in dilator pathways (e.g. EDHF and PG) that are not assessed by or comparable to the smooth muscle response to a nitric oxide donor.

Effects of Acute Sympathetic Stimulation on Popliteal FMD

In young women, superimposing CPT on FMD resulted in 1) a significant dilation during occlusion and reduction in peak diameter (Figure 4-3), 2) an alteration in the post-occlusion shear rate profile (Figure 4-5) and 3) an approximate 20% decrease in normalized popliteal FMD, when data were calculated relative to occlusion (Figure 4-6). When FMD was calculated relative to rest, normalized FMD was not significantly influenced by adrenergic stimulation in young women. By contrast, popliteal dilation estimated relative to rest or occlusion was not significantly blunted in older women following superimposition of CPT on FMD (Figure 4-6). These findings underscore the importance of analyzing FMD with respect to both resting and occlusion diameters since changes in conduit artery diameter during occlusion occur through different mechanisms as those evoked by reactive hyperemia and may affect estimation and assessment of endothelium-dependent dilation. Additionally, the application of CPT on FMD in the popliteal in young women did not yield results similar to what has been published in the brachial artery in young men (i.e., in young men, CPT blunts FMD with no change in shear rate or occlusion diameter; (Dyson *et al.*, 2005)), highlighting the importance of recognizing limb and sex differences in vascular regulation.

The differential effect of CPT on diameter measured during occlusion is puzzling, given that there were no changes in popliteal diameter during CPT alone in young women that would yield insight into a possible mechanism underlying the dilation, such as greater beta-adrenergic stimulation (Stratton *et al.*, 1992). An alternative possibility is that interactions between myogenic tone and norepinephrine-induced vasoconstriction, similar to what has been observed

in studies of isolated rat arterioles (Faber & Meininger, 1990; Meininger & Faber, 1991), lead to facilitation of the myogenic response to occlusion in young women that cannot occur in older women due to their already augmented myogenic response to occlusion and/or reduced adrenergic sensitivity. However, human data on this topic is lacking.

Contrary to our hypothesis, peak diameters were reduced in young but not older women. To investigate the possibility that this finding might be an artifact of a baseline effect in older women (i.e., the already reduced FMD observed in older women under normal conditions may be prohibitive of further reductions in the dilatory response when CPT is superimposed on FMD), we examined diameter changes in 7 young and 7 older women matched for baseline FMD. The observed findings (augmented dilation during occlusion, reduced peak popliteal diameter) persisted in young but not older women, suggesting that a baseline effect cannot completely explain our results. Alternative explanations are that the dilatory mechanisms underlying popliteal FMD differ in young vs older women such that CPT only influences the endothelium-dependent dilators utilized by young women (Wu *et al.*, 2001), vasodilation of the popliteal artery unmasks a reduction in conduit adrenergic sensitivity in older women that was not detectable at rest given the absence of conduit artery responses to CPT alone in either subject group, or the altered shear rate profile in young women provided a reduced dilatory stimulus even though total AUC was similar between conditions. Finally, given that there is evidence suggesting that the popliteal artery stiffens significantly with age in women (Tai *et al.*, 1999; Debasso *et al.*, 2004), it is possible that our findings represent a general, age-related reduction in popliteal vascular reactivity, as indicated by both the blunted responses to occlusion and to superimposition of CPT on FMD.

Experimental Considerations

During the primary study visit (Parts 1-4), the order of experiments was not randomized and did not change between subjects since we did not want the systemic release of epinephrine induced by CPT to affect our baseline measurements (Sinoway *et al.*, 1988). However, we do not believe that this study design limited interpretation of our findings as popliteal diameters and velocities always returned to baseline before the next protocol was started, and pilot testing demonstrated similar influence of CPT and/or FMD on the popliteal artery irrespective of time course.

We chose to administer CPT to acutely raise sympathetic tone due to previous results suggesting that the absolute increase in MSNA as well as norepinephrine and epinephrine release to CPT are not significantly altered by age in women (Ng *et al.*, 1994; Pascualy *et al.*, 1999; Koch *et al.*, 2003). Interestingly, we observed that our subset of older women displayed blunted changes in MSNA during CPT, resulting from higher resting MSNA accompanied by a similar peak response to CPT. Thus, it is possible that the current findings were influenced by the nature of the sympathetic stimulus. However, given the list of unknowns concerning age and adrenergic stimulation—the efficacy of the efferent signal that stimulates norepinephrine release, neuronal reuptake and spillover, transmission of norepinephrine into the intimal-medial layer, adrenergic receptor density and distribution, and downstream signaling efficacy-- we cannot distinguish between findings directly applicable to age and those attributable to an age-stimulus interaction.

Finally, our Doppler ultrasound machine samples the peak, or center, blood velocity envelope rather than an intensity-weighted mean blood velocity, as described previously (Parker *et al.*, 2006). Estimating popliteal blood flow, conductance and shear rate with peak blood velocity could introduce error into our measurements should velocity parabolas be different between the two subject groups. To this end, Silber et al. have collected MRI-based femoral

artery data on 63 young and older healthy subjects (men and women) suggesting that velocity profiles are similar (HA Silber, personal communication, unpublished data). Thus we do not believe that sampling peak blood velocity introduces age-related bias into our measurements.

Conclusions

This study provides evidence that reactivity of the lower leg conduit vasculature as well as inhibition of sympathetic vasconstriction in the leg resistance vasculature are diminished with age in women. In particular, the latter finding suggests that control of leg vascular conductance during conditions in which release of dilator and constrictor substances are acutely augmented is altered with age such that metabolic inhibition of sympathetic neural outflow is less effective in older compared to younger women.

Acknowledgements

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Table **4-1**: Baseline Subject Characteristics. Data are expressed as group averages ± S.E.M. * significant ($p < 0.05$) difference between young and older subjects.

	Young (n=16)	Older (n=14)
Age	23 ± 1	69 ± 1*
BMI ($kg \cdot min^{-2}$)	23.6 ± 0.2	24.7 ± 0.6
Body Fat (%)	29.7 ± 1.5	32.7 ± 1.4
Calf Muscle Mass (g)	1625 ± 58	1532 ± 89
$\dot{V}O_{2peak}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	32.8 ± 2.9	20.3 ± 1.2*
Hematocrit (%)	41.0 ± 0.6	41.0 ± 0.6
Triglycerides ($mg \cdot dL^{-1}$)	83.5 ± 6.3	93.6 ± 13.2
Total Cholesterol ($mg \cdot dL^{-1}$)	164.2 ± 8.1	203.7 ± 5.7*
HDL Cholesterol ($mg \cdot dL^{-1}$)	56.4 ± 2.5	69.5 ± 15.6
LDL Cholesterol ($mg \cdot dL^{-1}$)	90.8 ± 6.9	115.6 ± 4.2*
Systolic Pressure (mmHg)	109 ± 3	129 ± 4*
Diastolic Pressure (mmHg)	65 ± 1	72 ± 2*
Popliteal Blood Flow ($mL \cdot min^{-1}$)	151.4 ± 13.6	149.4 ± 12.4
Popliteal Conductance ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	1.9 ± 0.2	1.6 ± 0.2

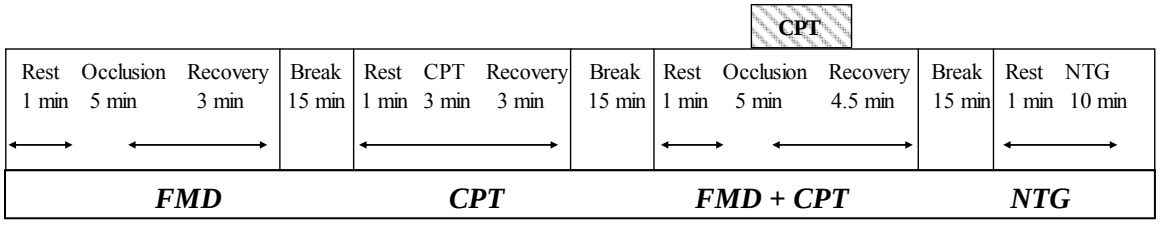

Figure **4-1**: Protocol schematic for Study Visit 3. FMD (Part 1): Doppler ultrasound measurements (represented by ◄——►) were taken during 1 minute rest, the last minute of occlusion, and 3 minutes following cuff release. CPT (Part 2): Doppler ultrasound measurements were taken during 1 minute rest, 3 minutes of 0-1º C ice water immersion, and 3 minutes of recovery. FMD + CPT (Part 3): Doppler ultrasound measurements were taken during 1 minute rest, the last minute of occlusion, and 4.5 minutes following cuff release. 3 minute CPT was applied during the last 1.5 minutes of occlusion and the first 1.5 minutes following cuff release. NTG (Part 4): Doppler ultrasound measurements were taken during 1 minute rest and 10 minutes following NTG administration.

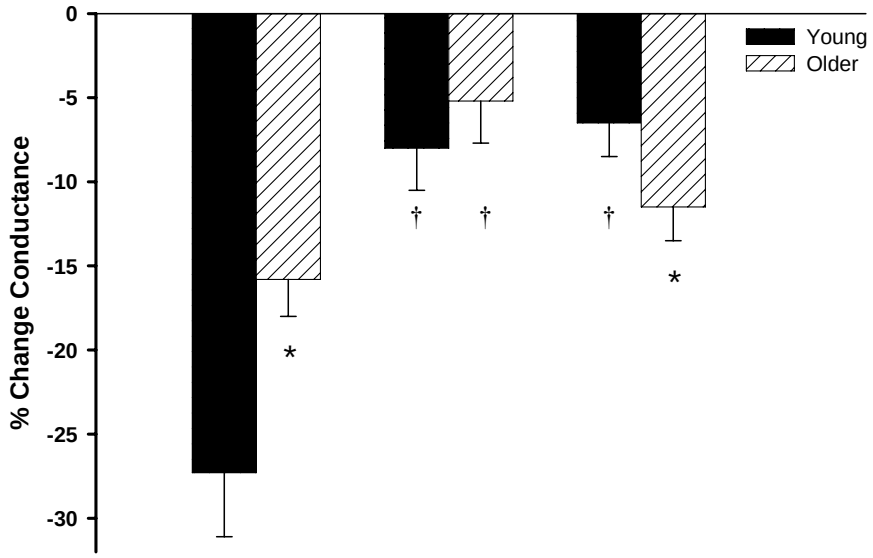

Figure **4-2**: *Left:* Resting percent reduction in conductance to CPT in young and older subjects. *Middle:* Percent reduction in peak popliteal (post-hyperemic) conductance when CPT was superimposed on FMD (all subjects). *Right:* Percent reduction in peak popliteal conductance following superimposition of CPT on FMD in 9 young and 9 older subjects matched for resting adrenergic sensitivity to CPT. Data are expressed as mean + S.E.M. † indicates significant difference relative to rest and * indicates significant difference between young and older subjects.

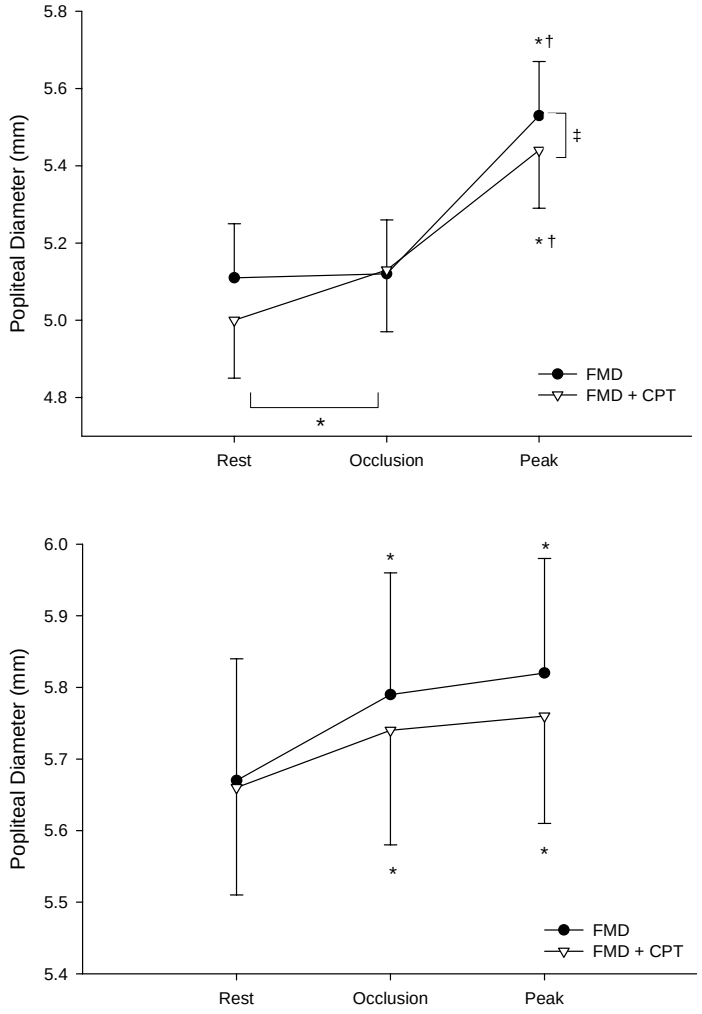

Figure **4-3**: Diameters at rest (mean + S.E.M.), during occlusion, and following cuff release (peak) in young (top) and older (bottom) subjects for FMD as well as FMD + CPT. * indicates significant ($p<0.05$) difference from resting diameters, † indicates significant difference from occlusion diameters, and ‡ indicates significant difference between conditions.

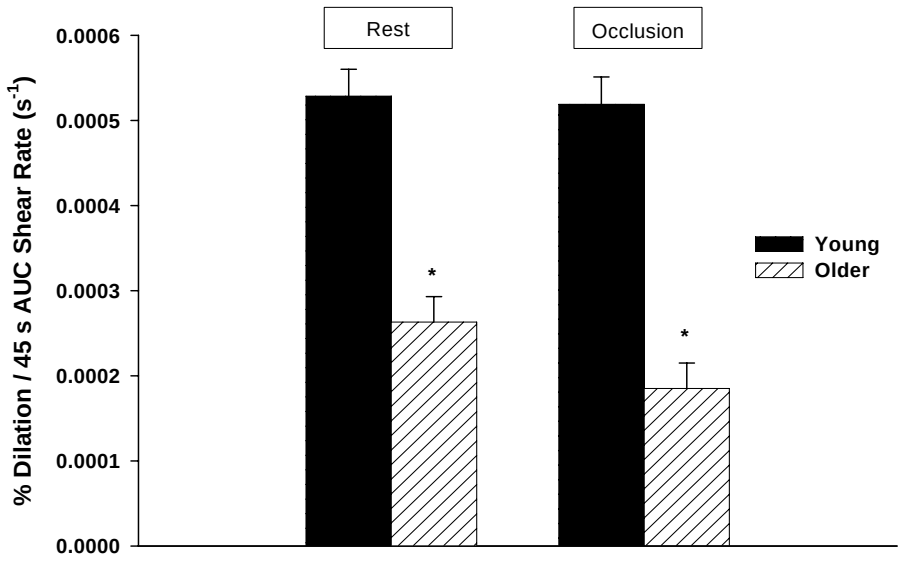

Figure **4-4**: Comparison of average normalized FMD responses (mean + S.E.M.) between young and older subjects. Dilation was calculated as the percentage increase above resting diameter (Rest) and diameter measured during the last minute of occlusion (Occlusion) divided by the 45 sec AUC shear rate. * indicates significant difference between young and older subjects ($p<0.01$). There was a significant age difference when diameters were calculated relative to rest or occlusion.

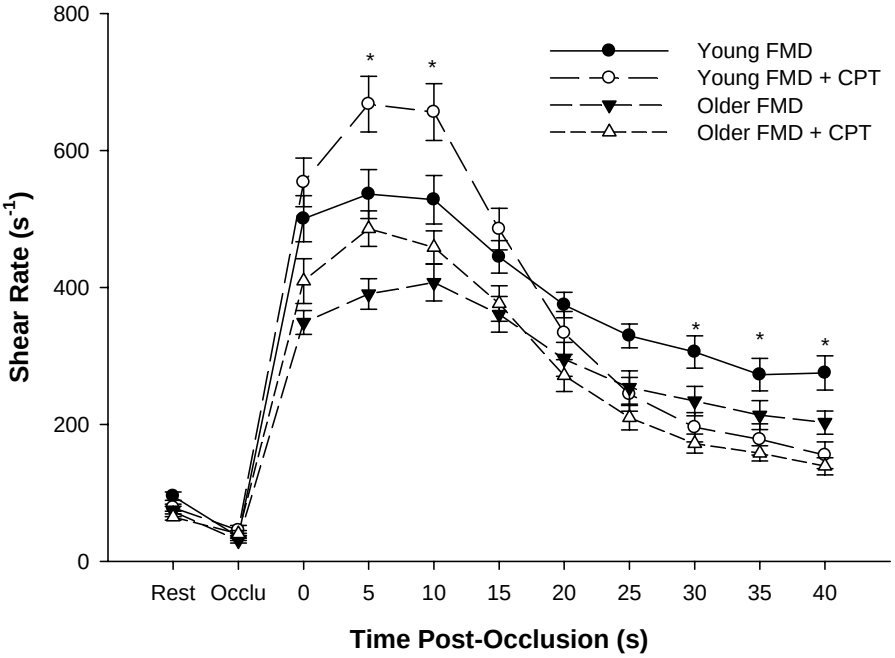

Figure **4-5**: Comparison of the 45 second AUC shear rate profile (data points expressed as mean + S.E.M.) immediately following 5 minutes calf occlusion (FMD) and a sympathetic stimulus superimposed on calf occlusion (CPT+FMD) in young and older subjects. * indicates significant difference ($p<0.05$) between conditions in young subjects. There were no condition differences observed in older subjects. Total AUC was not different between conditions in either group but was significantly higher in young vs older subjects in both FMD and FMD + CPT.

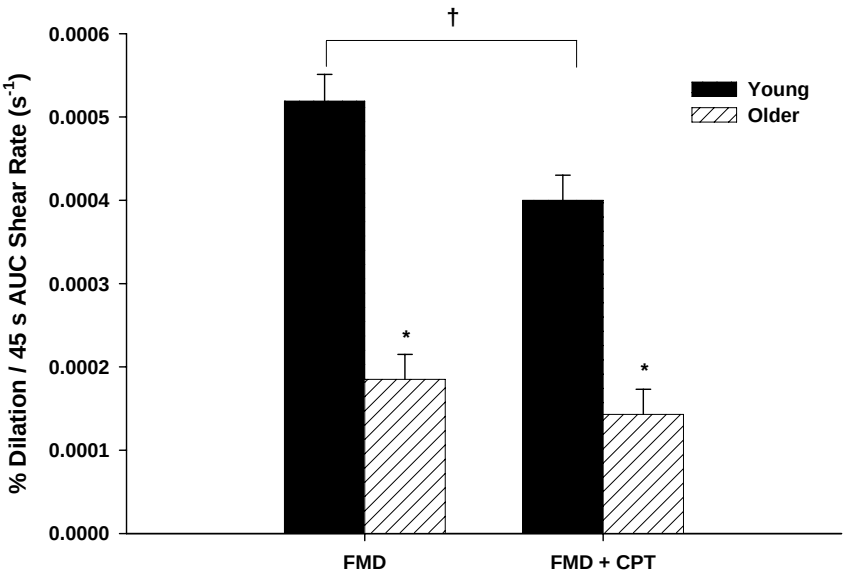

Figure **4-6**: Comparison of average normalized FMD responses (mean + S.E.M.) immediately following calf occlusion (FMD) and a sympathetic stimulus superimposed on calf occlusion (CPT + FMD) in young and older subjects. Dilation was calculated as the percentage increase above occlusion diameter divided by the 45 second AUC for shear rate immediately following cuff release. * indicates significant difference ($p<0.01$) between young and older subjects. † indicates significant difference ($p<0.01$) between conditions.

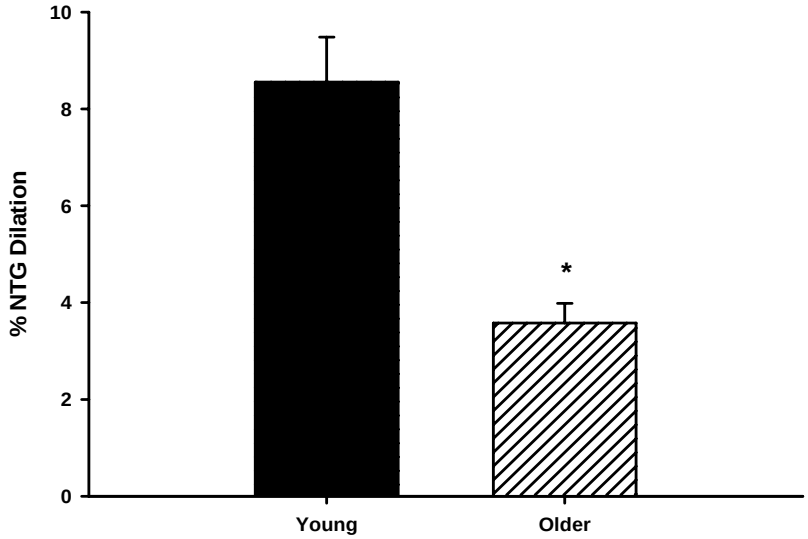

Figure **4-7**: Comparison of average popliteal responses (mean + S.E.M.) to nitroglycerin (NTG) in young and older subjects. Dilation was calculated as the percentage change from pre-NTG diameter to the maximum diameter measured during the 10 minutes following NTG administration. * indicates significant ($p < 0.01$) difference between young and older subjects.

Chapter 5

SEX DIFFERENCES IN LEG VASODILATION DURING GRADED KNEE EXTENSOR EXERCISE IN YOUNG ADULTS

Introduction

.There is substantial evidence to suggest that limb vasodilator responsiveness is sex-specific. For example, young women exhibit augmented brachial artery flow-mediated dilation (Sarabi *et al.*, 1999; Levenson *et al.*, 2001) and beta-adrenergic mediated forearm vasodilation (Kneale *et al.*, 2000) relative to young men. Moreover, the forearm vasodilatory response to acetylcholine (Dietz, 1999) as well as peak calf reactive hyperemia (Proctor *et al.*, 2005; Ridout *et al.*, 2005) tend to be higher in women. Collectively, these results suggest that women exhibit augmented dilatory responsiveness in the limbs.

Rogers and Sheriff (Rogers & Sheriff, 2004) recently reported that female rats exhibit greater hindlimb vascular conductance during incremental treadmill exercise than male rats, suggesting that limb vasodilator responses to dynamic exercise may also be sex-specific. The effect of sex on blood flow to active muscles and its regulation has not been systematically investigated in humans during dynamic exercise, although we recently observed evidence of greater leg blood flow responses in women compared to men during graded leg cycling (Koch *et al.*, 2005). One difficulty of using treadmill or conventional cycle ergometer exercise to examine sex differences in exercising leg blood flow, however, is that vasodilation of two or more limbs can exceed maximal cardiac output leading to sympathetic restraint of active muscle blood flow to preserve systemic blood pressure (Andersen & Saltin, 1985; Saltin *et al.*, 1998). Such effects could confound the comparison of limb vasodilatory responses to exercise in women vs. men due

to documented sex differences in baroreflex responsiveness (Christou *et al.*, 2005; Kimmerly *et al.*, 2007), alpha adrenergic responsiveness (Kneale *et al.*, 2000; Hogarth *et al.*, 2007) and the modulatory interaction between vasodilators and vasoconstrictors (McKee *et al.*, 2003).

Therefore, the purpose of this study was to investigate blood flow and vascular conductance responses in the femoral artery (the conduit inflow to the leg vasculature) during dynamic knee-extensor exercise, a mode of exercise involving a limited mass of active muscle (Andersen *et al.*, 1985; Andersen & Saltin, 1985), in healthy young men and women. Based on the work of Rogers and Sheriff (Rogers & Sheriff, 2004) as well as the precedent of greater limb vasodilator responsiveness in young women, we hypothesized that young women would exhibit augmented leg vascular conductance relative to young men during graded knee-kick exercise to maximal exertion.

Methods

Subject Characteristics and Initial Screening

Fifteen young men and 16 young women (ages 20-30) completed the study. All subjects were non-obese (BMI $\leq$ 30), nonsmokers, had clinically normal blood chemistry (i.e. hemoglobin concentrations ranged from 11.1-16.2 $g \cdot dL^{-1}$, total cholesterol $\leq$ 240 $mg \cdot dL^{-1}$, LDL cholesterol $\leq$ 150 $mg \cdot dL^{-1}$), and resting supine ankle-brachial index ratings (ABI between 0.90 and 1.30; VP2000, Colin Medical). All subjects were normotensive (resting blood pressure $\leq$ 140/90 mmHg) and were neither extremely sedentary nor extremely fit (i.e. had treadmill $\dot{V}O_{2max}$ values between 20 and 80% of age-predicted norms (ACSM, 2006)). Subjects were free of overt chronic diseases as evaluated by medical history questionnaire, a physical examination and resting ECG. Additionally, no subjects were taking medications having significant hemodynamic effects,

(including oral contraceptives) for at least the last 12 months. Young females were studied in days 1-7 of their menstrual cycle to standardize the influence of female hormones. On study day, subjects were asked to refrain from alcohol, exercise, caffeine, aspirin, ibuprofen, or herbal supplements for at least 12 hours prior to testing. All subjects gave their written, informed consent to participate. This study was approved by the Office for Research Protections and the Institutional Review Board at The Pennsylvania State University. Subject characteristics are presented in Table 5-1.

Subjects also completed a physical activity questionnaire to assess routine physical activity (Baecke Questionnaire of Habitual Physical Activity (Baecke *et al.*, 1982)). None of the subjects participated in moderate to high intensity aerobic exercise > 3 $d \cdot wk^{-1}$ or regular lower body resistance training > 2 $d \cdot wk^{-1}$during the past 12 months. To objectively quantify aerobic fitness status, all of the subjects performed a continuous incremental treadmill test (SensorMedics, Yorba Linda, CA) to maximal exertion to determine maximal oxygen uptake ($\dot{V}O_{2max}$).

Total and regional body composition was estimated using dual-energy X-ray absorptiometery (DXA; model QDR 4500W, Hologic, Waltham, MA) with subjects in the supine position as described previously (Proctor *et al.*, 2005). In addition, thigh volume was estimated by the anthropometric method described by Jones and Pearson from thigh patellar-pubic length, skinfold thicknesses, and circumference (Jones & Pearson, 1969). Quadriceps femoris muscle mass was then estimated from thigh volume as originally described by Andersen and Saltin (Andersen & Saltin, 1985).

Study Procedures

Exercise modality. Single leg knee extensor exercise, designed to isolate the quadriceps muscle group, was performed as described previously (Andersen & Saltin, 1985; Richardson *et al.*, 1993). Briefly, subjects were reclined in a seat in the supine position (to minimize cardiopulmonary baroreceptor-mediated decreases in MSNA during knee extensor exercise (Ray *et al.*, 1993) as well as sex-related differences in stroke volume responses to exercise (Fleg *et al.*, 1995)) with knees flexed at an angle of 90°. The subject's torso and both upper legs were fixed by straps attached to the chair to reduce extraneous movement and straining, and the left leg was strapped into a boot attached by lever arm to the pedal of a cycle ergometer. The right leg was allowed to hang free although subjects were instructed not to swing or move this leg. One extension of the quadriceps muscle moved the subject's lower leg 90°-170° and the ensuing flexion was a passive return pulled by the flywheel of the ergometer. Subjects kicked at a constant cadence of 40 kicks/minute (0.67 Hz), as our initial trials demonstrated that this cadence (rather than 60 kicks/minute) was easier for subjects to maintain proficiently with minimum motion artifact and consistent duty cycles, without straining the upper body or recruiting the active hamstring. Resistance was increased by increasing the weight attached to a belt surrounding the flywheel such that friction on the flywheel increased proportionately. Subjects participated in two familiarization visits totaling approximately one hour of kicking such that they could learn to avoid accessory muscle recruitment (i.e., of the hamstring, inactive leg, and upper body) and maintain cadence. The first familiarization visit consisted of just knee extension exercise while the second visit involved instrumentation and data acquisition identical to that described below for the actual study visit.

Exercise protocol. On the study day, the subjects began the protocol with 3 minutes of quiet rest, followed by 3 minutes of unloaded passive exercise (a research technician moved the

subject's leg at 40 kicks/min). The purpose of the passive bout was to investigate the increase in flow due to mechanical influences and tachycardia separate from metabolic stimuli (Wray *et al.*, 2005a). The subject was then instructed to begin kicking against no resistance (0W) for three minutes, after which resistance increased incrementally every three minutes until the subject could no longer maintain cadence. The workload increases were 8 W in men and 4.8 W in women. These increases were designed to produce similar time to exhaustion in both sexes, taking into account the reduced quadriceps muscle mass of women. Responses of men and women were compared at the same absolute workloads. Additionally, to take into account the different peak power outputs between men and women, each subject's maximal workload was then used to estimate the relative intensity of each workload at which measurements were collected.

Data Acquisition and Measurements

All variables were collected on-line at a sampling frequency of 400 Hz and stored using a Powerlab system (AD Instruments, Castle Hill, Australia). Heart rate and beat-to-beat systolic and diastolic blood pressure (radial tonometry of the right hand; Colin, Medical Instruments Corporation) were measured continuously throughout the study. In addition, manual auscultation was used every three minutes throughout the study to check the accuracy of the Colin during exercise. Electromyogram (EMG) signals of the active (left) biceps femoris and inactive (right) rectus femoris (to ensure that contraction was limited to the active quadriceps of the active leg, respectively) were collected with bipolar silver chloride surface electrodes (Bio-Tac; Tyco Healthcare Group LP; Mansfield, MA) fixed lengthwise over the middle of the muscle belly placed 10-20 cm apart. Reference electrodes were placed on the knee of each leg. Electrode signals were amplified (Gould Universal Amplifier Model 13 4615 55, Cleveland, OH and

Powerlab bioamplifier) with a bandwidth frequency ranging from 1.5 Hz to 2 kHz and simultaneously digitized using Powerlab. Knee kick cadence was captured using a Cateye Astrale 8 (Cateye, Boulder, CO) cycle computer attached to the flywheel. Kneekick force tracings were obtained using a load cell attached to the boot arm of the knee kick ergometer.

A Doppler ultrasound machine (HDI 5000, Philips, Bothell, Washington) equipped with a high resolution 7-4 MHz linear-array transducer was used to measure mean blood velocity and vessel diameter of the left common femoral artery, distal to the inguinal ligament but above the bifurcation into the superficial and profunda femoral branch. For velocity measurements, the artery was insonated at a constant angle of 60° with the sample volume adjusted to cover the width of the artery, while diameter measurements were obtained with the artery insonated perpendicularly. Velocity measurements were taken continuously during minutes 1 and 3 of rest, passive exercise, and each workload, while high-resolution diameter measurements (taken in 2D mode to optimize imaging) were taken during minute 2 of every workload (except the peak workload, during which diameter measurements were not taken). A custom interface unit processed the angle-corrected, intensity-weighted Doppler audio information from the ATL system into a flow velocity signal that was sampled in real time by Powerlab. Postprocessing using PowerLab'sChart application package yielded mean blood velocities.

Diameter measurements were stored on VHS tape and digitized at 4 frames/second using Brachial Imager software (Medical Imaging Applications; Iowa City, IA). Post-test analysis of diameters was performed using edge-detection software (Brachial Analyzer Software, Medical Imaging Applications; Iowa City, IA); briefly, the technician (always the same and blind to any subject information) selected a region of interest along the arterial wall and the edge of the wall was detected by pixel density and represented by a line of best fit. Each sequence of images was reviewed by the technician and adjusted to ensure that diameter measurements were always calculated from the intima-lumen interface at both the distal and proximal vessel wall. Images

affected by motion artifact were excluded based on visual inspection by the technician as well as a calculation by the software program that the confidence limit for the best-fit line was less than 80%. Diameter measurements for each workload comprised an average of approximately 80 frames.

Data Analysis and Computations

For all study variables, values were calculated as the average over the last minute of rest, passive exercise, and each workload (to allow hemodynamic parameters to reach steady-state conditions (Hughson *et al.*, 1997; MacDonald *et al.*, 1998)), with the exception of peak measurements, which were calculated with first and/or second minute data if the subject did not complete the peak workload. Mean arterial pressure (MAP, in mmHg) was calculated as (1/3 systolic pressure) + (2/3 diastolic pressure). The EMG signals were full-wave rectified, squared and median filtered, from which the root-mean square (RMS) value was derived. A spike-triggered average of EMG over the last minute was then derived from the cadence and calculated as a percent of the RMS value obtained from maximal isometric contraction (EMG averaged from three, five-second isometric contractions performed at the beginning of the study). Femoral artery blood flow (FBF) for each condition or workload was calculated by multiplying the cross-sectional area of the femoral artery (the diameter taken in minute 2 of each condition or workload) with mean blood velocity (V_{mean}; in $cm \cdot s^{-1}$), according to the formula:

$$\text{FBF: Blood velocity} \cdot \pi \cdot (\text{femoral diameter}/20)^2 \cdot 60$$

where the FBF is in $ml \cdot min^{-1}$, the blood velocity is in $cm \cdot s^{-1}$, the femoral diameter (averaged across the cardiac cycle) is in mm, and 60 is used to convert from $ml \cdot s^{-1}$ to $ml \cdot min^{-1}$. To validate the assumption that the diameter taken in minute 2 was representative of the diameter underlying the blood flow measured in minute 3 of each condition or workload, 4 subjects performed knee

extensor exercise at various 3 minute workloads with high resolution diameter imaged constantly in 2D mode. The within-subject correlation between minute 2 and minute 3 diameters was 0.99. In addition, since peak diameter measurements were not obtained, peak FBF measurements were calculated with the diameter from the previous workload. Femoral vascular conductance (FVC) was calculated as FBF/MAP and, in the case of normalized FVC, divided by each subject's anthropometric estimation of quadriceps muscle mass. Leg oxygen delivery ($L \cdot min^{-1}$) was estimated by multiplying the estimated arterial oxygen content ($1.34 \cdot Hb \cdot SaO_2$ (assuming SaO_2 of 97%) mL $O_2 \cdot dL^{-1}$ blood) by femoral blood flow. Finally, the force tracing for each duty cycle was used to analyze the time spent in quadriceps contraction (extension) and relaxation (return) as well as total FBF based on quadriceps contraction and relaxation. FBF was also separated into positive (anterograde) and negative (retrograde) flows per workload.

Statistical Analysis

Statistical analyses were performed using SAS (SAS 9.1, Cary, North Carolina) software. All data are reported as mean $\pm$ S.E.M. with significance set at $p<0.05$. A Student's t-test for independent groups and Tukey post-hoc analysis were used to compare baseline differences between male and female groups. An autoregressive, random-coefficients model (PROC MIXED) using a continuous predictor (either absolute workload or workload as a percent of maximal workload attained), fitting a random intercept and slope with workload as the within-individual factor and sex as the between-individual factor, was used to determine differences between subject groups in outcome variables (FVC, FBF, MAP, HR, EMG). This repeated-measures model fits the linear or curvilinear trend in responses based on the measured outcome variables at the absolute workload or calculated percent of maximal workload in all subjects, then estimates and compares the outcome variable at designated common values between subjects and

groups (Littell *et al.*, 2006). A Bonferroni post-hoc adjustment was performed when significant sex*workload differences were detected.

Results

Subject Characteristics. Subject characteristics are shown in Table 5-1. In addition to data presented in Table 5-1, there were no significant between-group differences in resting blood lipid parameters such as total cholesterol (Men: 159 ± 6 $mg \cdot dL^{-1}$; Women: 155 ± 7 $mg \cdot dL^{-1}$), LDL cholesterol (Men: 93 ± 7 $mg \cdot dL^{-1}$; Women: 82 ± 5 $mg \cdot dL^{-1}$), and triglycerides (Men: 90 ± 10 $mg \cdot dL^{-1}$; Women: 71 ± 6 $mg \cdot dL^{-1}$), although HDL cholesterol was significantly ($p = 0.03$) higher in women (Men: 49 ± 3 $mg \cdot dL^{-1}$; Women: 60 ± 4 $mg \cdot dL^{-1}$).

Peak power output. Men achieved a significantly higher peak knee extensor workload than women (Men: 38 ± 2 W; Women: 25 ± 2 W; $p < 0.01$), although there was no between-group difference in the number of three minute workloads taken to reach peak (Men: 5.8 ± 0.2 workloads; Women: 6.2 ± 0.3 workloads) such that the exercise protocols were of similar duration. Ensuing data are represented at either absolute workloads or as each subject's workloads normalized to his or her peak workload (% of maximal workload) to account for the different workload increases used in men vs. women.

Blood flow responses to incremental exercise (Figure 5-1). A) Femoral blood flow, B) estimated leg oxygen delivery, C) femoral blood flow normalized to estimated quadriceps muscle mass, and D) femoral blood flow partitioned by duty cycle into contraction and relaxation flows at rest, passive exercise, and incremental knee extensor exercise to exhaustion are shown in Figure 5-1. Femoral blood flow was significantly ($p < 0.05$) higher in women at workloads greater than or equal to 15W. In addition, the slope of the response was greater in women (47.1 ± 3.7 vs 29.1 ± 1.8 $mL \cdot min^{-1} \cdot W^{-1}$; $p < 0.01$). While there was no sex difference in estimated oxygen delivery at

workloads greater than 5W, the slope of the response was greater in women (0.0073 ± 0.0006 vs. 0.0056 ± 0.0003 $L \cdot min^{-1} \cdot W^{-1}$; $p = 0.02$). Normalizing femoral blood flow to estimated quadriceps muscle increased the sex difference such that women exhibited significantly ($p < 0.05$) greater normalized blood flow at workloads equal to or greater than 5W. Relaxation flows were greater ($p < 0.05$) in women than men at all workloads greater than or equal to 10 W, and contraction flows were greater ($p < 0.05$) in women than men at all workloads greater than or equal to 20W.

Femoral vascular conductance response to incremental exercise (Figure 5-2). Femoral vascular conductance at rest, passive exercise, and incremental knee extensor exercise to exhaustion are shown in Figure 5-2. Femoral vascular conductance was significantly ($p < 0.05$) higher in women at workloads greater than 5W. In addition, the slope of the response was greater in women (0.50 ± 0.06 vs 0.23 ± 0.03 $mL \cdot min^{-1} \cdot mmHg^{-1} \cdot W^{-1}$; $p < 0.01$), as was the conductance measured at each individual's maximal workload (Men: 18.0 ± 0.6 $mL \cdot min^{-1} \cdot mmHg^{-1}$; Women: 22.6 ± 1.4 $mL \cdot min^{-1} \cdot mmHg^{-1}$; $p < 0.01$).

Hemodynamic responses to incremental exercise expressed relative to maximal workload (Figure 5-3). A) Blood pressure (MAP), B) femoral vascular conductance, and C) femoral vascular conductance normalized to estimated quadriceps muscle mass responses at rest, passive exercise, and incremental knee extensor exercise to exhaustion are shown in Figure 5-3. MAP was significantly ($p < 0.01$) lower (10-15 mmHg) in women at every workload, and femoral vascular conductance was significantly ($p < 0.05$) higher at workloads greater than 40% of maximal workload in women. In addition, the slope of the conductance response was greater in women than men (0.12 ± 0.01 vs. 0.08 ± 0.01 $mL \cdot min^{-1} \cdot mmHg^{-1} \cdot \%\ maximal\ workload^{-1}$; $p = 0.02$). Normalizing to estimated quadriceps muscle exaggerated the sex difference such that with the exception of passive exercise, women had significantly ($p < 0.05$) higher normalized FVC at rest and every ensuing workload, and the slope of the normalized FVC response was again greater in

women (0.05 ± 0.01 vs. 0.03 ± 0.002 $mL \cdot min^{-1} \cdot mmHg^{-1} \cdot kg\ muscle^{-1} \cdot \%\ maximal\ workload^{-1}$ greater in women than men; $p = 0.01$).

Change in femoral diameter during passive and graded exercise (Figure 5-4). Femoral diameter changes (relative to rest) during incremental exercise are shown in Figure 5-4. Diameter measurements were not attained at each individual's peak workload. The change in diameter was greater in women at workloads equal to and greater than 0% of maximal workload.

Ipsilateral hamstring muscle and contralateral quadriceps muscle recruitment (Figure 5-5A and B). There were no sex differences in hamstring EMG, expressed as a percent of each subject's maximal isometric contraction, at any workload. In addition, inactive quadriceps EMG was significantly higher ($p < 0.05$) in women at workloads greater than 40% of maximal recruitment, although the magnitude of this difference was small (Quadriceps EMG at rest and at maximal workload in women vs. men: 2.7 ± 0.5 to 6.6 ± 1.0% vs 2.2 ± 0.5 to 4.2 ± 0.6%).

Discussion

The purpose of the present investigation was to test the hypothesis that vascular responses during dynamic exercise are sex-specific in healthy young humans. We utilized single-leg knee-extension exercise, thereby minimizing possible confounding effects of sex differences in cardiac output reserve and counterregulatory reflexes, to measure leg hemodynamics during incremental exercise to maximal exertion. Particular care was also taken to ensure that all subjects maintained the 40 contraction per minute kick rate, and effective isolation of the quadriceps (up to 80% of maximum workload) was confirmed by ipsilateral hamstring EMG activity. Accordingly, the major new findings of the current study were 1) the hyperemic response to graded small muscle leg exercise was greater in young women compared to men, 2) exercise-induced femoral artery dilation was greater in women than men, and 3) young women

exhibited lower blood pressure and augmented femoral vascular conductance during graded single-knee extensor exercise. These results suggest that the mechanisms by which leg vasodilation occurs during incremental exercise may be sex-dependent in healthy young humans.

Hyperemic Responses to Dynamic Leg Exercise in Young Men and Women

When compared as a function of absolute work intensity, the absolute leg blood flow responses during moderate and higher intensity knee kicking, as well as the overall slope of the blood flow response to this mode of exercise, were greater in women vs. men (Figure 5-1A). These results are in general agreement with our previous studies showing a steeper rise in cycle ergometer leg blood flow (via femoral thermodilution) per unit increase in leg $\dot{V}O_2$ in women compared to men. These findings could be explained by lower hemoglobin concentrations in women vs. men, as leg blood flow responses to exercise can be augmented when arterial O_2 content is reduced (Koskolou *et al.*, 1997; Roach *et al.*, 1999). In support of this possibility, estimated leg oxygen delivery (calculated with the assumptions that venous and arterial hemoglobin were similar, oxygen saturation was consistently 97%, and arterial oxygen content thus did not change during incremental exercise) was closely matched in men vs. women at most workloads (i.e. greater than 5W, Figure 5-1B). However, given that 1) the overall slope of the estimated leg oxygen delivery response was greater in women, 2) there was no relationship between venous hemoglobin and the change in femoral blood flow from 0 to 24W (data not shown) in men and women, and 3) the flow response (slope of blood flow vs. absolute workload) was still greater ($p < 0.01$) in four men and four women matched for hemoglobin (13.8-14.2 gm·dL^{-1}), we have concluded that hemoglobin is not the only factor contributing to the sex differences in exercising leg hyperemia. This conclusion is consistent with a previous study showing that moderate reductions in hemoglobin (from 14.7 to 13.1 gm·dL^{-1}) do not significantly

alter leg blood flow during single-knee extensor exercise (Gonzalez-Alonso *et al.*, 2006). Other potential mechanisms contributing to the sex difference in exercising leg hyperemia include an enhanced oxidative capacity representing greater oxygen demand in the leg muscles of women (Simoneau *et al.*, 1985; Hargreaves *et al.*, 1998; Russ & Kent-Braun, 2003), as well as greater mechanical compression of the muscle bed during contraction in men (Hicks *et al.*, 2001). This latter possibility is addressed below.

Augmented Vasodilator Responses to Dynamic Leg Exercise in Young Women

Comparisons of femoral vascular conductance were evaluated at both absolute workloads (Figure 5-2) as well as relative workloads (Figure 5-3B), given that arterial blood pressure responses to dynamic exercise are determined by relative exercise intensity (Hagberg *et al.*, 1988; Leuenberger *et al.*, 1993). The results were similar: given that women achieved similar or greater (dependent on workload) leg blood flow with lower MAP during graded knee extensor exercise, femoral vascular conductance was augmented in women at absolute workloads greater than 5W and relative workloads greater than 40% of maximal workload. This finding was in line with the previous observation in female rats of heightened hindlimb vascular conductance and lower MAP during treadmill exercise (Rogers & Sheriff, 2004). Leg vasodilator responsiveness (i.e. the slope of the conductance response) was also greater in women vs. men in the current study; normalizing to estimated quadriceps muscle enhanced these sex differences (Figure 5-3C).

In addition, while it has been reported that femoral artery diameter does not change during knee extensor exercise (Rådegran, 1997; Lutjemeier *et al.*, 2005), an unexpected finding was the statistically significant conduit dilation with increasing workload in both men and women, with the magnitude of the increase being almost five times as great in women (~ 0.5 mm over the course of the exercise trial in women vs. the ~0.1 mm in men). This finding underscores

the importance of measuring arterial diameter at every workload (Walther *et al.*, 2006) as the observation that conduit diameters remain uniform during exercise may be unique to the ergonomics of various knee extensor devices, femoral measurement site, and/or given populations. With respect to women in the current study, the augmented femoral dilation may be a mechanism through which the higher shear forces generated during exercise (i.e. greater blood velocity and smaller femoral diameter) are dissipated. We estimated the contribution of this diameter increase to femoral artery blood flow by comparing the observed blood flows to calculations of projected blood flow in the absence of any increase in femoral diameter; by this method, the observed dilation could have contributed up to 14% of the increase in blood flow achieved at the highest workloads in women but a rather insignificant 2% of the increase in blood flow in men under the same conditions. It should be noted that the extent to which conduit diameter changes influence downstream vascular control and blood supply to the working muscle may be minimal if the resistance vasculature is maximally dilated (Jasperse & Laughlin, 1997; Rådegran & Saltin, 2000); however, should the cross-sectional area of the quadriceps resistance vasculature be a limiting factor to vasodilation in women, then the conduit dilation may become more significant as exercise intensity increases.

Possible Determinants of Augmented Leg Vasodilator Responses in Young Women

Mechanical and Metabolic Influences

Certainly, given the numerous anatomic, metabolic and strength differences between men and women (Kent-Braun *et al.*, 2002; Pincivero *et al.*, 2003; Clark *et al.*, 2005), there is the possibility that between-sex differences in the mechanics and/or metabolic demands of knee kick exercise underlie the observed vasodilatory differences. For example, it is possible that the

reduced quadriceps muscle mass in women necessitated an increased hamstring recruitment during return to maintain cadence, resulting in a greater metabolic demand throughout the thigh. However, there were no differences in hamstring recruitment expressed relative to maximal isometric contraction (Figure 5-5A) which would support this theory. Moreover, greater inactive quadriceps recruitment by men could have evoked augmented counterregulatory and/or pressor responses; however, inactive quadriceps recruitment was only slightly (~2% of maximal isometric contraction) greater in women at higher workloads (Figure 5-5B). We also investigated the possibility that men, generating more absolute knee extensor power during each contraction, exhibited a greater retrograde blood flow due to the increasing impedance of the muscle pump at higher workloads (Hicks *et al.*, 2001; Lutjemeier *et al.*, 2005). There were no statistical differences between men and women in either the retrograde flows at any workload or the slope of the retrograde flow response (data not shown). In addition, flows partitioned by duty cycle closely mimicked the overall sex differences in flow responses (Figure 5-1D), and the length of the duty cycle was also similar ($p=0.85$) in men and women (0.83 ± 0.01 and 0.83 ± 0.02 seconds, respectively) providing no overt evidence of a contraction-dependent impedance to flow that was specific to this group of men.

Influence of Fatigue

During isometric knee extension to exhaustion, women exhibit less muscle fatigue since they generate less absolute force than men during contraction (Clark *et al.*, 2005). Under these conditions, fatigue appears coupled to blood flow as sex differences in muscle fatigue are eliminated under ischemia (Kent-Braun *et al.*, 2002; Russ & Kent-Braun, 2003). However, there is no evidence that greater fatigue is a cause of reduced blood flow during dynamic exercise (Hunter & Enoka, 2001; Kent-Braun *et al.*, 2002; Russ & Kent-Braun, 2003). This is supported

in the current study, where imposing greater fatigue on the women by doubling the length of the knee kick protocol (incremental workload increases were reduced to 2.4 W so that time to exhaustion was effectively doubled; data was collected on the second familiarization visit) did not alter observed sex-specific differences in vasodilatory responsiveness (Figure 5-6). Also, hamstring recruitment increased to a similar extent in men and women as maximal workload was approached, suggesting that the use of the hamstring to counteract quadriceps fatigue and maintain cadence at the highest workloads was occurring similarly between sexes.

Potential Estrogenic Factors

Through a series of inhibitory blockades and comparisons of ovariectomized and estrogen-replaced rats, Rogers and Sheriff determined that the nature of their sex-specific findings was attributable to estrogenic modulation of vascular responses mediated through nitric oxide- and prostaglandin-dependent pathways (Rogers & Sheriff, 2004). Besides its influence on endothelial-derived vasodilators, estrogen can also act as a direct smooth muscle vasorelaxant (Sudhir *et al.*, 1995), modulate reactive oxygen species generated through exercise (Brandes & Mugge, 1997; Barbacanne *et al.*, 1999; Bailey *et al.*, 2007), and mitigate blood pressure, myogenic contraction, and other smooth muscle cell contractile pathways (Ettinger *et al.*, 1998; Kahonen *et al.*, 1998; Crews & Khalil, 1999; David *et al.*, 2001), actions which may also serve to augment femoral vascular conductance. However, it must be noted that in the current study, we standardized study visits for women to coincide with days 1-7 of the menstrual cycle, when circulating estrogen is most likely to be lowest and similar to concentrations measured in men. Thus, any potential estrogenic mechanisms underlying the current observations in women would have to be exerted through the chronic, rather than acute, effects of estrogen on the exercising vasculature. While there are estrogen receptors located in both the vascular endothelium and

smooth muscle cells that may alter genetic transcription of many vasoactive metabolites, such as prostacyclin synthase, prostacyclin cyclooxygenase, endothelin-1, and endothelial nitric oxide synthase (Mendelsohn, 2002), additional research is necessary to determine whether chronic estrogen exposure alters dilator and constrictor pathways during exercise.

Experimental Considerations

It must be noted that our estimates of quadriceps muscle mass are based on previous work (Jones & Pearson, 1969; Andersen & Saltin, 1985) that has not been validated in large populations and specifically women. However, our estimates of quadriceps muscle mass in young men are similar to other published estimates in young men (Rådegran *et al.*, 1999; Lawrenson *et al.*, 2003; Krustrup *et al.*, 2004), there was a significant relation between DXA estimates of regional thigh volume and anthropometric estimates of quadriceps muscle mass in both men ($r^2 = 0.69$) and women ($r^2 = 0.84$) with no significant between-sex difference in these relations, and normalizing flow and conductance to DXA estimations of thigh muscle mass did not change the nature of our findings (data not shown). These comparisons lead us to believe that there was no sex-specific error associated with normalizing hemodynamic data to anthropometric estimates of quadriceps muscle mass.

Conclusions

The present study provides novel evidence for sex-specific leg blood flow and leg vascular conductance responses during single-knee extensor exercise in healthy young men and women, consistent with previous findings of augmented limb vasodilatory responses in women to other physiological and pharmacological stimuli.

Acknowledgements

We thank the GCRC clinicians and staff as well as Samuel Ridout and Dennis Koch for their assistance with data collection, and Dr. Chet Ray (Hershey Medical Center) for the use of the Colin blood pressure system. This research was supported by R01 AG18246 (D.N. Proctor), NIA Interdisciplinary Training in Gerontology Grant T32 AG00048 (B.A. Parker) and M01 RR10732 (GCRC).

Table **5-1**: Subject Characteristics. Data are expressed as group averages ± S.E.M. * significant ($p < 0.05$) difference between men and women. BMI = body mass index, FBF = femoral blood flow, FVC = femoral vascular conductance.

	Men (n=15)	Women (n=16)
Age (yrs)	24 ± 1	22 ± 1*
Systolic Pressure (mmHg)	128 ± 3	110 ± 2*
Diastolic Pressure (mmHg)	62 ± 2	55 ± 2*
Height (cm)	177 ± 2	165 ± 2*
Weight (kg)	77 ± 2	63 ± 3*
BMI ($kg \cdot m^{-2}$)	25 ± 1	23 ± 1
$\dot{V}O_{2max}$ (percentile)	54 ± 6	43 ± 5
Baecke Questionnaire Score	7.4 ± 0.3	7.4 ± 0.3
Quadriceps Muscle Mass (kg)	2.5 ± 0.1	2.1 ± 0.1*
Common Femoral Diameter (mm)	8.4 ± 0.2	7.2 ± 0.2*
Resting FBF ($mL \cdot min^{-1}$)	346 ± 35	303 ± 29
Resting FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	4.1 ± 0.3	4.2 ± 0.4
Hemoglobin ($gm \cdot dL^{-1}$)	15.0 ± 0.2	12.8 ± 0.2*

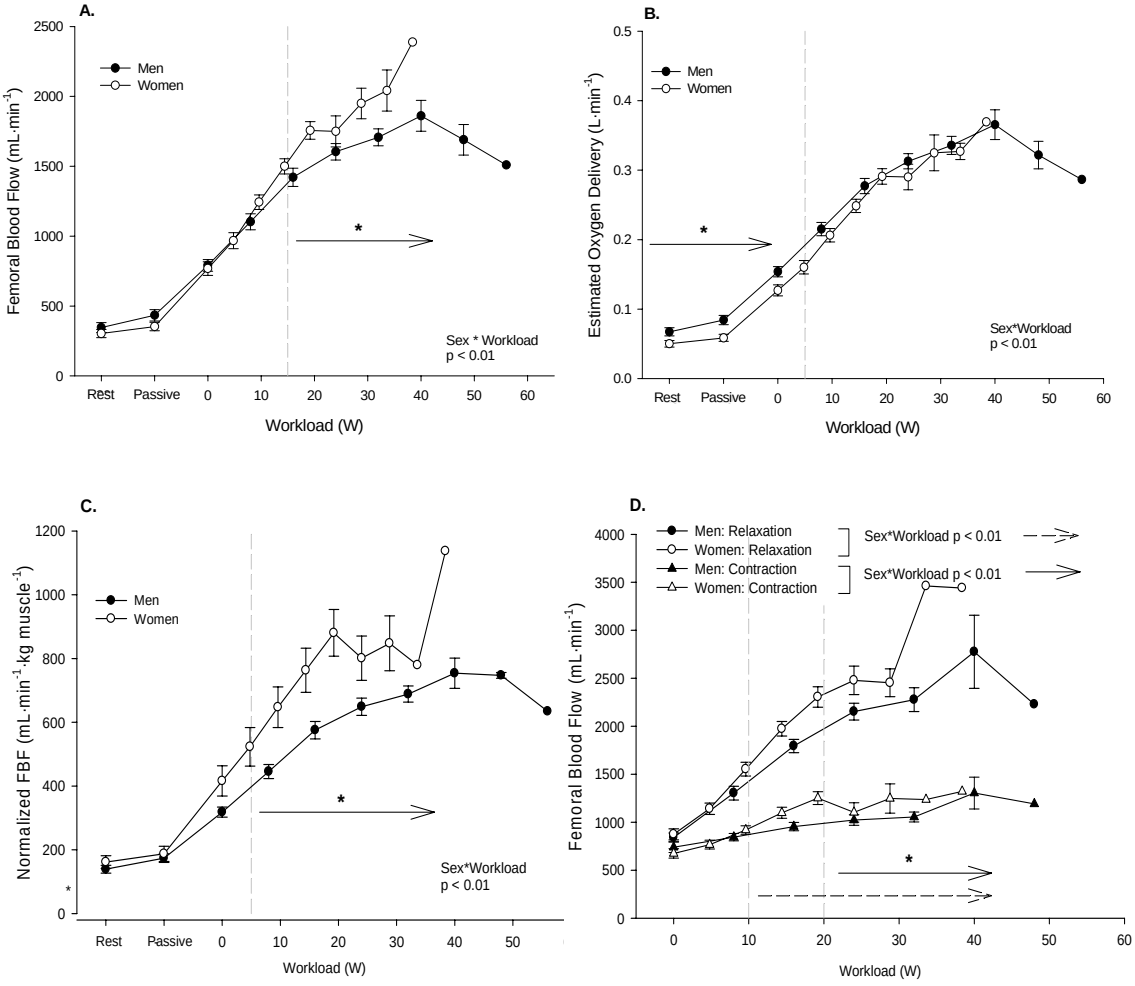

Figure **5-1**: A) Femoral blood flow (FBF), B) estimated oxygen delivery, C) Femoral blood flow normalized to estimated quadriceps muscle mass, and D) Contraction (quadriceps extension) and relaxation (passive quadriceps flexion) femoral blood flows expressed as group means ± S.E.M. at absolute workloads. * indicates significant ($p < 0.05$) difference between men and women at all workloads indicated by the arrow starting at (Graphs A, C and D) or ending with (Graph B) the dashed line. For men, sample size was n=15 until 24W, n=14 at 32W, n=10 at 40W, n=2 at 48W, and n=1 at 56W. For women, sample size was n=16 until 19.2W, n=11 at 24W, n=6 at 28.8W, n=2 at 33.6W, and n=1 at 38.4W.

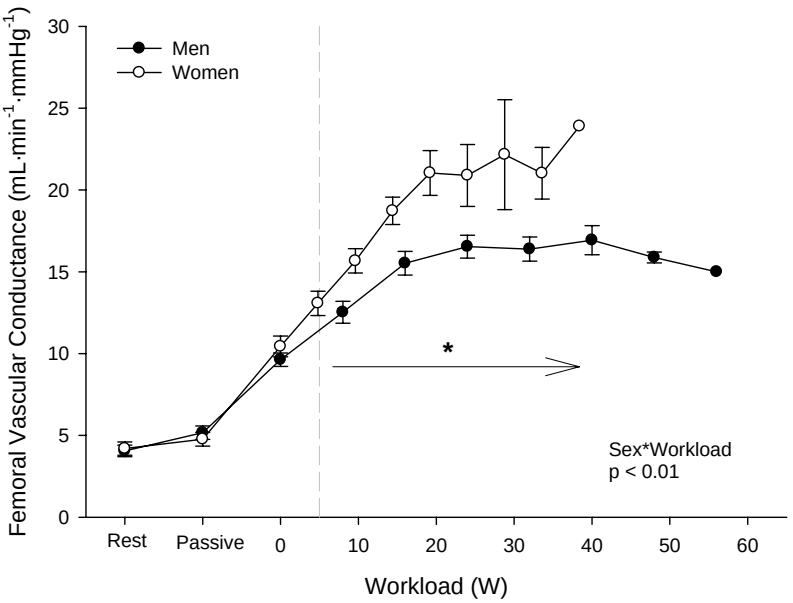

Figure **5-2**: Femoral vascular conductance (FVC) expressed as group means ± S.E.M. at absolute workloads. ***** indicates significant ($p < 0.05$) difference between men and women at all workloads indicated by the arrow starting at the dashed line. For men, sample size was n=15 until 24W, n=14 at 32W, n=10 at 40W, n=2 at 48W, and n=1 at 56W. For women, sample size was n=16 until 19.2W, n=11 at 24W, n=6 at 28.8W, n=2 at 33.6W, and n=1 at 38.4W.

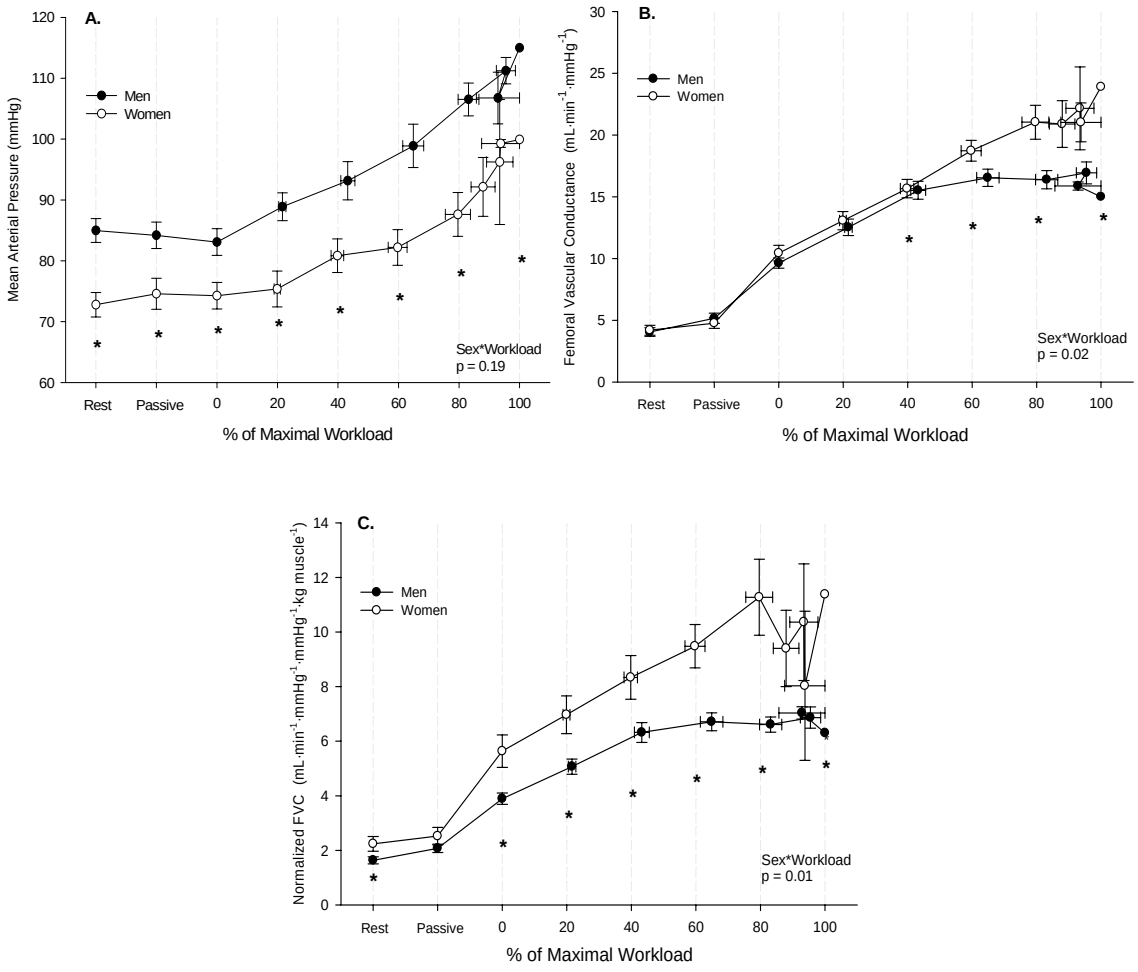

Figure **5-3**: A) Blood pressure (MAP), B) Femoral blood flow (FBF), and C) Femoral vascular conductance (FVC) normalized to estimated muscle mass during graded knee extensor exercise, expressed as group means ± S.E.M. at percent of maximal workload (% MW). * indicates significant ($p < 0.05$) difference between men and women at 0, 20, 40, 60, 80 and 100% of predicted responses. A significant sex*workload interaction indicates a between-sex slope difference. For men, sample size was n=15 until 83% MW, after which n=10 at 95% MW, n=2 at 93% MW, and n=1 at 100% MW. For women, sample size was n=16 until 80% MW, after which n=11 at 88% MW, n=6 at 93% MW, n=2 at 94% MW and n=1 at 100 % MW. Please note that these dropouts are reflected in the graph only; statistical comparisons were achieved by fitting curves to each individual's line and estimating responses at 0-100% maximal workload such that the entire sample size was utilized.

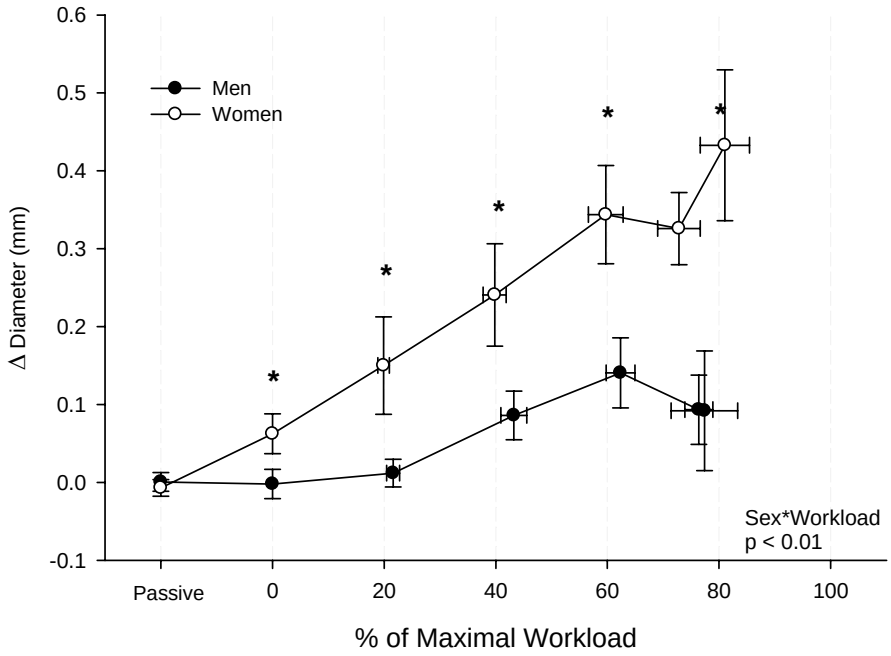

Figure **5-4**: Change in diameter relative to rest expressed as group means ± S.E.M. at percent of maximal workload (% MW). * indicates significant ($p < 0.05$) difference between men and women at 0, 20, 40, 60, 80 and 100% of predicted responses. A significant sex*workload interaction indicates a between-sex slope difference. Diameter measurements were not taken at each individual's peak workload. For men, sample size was n=15 until 62% MW, after which n=10 at 76% MW, and n=2 at 77% MW. For women, sample size was n=16 until 60% MW, after which n=11 at 72% MW; n=6 at 78% MW, and n=2 at 80% MW. Regarding sample size, please see the note in the legend of Figure 3.

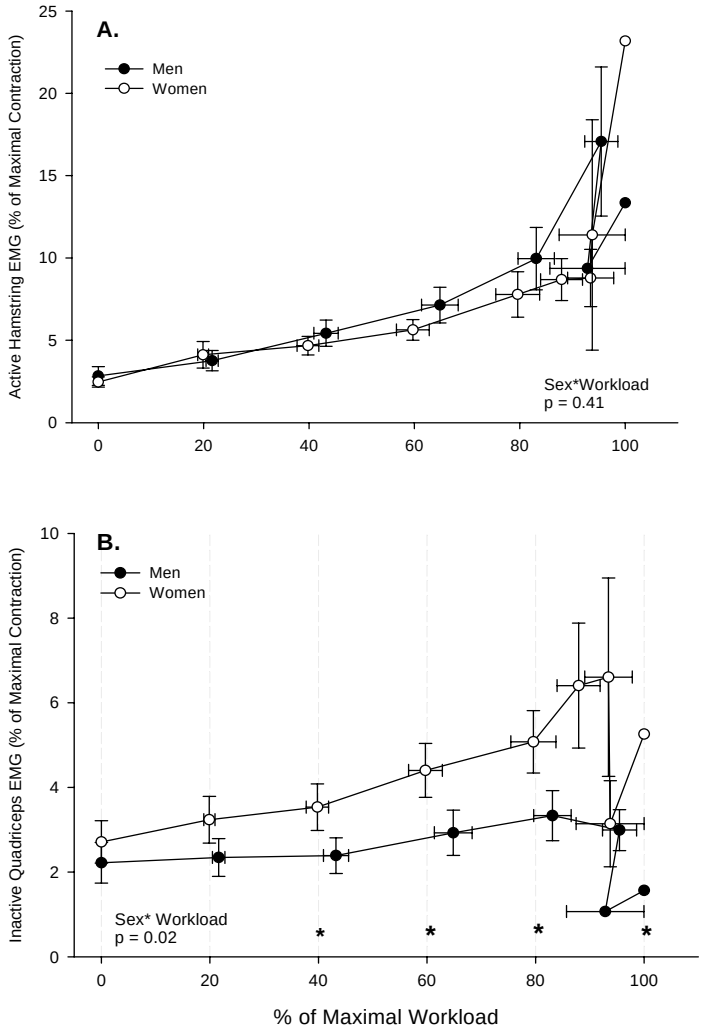

Figure **5-5**: A) Ipsilateral hamstring recruitment and B) contralateral quadriceps recruitment, as represented by electromyographical (EMG) activity normalized to each individual's maximal isometric contraction, during graded knee extensor exercise, expressed as group means ± S.E.M. at percent of maximal workloads (%MW). * indicates significant ($p < 0.05$) difference between men and women at 0, 20, 40, 60, 80 and 100% of predicted responses. A significant sex*workload interaction indicates a between-sex slope difference. For men, sample size was n=15 until 83% MW, after which n=10 at 95% MW, n=2 at 93% MW, and n=1 at 100% MW. For women, sample size was n=16 until 80% MW, after which n=11 at 88% MW, n=6 at 93% MW, n=2 at 94% MW and n=1 at 100 % MW. Regarding sample size, please see the note in the legend of Figure 3.

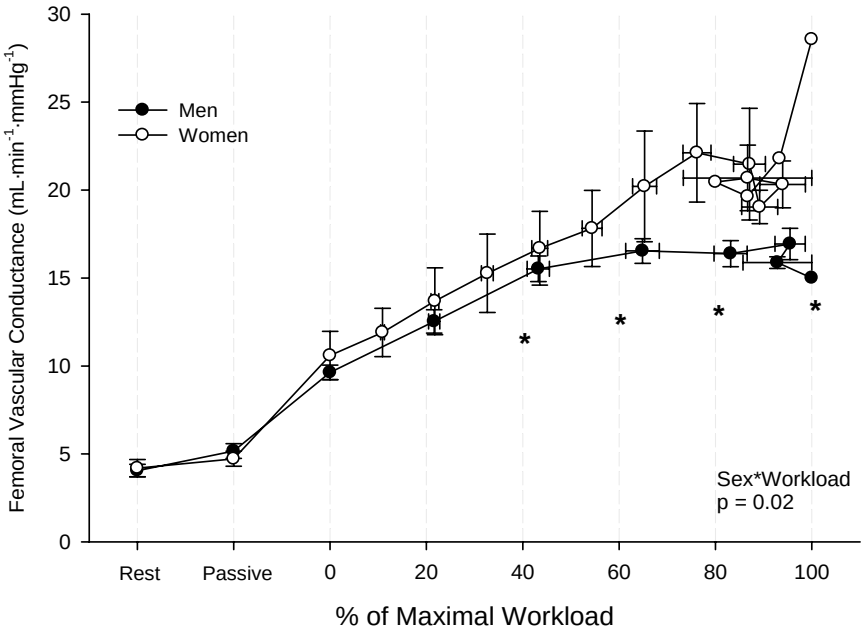

Figure **5-6**: Femoral vascular conductance (FVC) measured during a longer protocol in women (graded knee extensor exercise with workload increases of 2.4 W rather than 4.8W to the same approximate peak workload) compared with FVC measured during the normal (8W increase) protocol in men to investigate the influence of fatigue on observed sex differences. Data are expressed as group means ± S.E.M. at percent of maximal workloads (%MW). * indicates significant ($p < 0.05$) difference between men and women at 0, 20, 40, 60, 80 and 100% of predicted responses. A significant sex*workload interaction indicates a between-sex slope difference. For men, sample size was n=15 until 83% MW, after which n=10 at 95% MW, n=2 at 93% MW, and n=1 at 100% MW. For women, sample size was n=16 until 87% MW, after which n=10 at 89% MW, n=7 at 94% MW, n=2 at 87% MW and n=1 at all remaining points. Regarding sample size, please see the note in the legend of Figure 3.

Chapter 6

SEX-SPECIFIC INFLUENCE OF AGING ON EXERCISING LEG BLOOD FLOW

Introduction

In either chronically endurance-trained (Wahren *et al.*, 1974; Proctor *et al.*, 1998) or very sedentary (Beere *et al.*, 1999; Poole *et al.*, 2003) men, leg blood flow and vascular conductance during cycling exercise are reduced with age. By contrast, we previously observed that in normally active older men, submaximal two-leg cycling exercise was not associated with attenuated leg blood flow responses compared with young subjects (Proctor *et al.*, 2003b), whereas older normally active women demonstrated blunted hyperemic and vascular conductance responses during the same mode of submaximal exercise (Proctor *et al.*, 2003a). Cumulatively, these results suggest that 1) there may be a modulatory influence of fitness/physical activity on the relation between age and leg hemodynamic responses to exercise in men, and 2) in normally active humans, there may also be a sex-specific influence of aging that influences control of leg blood flow during dynamic exercise.

With respect to the latter premise, the use of upright two-leg cycling exercise in the aforementioned studies prohibits the ability to conclusively determine whether these sex-specific effects of aging are the result of central (cardiac) or peripheral (local) factors. For example, while leg vascular conductance was attenuated at all exercise intensities in older women, at the highest work rates utilized during submaximal cycling exercise, older women were also exercising at 85% of their peak cardiac output (Proctor *et al.*, 2003a). Thus, given that two-leg cycling utilizes a large muscle mass, it is possible that there was a sex-specific central limitation (i.e., supply

limitation) to leg blood flow that confounded interpretation of responses even during submaximal cycling exercise. Utilizing small muscle mass dynamic leg exercise, a model not limited by the pumping capacity of the heart and consequently eliciting less excitation of the sympathetic nervous system than two-leg exercise (Saito & Mano, 1991; Ray, 1993; Ray *et al.*, 1993; Saito *et al.*, 1993; Callister *et al.*, 1994), minimizes these central limitations on exercising leg hemodynamics in older men and women.

Thus, the major purpose of the present study was to determine if the blunted leg vasodilatory response in women persisted during graded single-knee extensor exercise, when the confounding influence of central cardiac limitations on leg hyperemia is minimized. Using a design statistically powered to detect age-sex interactions in healthy, normally active men and women, we hypothesized that there would be a significant age-by-sex interaction in the leg hemodynamic responses to graded exercise, with older women exhibiting a significantly greater age-related reduction in leg blood flow and vascular conductance relative to young controls than older men.

Methods

Subject Characteristics and Initial Screening

Fifteen young men, 16 young women (ages 20-30), 13 older men, and 18 older women (ages 60-79) completed the study. All subjects were non-obese (BMI $\leq$ 30), nonsmokers, had clinically normal blood chemistry (i.e. hemoglobin concentrations ranged from 11.1-16.2 $g \cdot dL^{-1}$, total cholesterol $\leq$ 240 $mg \cdot dL^{-1}$, LDL cholesterol $\leq$ 150 $mg \cdot dL^{-1}$), and resting supine ankle-brachial index ratings (ABI between 0.90 and 1.30; VP2000, Colin Medical). All subjects were normotensive (resting blood pressure $\leq$ 140/90 mmHg) and free of overt chronic diseases as

evaluated by medical history questionnaire, a physical examination and resting ECG. Additionally, no subjects were taking medications having significant hemodynamic effects (including oral contraceptives and hormone therapy) for at least the last 12 months. Young females were studied in days 1-7 of their menstrual cycle to standardize the influence of female hormones. On study day, subjects were asked to refrain from alcohol, exercise, caffeine, aspirin, ibuprofen, or herbal supplements for at least 12 hours prior to testing. All subjects gave their written, informed consent to participate. This study was approved by the Office for Research Protections and the Institutional Review Board at The Pennsylvania State University in agreement with the guidelines set forth by the *Declaration of Helsinki.* Subject characteristics are presented in Table 6-1.

Fitness and physical activity status. Subjects were neither extremely sedentary nor extremely trained or fit (as assessed by treadmill $\dot{V}O_{2max}$ values referenced to age-predicted norms (ACSM, 2006) and scores on either the Yale Physical Activity Questionnaire (Dipietro et al., 1993) for older subjects or the Baecke Questionnaire of Habitual Physical Activity (Baecke et al., 1982) for young subjects). None of the subjects participated in moderate to high intensity aerobic exercise > 3 $d \cdot wk^{-1}$ or regular lower body resistance training > 2 $d \cdot wk^{-1}$during the past 12 months. To objectively quantify aerobic fitness status, all of the subjects performed a continuous incremental treadmill test (SensorMedics, Yorba Linda, CA) to maximal exertion to determine maximal oxygen uptake ($\dot{V}O_{2max}$).

Total and regional body composition was estimated using dual-energy X-ray absorptiometery (DXA; model QDR 4500W, Hologic, Waltham, MA) with subjects in the supine position as described previously (Proctor *et al.*, 2005). In addition, thigh volume was estimated by the anthropometric method described by Jones and Pearson from thigh patellar-pubic length, skinfold thicknesses, and circumference (Jones & Pearson, 1969). Quadriceps femoris muscle

mass was then estimated from thigh volume as originally described by Andersen and Saltin (Andersen & Saltin, 1985).

Study Procedures

Exercise modality. Single leg knee extensor exercise, designed to isolate the quadriceps muscle group, was performed as described previously (Andersen & Saltin, 1985; Richardson *et al.*, 1993). Briefly, subjects were reclined in a seat in the supine position (to minimize cardiopulmonary baroreceptor-mediated decreases in MSNA during knee extensor exercise (Ray *et al.*, 1993) as well as age- and sex-related differences in stroke volume responses to exercise (Fleg *et al.*, 1995)) with knees flexed at an angle of 90°. The subject's torso and both legs were fixed by straps attached to the chair to reduce extraneous movement and straining, and the left foot was placed in a boot attached to a rod containing a strain gauge for force measurements and connected to the pedal arm of a cycle ergometer (Monark) placed behind the subject. The right leg was allowed to hang free although subjects were instructed not to swing or move this leg. One extension of the quadriceps muscle moved the subject's lower leg 90°-170° and the ensuing flexion was a passive return pulled by the flywheel of the ergometer. Subjects kicked at a constant cadence of 40 kicks/minute (0.67 Hz), as this cadence facilitated both young and older subjects' maintenance of cadence with minimum motion artifact and consistent duty cycles. Resistance was increased by increasing the weight attached to a belt surrounding the flywheel such that friction on the flywheel increased proportionately. Subjects participated in two familiarization visits totaling approximately one hour of kicking such that they could learn to minimize accessory muscle recruitment (i.e., of the hamstring, inactive leg, and upper body) and maintain cadence. The first familiarization visit consisted of constant-load knee extension

exercise while the second visit involved instrumentation and data acquisition identical to that described below for the actual study visit with the exception that work rate increases were halved.

Exercise protocol. On the study day, the subjects began the protocol with 3 minutes of quiet rest, followed by 3 minutes of unloaded passive exercise (a research technician moved the subject's leg at 40 kicks/min). The purpose of the passive bout was to investigate the increase in flow due to mechanical influences and tachycardia separate from metabolic stimuli (Wray *et al.*, 2005a). The subject was then instructed to begin kicking against no resistance (0W) for three minutes, after which resistance increased incrementally every three minutes until the subject could no longer maintain cadence. The work rate increases used were 8 W in young and older men and 4.8 W in young and older women (increases were designed to produce similar time to exhaustion in all groups, taking into account the reduced quadriceps muscle mass of women and the observation that maximal knee extension work rate does not decline with age (Lawrenson *et al.*, 2003)).

Data Acquisition and Measurements

All variables were collected on-line at a sampling frequency of 400 Hz and stored using a Powerlab system (AD Instruments, Castle Hill, Australia). Heart rate and beat-to-beat systolic and diastolic blood pressure (continuous assessment of arterial waveforms by piezo-electric pressure transducers through radial tonometry of the right hand; Colin CBM-7000, Medical Instruments Corporation) were measured continuously throughout the study. In addition, manual auscultation was used every three minutes throughout the study to check the accuracy of the Colin during exercise. Electromyogram (EMG) signals of the active (left) biceps femoris and inactive (right) rectus femoris (to ensure that contraction was limited to the active quadriceps of the active leg, respectively) were collected with bipolar silver chloride surface electrodes (Bio-

Tac; Tyco Healthcare Group LP; Mansfield, MA) fixed lengthwise over the middle of the muscle belly placed 10-20 cm apart. Reference electrodes were placed on the knee of each leg. Electrode signals were amplified (Gould Universal Amplifier Model 13 4615 55, Cleveland, OH and Powerlab bioamplifier) with a bandwidth frequency ranging from 1.5 Hz to 2 kHz and simultaneously digitized using Powerlab. Knee extensor cadence was captured using a Cateye Astrale 8 (Cateye, Boulder, CO) cycle computer attached to the flywheel. Knee extensor force tracings were obtained using a load cell attached to the boot arm of the knee extensor ergometer.

A Doppler ultrasound machine (HDI 5000, Philips, Bothell, Washington) equipped with a high resolution 7-4 MHz linear-array transducer was used to measure mean blood velocity and vessel diameter of the left common femoral artery, distal to the inguinal ligament but above the bifurcation into the superficial and profunda femoral branch. For velocity measurements, the artery was insonated at a constant angle of 60° with the sample volume adjusted to cover the width of the artery, while diameter measurements were obtained with the artery insonated perpendicularly. Velocity measurements were taken continuously during minutes 1 and 3 of rest, passive exercise, and each work rate, while high-resolution diameter measurements (taken in 2D mode to optimize imaging) were taken during minute 2 of every work rate (except the peak work rate, during which diameter measurements were not taken). A custom interface unit processed the high-resolution angle-corrected, intensity-weighted Doppler audio information (i.e., mean blood velocity) from the ATL system into a lower frequency velocity signal (frequency range 0-20 Hz) that could be sampled in real time by Powerlab. That is, whereas the ATL strips off the probe carrier frequency to get the audio signal, the converter processes the low frequency variations in the audio signal that carry information about the velocity of the blood flow. Postprocessing using PowerLab's Chart application package yielded mean blood velocities.

Diameter measurements were stored on VHS tape and digitized at 4 frames/second using Brachial Imager software (Medical Imaging Applications; Iowa City, IA). Post-test analysis of

diameters was performed using edge-detection software (Brachial Analyzer Software, Medical Imaging Applications; Iowa City, IA); briefly, the technician (always the same and blind to any subject information) selected a region of interest along the arterial wall and the edge of the wall was detected by pixel density and represented by a line of best fit. Each sequence of images was reviewed by the technician and adjusted to ensure that diameter measurements were always calculated from the intima-lumen interface at both the distal and proximal vessel wall. Images affected by motion artifact were excluded based on visual inspection by the technician as well as a calculation by the software program that the confidence limit for the best-fit line was less than 80%. Diameter measurements for each work rate comprised an average of approximately 80 frames.

Data Analysis and Computations

For all study variables, values were calculated as the average over the last minute of rest, passive exercise, and each work rate (to allow hemodynamic variables to reach steady-state conditions (Hughson *et al.*, 1997; MacDonald *et al.*, 1998)), with the exception of peak measurements, which were calculated with first and/or second minute data if the subject did not complete the peak work rate. Mean arterial pressure (MAP, in mmHg) was calculated as (1/3 systolic pressure) + (2/3 diastolic pressure). The EMG signals were full-wave rectified, squared and median filtered, from which the root-mean square (RMS) value was derived. A spike-triggered average of EMG over the last minute was then derived from the cadence and calculated as a percent of the RMS value garnered from maximal isometric contraction (EMG averaged from three, five-second isometric contractions performed at the beginning of the study). Femoral artery blood flow (FBF) for each condition or work rate was calculated by multiplying the cross-

sectional area of the femoral (the diameter taken in minute 2 of each condition or work rate) with mean blood velocity (V_{mean}; $cm \cdot s^{-1}$), according to the formula:

FBF: Blood velocity $\cdot \pi \cdot$ (femoral diameter/2)$^2 \cdot 60$

where the FBF is in $ml \cdot min^{-1}$, the blood velocity is in $cm \cdot s^{-1}$, the femoral diameter (averaged across the cardiac cycle) is in cm, and 60 is used to convert from $ml \cdot s^{-1}$ to $ml \cdot min^{-1}$. To validate the assumption that the diameter taken in minute 2 was representative of the diameter underlying the blood flow measured in minute 3 of each condition or work rate, 4 subjects performed knee extensor exercise at various 3 minute work rates with high resolution diameter imaged constantly in 2D mode. The within-subject correlation between minute 2 and minute 3 diameters was 0.99. In addition, since peak diameter measurements were not obtained, peak FBF measurements were calculated with the diameter from the previous work rate. Femoral vascular conductance (FVC) was calculated as FBF/MAP.

Statistical Analysis

Statistical analyses were performed using SAS (SAS 9.1, Cary, North Carolina) software. All data are reported as mean + S.E.M. with significance set at $p<0.05$. A two-way ANOVA (Proc GLM) and Tukey post-hoc analysis were used to compare baseline and peak differences between groups. For graded responses, data beyond 32W in men and 19.2W in women were excluded from analysis and graphic representation due to artifact of significant subject dropouts. For within-sex comparisons of responses to graded knee extensor exercise, a repeated measures ANOVA (Proc Mixed) model with work rate as the within-individual factor and age and sex as the between-individual factors and an auto-regressive variance-covariance structure was used to determine differences between young and older subjects in outcome variables. A Bonferroni post-hoc adjustment was performed when significant age*work rate differences were detected.

Slopes of each individual's linear regression line were determined mathematically (change in outcome variable / change in work rate), excluding rest and passive exercise and compared within sexes using a one-way ANOVA. To test for age*sex interactions (i.e., whether the effect of age on leg hemodynamic responses to exercise differed by sex), relative responses to graded knee extensor exercise were examined with a random-coefficients model (Proc Mixed), using a continuous predictor (work rate as a percent of maximal work rate attained) and fitting a random intercept and slope with work rate as the within-individual factor and age, sex and age*sex as the between-individual factors. An auto-regressive variance-covariance structure was used to determine differences between subject groups in outcome variables (Littell *et al.*, 2006).

Results

Characteristics of the Exercise Protocol. In addition to peak work rate data reported in Table 6-1, there were no significant age or sex differences ($p > 0.13$ for all comparisons) in the number of work rates taken to reach peak power output (Young Men: 5.8 ± 0.2 work rates; Older Men: 5.5 ± 0.2 work rates; Young Women: 6.2 ± 0.3 work rates; Older Women: 5.6 ± 0.2 work rates) such that the exercise protocols were of similar duration. There were also no age or sex differences ($p > 0.80$ for all comparisons) in the time spent in the contraction (Contraction Time in Young Men: 0.83 ± 0.01 sec; Older Men: 0.84 ± 0.01 sec; Young Women: 0.83 ± 0.02 sec; Older Women: 0.82 ± 0.01 sec) and relaxation (Relaxation Time in Young Men: 0.69 ± 0.01 sec; Older Men: 0.68 ± 0.01 sec; Young Women: 0.69 ± 0.02 sec; Older Women: 0.69 ± 0.01 sec) portions of the duty cycle.

Peak Leg Blood Flow and Vascular Conductance. Due to significant subject dropouts at higher work rates, data is not represented or included in analysis beyond 32W in men and 19.2W in women. However, peak leg blood flow and vascular conductance responses also demonstrated

significant age*sex interactions ($p < 0.01$ for both), with no age differences in peak leg blood flow (Y: 1886 ± 63 mL min^{-1} vs. O: 2032 ± 152 mL min^{-1}; $p = 0.30$) and peak leg vascular conductance (Y: 17.9 ± 0.7 mL min^{-1} $mmHg^{-1}$ vs. O: 19.0 ± 2.1 mL min^{-1} $mmHg^{-1}$; $p = 0.55$) in men, and significantly attenuated peak leg blood flow (Y: 1913 ± 72 mL min^{-1} vs. O: 1349 ± 92 mL min^{-1}; $p < 0.01$) and leg vascular conductance in older women relative to young women (Y: 22.6 ± 1.4 mL min^{-1} $mmHg^{-1}$ vs. O: 13.6 ± 1.0 mL min^{-1} $mmHg^{-1}$; $p < 0.01$). The age*sex interactions were still significant ($p < 0.01$ for both) when peak leg blood flow and vascular conductance were normalized to quadriceps muscle mass; that is, peak leg blood flow and leg vascular conductance normalized to quadriceps muscle were lower in older women relative to young women ($p= 0.02$ and $p < 0.01$, respectively) whereas peak leg blood flow normalized to quadriceps muscle was marginally higher in older men ($p = 0.05$) and peak leg vascular conductance was similar in young vs. older men ($p=0.24$).

Within-sex age group differences in leg hemodynamic responses to graded knee extensor exercise (Figure 6-1). Age-group differences in blood pressure, femoral blood flow, and femoral vascular conductance responses at rest, passive exercise, and absolute work rates during incremental knee extensor exercise to exhaustion are shown in Figure 6-1. For men, calculations of slopes of responses were conducted from 0-24W in men, when the responses exhibited a linear relation to work rate and a curvilinear function was not significant; both a linear and a curvilinear relation fit responses from 0-32W and therefore confounded interpretation of slopes. From 0-24W, older men had a significantly greater hyperemic (Slope of femoral blood flow vs. absolute work rate in Young: 35 ± 2 $mL \cdot min^{-1} \cdot W^{-1}$ vs Older: 49 ± 3 $mL \cdot min^{-1} \cdot W^{-1}$; $p < 0.01$) and vasodilatory response (Slope of femoral vascular conductance vs. absolute work rate in Young: 0.30 ± 0.03 $mL \cdot min^{-1} \cdot mmHg^{-1} \cdot W^{-1}$ vs Older: 0.44 ± 0.04 $mL \cdot min^{-1} \cdot mmHg^{-1} \cdot W^{-1}$; $p < 0.01$) to graded exercise than younger men. By contrast, comparisons of responses from 0-19.2W in women demonstrated that older women had a blunted hyperemic (Slope of femoral blood flow vs. absolute work rate in

Young: 52 ± 3 mL·min^{-1} W^{-1}; vs Older: 40 ± 4 mL·min^{-1}·W^{-1}; p = 0.02) and vasodilatory response (Slope of femoral vascular conductance vs. absolute work rate in Young: 0.56 ± 0.06 mL·min^{-1} ·mmHg^{-1}·W^{-1}vs Older: 0.37 ± 0.04 mL·min^{-1}·mmHg^{-1}·W^{-1}; p < 0.01) to graded exercise compared to younger women. There were no age differences in the pressor response (slope of mean arterial pressure vs. absolute work rate) calculated across the same range of work rates in men (p = 0.62) or women (p = 0.93).

Age-sex interactions in leg hemodynamic responses to graded knee extensor exercise (Figure 6-2). To investigate age*sex interactions in responses to graded exercise, data are represented as each subject's work rates normalized to his or her peak work rate (% of maximal work rate) to account for the different work rate increases used in men vs. women. Heart rate, mean arterial pressure (MAP), femoral blood flow (FBF), and femoral vascular conductance (FVC) responses at rest, passive exercise, and incremental knee extensor exercise to exhaustion are shown in Figure 6-2.

Hemodynamic responses normalized for muscle mass (Figure 6-3). Femoral blood flow was normalized to estimated quadriceps muscle (kg) in men and women (Figure 6-3). In men, normalizing to kg muscle did not influence interpretation of data significantly (age difference in hyperemic and vasodilatory slope was p < 0.01 and p = 0.01, respectively), although the age difference in normalized resting femoral blood flow was marginally significant (p=0.08) rather than significant and normalized femoral vascular conductance at 0W was not different with age. In women, normalizing to kg muscle altered the significance of slopes such that the slope of the hyperemic response was not significantly different with age (p=0.41); the slope of the vasodilatory response was marginally lower in older women (p < 0.09). In addition, in eight young and eight older women matched for anthropometrically estimated quadriceps muscle (1.79 ± 0.08 vs 1.79 ± 0.09 kg, respectively), femoral blood flow and femoral vascular conductance were attenuated (p < 0.05) at all absolute work rates from rest to maximal exertion. There was no

age difference ($p = 0.55$) in the slope of the hyperemic response, but the slope of the vasodilatory response was greater in young women ($p < 0.01$).

Change in femoral diameter during passive and graded exercise. In women, there was a significant age*work rate interaction ($p = 0.03$) such that femoral diameter changes relative to rest were greater ($p < 0.05$) in young women compared to older women at all exercise work rates greater than 0W. For example, from rest to 19.2W, diameter increased from 7.2 ± 0.2mm to 7.7 ± 0.1mm in young women and from 7.3 ± 0.2mm to 7.4 ± 0.2mm in older women. In men, the age*work rate interaction was not significant ($p = 0.94$); nor were there age-related differences in diameter changes at any work rate ($p > 0.50$ for all work rates). Diameter did not increase more than 0.1mm relative to rest in young or older men at any work rate.

Ipsilateral hamstring and contralateral quadriceps muscle recruitment (Figure 6-4). Older women exhibited significantly greater hamstring recruitment in the active leg (expressed as a percentage of maximal isometric contraction) at work rates greater than or equal to 14.4W relative to young women; by contrast, there were no age differences in quadriceps recruitment in the inactive leg ($p > 0.10$ at all work rates) in women (ranges of quadriceps EMG from 0W-19.2W in young women: 2.7 ± 0.5 to 5.1 ± 0.7 %, and older women: 2.6 ± 0.5 to 7.2 ± 1.3%). To investigate the influence of the hamstring recruitment in women, we compared femoral blood flow between young women and seven older women with minimal hamstring recruitment (i.e., recruitment was similar to young women; Figure 6-4b). There were no age differences in active hamstring recruitment (ranges of hamstring EMG from 0-32W in young men: 2.8 ± 0.6 to 10.0 ± 1.9%, vs. older men: 3.4 ± 0.5 to 10.6 ± 1.7%) or inactive quadriceps recruitment (ranges of quadriceps EMG from 0-32W in young men: 2.2 ± 0.5 to 5.4± 0.6%, vs. older men: 1.6 ± 0.4 to 3.4 ± 1.2%; $p > 0.22$ for all work rates).

Discussion

Previous studies have reported reduced leg hyperemic and/or vasodilator responses to both cycling (Wahren *et al.*, 1974; Proctor *et al.*, 1998; Beere *et al.*, 1999; Poole *et al.*, 2003) and knee extensor (Magnusson *et al.*, 1994; Lawrenson *et al.*, 2003) exercise in trained and extremely sedentary older men. By contrast, we reported well-preserved leg blood flow and vascular conductance responses to submaximal graded leg cycling with age in healthy, normally active men, but significantly blunted flow and conductance responses during the identical exercise modality in women of comparable health and fitness (Proctor *et al.*, 2003a; Proctor *et al.*, 2003b). In the present study we utilized the well-established single-knee extensor exercise model to minimize the potentially confounding influence of age-related limitations imposed by cardiac output, thereby allowing us to test the hypothesis that in men and women of comparable fitness, age differences in exercising leg vasodilation are sex-specific. The major new findings generated by this study are that there are significant age-by-sex interactions in leg hemodynamic responses during this small muscle exercise model such that 1) older women, but not men, exhibit blunted vasodilator and hyperemic responses to graded exercise relative to their young counterparts, 2) these findings are not solely attributable to age- associated differences in quadriceps muscle mass or the mechanics of knee extensor exercise, and 3) there is a potential modulatory influence of aerobic fitness on age-related differences in leg vasodilation of men, but not women.

Interactive Effects of Age and Sex on Leg Vasodilation

To the best of our knowledge, the present study is the first with a design statistically powered to detect interactive effects of age and sex on the rise in active muscle blood flow and vascular conductance during dynamic exercise in humans. Using this approach and comparing

men and women at relative work rates to take into account differences in peak power output (Figure 6-2), we observed significant age*sex*work rate interactions for leg blood flow and vascular conductance such that vasodilator responses to graded exercise were attenuated with age in women. Thus, despite similar age-associated reductions in resting leg vascular conductance in men and women, older women demonstrated blunted vasodilator responsiveness with graded contractions relative to young women that was not observed in older men (relative to young men). It is important to note that hemoglobin was marginally lower in young women relative to older women (Y: 12.8 ± 0.2 vs O: 13.4 ± 0.2 gm·dLl^{-1}; p = 0.05) and significantly higher in young men relative to older men ((Y: 15.0 ± 0.2 vs O: 14.4 ± 0.2 gm·dL^{-1}; p = 0.02). However, evaluating the influence of hemoglobin on the leg blood flow responses by estimating leg oxygen delivery (multiplying the estimated arterial oxygen content (1.34 · Hb · SaO_2 (assuming SaO_2 of 97%) mL O_2· dL^{-1} blood) by femoral blood flow) did not at all change the effect of age on absolute blood flow responses or the slope of the hyperemic responsiveness in women or men. Thus, the current observations do not appear to be attributable to sex-specific age differences in hemoglobin, and are in line with previous observations of reduced smooth muscle responsiveness (Parker et al., 2006b; Newcomer et al., 2005) and peak vascular conductance (Martin et al., 1991;; Proctor et al., 2005; Ridout et al., 2005) in the legs of older women but not older men. These results suggest a dramatic loss of leg vasodilatory responsiveness with healthy aging in women, the mechanisms of which could involve the menopause-induced loss of estrogen (Celermajer *et al.*, 1994; Virdis *et al.*, 2000; Moreau *et al.*, 2003; Fadel *et al.*, 2004). In addition, some of the sex difference in vasodilator responsiveness among older adults could further be explained by the modulatory influence of chronic aerobic fitness in men, but not women (Figure 6-5), a finding consistent with longitudinal training data (Martin *et al.*, 1990) suggesting that the influence of fitness on age group differences in leg vasodilatory capacity in humans is sex-specific.

Age and Exercising Leg Vascular Responses in Men

At rest, and during the initial stages of the knee extensor protocol (3 min of passive movement of the limb followed by 3 min of active unloaded kicking), older men exhibited lower vascular conductance than their younger counterparts (Figure 6-1). The attenuated rise in leg conductance among the older men during the early stages of this protocol could reflect the persistent effects of heightened baseline (tonic) vasoconstriction in the legs of older (vs. young) men (Dinenno *et al.*, 2001), in the absence of significant contraction-induced metabolic stimuli. Alternatively, the delayed rise in leg conductance exhibited by our older men during the early stages of this protocol could be explained by slower contraction-induced vasodilatory kinetics in the microcirculation of the leg, as recently observed in aged male rats (Bearden, 2007). Regardless, reducing the increases in work rate by 50% (4W; data collected on second familiarization visit) did not alter this pattern, i.e., conductance and blood flow during the initial stages of the protocol (< 4W) were attenuated in these older men (Figure 6-6). This latter finding suggests that the metabolic stimulus needed to counter the influence of heightened baseline vasoconstriction and/or slowed vasomotor kinetics in the legs of older men is minimal.

During subsequent knee extensor exercise work rates, the age difference in leg vascular conductance in these men was abolished. In fact, the slopes of both the hyperemic and vasodilatory responses were augmented in older men from 0-24W, although we did not calculate slopes for responses from 0-32W given that the increase from 24-32W better fit a curvilinear relation than linear relation. Thus, we would caution interpretation of these slope comparisons in men. However, previous studies involving healthy young and older men performing graded single limb exercise (Jasperse *et al.*, 1994; Magnusson *et al.*, 1994; Lawrenson *et al.*, 2003) have also supported the idea that active limb vasodilation is well preserved, or even augmented, with

age in men. Collectively, these findings suggest that the mechanisms underlying vasodilator responses to small muscle mass exercise in older men are intact and/or sufficiently redundant.

Our current results of preserved leg hemodynamic responses to exercise are similar to what we previously reported in normally active older men during two-leg cycling exercise (Proctor *et al.*, 2003b) and are again at odds with other published studies showing reduced leg blood flow and/or leg vascular conductance during one- or two-leg exercise (Magnusson *et al.*, 1994; Proctor *et al.*, 1998; Lawrenson *et al.*, 2003; Poole *et al.*, 2003). We have previously hypothesized that these differences may be attributable to the chronic fitness levels of the subjects studied such that reduced leg blood flow may be maladaptive in sedentary older men but adaptive in older trained men when variables such as oxygen extraction and leg $\dot{V}O_2$ are considered (Koch *et al.*, 2005). Accordingly, when we investigated the most fit older men (those with the highest $\dot{V}O_{2max}$, at or above the 60th percentile for age-group norms) versus the least fit older men (those with the lowest $\dot{V}O_{2max}$, at or below the 30th percentile for age-group norms) in the study population, we found that fitness significantly influenced leg vascular conductance in men, offering a possible explanation for the discrepancy within the literature (Figure 6-5). However, with respect to this topic, it should also be noted that a) leg blood flow may increase (Beere *et al.*, 1999) or remain unchanged (Lawrenson *et al.*, 2004) in older men following a training protocol, and b) Donato et al. also published results of reduced leg blood flow in normally active older men relative to young men during low-intensity knee extensor exercise (although aerobic fitness of the men was not reported and older men had a 50% reduction in maximal knee extensor work rate relative to young men, unlike the current study; (Donato *et al.*, 2006)); therefore, a more comprehensive investigation of the relation between chronic aerobic fitness, training, and leg hemodynamic responses to exercise in older men is merited.

Age and Exercising Leg Vascular Responses in Women

In comparison to young women, older women exhibited significantly lower femoral blood flow and vascular conductance responses at rest and throughout all stages of the graded knee extensor protocol. While the attenuated vascular responses observed in older women at the same absolute work rates may have been influenced by their smaller (~20% less muscle mass) quadriceps muscles, normalization to estimated quadriceps muscle did not completely abolish age differences (Figure 6-3), nor did comparing older vs. young women matched for muscle mass. The slope of the hyperemic response, however, was not significantly different between young vs. older women when muscle mass was taken into account in either analysis, suggesting that hyperemic responsiveness could be influenced by an age-related loss of muscle mass in women. However, normalization to total estimated quadriceps muscle mass is an admittedly limited analysis with respect to graded exercise, given that the actual volume, quality, location and metabolic activity of recruited quadriceps muscle at each work rate is not known, at least in women (Ray & Dudley, 1998; Rådegran *et al.*, 1999; Lanza *et al.*, 2007). In addition, although older women did recruit their hamstring muscles to a slightly greater extent at higher work rates, examining responses in older women for whom hamstring recruitment was similar to young women did not influence the effect of age on the leg blood flow response to exercise (Figure 6-4), suggesting that the hamstring did not increase knee extensor economy and lower the flow demand in older women. These findings collectively point to age and/or estrogen dependent alterations in leg vasodilator responsiveness with age in women.

One potential mechanism underlying the blunted leg vasodilation in older women is a progressively greater vasoconstriction in the active muscle bed during incremental exercise. Although older women exhibited higher systemic blood pressures compared to young women at every exercise work rate (~10-20 mmHg, twice the difference between young and older men), it

is unlikely that the observed blunted vasodilatory response is solely attributable to increasingly heightened vasoconstriction given that the slope of the pressor response (Figure 6-1) was not different between young and older women and metabolic inhibition of sympathoexcitation increases in proportion to exercise intensity (Buckwalter *et al.*, 2001). However, greater sympathetic outflow during exercise combined with attenuated functional sympatholysis (Fadel *et al.*, 2004) could certainly contribute to the reductions in leg blood flow and leg vascular conductance observed in older women at every work rate, although large increases in muscle sympathetic nerve activity have not been observed during single leg exercise, at least in younger subjects (Ray *et al.*, 1993; Saito *et al.*, 1993).

Additional explanations for the observed age difference in the vasodilatory response to small muscle mass exercise in women are either an age-associated alteration in the control of leg blood flow by local vasoregulatory mechanisms and/or a structural limitation to vasodilation in the quadriceps vasculature. Several studies, including our own, have documented age-related reductions in local vasoregulatory pathways in women (Taddei *et al.*, 1996; Fadel *et al.*, 2004; Parker *et al.*, 2006; Parker *et al.*, 2007a). In addition, as suggested by our previous findings of reduced peak leg vasodilator capacity and leg smooth muscle dilation (Ridout *et al.*, 2005; Parker *et al.*, 2007a), structural alterations in the quadriceps vasculature—stiffening, less responsive vessels and reductions in arterial cross-sectional area—are likely factors contributing to the blunted vasodilatory responses observed in the legs of older women.

One final point worthy of discussion is the finding that older women exercised at the same absolute work rates and for the same duration as young women with no difference in peak work rate attained, yet with reduced leg blood flow. There are several implications of this finding, given the expected close matching of muscle perfusion to metabolic demand (Richardson *et al.*, 1993; Rådegran & Saltin, 2000). The first is that if the leg $\dot{V}O_2$ to work rate relationship is preserved with age during knee extensor exercise in women, as has been reported during

comparable exercise in sedentary men (Lawrenson *et al.*, 2003), then older women must be compensating for reduced leg blood flow with augmented oxygen extraction. Following this line of reasoning, an alternative explanation of the present data could even be that the lower leg blood flow observed in older women is resultant from, rather than directive of, an augmented oxygen extraction. Regardless, however, Proctor et al. did not previously observe increased oxygen extraction during submaximal cycling exercise in older women (Proctor *et al.*, 2003a). Thus, if the more probable explanation is that the relation between leg $\dot{V}O_2$ and work rate is altered with age in women, then the preservation of work capacity with reduced leg blood flow could be reflective of age-related changes in parameters such as exercise efficiency, fiber type size and/or distribution, capillary recruitment, and/or muscle metabolism (Proctor *et al.*, 1995; Proctor *et al.*, 2003a; Short *et al.*, 2005; Lanza *et al.*, 2007). Additional research is necessary to address these possibilities.

Experimental Considerations

Previous studies have suggested that there may be an influence of contraction frequency on estimates of mean blood flow measured during knee extensor exercise, especially when contraction frequency alters the time spent in the relaxation portion of the duty cycle (Hoelting *et al.*, 2001; Osada & Rådegran, 2002). Thus, we cannot predict the effect that the 40 kicks/min cadence used in the current study vs. the more commonly used cadence of 60 kicks/min utilized in other knee extensor studies may have on comparing leg blood flow estimates between studies, since the time spent in contraction and relaxation within a duty cycle is not often reported. However, given that the purpose of the investigation was to examine age*sex interactions within a normally active population, and that our hemodynamic data are in agreement with several other published studies using Doppler ultrasound and comparable work rates

(MacDonald *et al.*, 1998; Hoelting *et al.*, 2001; Harper *et al.*, 2006), we do not believe using a cadence of 40 kicks/min significantly altered the interpretations and applicability of the study.

Finally, although we did not directly measure cardiac output, it is unlikely that cardiac reserve was significantly challenged in any of these four groups of healthy subjects. This is suggested by the fact that 1) heart rate increases were modest and similar among groups (< 40 beats/min; figure 2), 2) stroke volume was likely maximized by testing subjects in the supine posture (Rowell, 1993), and 3) previous studies involving cardiac limited older adults (e.g., heart failure) indicate that the rise in cardiac output during single-knee extensor exercise approximately doubles the rise in total active leg blood flow (Magnusson *et al.*, 1997).

Potential Significance and Conclusions

The present findings suggest that peripheral factors play a significant role in the sex-specific effects of aging on the leg hemodynamic responses to exercise in healthy humans. The nature of this age effect in women vs. men, and the modulatory influence of fitness in older men, also supports the idea that biological changes (i.e., the loss of estrogen, impact of exercise/physical activity, as well as reductions in muscle mass) have a greater impact on active muscle vasodilation in humans than the effect of aging per se. Further studies are needed to determine the precise mechanisms underlying the persistent attenuation of leg vasodilatory responses to exercise in older women, as well as the apparent plasticity of these vascular responses in aging men.

Acknowledgements

We thank the GCRC clinicians and staff as well as Samuel Ridout, Michael D. Herr, Doug Johnson, Denny Ripka and Dennis Koch for their assistance with data collection, and Dr. Chet Ray (Hershey Medical Center) for the use of the Colin blood pressure system. This research was supported by R01 AG18246 (D.N. Proctor), NIA Interdisciplinary Training in Gerontology Grant T32 AG00048 (B.A. Parker) and M01 RR10732 (GCRC).

Table **6-1**: Subject Characteristics. Data are expressed as group means ± S.E.M for M = men, W = women, Y = young, and O = older subjects. ***** denotes significant ($p<0.05$) within-sex difference between young and older subjects. † denotes significant ($p<0.05$) within-age difference between men and women. [1]Percentiles defined by age- and sex-specific normative values (ACSM, 2006).

	YM	YW	OM	OW
Sample Size	15	16	13	18
Age (yrs)	24 ± 1	22 ± 1	71 ± 2*	67 ± 1*†
Resting SBP (mmHg)	129 ± 3	110 ± 2†	131 ± 5	121 ± 3*
Resting DBP (mmHg)	62 ± 2	55 ± 2†	70 ± 2*	60 ± 2†
BMI ($kg \cdot m^{-2}$)	24.6 ± 0.6	23.1 ± 0.9	25.0 ± 0.8	24.6 ± 0.6
LDL Cholesterol ($mmol \cdot L^{-1}$)	2.4 ± 0.2	2.1 ± 0.1	3.0 ± 0.1*	2.8 ± 0.2*
Triglycerides ($mmol \cdot L^{-1}$)	1.0 ± 0.1	0.8 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
Quad Muscle (kg)	2.5 ± 0.1	2.1 ± 0.1†	2.3 ± 0.1	1.7 ± 0.1*†
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	45.7 ± 1.3	36.0 ± 1.0†	31.8 ± 1.6*	25.1 ± 1.0*†
$\dot{V}O_{2max}$ (Percentile)[1]	54 ± 6	43 ± 5	37 ± 7	40 ± 5
Resting FBF ($mL \cdot min^{-1}$)	346 ± 46	303 ± 29	240 ± 25*	184 ± 17*
Resting FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	4.1 ± 0.3	4.2 ± 0.4	2.7 ± 0.3*	2.4 ± 0.3*
Femoral Diameter (mm)	8.4 ± 0.2	7.2 ± 0.2†	9.6 ± 0.4*	7.3 ± 0.3†
Maximal Work Rate (W)	38 ± 2	25 ± 2†	36 ± 2	22 ± 1†
Hemoglobin ($g \cdot dL^{-1}$)	15.0 ± 0.2	12.8 ± 0.2†	14.4 ± 0.2*	13.4 ± 0.2†

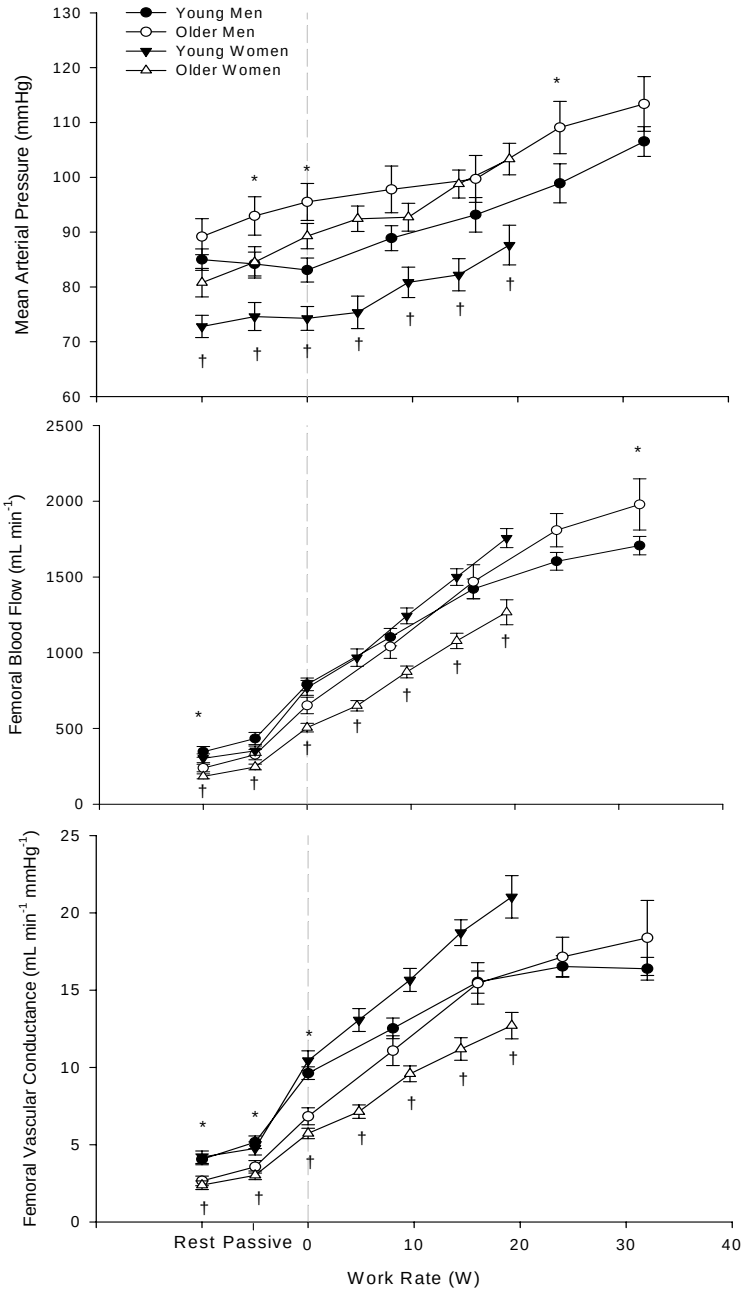

Figure **6-1**: Mean arterial pressure (top), femoral blood flow (middle), and femoral vascular conductance (bottom) expressed as group means ± S.E.M. at absolute work rates in young and older men and women. * indicates significant ($p < 0.05$) difference between young and older men. † indicates significant ($p<0.05$) difference between young and older women. The dashed line indicates the onset of active knee extensor exercise. For young men, sample size was n=15 until 24W and n=14 at 32W. For older men, sample size was n=13 until 24W and n=12 at 32W. For young women, sample size was n=16 at 19.2W. For older women, sample size was n=18 until 14.4W and n=17 at 19.2W.

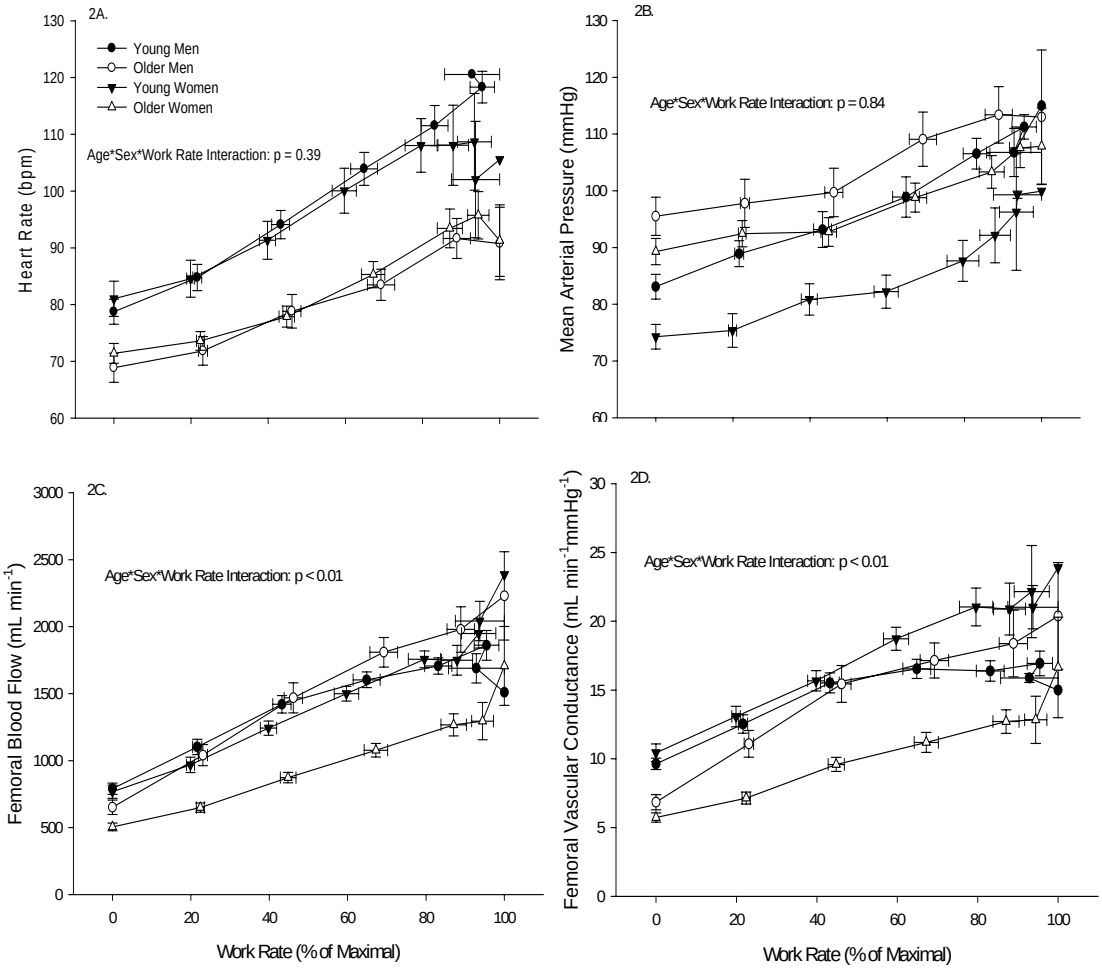

Figure **6-2**: Heart rate (2A), mean arterial pressure (2B), femoral blood flow (2C), and femoral vascular conductance (2D) expressed as group means ± S.E.M. at percent of maximal work rate (% MW) in young and older men and women. Graphically, portraying group averages of relative work rates (% of maximal work rate, or %MW), yielded the following sample sizes: for young men, sample size was n=15 until 83% MW, n=10 at 95% MW, n=2 at 93% MW, and n=1 at 100% MW; for older men, sample size was n=13 until 69%MW, n=12 at 89%MW, and n=6 at 100% MW; for young women, sample size was n=16 until 80% MW, n=11 at 88% MW, n=6 at 93% MW, n=2 at 94% MW and n=1 at 100 % MW; and for older women, sample size was n=18 until 67% MW, n=17 at 87%MW, n=9 at 94% MW, and n=3 at 100% MW. Please note that these dropouts are an artifact of graphical representation only; statistical comparisons were achieved by fitting curves to each individual's hemodynamic responses vs. the range of percent maximal work rate attributable to each work rate increase.

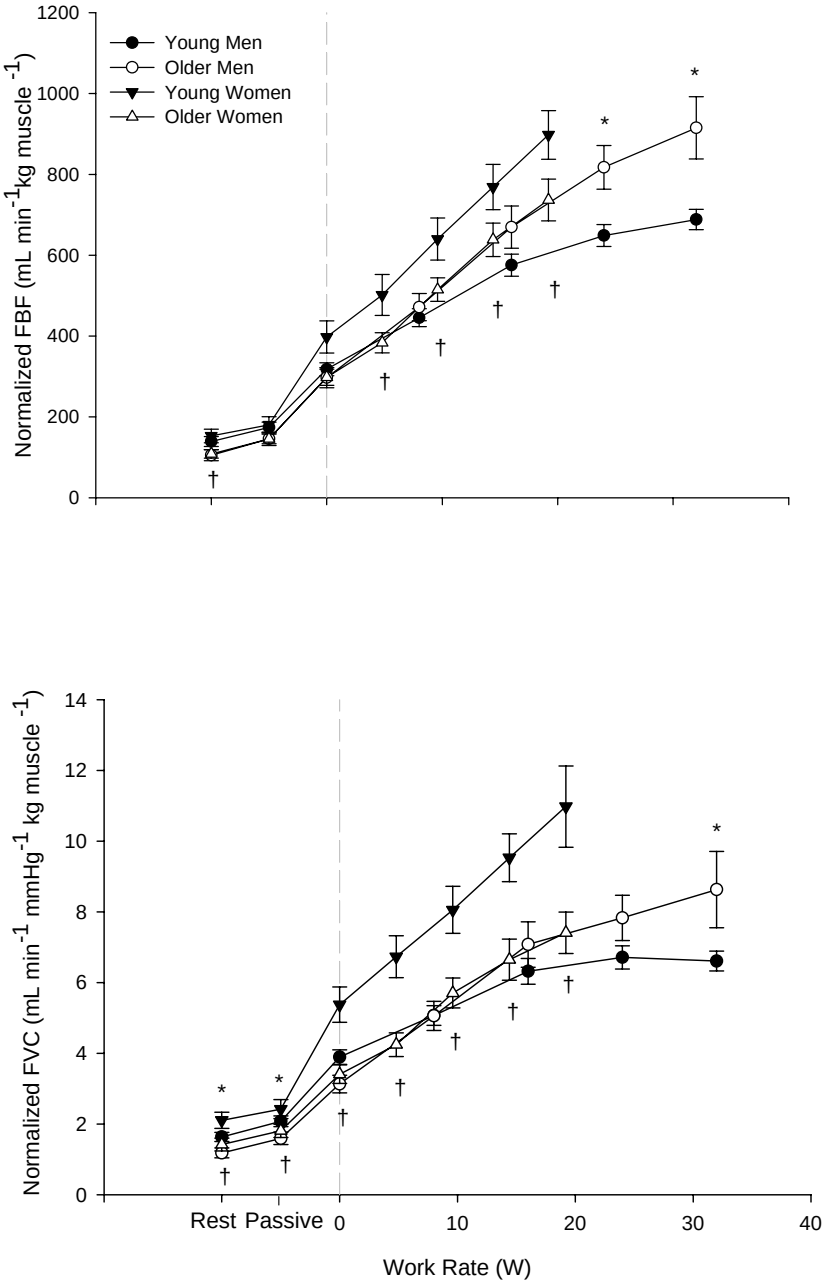

Figure **6-3**: Femoral blood flow (Normalized FBF; top), and femoral vascular conductance (Normalized FVC; bottom) normalized to estimated quadriceps muscle and expressed as group means ± S.E.M. at absolute work rates in young and older men and women. * indicates significant ($p < 0.05$) difference between young and older men. † indicates significant ($p<0.05$) difference between young and older women. The dashed line indicates the onset of active knee extensor exercise. Please see Figure 1 for sample sizes.

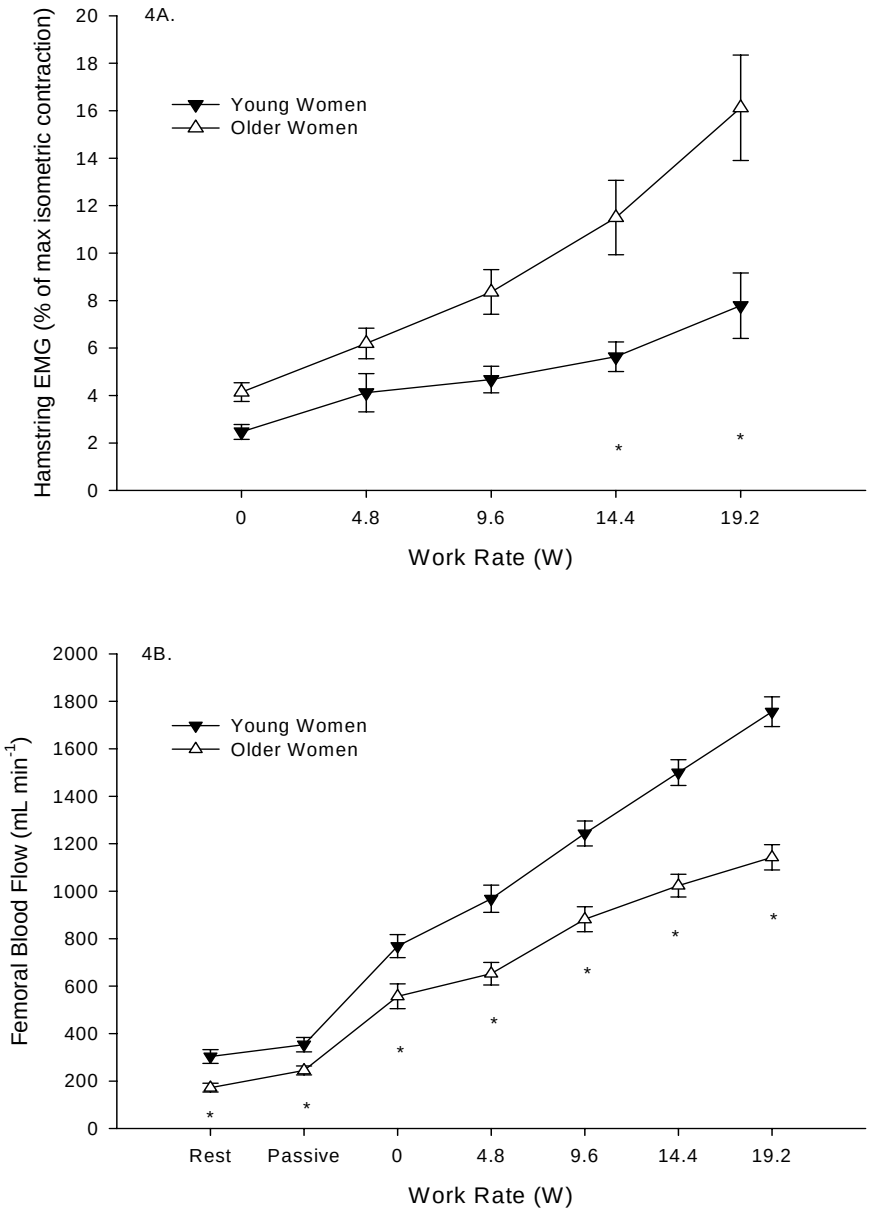

Figure **6-4**: Ipsilateral hamstring recruitment in women, as represented by electromyographical (EMG) activity normalized to each individual's maximal isometric contraction, during graded knee extensor exercise (4A), and age comparisons of femoral blood flow (4B) in seven older women with hamstring recruitment similar to young women. Data expressed as group means ± S.E.M. at absolute work rates in young and older women. * indicates significant ($p < 0.05$) difference between young and older subjects. Please see Figure 1 for sample sizes.

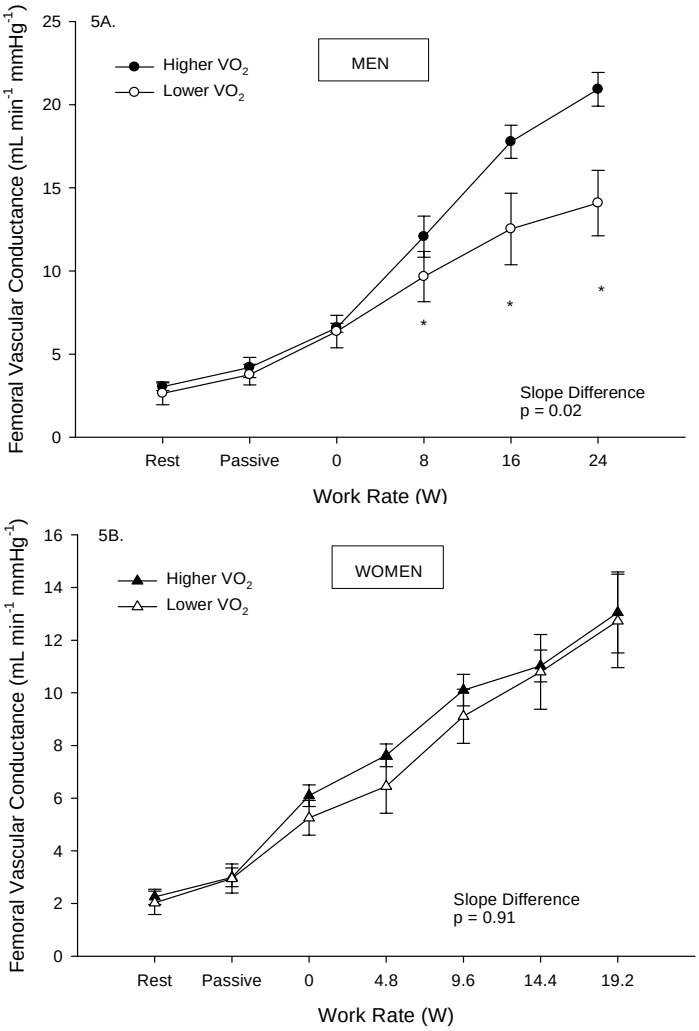

Figure **6-5**: Femoral vascular conductance expressed as group means ± S.E.M. at absolute work rates in older men (5A) and older women (5B) separated into groups by higher and lower $\dot{V}O_{2max}$ scores (i.e., most vs. least fit in study population). * indicates significant ($p < 0.05$) difference between young and older subjects. Slopes were calculated from 0-24W in men and 0-19.2W in women. For higher fitness men, sample size was n=4 until 24W. For lower fitness men, n=5 until 24W. 32W data was not included due to the undue influence that one subject drop-out between 24-32W had on the comparison. For higher fitness women, n=6 until 14.4W and n=5 at 19.2W. For lower fitness women, n= 6 until 19.2W.

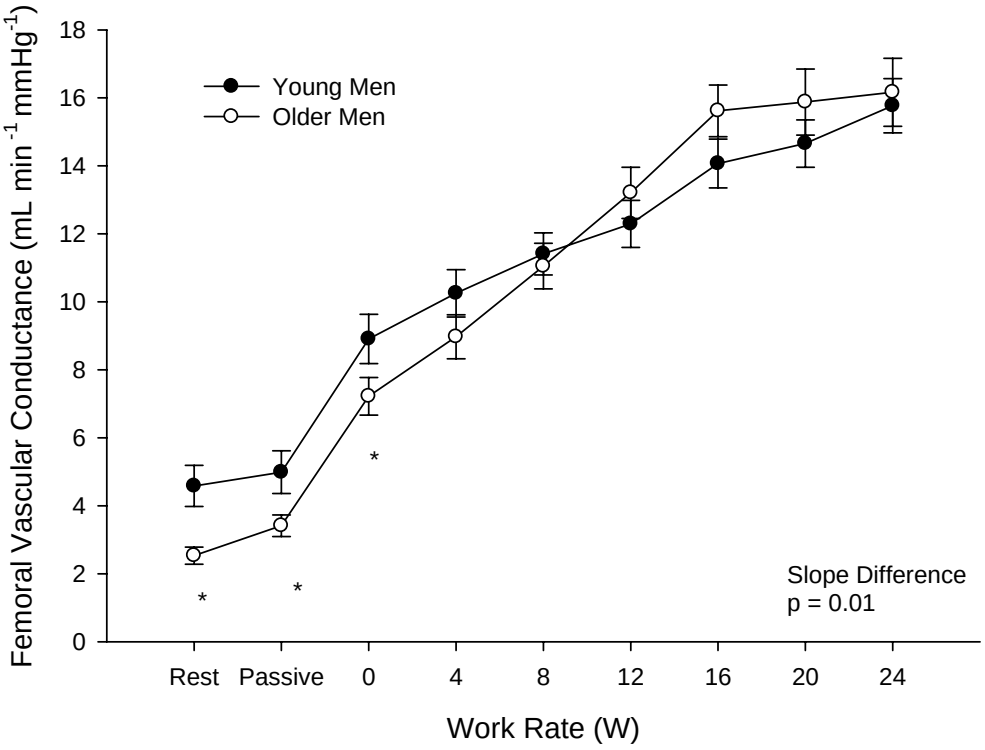

Figure **6-6**: Femoral vascular conductance measured during a longer protocol in young and older men (graded knee extensor exercise with work rate increases of 4W rather than 8W to the same approximate peak work rate; data collected on the second familiarization visit). Data are expressed as group means ± S.E.M. at absolute work rates. * indicates significant ($p < 0.05$) difference between young and older men. For young men, sample size was n=15. For older men, sample size was n=13 until 20W and n=11 at 24W.

Chapter 7

AGE AND MICROVASCULAR RESPONSES TO SUSTAINED KNEE EXTENSOR EXERCISE IN WOMEN

Introduction

Older women exhibit lower leg blood flow responses compared to young women during graded two-leg cycling (Proctor *et al.*, 2003a) and single-knee extensor exercise (Parker *et al.*, 2007c). In the latter study, older women exercised at the same absolute work rates as young women, and neither peak work rate attained nor the duration of the knee extensor exercise protocol (~18 min) differed with age. We hypothesized that older women may demonstrate compensatory alterations in local oxygen extraction to dynamic knee extensor exercise relative to young women to meet the sustained metabolic demand of the working muscle despite a dramatically lower conduit inflow (Lundgren *et al.*, 1988; Proctor *et al.*, 1998).

Accordingly, the purpose of the present study was to investigate oxygen extraction (estimated indirectly by near infrared spectroscopy, or NIRS) in the vastus lateralis (which has high proportional activation relative to the other quadriceps muscles during kicking exercise in women; (Heinonen *et al.*, 2007)) during an extended bout of strenuous (~17-18W) dynamic knee extensor exercise. We hypothesized that 1) there would be a significantly lower femoral artery hyperemic response to knee extensor exercise in older vs. young women, and 2) estimated oxygen extraction would thus be augmented in order for older women to continue knee extensor exercise at a similar absolute work rate as young women. To gain additional insight into the mechanisms underlying the vastus lateralis oxygen extraction response in older vs. young women, we also assessed microvascular blood volume and the heterogeneity of the microvascular blood volume

response to exercise, as blood flow heterogeneity is inversely associated with the efficacy of tissue oxygenation (Walley, 1996) and lower heterogeneity may thus improve oxygen extraction in adults exhibiting reduced leg blood flow (Kalliokoski *et al.*, 2001).

Methods

Inclusion/Exclusion Criteria and Initial Screening

9 young women (ages 20-30) and 13 older women (ages 60-79) completed the study. All subjects were non-obese (BMI $\leq$ 30), nonsmokers, had clinically normal blood chemistry (i.e. hemoglobin concentrations ranged from 111-162 $g \cdot L^{-1}$, total cholesterol $\leq$ 6.22 $mmol \cdot L^{-1}$, LDL cholesterol $\leq$ 3.89 $mmol \cdot L^{-1}$), and resting supine ankle-brachial index ratings (ABI between 0.90 and 1.30; VP2000, Colin Medical). All subjects were normotensive (resting blood pressure $\leq$ 140/90 mmHg) and free of overt chronic diseases as evaluated by medical history questionnaire, a physical examination and resting ECG. Additionally, no subjects were taking medications having significant hemodynamic effects (including oral contraceptives and hormone therapy) for at least the last 12 months. Young females were studied in days 1-7 of their menstrual cycle to standardize the influence of female hormones (Williams *et al.*, 2001). On study day, subjects were asked to refrain from alcohol, exercise, caffeine, aspirin, ibuprofen, or herbal supplements for at least 12 hours prior to testing. All subjects gave their written, informed consent to participate. This study was approved by the Office for Research Protections and the Institutional Review Board at The Pennsylvania State University in agreement with the guidelines set forth by the *Declaration of Helsinki.*

Fitness and physical activity status. Subjects were neither extremely sedentary nor extremely trained or fit (as assessed by treadmill maximal oxygen uptake, or $\dot{V}O_{2max}$, values

referenced to age-predicted norms (ACSM, 2006) and scores on either the Yale Physical Activity Questionnaire (Dipietro et al., 1993) for older subjects or the Baecke Questionnaire of Habitual Physical Activity (Baecke et al., 1982) for young subjects). None of the subjects participated in moderate to high intensity aerobic exercise > 3 $d \cdot wk^{-1}$ or regular lower body resistance training > 2 $d \cdot wk^{-1}$during the past 12 months. A continuous incremental treadmill test (SensorMedics, Yorba Linda, CA) to maximal exertion was used to determine $\dot{V}O_{2max}$.

Total and regional body composition was estimated using dual-energy X-ray absorptiometery (DXA; model QDR 4500W, Hologic, Waltham, MA) with subjects in the supine position as described previously (Proctor *et al.*, 2005). In addition, thigh volume was estimated as described previously (Parker *et al.*, 2007b).

Study Procedures

Exercise modality. Single leg knee extensor exercise, designed to isolate the quadriceps muscle group, was performed as described previously (Parker *et al.*, 2007b). Briefly, subjects were reclined in a seat in the supine position with knees flexed at an angle of 90°. The subject's torso and both legs were fixed by straps attached to the chair, and the left leg was strapped into a boot attached by lever arm to the pedal of a cycle ergometer placed behind the subject. The right leg was allowed to hang free. One extension of the quadriceps muscle moved the subject's lower leg 90°-170° and the ensuing flexion was a passive return pulled by the flywheel of the ergometer. Subjects kicked at a constant cadence of 40 kicks/minute (0.67 Hz). Resistance was increased by increasing the weight attached to a belt surrounding the flywheel such that friction on the flywheel increased proportionately. Subjects participated in three familiarization visits prior to the study visit.

Graded exercise protocol. To determine maximal knee extensor work rate, subjects completed a graded protocol as described previously (Parker *et al.*, 2007b). Following 3 minutes of quiet rest, and 3 minutes of unloaded passive exercise, the subject was instructed to begin kicking against no resistance (0W) for three minutes, after which resistance increased incrementally (by 4.8 W) every three minutes until the subject could no longer maintain cadence.

Study Protocol. On a separate day, again following 3 minutes of quiet rest and 3 minutes of unloaded passive exercise, subjects performed 0.67 Hz knee extensor exercise at an absolute work rate approximating 70-85% of the maximal work rate attained in the graded protocol. Subjects maintained a constant cadence at this work rate until they reached exhaustion. Subjects who did not reach exhaustion by 24 minutes were stopped to avoid quadriceps overuse and due to the technical difficulty of collecting continuous high-quality Doppler ultrasound measurements during sustained exercise.

Data Acquisition and Systemic Measurements

All systemic variables were collected on-line at a sampling frequency of 400 Hz and stored using a Powerlab system (AD Instruments, Castle Hill, Australia). Heart rate and beat-to-beat systolic and diastolic blood pressure (radial tonometry of the right hand; Colin, Medical Instruments Corporation) were measured continuously throughout the study. Mean arterial pressure (MAP, in mmHg) was calculated as (1/3 systolic pressure) + (2/3 diastolic pressure). Knee kick cadence was captured using a Cateye Astrale 8 (Cateye, Boulder, CO) cycle computer attached to the flywheel.

Femoral Artery Hemodynamic Measurements

A Doppler ultrasound machine (HDI 5000, Philips, Bothell, Washington) equipped with a high resolution 7-4 MHz linear-array transducer was used to measure mean blood velocity and vessel diameter of the left common femoral artery, as described previously (Parker *et al.*, 2007b). For velocity measurements, the artery was insonated at a constant angle of 60° with the sample volume adjusted to cover the width of the artery, while diameter measurements were obtained with the artery insonated perpendicularly. Velocity measurements were taken continuously during the protocol, with the exception of high-resolution diameter measurements (taken in 2D mode to optimize imaging) taken for 20 seconds every third minute starting at minute two of rest. A custom interface unit processed the high-resolution angle-corrected, intensity-weighted Doppler audio information (i.e., mean blood velocity) from the ATL system into a lower frequency velocity signal (frequency range 0-20 Hz) that could be sampled in real time by Powerlab.

Diameter measurements were stored on VHS tape and digitized at 4 frames/second using Brachial Imager software (Medical Imaging Applications; Iowa City, IA). Post-test analysis of diameters was performed using edge-detection software (Brachial Analyzer Software, Medical Imaging Applications) as described previously (Parker *et al.*, 2007b). Briefly, the technician (always the same and blind to any subject information) selected a region of interest along the arterial wall and the edge of the wall was detected by pixel density and represented by a line of best fit. Diameter measurements were calculated from the intima-lumen interface. Femoral artery blood flow (FBF), averaged at rest, during passive exercise, and during every three minutes of loaded kicking, was calculated according to the formula:

$$\text{FBF: Blood velocity} * \pi * (\text{femoral diameter}/2)^2 * 60$$

where the FBF is in $ml \cdot min^{-1}$, the blood velocity is in $cm \cdot s^{-1}$, the femoral diameter (averaged across the cardiac cycle) is in cm, and 60 is used to convert from $ml \cdot s^{-1}$ to $ml \cdot min^{-1}$. Femoral vascular conductance (FVC) was calculated as FBF/MAP.

Quadriceps Microvascular Oxygenation Measurements

Changes in oxygen levels in the vastus lateralis of both the active and inactive kicking leg were estimated noninvasively by NIRS (Near Infrared Spectroscopy; LEDI; NIM, Inc., Philadelphia). The NIRS unit uses a dual light source, continuous wavelength spectrophotometer with six detectors per probe and light-source separation of 30 mm to define the relationship between absorption of electromagnetic radiation and the relative concentration of deoxyhemoglobin (HHb) and oxyhemoglobin (O_2Hb) with respect to the Beer-Lambert Law. Light emitted into the larger arteries and veins (> 1mm) is almost completely absorbed by the large concentration of hemoglobin such that detectable changes in absorption at 730nm (HHb) and 850nm (O_2Hb) are attributable to changes in tissue oxygenation in the microcirculation (Kalliokoski *et al.*, 2006). Differences in oxygenation between hemoglobin and myoglobin cannot be distinguished; however, the NIRS signal is generally assumed to be coming primarily from HHb and O_2Hb (Wilson *et al.*, 1989; Mancini *et al.*, 1994). In addition, the assumption of a constant path length of photons (i.e., constant scattering) in this unit dictates that measurements be expressed as a relative measure.

Probes were wrapped in saran wrap, placed longitudinally on the belly of the vastus lateralis muscle of the active and inactive leg, and secured by wrapping elastic bandage around the leg and probe. After calibration and balancing, data were sampled at 3Hz continuously throughout the exercise protocol and stored offline in a text file. Total Hb was calculated as the sum of O_2Hb and HHb and used as an index of change in regional microvascular blood volume

(Belardinelli *et al.*, 1995; Van Beekvelt *et al.*, 2001b). Changes in the HHb signal were used to represent oxygen extraction (De Blasi *et al.*, 1994; Ferrari *et al.*, 1997). Measurements of total Hb and HHb were averaged in 30 second increments and normalized to reflect changes from rest (arbitrarily defined as 0 μM). Heterogeneity of the total Hb signal at each data point was calculated as relative dispersion, or the coefficient of variation of values from all eight detectors, and averaged for rest and exercise (Kalliokoski *et al.*, 2006).

Statistical Analysis

Statistical analyses were performed using SAS (SAS 9.1, Cary, North Carolina). All data are reported as mean ± S.E.M. with significance set at $p<0.05$. A one-way ANOVA (Proc GLM) and Tukey post-hoc analysis were used to compare baseline differences between groups. For comparisons of responses to knee extensor exercise, a repeated measures ANOVA (Proc Mixed) model with time as the within-individual factor and age as the between-individual factors and an auto-regressive variance-covariance structure was used to determine differences between young and older subjects in outcome variables. A Bonferroni post-hoc adjustment was performed when significant age*work rate differences were detected.

Results

Subject Characteristics. Subject characteristics are presented in Table 7-1.

Exercise Protocol Characteristics. During the graded knee extensor exercise test to exhaustion, there was no age difference in the peak exercise work rate attained in young vs older subjects (Y: 25 ± 2 W vs O: 22 ± 1 W; $p = 0.21$); accordingly, there was also no age difference in the absolute (Y: 18 ± 1 W vs O: 17 ± 1 W; $p = 0.47$) or relative (Y: 74 ± 3 % of max vs O: 78 ± 2

% of max; p = 0.28) work rates at which the subjects exercised. In addition, there was no age difference in the duration of the exercise protocol maintained (Y: 693 ± 75 s vs 0: 644 ± 58 s; p=0.67) among subjects who stopped due to exhaustion (n= 6Y and 9O). For the purpose of the ensuing leg hemodynamic comparisons, the first 9 minutes of measurements were analyzed to avoid the confounding influence of subject dropouts on data interpretation (all but one young and one older women remained in the sample until 9 minutes).

Femoral hemodynamic responses to exercise (Figure 7-1). Mean arterial pressure (MAP), femoral blood flow (FBF), and femoral vascular conductance (FVC) at rest, during passive exercise, and during loaded kicking exercise are shown in Figure 7-1. Older women exhibited higher MAP, lower FBF, and lower FVC at rest and during nine minutes of active kicking exercise.

Estimated changes in blood volume and oxygen extraction in the active and inactive leg (Figure 7-2a-d). One older subject's data was excluded from analysis due to questionable probe placement. The changes, relative to rest, in total hemoglobin (O_2Hb + HHb; estimated microvascular blood volume) and HHb (estimated oxygen extraction) in the active leg are shown in Figure 7-2a and b. For changes in blood volume (7-2a), there was no overall effect of age (p = 0.87) nor was there an age*time interaction (p= 0.27). For changes in oxygen extraction (7-2b), there was no significant overall effect of age (p = 0.13); however, the age*time interaction was significant (p < 0.01) as young women demonstrated a significantly more rapid increase in oxygen extraction in the first through fourth minutes of loaded kicking exercise. To test whether the above-described age differences in the active kicking leg were unique to the heightened metabolic demands of exercise (i.e., not attributable to age-related measurement artifact), changes in blood volume and oxygen extraction were also examined in the inactive leg (Figure 7-2c and d). There was no effect of age (p=0.76 and p=0.94, respectively) or age*time interaction (p=0.23 and p= 0.58, respectively) for either changes in blood volume (7-2c) or oxygen extraction (7-2d).

Heterogeneity of microvascular blood volume (Figure 7-3). There was no age difference in resting or exercise blood volume heterogeneity (p=0.45 and 0.14, respectively); however, blood volume heterogeneity significantly increased during exercise in young women (p=0.02) but not older women (p=0.71). Microvascular blood volume heterogeneity in the inactive leg was also not different between young and older women at rest or exercise ($p = 0.21$ and 0.63, respectively) and again increased significantly in young ($p < 0.05$) but not older ($p = 0.28$) women during exercise.

Discussion

The purpose of the study was to test the hypothesis that local oxygen extraction, as estimated indirectly by NIRS, is higher in older women who exhibit blunted femoral hyperemic responses to knee extensor exercise relative to young women. In contrast to our hypothesis, however, we did not find evidence of augmented oxygen extraction in older women, despite the observed age-related reduction in femoral artery blood flow. Several explanations could underlie this finding, including methodological aspects of NIRS-based estimates of muscle oxygen extraction as well as physiological mechanisms associated with aging, such as muscle metabolism, the matching of flow to metabolic demand, and microvascular blood volume.

Relation Between Femoral Blood Flow and Local Oxygen Extraction

We previously observed that the attenuated leg blood flow and vascular conductance responses exhibited by older women during knee extensor exercise did not influence their peak work rate attained or duration of the graded exercise protocol (Parker *et al.*, 2007c). In fact, doubling the length of the graded knee extensor protocol in that study by reducing the work rate

increments also did not influence the age group comparisons of peak work rate/duration of the exercise protocol. Similarly, in the current study, there was no age difference in the duration for which young and older women maintained ~17-18W knee extensor exercise to exhaustion, although not all of the women fatigued before measurements were stopped. Regardless, older women demonstrated reduced leg blood flow and leg vascular conductance relative to young women while exercising at a similar absolute work rate (Figure 7-1). While hemoglobin was lower in young women (Y: 127 ± 2 $g \cdot L^{-1}$ vs O: 133 ± 2 $g \cdot L^{-1}$; p = 0.03), estimated oxygen delivery ((multiplying the estimated arterial oxygen content (1.34 * Hb * SaO_2 (assuming SaO_2 of 97%) mL O_2/dL blood) by femoral blood flow) was still significantly higher in young women during exercise (data not shown). Collectively, these findings suggest that older women accomplish the knee extensor work with reduced oxygen delivery to the working muscle.

Data regarding compensatory changes in oxygen extraction in aged individuals who exhibit reduced leg blood flow during dynamic exercise are equivocal, with reports of both greater leg oxygen extraction during cycling exercise in middle-aged (Wahren *et al.*, 1974), older sedentary (Poole *et al.*, 2003) and older trained (Proctor *et al.*, 1998) men as well as similar oxygen extraction in older sedentary men (Beere *et al.*, 1999) and older normally active women (Proctor *et al.*, 2003a). Limited data regarding knee extensor exercise are similarly ambiguous (Lawrenson *et al.*, 2003; Donato *et al.*, 2006; Ferri *et al.*, 2007); discrepancies may be attributable to the age, fitness or sex of the subject populations studied as well as the methodology used to investigate oxygen extraction (i.e., direct vs. indirect measurements). Using NIRS based estimates of microvascular oxygenation, we did not find evidence of augmented oxygen extraction in older women (Figure 7-2a-b). In fact, estimated quadriceps oxygen extraction was lower in the initial minutes of exercise in older vs young women, suggestive of delayed kinetics and/or onset of metabolic demand in older women (DeLorey *et al.*, 2007).

Given the inverse relationship between blood flow heterogeneity and oxygen extraction (Walley, 1996; Kalliokoski *et al.*, 2001), we hypothesized that older women might also potentiate oxygen extraction through lower microvascular blood flow heterogeneity, as estimated through measurements of relative dispersion of microvascular blood volume (Kalliokoski *et al.*, 2006). Interestingly, while there were no age differences in heterogeneity of the total Hb signal at rest, microvascular blood volume heterogeneity increased significantly in the young but not older women during exercise in both the active and inactive leg (Figure 7-3). However, this age effect was observed in both the active and inactive leg, and did not appear to result in augmented oxygen extraction in older women.

Oxygen Extraction: Methodological Considerations

There are several methodological issues associated with NIRS-based estimates of oxygen extraction that must be considered in the present investigation. Given that arterial deoxygenated hemoglobin (HHb) concentrations are negligible at normal PO_2 (De Blasi *et al.*, 1994), it has been concluded that changes in HHb estimated by NIRS reflect changes in oxygen extraction independent of increased perfusion (Ferrari *et al.*, 1997). NIRS estimates of oxygenation cannot be considered a substitute for directly measured $a-\bar{v}O_2$ difference, especially given that the venous oxygen content draining from a whole limb (as sampled by a catheter) is quantitatively different from NIRS measures of microvascular oxygen content in the regional muscle vascular space, of which ~85% may be capillaries (Poole *et al.*, 1995; MacDonald *et al.*, 1999; DeLorey *et al.*, 2003). In addition, age- and exercise-associated effects on the microvasculature, with respect to microvessel rarefaction (Behnke *et al.*, 2006), arteriolar tortuosity (Bearden *et al.*, 2004), and the efficacy of the muscle pump, could also influence the HHb signal. However, the validity of NIRS is supported by studies showing that the magnitude and nature of the change in HHb is in

agreement with expected changes in oxygen extraction during exercise in young and older adults (DeLorey *et al.*, 2007) as well as diabetics (Bauer *et al.*, 2007).

Another consideration of using NIRS technology is that the propagation of light through tissue is influenced by both the muscle and subcutaneous fat through which it travels. A thicker adipose tissue layer, such as that observed in older subjects (caliper-derived measurements of quadriceps adipose thickness were 11.3 ± 1.6 mm in young and 16.0 ± 1.0 mm in older women), may confound NIRS measurements in muscle by preventing light from passing through the muscle tissue and biasing oxygenation measurements since adipose tissue metabolism differs from muscle metabolism (van Beekvelt *et al.*, 2001a). However, Homma et al. found that a source-detector distance of 30mm (the same as used in the current study) was sufficient to detect changes in leg tissue deoxygenation during exercise in subjects with varying thicknesses of adipose tissue (Homma *et al.*, 1996). In addition, since these same authors hypothesized that the NIR light penetrates muscle tissue at minimum deep enough to reach half the distance between source and detector, we also compared oxygenation characteristics of young vs. older women in the current study whose adipose tissue thickness was 15mm or less. In this subset analysis, there was still the same delayed onset of oxygen extraction in the initial minutes of exercise in older women yet no age differences in the following minutes. Finally, there were no age-differences in oxygenation parameters in the inactive leg (Figure 7-2c and d), which would be expected should adipose tissue thickness be significantly influencing NIR measurements.

Oxygen Extraction: Physiological Explanations

Leg exercise with similar oxygen extraction and lower leg blood flow in older women relative to young women, in agreement with our previous findings during cycling exercise (Proctor *et al.*, 2003a), suggests a lower exercising leg $\dot{V}O_2$ in older women, which could reflect

age-related changes in exercise efficiency and/or muscle metabolism. For example, greater reliance on oxidative metabolism has been observed in older vs. young adults (Lanza *et al.*, 2007). By contrast, however, some studies have shown that oxidative capacity declines by as much as 50% in the leg in older humans (Conley *et al.*, 2000). Changes in fiber type (Coggan *et al.*, 1992; Short *et al.*, 2005), motor unit recruitment (Ferri *et al.*, 2007) and reductions in flow directed to oxidative fibers (Musch *et al.*, 2004) with age may also influence muscle metabolism and alter the relation between leg $\dot{V}O_2$ and absolute work.

Alternatively, the NIRS-derived measure of microvascular blood volume—the estimated change in the total hemoglobin (HHb + O_2Hb) signal relative to rest—was not significantly different between young and older women during exercise in the current study (Figure 2). This finding does not likely reflect an augmentation of capillary recruitment with age in women (Coggan *et al.*, 1992; Fuglevand & Segal, 1997). Rather, the preserved microvascular blood volume observed in older women during the current study may be unique to knee extensor exercise, when the increase in cardiac output approximately doubles the increase in leg blood flow (Magnusson *et al.*, 1997), femoral venous oxygen content is still quite high even during intense exercise (Roach *et al.*, 1999) and there is an extremely high flow-to-quadriceps muscle mass ratio (Richardson *et al.*, 1993). Therefore, the increase in leg blood flow during higher-intensity single-knee extensor exercise in young women (greater even than that observed in young men; (Parker *et al.*, 2007b)) may exceed the metabolic demand of the working muscle, reflecting a "hyperperfused" condition (Rowell *et al.*, 1986) in which moderate reductions in leg blood in older women do not significantly influence capillary blood volume and consequently oxygen exchange. However, we would caution over-interpretation of microvascular blood volume given that the total hemoglobin signal derived by NIRS is influenced by numerous factors, including local muscle blood flow, the muscle pump, vasodilation, hemoconcentration, and capillary recruitment (DeLorey *et al.*, 2003).

One final possible explanation for the observed oxygen extraction responses in older women is that their reduced femoral blood flow was solely attributable to their ~15% reduction in quadriceps muscle mass, although we did not find this to be the case previously (Parker *et al.*, 2007c). We did not have the statistical power to fully examine the influence of quadriceps muscle mass in older women on leg blood flow and oxygen extraction given the smaller sample size utilized (i.e., age differences in leg blood flow normalized to quadriceps muscle were all non-significant due to low statistical power affected by increased variance associated with normalization). However, matching five young to five older women for quadriceps muscle (~1.84 kg) did not alter the observed age differences in leg blood flow and vascular conductance or microvascular oxygenation. Thus, the age difference in muscle mass does not seem a likely explanation for the current findings regarding estimated local oxygen extraction.

Conclusions

Use of near infrared spectroscopy to non-invasively assess muscle microvascular responses in the vastus lateralis during higher intensity knee extensor exercise in young vs. older women did not provide evidence that older women augment oxygen extraction to compensate for the reduced leg blood flow and vascular conductance measured in the femoral artery with Doppler ultrasound. While there are methodological limitations associated with NIRS-based estimates of oxygen extraction, the current study suggests that there may be metabolic and/or microvascular adaptations that result in the relatively well-preserved capacity for knee extensor exercise despite blunted conduit dilatory responses in older women.

Acknowledgements

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Table **7-1**: Subject Characteristics. Data are expressed as group means ± S.E.M for young and older women. * denotes significant ($p<0.05$) difference between young and older subjects. [1]Percentiles defined by age- and sex-specific normative values (ACSM, 2006).

	Young	Older
Sample Size	9	13
Age (yrs)	25 ± 1	67 ± 1*
Resting SBP (mmHg)	107 ± 5	123 ± 3*
Resting DBP (mmHg)	56 ± 1	59 ± 2
BMI ($kg \cdot m^{-2}$)	22.0 ± 1.0	24.1 ± 0.6
LDL Cholesterol ($mmol \cdot L^{-1}$)	2.5 ± 0.2	3.1 ± 0.1*
Triglycerides ($mmol \cdot L^{-1}$)	0.7 ± 0.1	0.9 ± 0.1
Quad Muscle (kg)	2.0 ± 0.1	1.7 ± 0.1*
$\dot{V}O_{2max}$ ($mL \cdot kg^{-1} \cdot min^{-1}$)	38.2 ± 1.6	24.6 ± 0.7*
$\dot{V}O_{2max}$ (Percentile)[1]	55 ± 9	40 ± 3
Resting FBF ($mL \cdot min^{-1}$)	199 ± 37	114 ± 12*
Resting FVC ($mL \cdot min^{-1} \cdot mmHg^{-1}$)	2.7 ± 0.4	1.4 ± 0.2*
Femoral Diameter (mm)	7.2 ± 0.2	7.1 ± 0.2

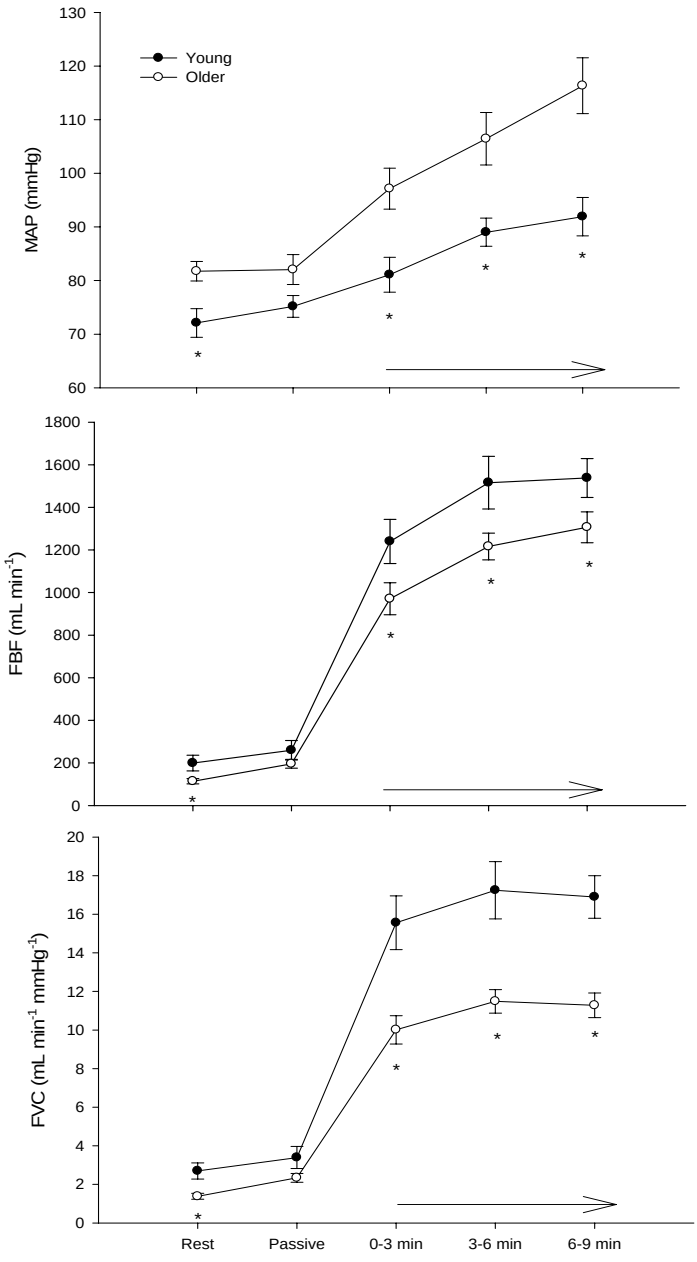

Figure **7-1**: Mean arterial pressure (MAP; top), femoral blood flow (FBF; middle), and femoral vascular conductance (FVC; bottom) expressed as group means ± S.E.M. at rest, passive exercise, and loaded kicking exercise in 9 young and 13 older women. * indicates significant ($p < 0.05$) difference between young and older women. From 6-9 minutes of exercise, one young and one older woman dropped out of the sample due to fatigue. Arrows indicate the onset of active kicking.

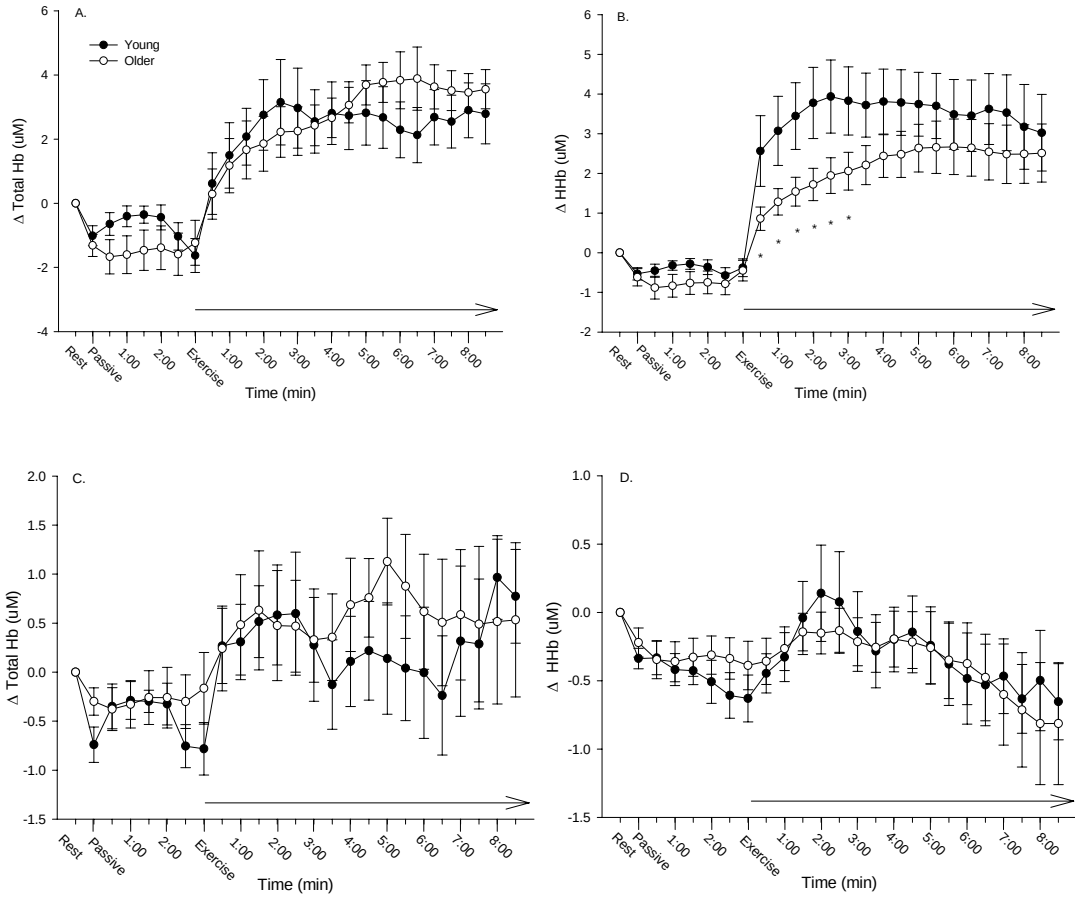

Figure **7-2**: Microvascular blood volume (Δ total Hb, or O_2Hb + HHb) and oxygen extraction (Δ HHb) in the active (kicking; 7-2a and b) and inactive (nonkicking; 7-2c and d) vastus lateralis expressed as group means ± S.E.M. averaged by thirty second intervals at rest, during passive exercise, and loaded kicking exercise in 9 young and 13 older women. * indicates significant ($p < 0.05$) difference between young and older women. From 6-9 minutes of exercise, one young and one older woman dropped out of the sample due to fatigue. Arrows indicate the onset of active kicking.

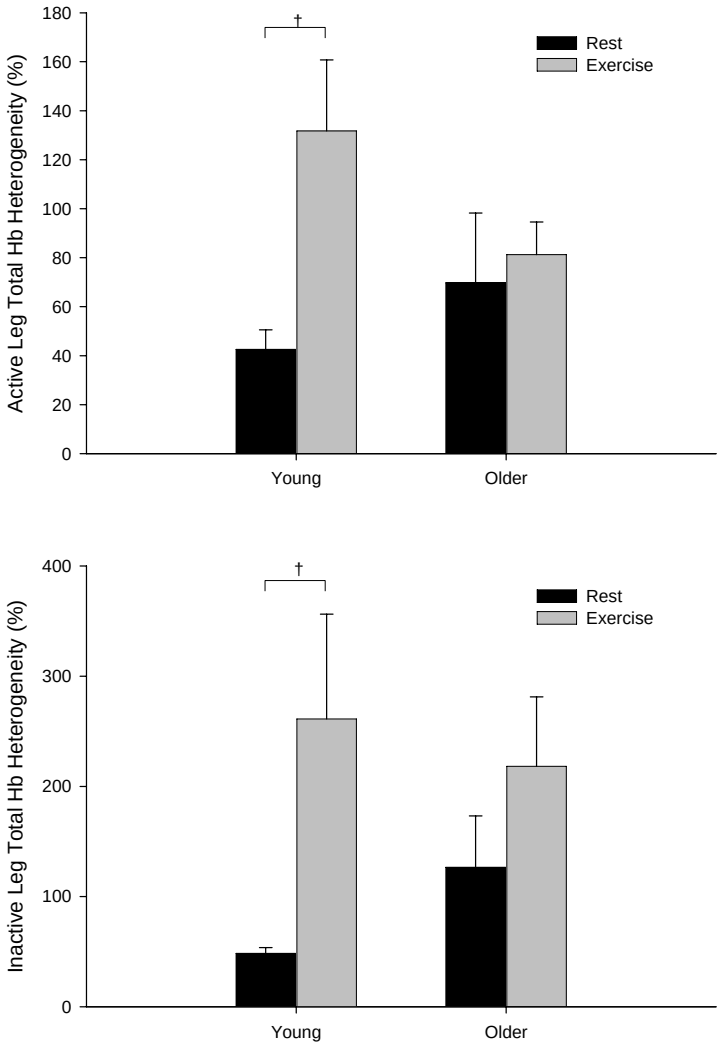

Figure **7-3**: Capillary blood volume heterogeneity expressed as group means ± S.E.M. at rest and during exercise in 9 young and 13 older women in the active (top) and inactive (bottom) leg. † indicates significant ($p < 0.05$) difference between rest and exercise within an age group. There were no age-group differences for comparisons at rest or during exercise.

Chapter 8

CONCLUSIONS AND FUTURE DIRECTIONS

This collection of studies investigated the influence of age and/or sex on local vasodilation through two different models: a non-exercising lower-extremity model (FMD), and an exercising knee extensor model. Collectively, the findings are as follows: 1) there are significant alterations in lower extremity vasodilation with age in women, 2) these alterations could underlie the dramatically reduced leg blood flow and leg vascular conductance observed in older women during small muscle mass dynamic leg exercise that is not apparent in older men, and 3) older women do not appear to compensate for reduced leg blood flow during sustained knee extensor exercise with augmented quadriceps oxygen extraction, suggesting that they accomplish the work with either a lower leg $\dot{V}O_2$ or other adaptive compensations.

Local Alterations in Leg Vasodilatory Responsiveness with Age in Women

Previous findings indicated no evidence of an age-related reduction in leg vasodilatory responsiveness in either the resistance vasculature (Newcomer *et al.*, 2005) or conduit artery (Wray *et al.*, 2006) of men. By contrast, investigations of calf and popliteal vasodilation in response to five minutes of distal cuff occlusion provided evidence of reduced vasodilation in older women. For example, in the first study, popliteal artery FMD, normalized to the peak shear stimulus, was blunted with age in women. Interestingly, the peak shear stimulus, representative of the ischemically-mediated dilatory response of the downstream resistance vasculature, was not different between young and older women, although data have emerged since then suggesting that

the shear stimulus for FMD is more closely approximated by measuring the area-under-the-curve shear response until the time of peak conduit dilation (Pyke *et al.*, 2004; Pyke & Tschakovsky, 2005). Indeed, in the second study, where popliteal FMD was measured before and during ice water stimulation of the sympathetic nervous system, the shear stimulus (estimated as the 45 second area-under-the-curve shear response) was reduced with age in older women. These data revealed a blunted conduit artery response to ischemia with age in women, the magnitude of which paralleled the reduced response to nitroglycerin (a smooth muscle NO donor). It is attractive to assume a causal link between blunted FMD and reduced response to NO in the smooth muscle, given that the assumption is that FMD evokes NO-dependent dilation (Joannides *et al.*, 1995; Doshi *et al.*, 2001). However, the vasodilators underlying FMD in the leg, in women, and in aged humans have not been elucidated and thus reduced popliteal FMD in older women cannot be attributed solely to the reduced smooth muscle response to NO. While the role of conduit artery dilation in vasoregulatory control of exercise hyperemia is not clear (Jasperse & Laughlin, 1997; Rådegran & Saltin, 2000; Eskurza *et al.*, 2001), these data provide evidence that there is reduced vasodilation in the leg vasculature in women in a non-exercising model. These results further suggest that the observed heterogeneity in vasodilation with respect to limbs (arm vs. leg) and aging (Newcomer *et al.*, 2005; Thijssen *et al.*, 2006; Wray *et al.*, 2006; Nishiyama *et al.*, 2007) may also be sex-specific.

The second local alteration in vasodilation investigated in women was the metabolic inhibition of increased sympathetic outflow (i.e., sympatholysis) evoked by pairing the vasodilatory response to cuff ischemia in both the resistance vasculature (post-ischemic popliteal conductance) and conduit artery (popliteal FMD) in young and older healthy subjects. Superimposing the cold pressor test on peak popliteal conductance yielded a similar reduction in peak popliteal conductance in young and older women despite demonstrated reductions in the resting vasoconstrictor response and muscle sympathetic nerve activity to cold pressor

stimulation in older women. This observation, paired with the finding that the reduction in peak popliteal conductance was greater in older women matched to young women for resting adrenergic sensitivity, provided evidence for a reduction in sympatholysis with age in the lower extremity in women. While Fadel attributed findings in the forearm in older women to an estrogen-dependent mechanism potentially mediating nitric oxide production (Fadel *et al.*, 2003; Fadel *et al.*, 2004), discrepancy in the literature involving the role of nitric oxide in sympatholysis (Dinenno & Joyner, 2003; Rosenmeier *et al.*, 2003; Buckwalter *et al.*, 2004) as well as the paucity of knowledge on dilatory pathways mediating reactive hyperemia in the leg preclude definitively explaining the reduced sympatholysis observed in older women. In addition, the influence of cold pressor stimulation on popliteal artery FMD in young vs. older women differed in that cold pressor stimulation evoked alterations in the shear stimulus following cuff release as well as the popliteal artery dilation observed during and following occlusion in young but not older women. Although specific mechanisms for these observations cannot be elucidated, the diminished popliteal response to a combination of two opposing stimuli in older women could be attributable to alterations in interactions between dilator and constrictor pathways (Faber & Meininger, 1990; Sullivan & Davison, 2001), smooth muscle responsiveness, and/or general stiffening of the popliteal artery (Debasso *et al.*, 2004). Collectively, results from these first two studies provided evidence of differences in vasodilation in the leg in aged women that a) extended the growing body of literature regarding sex-specific vasodilatory responsiveness in young and older humans, and b) provided further support for the premise that the observed reduction in leg blood flow and leg vascular conductance in older women during two-leg cycling may be influenced by peripheral alterations in vasodilation.

Sex Differences in Leg Hemodynamic Responses to Exercise in Young Humans

There is an emerging body of work suggesting that young women exhibit greater vasodilator and blunted vasoconstrictor responses to stimuli than their male counterparts, and the results from the third study are in agreement with these general findings. Across the higher range of work rates during knee extensor exercise, young women exhibited lower mean arterial pressure, higher leg blood flow, and higher leg vascular conductance responses to exercise than men. There were no overt differences in the mechanics of knee extensor exercise (duty cycle, fatigue, accessory muscle recruitment) between men and women that could explain these findings. These sex differences do not appear to be attributable to the lower estimated quadriceps muscle mass and/or reduced hemoglobin concentrations in women, as estimated oxygen delivery per kilogram muscle was still higher in young women than men at all workloads greater than 10W (Figure 8-1; bottom). Given the close matching of muscle blood flow to metabolic demand of the working muscle (Andersen & Saltin, 1985) and the relation between oxygen uptake and work (Richardson *et al.*, 1993), differences in the metabolic capacity of the working muscle between men and women (Simoneau *et al.*, 1985; Hargreaves *et al.*, 1998) may underlie the greater oxygen delivery occurring per kilogram muscle in young women.

The finding that leg vascular conductance responses to dynamic exercise are augmented in young women is in line with findings in rats (Rogers & Sheriff, 2004) and a very recent investigation in the forearm (Gonzales *et al.*, 2007) in humans. While previous mechanistic investigations regarding factors that mediate sex differences in vasodilation in animal models have almost uniformly focused on estrogenic influences, providing strong evidence for a potentiating role of estrogen on vasoregulatory factors (Fulton & Stallone, 2002; McKee *et al.*, 2003; Rogers & Sheriff, 2004), it cannot be determined from the current study whether the chronic and cyclical influence of female sex hormones underlies the observed sex differences in

the leg vasodilatory responses to exercise. There may be other characteristics that distinguish young women from men—greater beta-receptor density in the peripheral vasculature (Kneale *et al.*, 2000), greater nitric oxide biosynthesis (Forte *et al.*, 1998), chronic influences of other hormones (Morrison & Pickford, 1971; Yoshioka *et al.*, 2007)—that underlie the observed sex differences in vasodilation.

Age By Sex Interactions in Leg Hemodynamic Responses to Exercise in Humans

Despite lower leg blood flow and vascular conductance at rest and during unloaded passive and active knee extension, older men exhibited relatively preserved leg vasodilatory responses to exercise relative to young men, suggesting that the regulation of hyperemia is preserved or even augmented with age (i.e., slope of the hyperemic and vasodilatory responses from 0-24W were greater in older men). This latter finding is in agreement with previous observations during small muscle mass dynamic exercise (Jasperse *et al.*, 1994; Lawrenson *et al.*, 2003). By contrast, older women exhibited similarly low leg blood flow and leg vascular conductance at rest and during unloaded passive and active knee extension relative to young women, but these lower hemodynamics persisted at all ensuing knee extension work rates. In addition, the slopes of the hyperemic and vasodilatory responses were lower in older women. The statistical test for an age by sex interaction (comparing the four groups at relative, or % of maximal work rates) was also significant. Taking into account age and sex differences in hemoglobin and quadriceps muscle by expressing data as the estimated oxygen delivery per kg muscle (Figure 8-2) yielded similar findings: older women exhibited lower estimated oxygen delivery per kg muscle than young women at almost all knee extensor work rates, whereas older men demonstrated similar estimated oxygen delivery per kg muscle as young men at all but the two highest work rates, where muscle perfusion was greater in older men. These findings suggest

that the influence of age on the local mechanisms underlying the regulation of leg vasodilation is sex-specific, with older women exhibiting unique and significant alterations with age in dilator and/or constrictor vasoregulatory responses. Given the significant augmentation in blood pressure during exercise in older women relative to young women as well as previous evidence regarding structural limitations to vasodilation (Ridout *et al.*, 2005) and sympatholysis (Study 2) in the leg vasculature, the potential mechanisms underlying the observed-sex differences may well be multiple and interactive. Finally, evidence in animal and isolated vessel models as well as hormone therapy investigations suggests that menopause and the loss of female sex hormones may exacerbate the aging process in women (Taddei *et al.*, 1996; Gonzales *et al.*, 2001; Moreau *et al.*, 2003; Kirwan *et al.*, 2004), although this explanation could not be investigated in the present study population. It should be noted, however, that investigating leg hemodynamic responses to the knee extensor exercise protocol in three women with a continuous history of hormone therapy since menopause did not indicate a beneficial effect of hormone therapy (Figure 8-3). This is in contrast to results from a previous investigation finding that continuous hormone therapy was associated with preserved intima-medial thickness and smooth muscle dilatory responsiveness in the leg in older women (Parker *et al.*, 2007d).

Isolated Knee Extensor Exercise: Assumptions of the Model Relevant to Aging and Sex Differences

For the studies involving sex and age differences in local leg vasodilatory responses to dynamic exercise, the purpose of using the single-knee extension exercise model was to isolate exercise-induced vascular responses to a well-defined muscle group and to reduce the potential for age- or sex-related limitations in cardiac reserve and consequent counterregulatory adjustments. Although cardiac output was not directly measured, there is no compelling reason to

suspect that cardiac reserve was significantly challenged in any of these four groups of healthy subjects. This is suggested by the fact that 1) heart rate increases were moderate and similar among groups (< 40 beats/min), 2) stroke volume was likely maximized by testing subjects in the supine posture (Rowell, 1993), and 3) previous studies involving cardiac limited older adults (e.g., heart failure) indicate that the rise in cardiac output during single-knee extensor exercise still approximately doubles the rise in total active leg blood flow (Magnusson *et al.*, 1997). Evidence against a major central limitation to knee extensor exercise is further provided by the small age group differences in peak power output in Study 4 (less than 12 and 7% in women and men), which were much lower than those observed in previous cycle ergometer studies (i.e., 30 and 20% in women and men; (Proctor *et al.*, 2004b)). A second potentially confounding variable of this model is group differences in total or active muscle (i.e., quadriceps) mass. Attempts to minimize group differences in active and inactive muscle recruitment involved providing subjects with adequate familiarization to the exercise protocol (i.e., 2-4 pre-test sessions per subject) and providing constant verbal feedback regarding kick cadence and inactive leg/upper body movement. Accordingly, during submaximal (<70-80% of peak) work rates, minimal hamstring recruitment was observed, although older women exhibited statistically higher hamstring EMG activity than young subjects at all relative work rates. However, matching older and younger women for hamstring recruitment did not abolish or influence observed age differences. In addition, there were no obvious age by sex interactions in the mechanical influences of muscle contraction on muscle perfusion as reflected by a) a similar time spent in the contraction or relaxation phases of the duty cycle and b) the relative proportion of flow increase that occurred during relaxation vs. contraction (~100% greater during relaxation than contraction) (Osada & Rådegran, 2006). Therefore, the use of single-knee extensor exercise to evoke a non-centrally limited exercise hyperemia primarily influenced by the metabolic demand of the working quadriceps was valid in the current series of studies.

The Influence of Fitness on Leg Vasodilatory Responses to Dynamic Exercise

As mentioned previously, there are conflicting results in the literature regarding the influence of age on leg hemodynamic responses to large muscle mass cycling exercise, with findings from studies regarding normally active men (Proctor *et al.*, 2003b) differing from those involving either very sedentary (Poole *et al.*, 1992) or highly trained (Proctor *et al.*, 1998) men. In addition, aerobic training also appears to differentially influence improvements in peak calf conductance in older men and women as modulated through weight loss (men) and adipose tissue loss (women) (Martin *et al.*, 1990). In agreement with both of these lines of evidence, aerobic fitness ($\dot{V}O_{2max}$) differentiated leg hemodynamic responses to exercise in all subject groups except older women in Study 4. For example, modeling $\dot{V}O_{2max}$ as a covariate in the blood flow and conductance responses to increasing knee extension work rates in each subject group revealed that fitness was a significant covariate in young women ($p < 0.01$ for both comparisons) and a marginal covariate in older ($p = 0.09$ for both comparisons) and younger ($p = 0.02$ and 0.08, respectively) men, but a highly insignificant covariate in older women ($p = 0.94$ and 0.87, respectively). This analysis provides insight into the differing findings in the literature with respect to age and leg hyperemia in men, and also suggests that aging-related changes in leg hyperemia in women may be immutable to the modifying influence of fitness.

The Influence of Additional Covariates on Leg Vasodilatory Responses to Dynamic Exercise in Women

To determine whether the significant influence of age on leg blood flow and vascular conductance in women was modified through any baseline characteristics which would yield insight into particular mechanisms, the following variables were modeled as covariates in the repeated measures model used to compare leg vascular conductance with respect to age in women

at each knee extensor work rate: hemoglobin, triglycerides, cholesterol (total, HDL and LDL), % body fat, pulse wave velocity (carotid-femoral, femoral-ankle, and brachial-ankle), resting blood pressure (systolic, diastolic, and MAP), muscle mass (thigh and quadriceps), height, weight and BMI. In addition, through a collaborative effort of the author and Martha Kalasky, an honor's student in Dr. Proctor's laboratory, accelerometer-derived measures of physical activity (activity calories/day and moderate-vigorous activity calories/day or bouts/day) were also investigated (data were collected on 10 young and 12 older women who participated in the knee extensor study). Hemoglobin, triglycerides, cholesterol, body fat, carotid-femoral pulse wave velocity, and resting systolic pressure were not significant covariates in the model. BMI, femoral-ankle pulse wave velocity, resting diastolic pressure, and resting MAP were significant covariates ($p < 0.05$) with no age or work rate interactions in the relation to leg vascular conductance such that lower resting values for each variable were associated with higher femoral vascular conductance responses to knee extensor exercise. The variables associated with body size and muscle—height, weight, thigh muscle mass, and quadriceps muscle mass—were all significant ($p < 0.05$) covariates in the model with specificity to work rate (a significant variable*work rate interaction was detected for each measure). In all cases, a smaller baseline body size/muscle mass indicator variable was associated with greater femoral vascular conductance at the highest knee extensor work rates. In addition, the magnitude of the covariate effect was driven primarily by the young women (when compared separately in young and older women, all four variables were significant covariates only in young women), although the only significant variable*work rate*age interaction detected statistically ($p = 0.04$) was with respect to quadriceps muscle mass. In addition, the interaction of brachial-ankle pulse wave velocity*age*work rate was significant ($p = 0.03$) such that in young women only, higher resting pulse wave velocity was associated with lower femoral vascular conductance, with the magnitude of the effect increasing with increasing work rate. Finally, as described above with respect to $\dot{V}O_{2max}$ and femoral hemodynamics during

exercise, accelerometer-derived indices of physical activity (activity calories/day, and moderate-vigorous physical activity measured in either minutes/day or bouts/day) demonstrated an interaction with age ($p < 0.05$) such that daily physical activity was a significant and positive covariate in young ($p < 0.01$ for all three analyses) but not older ($p = 0.19$, 0.30, and 0.28, respectively) women. In every covariate analysis, however, the influence of age and the age*work rate interaction with respect to femoral vascular conductance during knee extensor exercise remained highly significant ($p < 0.01$ for every analysis), indicating that the primary effect of aging was not mediated solely through any of these investigated variables. Conclusions from covariate analysis, therefore, while suggesting interesting hypotheses (e.g., larger quadriceps muscle mass/body size may be related to a contraction impedance to flow at the highest work rates in young women, daily physical activity may modify leg vasodilatory responses to exercise in young women) do not provide additional information on mechanisms underlying the age-related vasodilatory deficit in women except to suggest again that aging-related processes in leg exercise hyperemia in women may be largely independent of other lifestyle/physiological characteristics such as fitness and physical activity.

The Influence of Age on Vasodilatory Responses to Large vs. Small Muscle Mass Exercise

The current findings in men are qualitatively similar to results observed during two-leg cycling exercise (i.e., preserved leg blood flow and leg vascular conductance), although the smaller range of work rates studied previously did not allow for measurement of the initial responses to exercise for direct comparison to the present study (Proctor *et al.*, 2003b). It is of note, however, that while findings in women were also qualitatively similar to results observed previously, one difference with respect to women is the finding that leg blood flow during knee extensor exercise was reduced at all work rates rather than only at the higher work rates observed

during cycling exercise (Proctor *et al.*, 2003a). Although the lowest work rates used in the cycling study were not directly comparable to the resting/lowest work rates used in the knee extensor study, this finding suggests that there are persistent local mechanisms underlying the blunted vasodilatory response in women that may be masked during large muscle mass dynamic exercise where greater increases in cardiac output may be sufficient to augment leg blood flow at the lower cycling work rates. Cumulatively, however, these results in women suggest that the model used to study vasodilation—submaximal cycling vs. single-knee extensor exercise—does not incorporate unique mechanical or metabolic confounding factors that limit interpretations to a specific model; rather the blunted hyperemic and vasodilatory responses unique to older women are observed during both large and small muscle mass exercise.

Age and Microvascular Responses to Exercise in Women

Evidence suggests that in circumstances where leg blood flow is reduced, healthy humans maintain leg $\dot{V}O_2$ with augmented oxygen extraction (Lundgren *et al.*, 1988; Kalliokoski *et al.*, 2001; Gonzalez-Alonso & Calbet, 2003). While Proctor et al. did not previously observe this adaption in aged women during cycling exercise (Proctor *et al.*, 2003a), where peak power output is significantly reduced with age (Proctor *et al.*, 2004b), the seeming maintenance of knee extensor work capacity in older women indicated that augmentation of oxygen extraction might be more likely to occur during this model of exercise. However, while once again older women performed similarly during a sustained, higher-intensity bout of knee extensor exercise despite reduced femoral blood flow and vascular conductance (Study 5), oxygen extraction was not higher in older women. In fact, there was a delayed rise in the increase in oxygen extraction in the first three minutes of exercise in older women, although there were no age differences after this point. Surprisingly, though, microvascular blood volume was preserved in older women,

despite the dramatically lower conduit inflow. While there is no evidence to support an increased capillary recruitment in older women, given the age-related reductions in leg muscle capillarity (Coggan *et al.*, 1992; Proctor *et al.*, 1995), there is evidence suggesting that knee extensor exercise may be a "hyperperfused" condition (particularly in light of the high perfusion observed in young women in Study #3), given the high femoral venous oxygen content (Roach *et al.*, 1999), relatively minor impact of anemia (Gonzalez-Alonso *et al.*, 2006), and increase in leg blood flow but not oxygen extraction with adenosine infusion (Barden *et al.*, 2007) during knee extensor exercise. Thus, reductions in leg blood flow during knee extensor exercise in older women may not significantly impact microvascular blood volume or oxygen extraction and consequently exercise capacity. However, this hypothesis should be interpreted with caution, given the limitations of measuring oxygen extraction with NIRS (e.g., contribution and/or influence of adipose tissue, assumed photon path length, relative rather than absolute measurements, and unknown structure and histochemical composition of the microvasculature). Further research involving more direct measurement of oxygen extraction, multiple work rates, and/or different exercise modalities will better address the consequences of reduced leg blood flow during dynamic exercise in older women.

Summary

Cumulatively, the findings from this series of studies suggest that there is a substantial influence of age on peripheral vasodilation in women that persists after factors such as cardiac performance, leg muscle mass, hemoglobin, and physical fitness are taken into account and/or minimized. The collective observations of reduced popliteal artery FMD, smooth muscle dilation to an NO-donor, and metabolic modulation of a sympathetic stimulus (Studies 1 and 2), lower peak vascular conductance following 10 minutes of ischemia (Ridout *et al.*, 2005), augmented

blood pressure and blunted hyperemic responses to single-knee extensor exercise (Study 4), and insignificant modulation of leg hemodynamic responses by myriad age-associated covariates in older women provide evidence of a reduction in leg vasodilatory responsiveness attributable to local alterations in dilation and/or constriction. However, the functional implication of reduced vasodilation on exercise capacity remains unclear, as older women are able to exercise at similar absolute knee extensor work rates as young women for a sustained duration of time without augmented oxygen extraction or diminished microvascular blood volume (Study 5). Finally, the differing leg hemodynamic responses to knee extensor exercise in young and older men vs. women suggest that both the peripheral pathways and mechanisms underlying control of leg hyperemia and vasodilation in young adults as well as the influence of aging on these pathways and mechanisms are sex-specific in healthy humans.

Future Directions

Significant new questions were generated following this series of thesis projects. They are described in detail below.

Pathways Underlying Flow-Mediated Dilation and Relevance of the Model

What are the dilator/constrictor pathways underlying FMD in the arm and leg of women, and how do they change with age? Is FMD relevant to exercise hyperemia, or is the utility of the model more appropriate for clinical pathologies and/or studying alterations to and manipulations of individual dilator and constrictor pathways? To date, the pathways evoking reactive hyperemia and FMD remain primarily unknown in humans (Tagawa *et al.*, 1994; Engelke *et al.*, 1996; Duffy *et al.*, 1999). While evidence in young males demonstrates that blocking nitric oxide

synthesis abolishes brachial and radial artery flow-mediated dilation (Joannides *et al.*, 1995; Doshi *et al.*, 2001), this research has not been replicated in populations that differ by age and sex and in the lower limb. Given that blocking nitric oxide synthesis also evokes dilatory compensation through other pathways (Bellien *et al.*, 2007) in a manner that is shown to be hormone- and sex-specific (Huang *et al.*, 2001; Wu *et al.*, 2001), it is likely that FMD is mediated in part by additional pathways in aged adults and women. Thus, comprehensive understanding of the release of vasoactive metabolites in response to ischemia in both the resistance and conduit vasculature of young and older men and women is necessary to fully utilize FMD as a model with which to study the influence of age and sex on specific dilator and constrictor pathways. Moreover, while there is significant evidence to suggest that FMD has clinical relevance to many pathologies (Anderson *et al.*, 1995; Angerer *et al.*, 2001; Spacil *et al.*, 2002), the relevance of reactive hyperemia and conduit artery dilation to exercise is unclear. It is thought that the conduit arteries do not regulate exercise hyperemia (Jasperse & Laughlin, 1997; Rådegran & Saltin, 2000); however, the significant conduit dilation observed in young but not older women in Study 4 may indicate FMD plays a functional role during exercise.

Sex-Specific Aging and Leg Vascular Responses to Exercise

With respect to the sex-specific nature of aging on the vascular responses to dynamic exercise, is the effect of age in women truly maladaptive, or is it exaggerated in part by the high perfusion observed in young women? Given that the increase in leg blood flow during exercise serves to match oxygen delivery to oxygen demand, the most insightful way to answer this question is to consider hyperemic responses to graded knee extensor exercise expressed as estimated oxygen delivery and normalized to muscle mass to take into account age and sex differences in hemoglobin (i.e., arterial oxygen content) and active muscle. This analysis

(Figures 8-1 and 8-2) demonstrates effectively that the influence of age on leg oxygen delivery is different in men and women. However, what is unclear is whether the effect of age in women is attributable to a "higher" oxygen delivery per kg muscle in young women relative to young men (Figure 8-1) or an "attenuated" oxygen delivery per kg muscle in older women relative to young women (Figure 8-2) given that the actual metabolic demand of the working muscle in the four subject groups is not known. For example, there are known age and sex differences in metabolism during ankle dorsiflexion exercise, with greater reliance on oxidative metabolism observed in older vs. young adults and women vs. men (Kent-Braun *et al.*, 2002; Lanza *et al.*, 2007). By contrast, relative to young rats, exercising hindlimb blood flow is redistributed in older rats from highly oxidative to highly glycolytic muscles (Musch *et al.*, 2004). To date, there are not published data on muscle metabolic responses to graded quadriceps exercise in young and older men and women. Examining the relation between leg $\dot{V}O_2$ and work during graded exercise in the leg in young and older men and women can provide an estimate of the increased metabolism attributable to exercise but is confounded by the dependence between leg blood flow and leg $\dot{V}O_2$. A more direct approach in humans utilizes magnetic resonance spectroscopy to determine the rates of oxidative and glycolytic flux as well as phosphocreatine hydrolysis in humans *in vivo* (Lanza *et al.*, 2007), since the alternative approach, obtaining muscle biopsies to measure enzymes associated with energetic pathways, does not provide information on the graded metabolic response to exercise (Short *et al.*, 2005). Comparing the rates of ATP synthesis vs. oxygen delivery in all four subject groups will answer the question of whether the relation between oxygen demand and oxygen supply differs with age and sex, thus contributing to age and sex differences in hyperemic responses to exercise.

Additional mechanisms underlying the sex-specific effect of age on leg vasodilation in humans could involve the influence of male and/or female sex hormones and the menopausal transition, as well as other factors, such as adrenergic receptor distribution, body fat composition,

and age-related loss of muscle mass. Following women across the menopausal transition, investigating women immediately post-menopause or post-hysterectomy who use hormone therapy, using a hormone control/suppression/replacement model in young women to manipulate female sex hormones (Stachenfeld & Taylor, 2005), and/or manipulating hormones in animal models may be sufficient approaches with which to examine the influence of sex hormones on age by sex interactions in leg hemodynamic responses to exercise. Alternative approaches could include matching older men and women for body composition or comparing vasoconstrictor responsiveness in adipose tissue and the muscle vasculature, given that age is associated with reduced adrenergic receptor sensitivity in adipose tissue in rats (Ramsey *et al.*, 2007) and leg muscle vasculature in men (Smith *et al.*, 2007) with ensuing possible consequences for the successful direction of blood flow to active muscle. As a final note on this topic, it is worth mentioning that in Study 2, estimated sympathetic transduction (the change in calf vascular resistance relative to the change in MSNA) was not reduced in older women (unpublished data), as has been shown in men in the forearm (Davy *et al.*, 1998); this finding may have relevance to sympathetic influences on leg hyperemia in older men and women.

Regulation of Leg Hemodynamic Responses to Exercise in Older Men

With respect to normally active older men, what are the regulatory mechanisms underlying the preserved leg hemodynamic responses to exercise? What adaptations (i.e., vasoconstriction of inactive vasculature, vasodilation of active vasculature) allow older men to successfully distribute blood flow to the working muscle? During cycling exercise, older men direct less blood from the renal and splanchnic circulations to the skin circulation (Kenney & Ho, 1995); on the other hand, vasoconstriction of the forearm is greater (Taylor *et al.*, 1992). However, the importance of efficiently redistributing blood from inactive circulations to the leg

during single-knee extensor exercise, when adding additional volume of active muscle (i.e., arm and contralateral limb exercise) does not reduce leg blood flow in the original kicking leg given the substantial cardiac reserve (Richardson *et al.*, 1995), is less certain. Therefore, it is not clear to what extent older men achieve preserved leg hemodynamics through redistribution of cardiac output. Alternatively, adrenergic sensitivity in the leg is diminished with age in men (Smith *et al.*, 2007). Thus, it is possible that metabolic inhibition of the sympathetic outflow associated with exercise is more effective with age in men due to diminished adrenergic sensitivity, although the MSNA responses to knee extensor exercise measured in young men (Saito & Mano, 1991; Ray, 1993) do not support the concept that there are large increases in sympathetic outflow with this exercise modality and sympatholysis is reduced with age in the forearm in men (Dinenno *et al.*, 2005). Finally, given that the slope of the hyperemic response to knee extensor exercise appears preserved with age (Lawrenson *et al.*, 2003), Study 4), the similar leg blood flow responses in young vs. older men may also be indicative of relatively well-preserved and/or redundant dilatory pathways with age in men. These questions could be answered by a series of studies that measure cardiac output, regional circulatory responses and muscle sympathetic nerve activity during knee extensor exercise in old vs. younger men and by using blockades of local dilator pathways associated with muscle hyperemia.

Regulation of Leg Hemodynamic Responses to Exercise in Older Women

With respect to normally active older women, what are the regulatory mechanisms underlying the lower leg vasodilatory responses to exercise? In addition to the previously mentioned possibility of an age-associated metabolic difference that may influence leg blood flow, what additional factors (i.e., heightened vasoconstriction, structural alterations in the microvasculature, and/or reduced vasodilation) contribute to the reduced leg vascular

conductance observed in older women? There is evidence for all of these possibilities in the current animal and human literature. A reductionist approach needs to be assumed in order to understand the contribution of each possible mechanism to the lower leg vascular conductance responses observed in older women. For example, reducing sympathetic outflow through neck suction (stimulating baroreceptors) or infusing alpha-adrenergic blockers will provide information about the contribution of augmented sympathetic stimulation to the lower leg vascular conductance responses in older women. Combining this experimental maneuver with infusion of a substance that promotes dilation (e.g., an acute administration of ascorbate or ATP; (Rosenmeier *et al.*, 2004; Moreau *et al.*, 2007)) will provide additional information concerning a potential alteration in dilatory pathways. Moreover, should reducing sympathetic tone and increasing dilation not increase leg vasodilatory responses in older women such that these responses are comparable to those observed in young women, then there is a strong possibility that there is a structural limitation to vasodilation in older women, particularly if administration of vasodilatory substances alone does not augment leg blood flow/vascular conductance in older women to the same extent it does in young women. Additional approaches might involve blocking local vasodilatory pathways (to investigate the contribution of various dilators to exercise hyperemia in the leg in young vs. older women) or augmenting sympathetic outflow in young women (via single leg LBNP).

Consequences of Reduced Leg Hemodynamic Responses to Exercise in Older Women

With respect to the reduced leg blood flow and vascular conductance observed in older women during dynamic exercise, what are the consequences of this observation? In Studies 4 and 5, as well as a longer knee extensor bout conducted in an earlier visit in Study 4, older women exhibited well-preserved ability to perform knee extensor exercise at the same absolute work

rates, to the same peak power output, and for the same duration of time as young women. This finding is surprising given the close matching of muscle blood flow to metabolic demand of the working muscle (Andersen & Saltin, 1985) and the relation between oxygen uptake and work (Richardson *et al.*, 1993), and suggests that, as mentioned previously, changes in muscle metabolism with age in women may explain the reduced leg blood flow observed in older women. Alternatively, preliminary investigation into this topic using NIRS (Study 5) suggested that preserved microvascular blood volume may contribute to the preserved exercise performance in older women, especially in the absence of an observable augmentation of oxygen extraction. However, more direct estimates of microvascular blood volume (e.g., contrast-enhanced ultrasound with albumin microbubbles; (Coggins *et al.*, 2001)) and oxygen extraction (e.g., radial artery and femoral venous catheter) are necessary to support this initial finding.

In the absence of a metabolic/microvascular mechanism underlying changes in leg hemodynamic responses to exercise in older women, an alternative explanation is that exercise performance during single-knee extensor exercise may be well-preserved in older women given the relatively high perfusion of young women observed during single-knee extensor exercise. It is thus possible that the consequences of reduced leg blood flow and vascular conductance are more readily apparent during sustained two-leg exercise and/or repeated bouts of exercise. Challenging cardiac output through exercise in the heat (which does not result in lower leg blood flow in young, healthy humans; (Savard *et al.*, 1988)) or dehydration (which does result in lower leg blood flow in young adults; (Gonzalez-Alonso *et al.*, 1998)) may also reveal a condition in which the reduced leg blood flow in older women becomes functionally limiting with respect to exercise tolerance and capacity. In addition, older women may exercise with greater fatigue, which could be examined with femoral nerve stimulation to assess twitch force after sustained dynamic exercise (Amann *et al.*, 2006; Katayama *et al.*, 2007). Given that older women self-report more functional decline and disability than older men (Morey & Zhu, 2003; Borglin *et al.*,

2005), investigating correlations between physical function (e.g., stair-climbing, gait, balance, and strength tasks relevant to daily independent living) and reduced leg blood flow could unmask additional functional consequences in older women. Finally, implications of the reduced hemodynamic responses to exercise in women could be investigated indirectly by observing exercise responses in populations of women more sedentary, more hypertensive, more obese or with other aging-related pathologies to assess whether reduced exercise hyperemia becomes more limiting with successive decrements in overall health and/or leg blood flow. It also should be noted that there may be indirect consequences of the blunted vasodilatory response to dynamic leg exercise in older women; the exaggerated exercising blood pressure responses observed in older women (Ogawa *et al.*, 1992; Fleg *et al.*, 1995; Proctor *et al.*, 2003a) may be due in part to reduced vasodilation in the active leg vasculature and thus have implications for exercise in older women with hypertension and/or cardiovascular disease.

Finally, will training older women result in an improvement in leg hemodynamic responses to exercise if in fact reduced leg blood flow is maladaptive? Lawrenson et al. found no improvement in leg blood flow and oxygen extraction following eight weeks of knee extensor exercise training in older sedentary men (Lawrenson *et al.*, 2004), Thijssen et al. found that eight weeks of training improved structural and functional characteristics of the femoral artery in older men (Thijssen *et al.*, 2007a), and Martin et al. found that calf reactive hyperemia was significantly improved by training in older men but not women (Martin *et al.*, 1990). With respect to young adults, Proctor et al. noted that young women appeared to show a greater decrease in leg blood flow following high-intensity endurance training, although the sex difference was not significant (Proctor *et al.*, 2001). Moreover, in the porcine model, Laughlin et al. found that exercise training influenced contractile and dilatory responses of isolated femoral and brachial arterial rings in a sex-dependent manner (Laughlin *et al.*, 2001). In light of this evidence, the ability of exercise to affect improvements in leg blood flow, vascular conductance

and leg $\dot{V}O_2$ in older women is unclear. Certainly, evidence to date from the current study regarding physical fitness and physical activity in young vs. older women suggests that the blunted leg hemodynamic responses to exercise in older women are not modulated by either the subject's chronic fitness or daily physical activity. However, a comprehensive training study needs to be conducted to determine whether older women can in fact improve leg vasodilation during more acute exercise interventions and if so, by which mechanisms, to what extent, and to what benefit.

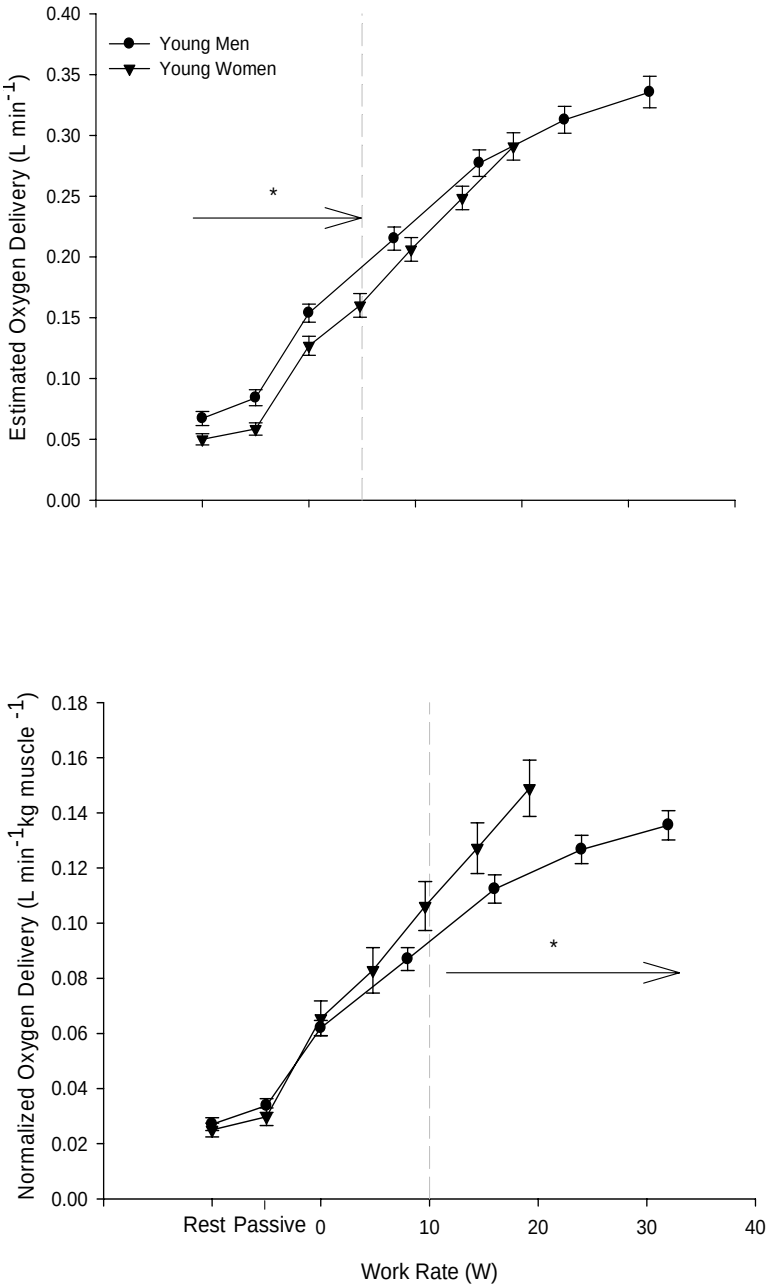

Figure **8-1**. Estimated oxygen delivery (top) and estimated oxygen delivery normalized to estimated quadriceps muscle mass (bottom) in young men and women expressed as group means ± S.E.M. at absolute workloads. * indicates significant ($p < 0.05$) difference between men and women at all workloads indicated by the arrow ending with (top) or starting at (bottom) the dashed line. For men, sample size was n=15 until 24W and n=14 at 32W. For women, sample size was n=16 until 19.2W. Statistical analysis used in these plots has been described in Chapter 5.

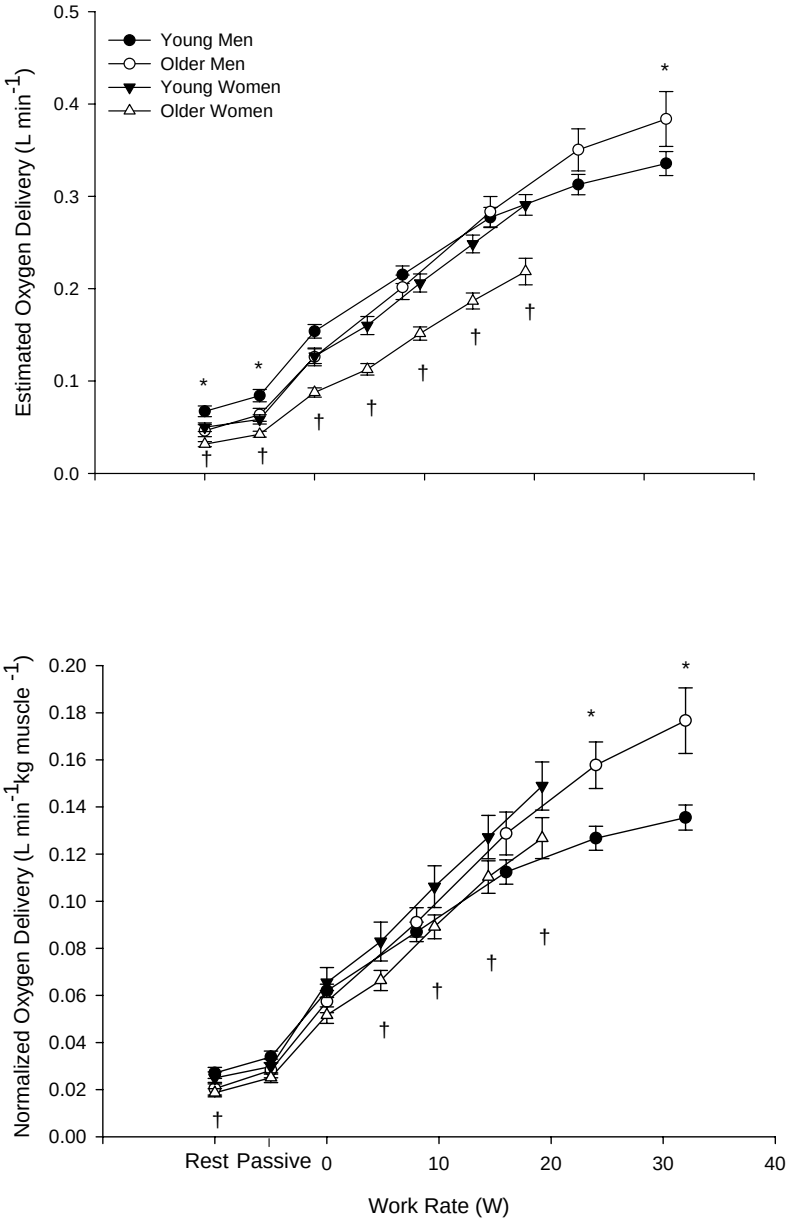

Figure **8-2**: Estimated oxygen delivery (top), and estimated oxygen delivery normalized to estimated quadriceps muscle (bottom) and expressed as group means ± S.E.M. at absolute work rates in young and older men and women. * indicates significant ($p < 0.05$) difference between young and older men. † indicates significant ($p<0.05$) difference between young and older women. For young men, sample size was n=15 until 24W and n=14 at 32W. For older men, sample size was n=13 until 24W and n=12 at 32W. For young women, sample size was n=16 at 19.2W. For older women, sample size was n=18 until 14.4W and n=17 at 19.2W. Statistical analysis used in these plots has been described in Chapter 6.

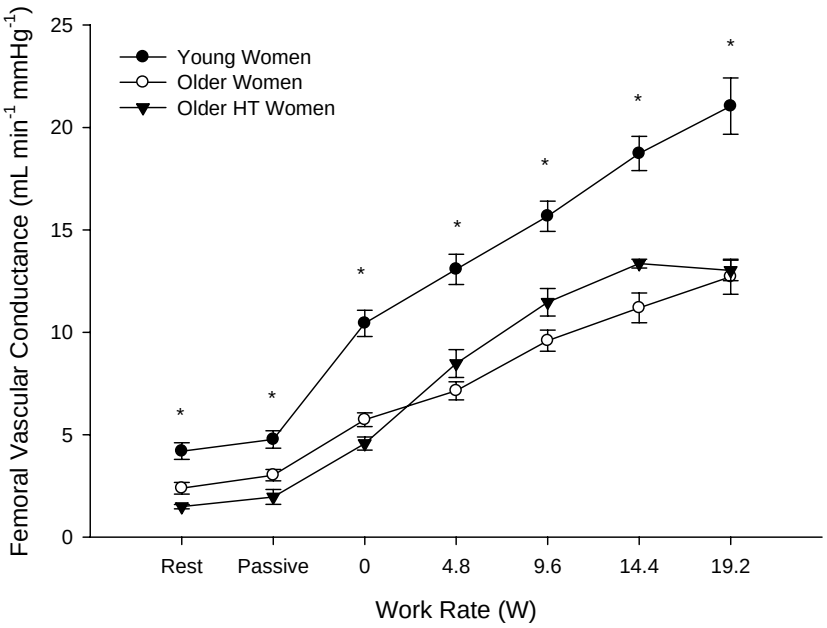

Figure **8-3**: Femoral vascular conductance (FVC) expressed as group means ± S.E.M. at absolute work rates. ***** indicates significant ($p < 0.05$) difference between young women and either group of older women. There were no significant differences between the two groups of older women. Statistical analysis used in these plots has been described in Chapter 6.

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Appendix

INFORMED CONSENTS

Informed Consent for Study 1 (arm vs. leg FMD study):

Informed Consent Documentation For Research Study: The Pennsylvania State University

Study Title: "Influence of Age on Limb Vasodilator Capacity"
IRB # 15122

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This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. David Proctor, Assistant Professor of Kinesiology.

1. Purpose of the Study:
The purpose of this study is to determine if aging impairs blood vessel function in the arms or legs. Volunteers will be healthy women who are taking no medications that significantly alter the function of the heart or blood vessels and who are not currently involved in a vigorous exercise program. Pre-menopausal women cannot currently be taking birth control medication. Post-menopausal volunteers must either a.)not currently be taking any form of hormone replacement therapy (i.e.: for the last 12 months) or b.) must have a recent history of taking estrogen only hormone replacement (i.e.: for at least 12 months.) We are planning to study volunteers ranging from 20 to 80 years of age (14 women per decade).

2. Procedures to be followed: If you agree to be a subject in this research, you will be asked to complete several tests which include a physical examination, laboratory assessments of your cardiovascular health and fitness, body composition (how much fat and muscle you have), handgrip and calf strength, and resting and peak (post-exercise) limb blood flow. First, you will be asked to perform several screening procedures outlined below. If you meet the eligibility requirements, you will then be asked to return for three additional visits. The major purpose of these three visits is to measure the maximal blood flow through the vessels in your forearm (measured at the elbow) and your calf (measured behind your knee), in response to forearm and calf exercise. The procedures for all visits will be explained below.

Screening Procedures:

Medical History Questionnaire: You will be asked to fill out a form about your medical history (i.e. major injuries, illnesses, etc.).

Physical Activity Questionnaire: You will be asked to fill out a form about your daily/weekly physical activity (i.e. walking, running, lifting, yard work, etc.)

Blood and Urine Analysis: You will be asked to provide a small sample of your blood (approximately *2 Tablespoons*) during your screening visit. Blood will be drawn from your arm using a needle. Pre-menopausal women will have a urine pregnancy test done prior to the graded exercise test and DEXA scan. (A positive pregnancy test would exclude you from this study.)

Physical Examination: A clinician at the General Clinical Research Center (GCRC) will review your medical history and conduct a physical examination.

Graded Exercise Test: You will be asked to perform an exercise test on a research stationary bicycle to assess your cardiovascular fitness and to rule out any blood pressure or heart abnormalities. During this test, the tension on the pedals will gradually increase every minute until you reach maximal effort. For those of you 50 years and older, your heart will be continuously monitored via a 12 lead EKG (electrical tracing of your heart's activity). For those of you under 50 years of age, your heart rate will be monitored using a Polar Heart Monitor. Ratings of perceived effort and your blood pressure will be recorded each minute. You will also be asked to wear a mouth piece and a nose clip during this test so that your expired air can be measured. This test will be excluded for those of you who have performed one within the past year for our lab.

Testing Procedures:

Body Composition Test: Your body composition (% fat, muscle, and bone) will be estimated during this study using a Dual-Energy X-Ray Absorptiometry (DEXA) test. This whole body scan requires that you lie flat on a padded table without moving for approximately 10 minutes while an X-ray scanner moves over your body. If you have recently (within the last 6 months) had a DEXA scan while participating in the Coefficient of Variation for DEXA study (# 151), Macronutrient study (# 137), or the Bioenergetics study (# 105), we may be able to use the data from that scan if you provide your permission. If applicable please check one of the following statements.

_____Yes, I give my permission for my DXA scan results to be shared with Dr. Proctor's research team.

____ No, I do not give my permission for my DXA scan results to be shared with Dr. Proctor's research team.

Vascular Profiling: The health of your blood vessels will be assessed using the VP2000. This device measures the blood pressures at your ankles and arms. In addition, it will measure the time it takes for a pulse of blood to travel from your heart to your neck and legs. You will be asked to lie still on a bed with blood pressure cuffs placed on you arms and ankles. Sensors are then applied to your chest, wrists, neck and upper thigh. Measurements are then taken over a period of less than 2 minutes.

Resting Blood Vessel Diameter and Velocity Test: The resting size of a blood vessel in your arm (at your elbow) and leg (behind your knee) will be measured using an Ultrasound probe (pencil-like device) placed firmly against your skin. We will also record the pulse wave velocity of your blood flow at the same time. This procedure requires that you lie flat on a padded table without moving for approximately 10 minutes.

Resting Limb Blood Flow Test: Venous Occlusion Plethysmography (VOP) will be used to determine your resting forearm and calf blood flow. VOP is based on measuring the volume (size) of your limb segment when blood going away from your limb is temporarily stopped, but blood going into your limb continues. The rate of limb swelling is proportional to the rate of blood flow. A pressure cuff (similar to a blood pressure cuff) will be placed around your upper arm and thigh. Similar cuffs will also be placed around your wrist and ankle. These pressure cuffs will be used to stop the flow of blood. A mercury-in-silastic gauge (strain gauge) is placed around the widest portion of the limb segment (forearm and calf). This strain gauge is used to sense the volume change in the limb. This procedure requires that you lie flat on a padded table without moving for approximately 10 minutes.

Resting Calf Compliance Test: A blood pressure cuff will be placed around you upper arm. A mercury-in-silastic gauge (strain gauge) is placed around the widest portion of the calf. This strain gauge is used to

sense the volume change in the limb. A strap with a blood pressure sensor will be placed around your foot to measure pressure in your foot. This procedure requires that you lie flat on a padded table without moving for approximately 10 minutes.

Peak Limb Blood Flow Test: VOP will also be used to measure a peak blood flow from your forearm and calf. For this test, blood flow to and from your limb will be stopped for approximately 10 minutes. You will be asked to lie still on a padded table. Immediately following the 10 minutes, blood flow measurements will be made for approximately six minutes. Protocol #1 involves the forearm. Protocol #2 involves the calf.

Peak Blood Vessel Diameter and Velocity Test: For this test, blood flow to and from your arm and leg will be stopped for approximately 5 minutes by inflating a blood pressure cuff around your forearm or calf. This is a different site than before, when the cuff was inflated around the upper arm or upper leg. You will be asked to lie still on a padded table. Immediately following the 5 minute occlusion, an ultrasound probe will be placed at your elbow and knee to measure your blood vessel diameter and pulse wave velocity of blood flow.

Peak Calf Compliance Test: For this test, blood flow to and from your limb will be stopped for approximately 5 minutes by inflating a cuff (similar to a blood pressure cuff) around your upper leg. You will be asked to lie still on a padded table. Immediately following the 5 minutes, compliance measurements (same as resting) will be taken.

Strength Testing: You will be asked to perform tests to measure the strength of your forearm and calf muscles. Forearm strength will be determined by having you squeeze a hand grip device as hard as you can. Calf strength will be determined by having you press your foot against a pedal as hard as you can. These tests will be performed three times during one of your visits.

Anthropometric Testing: Your arm and leg dimensions will be determined by measuring your segment perimeters, width, and skinfold.

3. Discomfort and Risk:

Resting Blood Sample: The risk associated with single blood samples obtained with a needle and syringe may include one or all of the following: local discomfort at the puncture site, occasional dizziness and nausea, and bruising. Thrombosis (blood clots attached to walls of a blood vessel), embolism (blood clot in the circulation), and infections are very rare but are also potential risks. The risks will be minimized or eliminated by having only trained medical personnel from the GCRC who use sterile techniques to draw blood. Additionally, trained assistants will monitor you while blood is being obtained in a lying down position. All blood samples will be taken in the laboratory or clinic under sterile conditions using "one-time use" needles and containers. Proper procedures will be followed for the protection and safety of those individuals taking blood, and for the disposal of biohazardous waste.

Urine Sample: There are no known risks associated with the self collection of one's urine. You will be given screw top, air tight vials in which to store your urine.

Graded Exercise Testing: There is discomfort associated with graded exercise testing to maximum effort, including temporary muscle fatigue and shortness of breath. These feelings go away very quickly after exercise is stopped. It is possible that you may also experience lightheadedness, chest discomfort, cramping in the legs, irregular heart beats, and irregular blood pressures during this test. The risk of life-threatening problems (such as a heart attack) is very rare (1 in 2500 tests). Other potential risks, including fainting, nausea, muscle strain, and muscle soreness, will be minimized by proper warm-up, familiarization procedures, and cool-down. A research assistant will closely watch you throughout exercise and recovery.

Every attempt will be made to reduce the risk associated with exercise testing by using proper medical screening procedures. Pre-exercise screening will be done according to the guidelines of the American College of Sports Medicine (ACSM). A review of your medical history, as well as a physical exam with a resting 12 lead EKG will be conducted by a GCRC clinician prior to the graded exercise test. Proper procedures for stopping the test will be observed should you become lightheaded or faint. Should an emergency situation occur, access to further medical care at the GCRC or Mount Nittany Medical Center is available via a telephone located in the testing laboratory. Overall, the risks of this exercise test are minimal and probably less than if you were to exercise outside of a medical facility by yourself.

Body Composition Test: The DXA bone density procedure exposes an individual to a small amount of radiation where the x-ray beam crosses the body. The radiation involved is equivalent to a whole body

radiation dose of approximately 1.5 mrem (millirem). A mrem is a unit of whole-body radiation dose. For comparison purposes, 1.5 mrem is less than you would receive from a routine chest x-ray, or from cosmic rays during a coast-to-coast flight, or from 5 days worth of natural background radiation in central Pennsylvania.

Vascular Profiling: The risks of this vascular stiffness test are similar to that of taking blood pressure and an ECG. There may be minor discomfort when the cuffs are inflated.

Resting Blood Vessel Diameter Test: The risk of resting blood vessel diameter testing using Ultrasound is minimal. You may experience minor redness at the point were the Ultrasound probe is pressed against your skin. This redness is due to the pressure on your skin from the probe. The redness is temporary and quickly goes away.

Resting Limb Blood Flow Test: The risk involved with resting limb blood flow testing is minor. There may be slight bruising from the wrist and ankle cuffs. In addition, there may be minor discomfort when the cuffs are inflated. This discomfort includes tingling and numbness (hand or foot falling asleep).

Resting Calf Compliance Test: The risks involved with resting calf compliance are minimal. There may be slight discomfort/redness at the site where the blood pressure monitor rests on the surface of the foot. In addition, there may be minor discomfort when the arm blood pressure cuff is inflated.

Peak Limb Blood Flow Test: The risks associated with peak limb blood flow testing include slight bruising, tingling and numbness, moderately elevated blood pressure, minor elevation of heart rate, and discomfort from the cuff pressure. The discomfort caused by the cuff pressure quickly goes away immediately after the test. Women on estrogen replacement therapy have an increased risk of developing blood clots and occlusion of arm or leg blood flow may increase this risk. However, to our knowledge, there have been no actual cases of blood clots reported in studies involving blood flow occlusion.

Peak Blood Vessel Diameter Test: The risks associated with peak blood vessel diameter testing include small bruising, tingling and numbness, moderate rise in blood pressure, minor rise of heart rate, and discomfort from the cuff pressure. The discomfort caused by the cuff pressure goes away immediately after the test. In addition, minor redness may occur on your skin at the point where the probe is placed.

Peak Calf Compliance Test: The risks associated with peak calf compliance testing include slight bruising, tingling and numbness, moderately elevated blood pressure, minor elevation of heart rate, and discomfort from the cuff pressure. The discomfort caused by the cuff pressure quickly goes away immediately after the test. Women on estrogen replacement therapy have an increased risk of developing blood clots and occlusion of arm or leg blood flow may increase this risk. However, to our knowledge, there have been no actual cases of blood clots reported in studies involving blood flow occlusion. Additionally, there may be slight discomfort/redness at the site where the blood pressure monitor rests on the surface of the foot.

Strength Testing: There is minimal risk involved with strength testing. You may experience cramping, tightness, or temporary weakness in the muscles required for the test. Additionally, muscle strains or delayed muscle soreness (24 – 48 hours after testing) may occur. To reduce these risks, warm-up and stretching will be done prior to testing. All procedures will be demonstrated prior to testing.

Anthropometric Testing: There is no risk associated with limb segment measurements. The measurements are not different to those used by a tailor when measuring somebody for a suit or dress etc.

Abnormal Test Results: In the event that abnormal test results are obtained, either during the initial screening tests or during the bike exercise test, you will be apprised of the results and recommended to contact your medical provider for follow-up.

Stopping the Test: If you should feel that you cannot continue a test, you may stop the experiment at any time. It will be considered an uncompleted test.

Injury Clause:

I,_____ understand that medical care is available in the event of injury from the research but that neither financial compensation nor free medical treatment is provided. I also understand that I am not waiving any rights that I may have against Pennsylvania State University for injury from negligence of the University or investigators.

4. Benefits:

a. To You: Although there are no clinical (i.e. treatment) benefits associated with participating in these studies, you will receive a complete report of your results on the tests and procedures, and an

explanation of the meaningfulness of these results. You will receive a wealth of information regarding your overall health and fitness, and how you compare to others in your age group.

b. To Society: The ability to exercise declines with age. The results of these studies will tell us whether altered blood vessel structure or function is, in part, responsible for this. By participating in this research, you will help increase the knowledge of how aging and estrogen affect the flow of blood in women.

5. Time and Duration of the Study:

Your involvement in this study will require a series of screening tests to determine your eligibility. This will be followed by 3 visits that involve blood vessel diameter and blood flow testing. The first is a screening visit to determine your eligibility. The last three will be the blood vessel diameter and blood flow measurement testing. Each visit will last approximately 2 hours. Scheduling of these visits will depend on your availability. All visits can be completed within 4 weeks or less.

Screening Visits	**Experimental Test Visit**		
	Visit #1	Visit #2	Visit #3
• Medical History • Blood Draw • Physical Exam • Bike Test *(if age 45 or older, this will take place on a different day)*	• Body Composition / Vascular Profiling • Forearm Strength Test • Resting and Peak Blood Flow (forearm & calf)	• Vascular Profiling • Forearm Strength Test • Resting and Peak Blood Flow (forearm & calf)	• Resting and Peak Arterial Diameter and Velocity (forearm & calf) • Calf Strength Test • Resting and Peak Calf Compliance

6. Statement of Confidentiality:

All records associated with your participation in the study will be subject to the usual confidentiality standards similar to medical records (e.g., such as records maintained by physicians, hospitals, etc.) and in the event of any publication resulting from the research, no personally identifiable information will be disclosed. The Office for Research protections and the Biomedical Institutional Review Board (IRB) may review records related to this project

Throughout the study, a number randomly assigned to you will be used as an identifier for all forms, records and data compiled. Consistent with the conduct of human research studies, the data will not be available to anyone outside of the experimental research team.

7. Rights to Ask Questions:

Feel free to contact the person below at any time if you have questions regarding your participation in this study. You may ask any questions about the research procedures, and these questions will be answered. All questions should be directed to the person below. He may be contacted at any time about the nature, conduct, or a problem with the study. The Office for Research Protections can be contacted at 212 Kern Building, University Park, PA 16802 or by calling (814) - 865 - 1775 with regards to questions about the

rights of research participants. In the event of a research-related emergency, you may contact the GCRC medical staff (7AM – 5PM) at (814) - 865 - 7103.

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I, _____ have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

8. Compensation:

The cost of all tests and procedures directly related to participation in this study will be paid for by the study. You will be paid $25 dollars for completing each of the three blood vessel diameter and blood flow measurement testing visits. No payment will be made for the screening visit. If you need to withdraw from the study before all tests are completed, you will be paid only for the test you completed. These payments compensate you for the inconvenience and time associated with participating in this study. If you live out of town (>30 miles driving distance from State College), we will pay for any travel expense incurred or provide transportation if needed.

If you are an employee of Pennsylvania State University, the compensation you receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If you are not employed by Pennsylvania State University, total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

9. Voluntary Participation:

I, _____ understand that my participation in this study is voluntary, and that I may withdraw from this study at any time by notifying the investigator. My withdrawal from this study or my refusal to participate will in no way affect my care or access to medical services. I can decline to answer specific questions.

You must be 18 years of age or older to consent to participate in this research study. If you consent to participate in this research study and to the terms above, please sign your name and indicate the date below. You will be given a copy of this consent form to keep for your records.

This is to certify that I, _____, consent to give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

I, _____, have defined and explained the procedures and protocols of the study to the above volunteer.

Volunteer: _____ Date: _____

Investigator: _____ Date: _____

Informed Consent for Study 2 and addendum for Study 1 (FMD + CPT study):

Informed Consent Documentation For Research Study: The Pennsylvania State University

Study Title: "Influence of Age and Estrogen on Popliteal Vasoactivity In Women"
IRB # 21057

Investigator: *Beth Parker*

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James Pawelczyk, PhD
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This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. David Proctor, Assistant Professor of Kinesiology.

2. Purpose of the Study:
The purpose of this study is to determine if aging impairs blood vessel function in the legs. Volunteers will be healthy women who are taking no medications that significantly alter the function of the heart or blood vessels and who are not currently involved in a vigorous exercise program. Pre-menopausal women cannot currently be taking birth control medication. Post-menopausal volunteers must either a.) not currently be taking any form of hormone replacement therapy (i.e.: for the last 12 months) or b.) must have a recent history of taking hormone replacement therapy (i.e.: for at least 12 months.) We are planning to study 16 young (ages 20-30) and 32 older (ages 60-79) women.

2. Time and Duration of the Study:
Your involvement in this study will require a series of study visits. The first is a screening visit to determine your eligibility (1 hour). The second is another screening visit consisting of a graded exercise test visit to assess your cardiovascular fitness (1-1.5 hours). The third is the study visit to measure diameter/velocity responses in your leg artery (2-3 hours). The final visit, ***which will only be conducted in a subset of subjects***, examines the response of your nervous system to the cold stimulus (2 hours). Please note that for the first screening visit, you must avoid all food and drink except for water for 12 hours prior to the visit. For Visits 3 and 4, you must avoid caffeine, alcohol, and certain medications after 10 PM the night before and morning prior to all study visits. Scheduling of these visits will depend on your availability. All visits can be completed within 4 weeks or less. The details of each visit are described below:

Visit 1:

- During this visit, you will fill out your informed consent as well as a health screening form, admission form, release of information form, and physical activity questionnaire. You will receive a copy of your informed consent.
- Following completion of this paperwork, you will provide a blood sample.

Visit 2:

- Vascular Profiling: We will screen you for evidence of peripheral vascular disease.
- Physical Exam: A clinician will conduct a physical examination and review your medical history.
- Graded Exercise Test: You will perform an exercise test on a stationary bicycle to determine peak aerobic capacity.

Visit 3:

- Pregnancy test for younger subjects (you will be excluded if the result is positive)
- DXA scan: We will assess your total body and limb composition.
- Instrumentation: We will instrument you with a blood pressure cuff and an ECG monitor/electrodes to measure blood pressure and heart rate.
- Resting/Peak Diameter and Velocity Test: Using Doppler ultrasound, we will assess the response of your popliteal artery to 5 minutes of calf arterial occlusion.
- ½ hour rest
- Cold Stimulus with Resting Diameter and Velocity Test: We will assess the response of your popliteal artery to a cold stimulus (immersing your hand in ice water for 3 minutes).
- ½ hour rest
- Cold Stimulus with Resting/Peak Diameter and Velocity Test: We will assess the response of your popliteal artery to 5 minutes of calf arterial occlusion combined with a cold stimulus.
- 10 min rest
- Nitroglycerin Tablet with Resting Diameter and Velocity Test: We will measure the response of your popliteal artery to one 0.4 mg sublingual (dissolved under your tongue) nitroglycerin tablet.

Visit 4 *(only a subset of subjects will be selected to return for this visit):*

- Instrumentation: We will instrument you with heart rate electrodes, a blood pressure cuff, electrodes for nerve activity measurement (MSNA), and pressure cuffs for measurement of calf blood flow (VOP).
- Neural Activity: We will measure the response of a leg nerve to cold stimulus and 5 minutes of calf occlusion
- Resting Limb Blood Flow Test: We will measure the effect of cold stimulus of resting calf blood flow
- Peak Limb Blood Flow Test: We will measure the effect of 5 minutes of calf occlusion on calf blood flow

3. Procedures to be followed:

If you agree to be a subject in this research, you will be asked to complete several tests which include a physical examination, laboratory assessments of your cardiovascular health and fitness, body composition (how much fat and muscle you have), and resting and peak (post-exercise) limb blood flow. First, you will be asked to perform several screening procedures (Visits 1 and 2 as outlined above and described below). If you meet the eligibility requirements, you will then be asked to return for a third and possibly fourth visit. The major purpose of the third visit is to measure the diameter and blood flow velocity of the artery in your lower leg (measured behind your knee), in response to a cold stimulus, increased blood flow and nitroglycerin. The purpose of the fourth visit is to measure the response of your peripheral nervous system to the cold stimulus . *This visit will only be conducted in a randomly selected subset of subjects. During your screening visit, we will inform you if you have been selected for Visit 4.* The procedures for all visits will be explained below.

Screening Procedures:

Medical History Questionnaire: You will be asked to fill out a form about your medical history (i.e. major injuries, illnesses, etc.).

Physical Activity Questionnaire: You will be asked to fill out a form about your daily/weekly physical activity (i.e. walking, running, lifting, yard work, etc.)

Blood and Urine Analysis: You will be asked to provide a small sample of your blood (approximately *2.5 Tablespoons*) during your screening visit to evaluate the chemical profile (including estrogen level) of your blood. Blood will be drawn from your arm using a needle. Pre-menopausal women will have a urine pregnancy test done prior to the graded exercise test and DEXA scan. (A positive pregnancy test would exclude you from this study.) You must be fasted for this visit, which means that you should avoid all food and drink except water for 12 hours prior to the visit.

Physical Examination: A clinician at the General Clinical Research Center (GCRC) will review your medical history and conduct a physical examination.
Graded Exercise Test: You will be asked to perform an exercise test on a research stationary bicycle to assess your cardiovascular fitness and to rule out any blood pressure or heart abnormalities. During this test, the tension on the pedals will gradually increase every minute until you reach maximal effort. For those of you 50 years and older, your heart will be continuously monitored via a 12 lead EKG (electrical tracing of your heart's activity). For those of you under 50 years of age, your heart rate will be monitored using a Polar Heart Monitor. Ratings of perceived effort and your blood pressure will be recorded each minute. You will also be asked to wear a mouth piece and a nose clip during this test so that your expired air can be measured. This test will be excluded for those of you who have performed one within the past year in our lab or have the results from a treadmill test (also performed within the last year) that you can share with us.
Vascular Profiling: The health of your blood vessels will be assessed using the VP2000. This device measures the blood pressures at your ankles and arms. In addition, it will measure the time it takes for a pulse of blood to travel from your heart to your neck and legs. You will be asked to lie still on a bed with blood pressure cuffs placed on you arms and ankles. Sensors are then applied to your chest, wrists, neck and upper thigh. Measurements are then taken over a period of less than 2 minutes.

Testing Procedures:
Body Composition Test: Your body composition (% fat, muscle, and bone) will be estimated during this study using a Dual-Energy X-Ray Absorptiometry (DEXA) test. This whole body scan requires that you lie flat on a padded table without moving for approximately 10 minutes while an X-ray scanner moves over your body. This test will be excluded for those of you who have had one done within the past year for our lab.
Heart Rate/Blood Pressure Monitoring: Electrodes (sticky patches) will be applied to the skin of your chest to measure your heart's electrical activity. Also, a blood pressure cuff will be inflated around your upper arm periodically to measure your blood pressure.
Resting Blood Vessel Diameter and Velocity Test: The resting size of a blood vessel in your leg (behind your knee) will be measured using an Ultrasound probe (pencil-like device) placed firmly against your skin. We will also record the pulse wave velocity of your blood flow at the same time. This procedure requires that you lie flat on a padded table without moving for approximately 10 minutes.
Resting Limb Blood Flow Test ***(Visit 4 Only):*** Venous Occlusion Plethysmography (VOP) will be used to determine your resting calf blood flow. VOP is based on measuring the volume (size) of your limb segment when blood going away from your limb is temporarily stopped, but blood going into your limb continues. The rate of limb swelling is proportional to the rate of blood flow. A pressure cuff (similar to a blood pressure cuff) will be placed around your upper thigh. Similar cuffs will also be placed around your ankle. These pressure cuffs will be used to stop the flow of blood. A mercury-in-silastic gauge (strain gauge) is placed around the widest portion of the calf. This strain gauge is used to sense the volume change in the limb. This procedure requires that you lie flat on a padded table without moving for approximately 10 minutes.
Peak Blood Vessel Diameter and Velocity Test: For this test, blood flow to and from your leg will be stopped for approximately 5 minutes by inflating a blood pressure cuff around your calf. You will be asked to lie still on a padded table. Immediately following the 5 minute occlusion, an ultrasound probe will be placed at your knee to measure your blood vessel diameter and pulse wave velocity of blood flow.
Neural Activity (Microneurography) ***(Visit 4 Only):*** We will record activity (nerve signals) in the peroneal nerve (located near your knee). First, we will map the course of the nerve by stimulating through the skin with a pencil-shaped electrode. When we electrically stimulate the nerve, you will notice involuntary twitching or tingling sensations in the lower region of your leg. The twitching or tingling sensations will disappear when the stimulation is stopped. Once we have located the nerve, we will insert two tiny, sterile, needle electrodes through the skin. This is done without local anesthesia since the needle electrodes are so small they do not produce appreciable pain when inserted. One needle electrode is inserted just under the skin a short distance away from the nerve. The other needle will be positioned to contact your nerve. When the needle enters the nerve you will once again notice involuntary twitching or tingling. We will position the needle electrode to cause twitches without tingling sensations, and move it to obtain a good

recording of your neural activity. These adjustments may take up to 1 hour. When we have obtained a good recording, we will begin the experiment. You will probably be unaware of the needle electrode once we stop adjusting its position (your leg may feel like it is falling asleep). It is important that you keep your leg very still to maintain good recording throughout the experiment, which will last approximately 1 hour. After the experiment, the needle electrodes will be removed by simply pulling them out of the skin. Since they are very small, there is no need to numb your skin before the needles are inserted or removed.
Cold Stimulus: Your hand (up to the wrist) will be immersed in an ice water bath (32 º F) for 3 minutes.
Nitroglycerin Tablet*:* 0.4 mg nitroglycerin tablet will be inserted under your tongue; you will close your mouth immediately afterwards. The tablet will dissolve in 15-90 seconds; you will be asked to avoid swallowing until it dissolves. Nitroglycerin causes your blood vessels to dilate (open up); the effects last for 5-10 minutes. You will be asked to remain lying down for 20 minutes following the nitroglycerin administration. You will not be allowed to leave until 20 minutes have passed since you were given the nitroglycerin. Should you have an adverse reaction to the nitroglycerin, you will be monitored for up to 60 minutes following nitroglycerin administration. This is so that we can monitor your blood pressure following administration of the tablet.

4. Discomfort and Risk:
Resting Blood Sample: The risk associated with single blood samples obtained with a needle and syringe may include one or all of the following: local discomfort at the puncture site, occasional dizziness and nausea, and bruising. Thrombosis (blood clots attached to walls of a blood vessel), embolism (blood clot in the circulation), and infections are very rare but are also potential risks. The risks will be minimized or eliminated by having only trained medical personnel from the GCRC who use sterile techniques to draw blood. Additionally, trained assistants will monitor you while blood is being obtained in a lying down position. All blood samples will be taken in the laboratory or clinic under sterile conditions using "one-time use" needles and containers. Proper procedures will be followed for the protection and safety of those individuals taking blood, and for the disposal of biohazardous waste.
Urine Sample: There are no known risks associated with the self collection of one's urine. You will be given screw top, air tight vials in which to store your urine.
Graded Exercise Testing: There is discomfort associated with graded exercise testing to maximum effort, including temporary muscle fatigue and shortness of breath. These feelings go away very quickly after exercise is stopped. It is possible that you may also experience lightheadedness, chest discomfort, cramping in the legs, irregular heart beats, and irregular blood pressures during this test. The risk of life-threatening problems (such as a heart attack) is very rare (1 in 2500 tests). Other potential risks, including fainting, nausea, muscle strain, and muscle soreness, will be minimized by proper warm-up, familiarization procedures, and cool-down. A research assistant will closely watch you throughout exercise and recovery.
Every attempt will be made to reduce the risk associated with exercise testing by using proper medical screening procedures. Pre-exercise screening will be done according to the guidelines of the American College of Sports Medicine (ACSM). A review of your medical history, as well as a physical exam with a resting 12 lead EKG will be conducted by a GCRC clinician prior to the graded exercise test. Proper procedures for stopping the test will be observed should you become lightheaded or faint. Should an emergency situation occur, access to further medical care at the GCRC or Mount Nittany Medical Center is available via a telephone located in the testing laboratory. Overall, the risks of this exercise test are minimal and probably less than if you were to exercise outside of a medical facility by yourself.
Body Composition Test: The DXA bone density procedure exposes an individual to a small amount of radiation where the x-ray beam crosses the body. The radiation involved is equivalent to a whole body radiation dose of approximately 1.5 mrem (millirem). A mrem is a unit of whole-body radiation dose. For comparison purposes, 1.5 mrem is less than you would receive from a routine chest x-ray, or from cosmic rays during a coast-to-coast flight, or from 5 days worth of natural background radiation in central Pennsylvania. A DXA procedure also creates risks for an unborn fetus. To eliminate these risks, we will administer a pregnancy test prior to DXA testing. A positive pregnancy test will exclude you from the study.
Vascular Profiling: The risks of this vascular stiffness test are similar to that of taking blood pressure and an ECG. There may be minor discomfort when the cuffs are inflated.

Heart Rate/Blood Pressure Monitoring: Other than skin irritation from the sticky electrodes, there are no known risks involved with these procedures.
Resting Blood Vessel Diameter/Velocity Test: The risk of resting blood vessel diameter testing using ultrasound is minimal. You may experience minor redness at the point were the Ultrasound probe is pressed against your skin. This redness is due to the pressure on your skin from the probe. The redness is temporary and quickly goes away.
Resting Limb Blood Flow Test ***(Visit 4 Only):*** The risk involved with resting limb blood flow testing is minor. There may be slight bruising from the ankle cuff. In addition, there may be minor discomfort when the cuffs are inflated. This discomfort includes tingling and numbness (foot falling asleep).
Peak Blood Vessel Diameter/Velocity Test: The risks associated with peak blood vessel diameter testing include small bruising, tingling and numbness, moderate rise in blood pressure, minor rise of heart rate, and discomfort from the cuff pressure. The discomfort caused by the cuff pressure goes away immediately after the test. In addition, minor redness may occur on your skin at the point where the probe is placed. Women on estrogen replacement therapy have an increased risk of developing blood clots and occlusion of arm or leg blood flow may increase this risk. However, to our knowledge, there have been no actual cases of blood clots reported in studies involving blood flow occlusion. Should you feel pain or swelling in the limb we tested (or any other limb), shortness of breath, or any other unfamiliar symptoms such as persistent headaches, please notify the principal investigator immediately.
Cold Stimulus: Immersion of the hand in ice water can cause discomfort as well as temporarily elevate blood pressure (15-20 mmHg). In instances of extreme discomfort or excessive increase in blood pressure (> 50 mmHg increase) the test will be stopped immediately. In very rare circumstances, fainting may occur. We will monitor your heart rate and blood pressure throughout the study to minimize this risk.
Neural Activity (Microneurography) ***(Visit 4 Only):*** There have been no significant medical complications resulting from this procedure. About 7-10% of subjects experience some aching at the recording site or "pins and needles" sensations below the recording site for a few days after the procedure. Some people report their lower leg to be slightly weaker for a few days (similar to sensations you might feel after jogging), probably because of the muscle twitches they experienced. To minimize chances of any problems, you should not rub the site or perform heavy leg activity for at least 24 hours after the experiment. Additionally, to minimize the risk of infection, needle electrodes are sterilized and your skin will be cleansed with alcohol prior to insertion and after removal of the needle electrodes.
Nitroglycerin Tablet: Nitroglycerin may be associated with headache, lightheadedness, dry mouth, flushing, transient (5-10 minutes) lowering of blood pressure, dizziness, irregular heart beat, weakness, nausea, vomiting, sweating and fainting. You may also notice a sweet, tingling sensation in your mouth while the tablet dissolves. These effects are usually short-lived and will be minimized by having you lie down for 20 minutes (while the study staff monitors your heart rate and blood pressure) after receiving the nitroglycerin. Should you have an adverse reaction, you will be monitored for up to an hour following administration of nitroglycerin. As with any medication, nitroglycerin may cause an allergic reaction, such as a rash, in some individuals. The use of nitroglycerin for popliteal artery measurements is not an approved use of this drug by the Food and Drug Administration (FDA). However, nitroglycerin has been used in many other similar research studies without any problem. In addition, nitroglycerin is a common drug that is prescribed for heart patients who have, or are at risk for, angina (heart pain). The influence of nitroglycerin has not been researched with respect to pregnant or nursing women; therefore, the effects of nitroglycerin on pregnant or nursing women are unknown.
Abnormal Test Results: In the event that abnormal test results are obtained, either during the initial screening tests or during the bike exercise test, you will be apprised of the results and recommended to contact your medical provider for follow-up.
Stopping the Test: If you should feel that you cannot continue a test, you may stop the experiment at any time. It will be considered an uncompleted test.

Injury Clause:
I,_____ understand that medical care is available in the event of injury from the research but that neither financial compensation nor free medical treatment is provided.

I also understand that I am not waiving any rights that I may have against Pennsylvania State University for injury from negligence of the University or investigators.

5. Benefits:

a. To You: Although there are no clinical (i.e. treatment) benefits associated with participating in these studies, you will receive a complete report of your results on the tests and procedures, and an explanation of the meaningfulness of these results. You will receive information regarding your overall health and fitness, and how you compare to others in your age group.

b. To Society: The ability to exercise declines with age. The results of these studies will tell us whether altered blood vessel structure or function is, in part, responsible for this. By participating in this research, you will help increase the knowledge of how aging and estrogen affect the flow of blood in women.

6. Statement of Confidentiality:

All records associated with your participation in the study will be subject to the usual confidentiality standards similar to medical records (e.g., such as records maintained by physicians, hospitals, etc.) and in the event of any publication resulting from the research, no personally identifiable information will be disclosed. The following may review and copy records related to this research: The Office of Human Research Protections in the U.S. Dept. of Health and Human Services; The U.S. Food and Drug Administration (FDA) if applicable; The Penn State University Biomedical Institutional Review Board (IRB); The Penn State University Office for Research Protections.
Throughout the study, a number randomly assigned to you will be used as an identifier for all forms, records and data compiled. Blood samples will be labeled with the personal numerical identifier, stored in a freezer in a locked laboratory, and destroyed after one month. Consistent with the conduct of human research studies, the data will not be available to anyone outside of the experimental research team.

7. Rights to Ask Questions:

Feel free to contact the person below at any time if you have questions regarding your participation in this study. You may ask any questions about the research procedures, and these questions will be answered. All questions should be directed to the person below. He may be contacted at any time about the nature, conduct, or a problem with the study. The Office for Research Protections can be contacted at 212 Kern Building, University Park, PA 16802 or by calling (814) - 865 - 1775 with regards to questions about the rights of research participants. In the event of a research-related emergency, you may contact the GCRC medical staff (7AM – 5PM) at (814) - 865 - 7103.

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I, _____ have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

8. Compensation:

The cost of all tests and procedures directly related to participation in this study will be paid for by the study. You will be paid $100 dollars for completing this study ($50 for Visit 3 and $50 for Visit 4). No payment will be made for the screening visits. If you need to withdraw from the study before all tests are

completed, you will be paid only for the tests you completed. These payments compensate you for the inconvenience and time associated with participating in this study. If you live out of town (>30 miles driving distance from State College), we will pay for any travel expense incurred or provide transportation if needed.

If you are an employee of Pennsylvania State University, the compensation you receive for participation will be treated as taxable income and therefore taxes will be taken from the total amount. If you are not employed by Pennsylvania State University, total payments within one calendar year that exceed $600 will require the University to annually report these payments to the IRS. This may require you to claim the compensation that you receive for participation in this study as taxable income.

9. Voluntary Participation:

I, _____ understand that my participation in this study is voluntary, and that I may withdraw from this study at any time by notifying the investigator. My withdrawal from this study or my refusal to participate will in no way affect my care or access to medical services. I can decline to answer specific questions.

You must be 18 years of age or older to consent to participate in this research study. If you consent to participate in this research study and to the terms above, please sign your name and indicate the date below. You will be given a copy of this consent form to keep for your records.

This is to certify that I, _____, consent to give permission for my participation as a volunteer in this program of investigation. I understand that I may be randomly selected to return for a 4th visit as specified above. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

I, _____, have defined and explained the procedures and protocols of the study to the above volunteer.

Volunteer: _____ Date: _____

Investigator: _____ Date: _____

<u>Informed Consent Documentation For Research Study:</u> The Pennsylvania State University

Study Title: **Addendum** to: "Influence of Age and Estrogen on Popliteal Vasoactivity In Women"
IRB# 21057

Investigator:
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Aaron Mishkin
email: adm193@psu.edu

This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. David Proctor, Assistant Professor of Kinesiology.

1. Purpose of the Study:

The purpose of this additional study visit is to determine if aging impairs smooth muscle function in the arm. Also, pilot data on resting and peak limb compliance will be gathered. Volunteers who have already participated in our study, "Influence of Age and Estrogen on Popliteal Vasoactivity In Women," will be called back to participate in this additional test. Information in the original consent form remains the same unless noted otherwise in this form.

2. Time and Duration of the Study:

Your involvement in this additional study will consist of one additional, ½ hour -1 hour visit. You will be asked to avoid caffeine 12 hours before the visit, as well as the following substances 24 hours before the visit: migraine medication, aspirin and alcohol. In addition, you will not be able to participate in the visit if you have glaucoma, nitroglycerin allergies or are taking impotence drugs. If it has been more than a year since your initial screening for this study, you will be asked to repeat the vascular screening as well as a fasting blood draw to measure your coronary risk profile. The details of the visits are as follows:

Screening Visit **(only if it has been more than a year since the initial screening visit):**

- You must avoid all food and drink except for water for 12 hours prior to this visit.
- During this visit, you will fill out your informed consent as well as a modified health screening form. You will receive a copy of your informed consent. You must avoid all food and drink except for water for 12 hours prior to the visit
- Following completion of this paperwork, you will provide a blood sample.
- Vascular Profiling: We will screen you for evidence of peripheral vascular disease.

Study Visit:

- Administration of questionnaire and modified health screening form: We will briefly ask you questions about your alcohol and medication intake for the 24 hours prior to the study, as well as about any changes in your health/medication status since you last participated in the study. Should you have taken anything that will interact with the nitroglycerin, we will reschedule the study for another day. Additionally, young subjects will be given a pregnancy test. A positive test will prohibit participation in the study visit.

- Instrumentation: We will instrument you with a blood pressure cuff and ECG monitor/electrodes to measure blood pressure and heart rate. Also, your leg will be instrumented with a strain gauge (rubber tube that loops around the widest portion of the calf) and a blood pressure monitor that wraps around your foot and noninvasively measures beat-to-beat blood pressure.

- We will then measure resting compliance, which takes approximately 2-3 minutes. The popliteal artery (behind the knee) will be imaged with the Doppler ultrasound machine and measurements of vessel compliance will be made, which takes approximately 2-3 minutes.

- Nitroglycerin Tablet with Resting Diameter and Velocity Test: We will measure the response of your brachial artery (in the arm) to one 0.4 mg sublingual (dissolved under your tongue) nitroglycerin tablet, using Doppler ultrasound.

- Following 10 minutes of measurements in the arm, we will again measure compliance in the popliteal artery, which takes approximately 2-3 minutes.

3. Testing Procedures to be followed: Please note that these procedures are identical to the nitroglycerin portion of the study visit in which you previously participated, except that we will be taking Doppler ultrasound measurements of your brachial artery in your arm instead of your popliteal artery in your leg.

Blood Analysis *(Only if it has been more than one year since your initial screening):* You will be asked to provide a small sample of your blood (approximately *2.5 Tablespoons*) during your screening visit to evaluate your coronary risk level. Blood will be drawn from your arm using a needle. You must be fasted for this visit, which means that you should avoid all food and drink except water for 12 hours prior to the visit.

Vascular Profiling *(Only if it has been more than one year since your initial screening):* The health of your blood vessels will be assessed using the VP2000. This device measures the blood pressures at your ankles and arms. In addition, it will measure the time it takes for a pulse of blood to travel from your heart to your neck and legs. You will be asked to lie still on a bed with blood pressure cuffs placed on you arms and ankles. Sensors are then applied to your chest, wrists, neck and upper thigh. Measurements are then taken over a period of less than 2 minutes.

Nitroglycerin Tablet*:* 0.4 mg nitroglycerin tablet will be inserted under your tongue; you will close your mouth immediately afterwards. The tablet will dissolve in 15-90 seconds; you will be asked to avoid swallowing until it dissolves. Nitroglycerin causes your blood vessels to dilate (open up); the effects last for 5-10 minutes. We will monitor the diameter and velocity response of your brachial artery in your non-dominant (non-writing) arm with Doppler ultrasound for 10 minutes following the administration of nitroglycerin. You will be asked to remain lying down for 20 minutes following the nitroglycerin administration. You will not be allowed to leave until 20 minutes have passed since you were given the nitroglycerin. Should you have an adverse reaction to the nitroglycerin, you will be monitored for up to 60 minutes following nitroglycerin administration with a 12-lead ECG. This is so that we can monitor your blood pressure following administration of the tablet.

Calf Compliance Test: A blood pressure cuff will be placed around your upper arm. A mercury-in-silastic gauge (strain gauge) is placed around the widest portion of the calf. This strain gauge is used to sense the volume change in the limb. A strap with a blood pressure sensor will be placed around your foot to measure pressure in your foot. In addition, Doppler ultrasound measurement of the popliteal artery will be recorded to measure diameter of your artery. This procedure requires that you lie flat on a padded table without moving for approximately 2-3 minutes.

4. Discomfort and Risk:

Resting Blood Sample: The risk associated with single blood samples obtained with a needle and syringe may include one or all of the following: local discomfort at the puncture site, occasional dizziness and nausea, and bruising. Thrombosis (blood clots attached to walls of a blood vessel), embolism (blood clot in the circulation), and infections are very rare but are also potential risks. The risks will be minimized or eliminated by having only trained medical personnel from the GCRC who use sterile techniques to draw blood. Additionally, trained assistants will monitor you while blood is being obtained in a lying down position. All blood samples will be taken in the laboratory or clinic under sterile conditions using "one-time use" needles and containers. Proper procedures will be followed for the protection and safety of those individuals taking blood, and for the disposal of biohazardous waste.

Vascular Profiling: The risks of this vascular stiffness test are similar to that of taking blood pressure and an ECG. There may be minor discomfort when the cuffs are inflated.

Nitroglycerin Tablet: Nitroglycerin may be associated with headache, lightheadedness, dry mouth, flushing, transient (5-10 minutes) lowering of blood pressure, dizziness, irregular heart beat, weakness, nausea,

vomiting, sweating and fainting. You may also notice a sweet, tingling sensation in your mouth while the tablet dissolves. These effects are usually short-lived and will be minimized by having you lie down for 20 minutes (while the study staff monitors your heart rate and blood pressure) after receiving the nitroglycerin. Should you have an adverse reaction, you will be monitored for up to an hour following administration of nitroglycerin. As with any medication, nitroglycerin may cause an allergic reaction, such as a rash, in some individuals. The use of nitroglycerin for brachial artery measurements is not an approved use of this drug by the Food and Drug Administration (FDA). However, nitroglycerin has been used in many other similar research studies without any problem. In addition, nitroglycerin is a common drug that is prescribed for heart patients who have, or are at risk for, angina (heart pain). The influence of nitroglycerin has not been researched with respect to pregnant or nursing women; therefore, the effects of nitroglycerin on pregnant or nursing women are unknown.

Calf Compliance Test: The risks involved with resting calf compliance are minimal. There may be slight discomfort/redness at the site where the blood pressure monitor rests on the surface of the foot or where the Doppler probe contacts the back of your knee. In addition, there may be minor discomfort when the arm blood pressure cuff is inflated.

Stopping the Test: If you should feel that you cannot continue this test, you may stop the experiment at any time. It will be considered an uncompleted test.

Injury Clause:

I,_____ understand that medical care is available in the event of injury from the research but that neither financial compensation nor free medical treatment is provided. I also understand that I am not waiving any rights that I may have against Pennsylvania State University for injury from negligence of the University or investigators.

5. Benefits:

a. To You: There are no clinical benefits involved in participating in this experiment.

b. To Society: This will better help us define whether the smooth muscle function in your arm is altered with age in women.

6. Statement of Confidentiality:

All records associated with your participation in the study will be subject to the usual confidentiality standards similar to medical records (e.g., such as records maintained by physicians, hospitals, etc.) and in the event of any publication resulting from the research, no personally identifiable information will be disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), the Office for Research Protections at Penn State and the Biomedical Institutional Review Board (IRB) may review records related to this project.

Throughout the study, a number randomly assigned to you will be used as an identifier for all forms, records and data compiled. Consistent with the conduct of human research studies, the data will not be available to anyone outside of the experimental research team.

7. Rights to Ask Questions:

Feel free to contact the person below at any time if you have questions regarding your participation in this study. You may ask any questions about the research procedures, and these questions will be answered. All questions should be directed to the person below. He may be contacted at any time about the nature, conduct, or a problem with the study. The Office for Research Protections can be contacted a 201 Kern Building, University Park, PA 16802 or by calling (814) - 865 - 1775 with regards to questions about the rights of research participants. In the event of a research-related emergency, you may contact the GCRC medical staff (7AM – 5PM) at (814) - 865 - 7103.

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I, _____ have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

8. Compensation:

The cost of all tests and procedures directly related to participation in this study will be paid for by the study. You will be paid $15 dollars for completing this additional study.

9. Voluntary Participation:

I, _____ understand that my participation in this additional study is voluntary, and that I may withdraw from this study at any time by notifying the investigator. My withdrawal from this study or my refusal to participate will in no way affect my care or access to medical services. I can decline to answer specific questions.

You must be 18 years of age or older to consent to participate in this research study. If you consent to participate in this research study and to the terms above, please sign your name and indicate the date below. You will be given a copy of this consent form to keep for your records.

This is to certify that I, _____, consent to give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

I, _____, have defined and explained the procedures and protocols of the study to the above volunteer.

Volunteer: _____ Date: _____

Investigator: _____ Date: _____

Informed Consent Documentation For Research Study: The Pennsylvania State University

Study Title: Influence of Age and Sex on Acute Vasoactive Responsiveness During Small Muscle Dynamic Exercise (IRB #22437)

Investigator: *Beth Parker*
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This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. David Proctor, Associate Professor of Kinesiology.

9. Purpose of the Study:

The capacity to perform physical activities of daily living and the ease with which physical activity is performed declines with advancing age. This may be due in part to changes in the regulation of blood flow to the legs. The purpose of this research is to determine whether there are age-related changes in the control of blood flow to exercising muscles. These age-related changes in the control of blood flow may also depend in part on the sex and/or fitness level of the older individual. Understanding the factors regulating blood flow to active muscles also has important implications for chronic diseases associated with old age. We are planning to study young (ages 20-30) sedentary men and women and older (ages 60-80) men and women of varying fitness levels.

2. Time and Duration of the Study:

Your involvement in this study will require a series of study visits. The first is a screening visit to determine your eligibility (1 hour). The second is another screening visit consisting of a graded exercise test visit to assess your cardiovascular fitness (1-2 hours). The third is the study visit to measure leg blood flow responses in your leg during graded single-knee kicking (1.5-2 hours). The fourth is the study visit to measure leg blood flow responses to graded single-knee kicking in which work rate increases are twice as great as those used in the first study visit (1 hour). Please note that for the first screening visit, you must avoid all food and drink except for water for 12 hours prior to the visit. For the study visits, you must

avoid caffeine, alcohol, and certain medications after 10 PM the night before and morning prior to all study visits. Scheduling of these visits will depend on your availability. All visits can be completed within 4 weeks or less. The details of each visit are described below:

Screening Visit 1:

- During this visit, you will fill out your informed consent as well as a health screening form, admission form, release of information form, and physical activity questionnaire. You will receive a copy of your informed consent.
- Following completion of this paperwork, you will provide a blood sample and have a resting ECG (electrocardiogram).

Screening Visit 2:

- Pregnancy test for younger female subjects (you will be excluded from the study if the result is positive)
- Vascular Profiling: We will screen you for evidence of peripheral vascular disease.
- Physical Exam: A clinician will conduct a physical examination and review your medical history.
- Graded Exercise Test: You will perform an exercise test on a treadmill to determine peak aerobic capacity.
- Knee Kick Familiarization: You will be briefly familiarized with the knee extensor device and will practice kicking at the cadence of 40 kicks/minute.

Study Visit 1:

- Pregnancy test for younger female subjects (you will be excluded from the study if the result is positive)
- DXA (Dual X-Ray Absorptiometry) scan: We will assess your total body and limb composition.
- Instrumentation: We will instrument you with a blood pressure cuff/wrist or finger brace, EMG (electromyography) electrodes (sticky patches), and an ECG monitor/electrodes to measure inactive muscle recruitment, blood pressure and heart rate.
- Strength Testing: Maximal leg extension force will be measured three times.
- Graded Single-knee Kick Exercise Test: We will assess the blood flow response (using Doppler ultrasound) in your non-dominant leg during single-knee extensor exercise to maximum exertion.

Study Visit 2:

- Thigh muscle measurement: We will use a measuring tape and skinfold calipers to determine the amount of muscle in the front of your upper leg.
- Instrumentation: We will instrument you with a blood pressure cuff/wrist or finger brace, EMG electrodes, and an ECG monitor/electrodes to measure blood pressure, inactive muscle recruitment, and heart rate.
- Graded Single-knee Kick Exercise Test: We will assess the blood flow response (using Doppler ultrasound) in your non-dominant leg during single-knee kick exercise to maximum exertion. The resistance against which you are kicking will increase twice as fast as the protocol in which you participated in Study Visit 1.

3. Procedures to be followed:

If you agree to be a subject in this research, you will be asked to complete several tests which include a physical examination, laboratory assessments of your cardiovascular health and fitness, body composition (how much fat and muscle you have), and resting and exercising limb blood flow. First, you will be asked to perform several screening procedures (Screening Visits 1 and 2 as outlined above and described below). If you meet the eligibility requirements, you will then be asked to return for a

third and fourth visit. The major purpose of Study Visit 1 is to measure the leg blood flow response to graded single-knee kick exercise to maximum exertion. The purpose of Study Visit 2 is to measure the leg blood flow response to graded single-knee kick exercise with larger increases in work rates. The procedures for the visits will be explained below.

Screening Procedures:

Medical History Questionnaire: You will be asked to fill out a form about your medical history (i.e. major injuries, illnesses, etc.).

Physical Activity Questionnaire: You will be asked to fill out a form about your daily/weekly physical activity (i.e. walking, running, lifting, yard work, etc.)

Blood and Urine Analysis: You will be asked to provide a small sample of your blood (approximately *2.5 Tablespoons*) during your screening visit to evaluate the chemical profile of your blood. Blood will be drawn from your arm using a needle. Pre-menopausal women will have a urine pregnancy test done prior Screening Visit 2 and Study Visit 1. (A positive pregnancy test would exclude you from this study.) You must be fasted for this visit, which means that you should avoid all food and drink except water for 12 hours prior to the visit.

Physical Examination: A clinician at the General Clinical Research Center (GCRC) will review your medical history and conduct a physical examination.

Graded Exercise Test: You will be asked to perform an exercise test on a research treadmill to assess your cardiovascular fitness and to rule out any blood pressure or heart abnormalities. During this test, the grade and/or speed of the treadmill will gradually increase until you reach maximal effort. Your heart will be continuously monitored via a 12 lead ECG (electrical tracing of your heart's activity). Ratings of perceived effort and your blood pressure will be recorded each minute. You will also be asked to wear a mouth piece and a nose clip during this test so that your expired air can be measured. This test will be excluded for those of you who have performed a graded exercise test within the past year in our lab or have the results from a treadmill test (also performed within the last year) that you can share with us.

Vascular Profiling: The health of your blood vessels will be assessed using the VP2000. This device measures the blood pressures at your ankles and arms. In addition, it will measure the time it takes for a pulse of blood to travel from your heart to your neck and legs. You will be asked to lie still on a bed with blood pressure cuffs placed on you arms and ankles. Sensors are then applied to your chest, wrists, neck and upper thigh. Measurements are then taken over a period of less than 2 minutes.

Testing Procedures:

Body Composition Test: Your body composition (% fat, muscle, and bone) will be estimated during this study using a Dual-Energy X-Ray Absorptiometry (DEXA) test. This whole body scan requires that you lie flat on a padded table without moving for approximately 10 minutes while an X-ray scanner moves over your body. This test will be excluded for those of you who have had one done within the past year for our lab.

Heart Rate/Blood Pressure Monitoring: Electrodes (sticky patches) will be applied to the skin of your chest to measure your heart's electrical activity. Blood pressure will be measured continuously using a Colin or Portapres system. Your arm will be placed in a wrist brace for stabilization and a sensor will be gently strapped on your wrist (Colin) or two fingers (Portapres). The wrist or finger sensor pushes against your wrist/finger periodically. A cuff will also be placed on the upper arm and will inflate periodically (Colin instrument only). Alternatively, a small heart rate electrode will be placed on your chest if the Portapres is being used. You will be asked to keep your dominant arm relaxed throughout the protocols to ensure the validity of the blood pressure measurements.

Strength Testing: Your maximal knee kick force will be measured by having you extend your non-dominant knee while force is measured. If you have a current or chronic knee injury in the non-dominant leg, the dominant leg will be tested and used for the single leg knee kick exercise throughout the experiment. Each test will be performed three times, and the highest force measured in the three trials will be considered your peak force.

EMG: Surface electromyography (EMG) is a tool used to measure muscle activity since it measures the voltage associated with muscle use. EMG electrodes will be placed over the thigh of your inactive leg during the graded exercise test protocol to ensure that the inactive muscles are not being used. We may also place

electrodes on the back of the thigh of your exercising leg to ensure that you are not using these muscles during the single-knee kick exercise trials. To prepare you for EMG surface-electrode placement, we will shave the skin at each electrode location and clean the site with alcohol.

Leg Blood Flow Measurements*:* Blood flow measurements at the femoral artery will be made before exercise, during exercise, and during recovery. Femoral artery diameter and mean blood velocity will be measured using an ATL 5000 Doppler Ultrasound probe placed firmly against the skin of one leg. The Ultrasound probe is a pencil-like device that is placed firmly against your skin and held in place at specific time points during the study visits.

Graded Knee Kick Maximal Exercise Test*:* The single leg knee kick device is a special chair that will be placed in a reclined position, and one of your legs will be strapped into a "boot" through which resistance can be applied. This exercise consists of extending your leg so that your thigh moves from a 90 degree angle to your knee to a 0 degree angle to your knee. You will then relax your leg so that your thigh passively returns to the starting position. This cycle represents one kick. You will be asked to maintain a specific cadence (40 kicks/minute) throughout each exercise protocol. You will be familiarized with the knee extensor device and will practice unloaded kicking (kicking against no resistance) at the cadence of 40 kicks/minute both during Screening Visit 2 and Study Visit 1. After your second familiarization practice at Study Visit 1 you will perform a graded exercise test to maximal exertion. After resting measurements are taken, you will perform passive single-knee kick exercise. For this, you will be asked to relax your leg while the researcher moves your leg in the knee kick device at the same cadence and work rate (power output) used for active kicking exercise. You will then begin unloaded kicking for 3 minutes to establish baseline measurements. Then, the work rate will be increased by 3-10 units of resistance (depending on your maximal measured leg extension force) every 3 minutes until exhaustion. Heart rate, blood pressure, blood flow, arterial diameter, and EMG will be monitored during each condition.

Thigh Muscle Measurement: A measuring tape will be used to measure the length of your thigh as well as the circumference of your thigh in three places (top, middle and bottom of thigh). We will also use a skinfold caliper (small device with two tips used to measure the thickness of your skin and the fat below the skin) to estimate the amount of superficial fat in your thigh in the same three places.

4. Discomfort and Risk:

All of the risks associated with this study are extremely unlikely and not at all serious except for the risks associated with graded exercise testing. The risks associated with graded exercise testing are somewhat more serious. However, we have taken significant precautions to minimize all risks, as explained below.

Resting Blood Sample: The risk associated with single blood samples obtained with a needle and syringe may include one or all of the following: local discomfort at the puncture site, occasional dizziness and nausea, and bruising. Thrombosis (blood clots attached to walls of a blood vessel), embolism (blood clot in the circulation), and infections are very rare but are also potential risks. The risks will be minimized or eliminated by having only trained medical personnel from the GCRC who use sterile techniques to draw blood. Additionally, trained assistants will monitor you while blood is being obtained in a lying down position. All blood samples will be taken in the laboratory or clinic under sterile conditions using "one-time use" needles and containers. Proper procedures will be followed for the protection and safety of those individuals taking blood, and for the disposal of biohazardous waste.

Urine Sample: There are no known risks associated with the self collection of one's urine. You will be given screw top, air tight vials in which to store your urine.

Graded Exercise Testing: There is discomfort associated with graded exercise testing to maximum effort, including temporary muscle fatigue and shortness of breath. These feelings go away very quickly after exercise is stopped. It is possible that you may also experience lightheadedness, chest discomfort, cramping in the legs, irregular heart beats, and irregular blood pressures during this test. The risk of life-threatening problems (such as a heart attack) is very rare (1 in 2500 tests). Other potential risks, including fainting, nausea, muscle strain, and muscle soreness, will be minimized by proper warm-up, familiarization procedures, and cool-down. A research assistant will closely watch you throughout exercise and recovery.

Every attempt will be made to reduce the risk associated with exercise testing by using proper medical screening procedures. Pre-exercise screening will be done according to the guidelines of the American College of Sports Medicine (ACSM). A review of your medical history, as well as a physical exam with a resting 12 lead ECG will be conducted by a GCRC clinician prior to the graded exercise test. Proper

procedures for stopping the test will be observed should you become lightheaded or faint. Should an emergency situation occur, access to further medical care at the GCRC or Mount Nittany Medical Center is available via a telephone located in the testing laboratory. Overall, the risks of this exercise test are minimal and probably less than if you were to exercise outside of a medical facility by yourself.

Body Composition Test: The DXA bone density procedure exposes an individual to a small amount of radiation where the x-ray beam crosses the body. The radiation involved is equivalent to a whole body radiation dose of approximately 1.5 mrem (millirem). A mrem is a unit of whole-body radiation dose. For comparison purposes, 1.5 mrem is less than you would receive from a routine chest x-ray, or from cosmic rays during a coast-to-coast flight, or from 5 days worth of natural background radiation in central Pennsylvania. A DXA procedure also creates risks for an unborn fetus. To eliminate these risks, we will administer a pregnancy test prior to DXA testing. A positive pregnancy test will exclude you from the study.

Vascular Profiling: The risks of this vascular stiffness test are similar to that of taking blood pressure and an ECG. There may be minor discomfort when the cuffs are inflated.

ECG: There is a minimal risk due to potential allergic reaction to adhesive on the ECG electrodes.

EMG: There is a minimal risk due to potential allergic reaction to adhesive on the electrodes.

Blood pressure: The inflated arm cuff and wrist sensor may cause temporary tingling and numbness (Colin). The finger cuffs may cause temporary tingling and numbness (Portapres).

Strength Testing: There is a small chance of muscle strain or cramping associated with testing maximal force production.

Ultrasound Doppler: You may experience minor risk of redness at the point where the ultrasound probe is pressed against the skin.

Single-knee Kick Exercise: You may experience temporary muscle fatigue and shortness of breath. These feelings go away very quickly after exercise is stopped. It is possible that you may also experience cramping in the legs, muscle strain, and muscle soreness, which will be minimized by proper warm-up, familiarization procedures, and cool-down. In addition, at the second visit you may experience greater fatigue and soreness than that experienced in your first study visit; the risk of muscle and/or joint injury may be greater since we are increasing the resistance more quickly. At any point during the study, should you feel muscle and joint pain, please tell us and we will stop the study immediately.

Thigh Muscle Measurement: You may feel a slight pinching sensation when we use the skinfold calipers on your thigh.

Abnormal Test Results: In the event that abnormal test results are obtained, either during the initial screening tests or during the exercise test, you will be apprised of the results (within one week of obtaining the results) and recommended to contact your medical provider for follow-up.

Stopping the Test: If you should feel that you cannot continue a test, you may stop the experiment at any time. It will be considered an uncompleted test.

Injury Clause:
Medical care is available in the event of injury from the research but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against Pennsylvania State University for injury from negligence of the University or investigators.

5. Benefits:

A. To You: Although there are no clinical (i.e. treatment) benefits associated with participating in these studies, you will receive a complete report of your results on the tests and procedures, and an explanation of the meaningfulness of these results. You will receive information regarding your overall health and fitness, and how you compare to others in your age group.

B. To Society: The ability to exercise declines with age. The results of these studies will tell us whether altered regulation of leg muscle blood flow during exercise is, in part, responsible for this. By participating in this research, you will help increase the knowledge of how aging, sex and fitness status influence leg blood flow in older adults.

6. Statement of Confidentiality:

All records associated with your participation in the study will be subject to the usual confidentiality standards similar to medical records (e.g., such as records maintained by physicians, hospitals, etc.) and in the event of any publication resulting from the research, no personally identifiable information will be disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), the Office for Research Protections at Penn State and the Biomedical Institutional Review Board (IRB) may review records related to this project.
Throughout the study, a number randomly assigned to you will be used as an identifier for all forms, records and data compiled. Consistent with the conduct of human research studies, the data will not be available to anyone outside of the experimental research team.

7. Rights to Ask Questions:

Feel free to contact the person below at any time if you have questions regarding your participation in this study. You may ask any questions about the research procedures, and these questions will be answered. All questions should be directed to the person below. He may be contacted at any time or via phone about the nature, conduct, or a problem with the study. The Office for Research Protections can be contacted at 201 Kern Building, University Park, PA 16802 or by calling (814) - 865 - 1775 with regards to questions about the rights of research participants. In the event of a research-related emergency, you may contact the GCRC medical staff (7AM – 5PM) at (814) - 865 - 7103. In the event of a research-related injury, please call:

David N. Proctor, PhD
Department of Kinesiology and Physiology
105 Noll Physiological Research Center
Pennsylvania State University
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I, _____ have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

8. Compensation:
9.
The cost of all tests and procedures directly related to participation in this study will be paid for by the study. You will be paid $80 dollars for completing this study ($40 for each study visit). No payment will be made for the screening visits. If you need to withdraw from the study before all tests are completed, you will be paid only for the tests you have completed. These payments compensate you for the inconvenience and time associated with participating in this study. If you live out of town (>30 miles driving distance from State College), we will pay for any travel expense incurred or provide transportation if needed.
Total payments within one calendar year will be treated as taxable income and therefore taxes will be taken from the total amount. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. Voluntary Participation:
11.
I, _____ understand that my participation in this study is voluntary, and that I may withdraw from this study at any time by notifying the investigator. My

withdrawal from this study or my refusal to participate will in no way affect my care or access to medical services. I can decline to answer specific questions.

You must be 18 years of age or older to consent to participate in this research study. If you consent to participate in this research study and to the terms above, please sign your name and indicate the date below.You will be given a copy of this consent form to keep for your records.

This is to certify that I, _____, consent to give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

I, _____, have defined and explained the procedures and protocols of the study to the above volunteer.

Volunteer: _____ Date: _____

Investigator: _____ Date: _____

Informed Consent Documentation For Research Study: The Pennsylvania State University

Study Title: **Addendum** to: Influence of Age and Sex on Acute Vasoactive Responsiveness During Small Muscle Dynamic Exercise (IRB #22437)

Investigator:
Beth Parker
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201 Noll Physiological Research Center
Pennsylvania State University
University Park, PA 16802
(814) 863 - 3182
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Other Investigators:
David Proctor, PhD (Faculty Advisor)
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Aaron Mishkin
email: adm193@psu.edu
Samuel Ridout
email: sjr210@psu.edu
Martha Kalasky
email: mjk5013@psu.edu

This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. David Proctor, Associate Professor of Kinesiology.

1. Purpose of the Study:

The purpose of this research is to determine whether there are age-related changes in the control of blood flow to exercising muscles. These age-related changes in the control of blood flow may also depend in part on the sex and/or fitness level of the older individual. You previously gave your consent to participate in two study visits in which we measured your leg blood flow response to single-knee extensoring exercise. You have also completed a treadmill test to assess your cardiovascular fitness and filled out a questionnaire about your daily physical activity. However, to better understand the relationship between your fitness, daily physical activity, and leg blood flow responses to exercise, we plan to measure your daily physical activity using questionnaires and an accelerometer (a small device worn on your hip that measures motion). This additional protocol will help us determine whether changes in leg blood flow responses with age are due in part differences in daily physical activity between young and older subjects. You will be compensated for your involvement in this additional study protocol. All information from the original consent form for this study remains the same.

2. Time and Duration of the Additional Protocol:

Your involvement in this additional protocol will require a laboratory visit of about 1 hour during which we will teach you how to use and wear the accelerometer, describe the daily information you are to complete while wearing the accelerometer, and administer several questionnaires on your daily physical activity. You will then be asked to wear the accelerometer for the next 7 days (beginning with the time of your initial visit). In addition, each day that you wear the accelerometer, you will be asked to fill out a daily activity log concerning accelerometer use and various activities you perform throughout the day. This will take approximately ½ hour each day. Finally, we will ask you to return to the lab after 7 days to return the accelerometer and fill out another physical activity questionnaire. This final visit will take approximately ½ hour. The total time required to complete the study will be 5 hours (1.5 at Noll Laboratory and 3.5 at home).

3. Procedures to be followed for Additional Protocol:
Testing Procedures:

Physical Activity Questionnaires: We will administer 3 physical activity questionnaires at the beginning of the study during the initial laboratory study, and one questionnaire at the end of the study. These questionnaires are designed to assess your daily physical activity and health as well as your attitudes towards exercise. For two questionnaires, we will ask you the questions and record your answers. For the third questionnaire, you will be asked to circle the answer that best completes the statement. For all of the questionnaires, we will ask you to answer each question as accurately as possible. There are no right or wrong answers.

Accelerometer Use: An accelerometer is a small, portable device (similar to a pedometer) that is worn on the hip to measure motion. It allows us to estimate your daily physical activity. We ask that you wear the accelerometer clipped to your pants or belt above your left hip. You will be asked to wear the accelerometer during both waking and sleeping hours, with the exception of times when you are bathing/showering, exercising intensely, and participating in sexual activity. You will be given a phone number to call each day should you have any questions about wearing the accelerometer.

Daily At-Home Activity Log*:* We will ask you to fill out a daily activity log each day. You will be asked to fill out these questions as accurately as possible. You will be asked to keep track of the times when you put on and take off the accelerometer each day as well as the times you sleep, perform physical activity, and ride in a car or bus. You will be given a phone number to call each day should you have any questions about filling out the daily activity log.

4. Discomfort and Risk:

All of the risks associated with this study are extremely unlikely and not at all serious.

Questionnaires: You may feel mildly fatigued after filling out the questionnaire and/or being asked questions.

Accelerometer: There is a minimal risk of discomfort associated with wearing the accelerometer during sleep, sitting, or lying on your stomach. This discomfort can be minimized by changing positions.

Daily logs: You may feel mildly fatigued after filling out the daily activity log.

Stopping accelerometer use: If you should feel that you cannot continue wearing the accelerometer, you may stop wearing it at any time. We ask that you notify us immediately should this occur.

Injury Clause:

Medical care is available in the event of injury from the research but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against Pennsylvania State University for injury from negligence of the University or investigators.

5. Benefits:

A. To You: There are no clinical (i.e. treatment) benefits associated with this protocol.
B. To Society: This study will help us determine whether changes in leg blood flow responses with age are due in part differences in daily physical activity between young and older subjects.

6. Statement of Confidentiality:

*A*ll records associated with your participation in the study will be subject to the usual confidentiality standards similar to medical records (e.g., such as records maintained by physicians, hospitals, etc.) and in the event of any publication resulting from the research, no personally identifiable information will be disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), the Office for Research Protections at Penn State and the Biomedical Institutional Review Board (IRB) may review records related to this project.

Throughout the study, a number randomly assigned to you will be used as an identifier for all forms, records and data compiled. Consistent with the conduct of human research studies, the data will not be available to anyone outside of the experimental research team.

7. Rights to Ask Questions:

Feel free to contact the person below at any time if you have questions regarding your participation in this additional protocol. You may ask any questions about the research procedures, and these questions will be answered. All questions should be directed to the person below. He may be contacted at any time or via phone about the nature, conduct, or a problem with the protocol. The Office for Research Protections can be contacted at 201 Kern Building, University Park, PA 16802 or by calling (814) - 865 - 1775 with regards to questions about the rights of research participants. In the event of a research-related emergency, you may contact the GCRC medical staff (7AM – 5PM) at (814) - 865 - 7103. In the event of a research-related injury, please call:

David N. Proctor, PhD
Department of Kinesiology and Physiology

105 Noll Physiological Research Center
Pennsylvania State University
University Park, PA 16802
Work: (814) - 863 – 0724
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I, _____ have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

8. Compensation:

9.

The cost of all tests and procedures directly related to participation in this additional protocol will be paid for by the study. You will be paid $75 dollars for completing this additional protocol. If you live out of town (>30 miles driving distance from State College), we will pay for any travel expense incurred or provide transportation if needed.

Total payments within one calendar year will be treated as taxable income and therefore taxes will be taken from the total amount. This may require you to claim the compensation that you receive for participation in this study as taxable income.

10. Voluntary Participation:

I, _____ understand that my participation in this additional protocol is voluntary, and that I may withdraw from this protocol at any time by notifying the investigator. My withdrawal from this protocol or my refusal to participate will in no way affect my care or access to medical services. I can decline to answer specific questions.

You must be 18 years of age or older to consent to participate in this research study. If you consent to participate in this research study and to the terms above, please sign your name and indicate the date below. You will be given a copy of this consent form to keep for your records.

This is to certify that I, _____, consent to give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed copy of this consent form. I have read this form, and understand the content of this consent form.

I, _____, have defined and explained the procedures and protocols of the study to the above volunteer.

Volunteer: _____ Date: _____

Investigator: _____ Date: _____

Informed Consent Documentation For Research Study: The Pennsylvania State University

Study Title: **Addendum** to: Influence of Age and Sex on Acute Vasoactive Responsiveness During Small Muscle Dynamic Exercise (IRB #22437)

Investigator: *Beth Parker*
Department of Kinesiology

201 Noll Physiological Research Center
Pennsylvania State University
University Park, PA 16802
(814) 863 - 3182
email: bap202@psu.edu

Other Investigators:

Sandra L. Smithmyer
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David Proctor, PhD
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Aaron Mishkin
email: adm193@psu.edu
Samuel Ridout
email: sjr210@psu.edu

This is to certify that I, _____ have been given the following information with respect to my participation as a volunteer in a program of investigation under the supervision of Dr. David Proctor, Associate Professor of Kinesiology.

12. Purpose of the Study:
You previously participated in IRB Study #22437, in which you completed two knee kick protocols where you kicked to exhaustion as resistance increased. We are now interested in examining your blood flow responses during kicking at a constant resistance to exhaustion. In addition, if you participated in IRB Study #24538 (Samuel Ridout was the principle investigator), and successfully completed the screenings and knee kick protocol for that study, you are also eligible to complete this additional protocol.

2. Time and Duration of the Study:
Your involvement in this study will require one possible screening visit as well as one study visit to measure leg blood flow responses to single-knee kicking in which the work rate at which you kick will be constant (it will be 75-85% of the maximal work rate you attained in your previous short study visit). This protocol will take approximately 1 hour. You must avoid caffeine, alcohol, and certain medications after 10 PM the night before and morning prior to all study visits.
Screening Visit: If your bloodwork from either study (#22437 or #24538) is greater than 6 months old, you will provide a blood sample. You must avoid all food and drink except for water for 12 hours prior to the visit. In addition, subjects from Sam Ridout's study (#24538) will have a physical exam and a peripheral vascular exam on this visit to be admitted to the current study.

Study Visit:

- Instrumentation: We will instrument you with a blood pressure cuff/wrist brace, EMG electrodes, and an ECG monitor/electrodes to measure blood pressure, muscle recruitment, and heart rate.
- We will also instrument you with NIRS (Near Infrared Spectroscopy) electrodes.
- Constant Single-knee Extensor Exercise Test: We will assess the blood flow response (using Doppler ultrasound) in your non-dominant leg during single-knee kick exercise at a constant resistance (50% of the peak work rate you achieved in your previous knee kick exercise visit).

3. Procedures to be followed:

The procedures for this visit are identical (with the exception of the NIRS data) to the procedures for your previous study visit. The purpose of modified visit is to measure the leg blood flow response to graded single-knee kick exercise with larger increases in work rates.

Testing Procedures:

Blood Analysis: If your bloodwork is more than 6 months old, you will be asked to provide a small sample of your blood (approximately *2.5 Tablespoons*) during your screening visit to evaluate the chemical profile of your blood. Blood will be drawn from your arm using a needle. You must be fasted for this visit, which means that you should avoid all food and drink except water for 12 hours prior to the visit.

Physical Examination: If you were in Samuel Ridout's study, a clinician at the General Clinical Research Center (GCRC) will review your medical history and conduct a physical examination to admit you into the current study.

Vascular Profiling: If you were in Samuel Ridout's study, the health of your blood vessels will be assessed using the VP2000. This device measures the blood pressures at your ankles and arms. In addition, it will measure the time it takes for a pulse of blood to travel from your heart to your neck and legs. You will be asked to lie still on a bed with blood pressure cuffs placed on you arms and ankles. Sensors are then applied to your chest, wrists, neck and upper thigh. Measurements are then taken over a period of less than 2 minutes.

Heart Rate/Blood Pressure Monitoring: Electrodes (sticky patches) will be applied to the skin of your chest to measure your heart's electrical activity. Blood pressure will be measured continuously using a Colin system. Your arm will be placed in a wrist brace for stabilization and a sensor will be gently strapped on your wrist. The wrist sensor pushes against your wrist periodically. A cuff will also be placed on the upper arm and will inflate periodically. You will be asked to keep your dominant arm relaxed throughout the protocols to ensure the validity of the blood pressure measurements.

EMG: Surface electromyography (EMG) is a tool used to measure muscle activity since it measures the voltage associated with muscle use. EMG electrodes will be placed over the front of your kicking leg during the graded exercise test protocol to ensure that you are using these muscles. We will also place probes over the back of your exercising leg to ensure that you are not using these muscles during the single-knee kick exercise trials.

Leg Blood Flow Measurements*:* Blood flow measurements at the femoral artery will be made before and during exercise. Femoral artery diameter and mean blood velocity will be measured using an ATL 5000 Doppler Ultrasound probe placed firmly against the skin of one leg. The Ultrasound probe is a pencil-like device that is placed firmly against your skin and held in place at specific time points during the study visits.

Single-knee extensor Exercise (Constant Work rate)*:* For the exercise trials, you will perform active single leg knee extension exercise tests against a certain, constant resistance (75-85% of the peak power output you achieved in your previous study visit) on the knee kick ergometer used in your previous visit.

Tissue Oxygenation Measurements*:* Measurements of tissue oxygenation will be performed using near infrared spectroscopy (NIRS). Near infrared light is emitted and detected by probes (each probe is approximately the size of a bar of soap) placed against the skin. These probes then provide an estimate of tissue oxygenation. For each protocol, probes will be placed over the quadriceps muscle to measure changes in oxygenation in response to knee extensor exercise.

4. Discomfort and Risk:

Resting Blood Sample: The risk associated with single blood samples obtained with a needle and syringe may include one or all of the following: local discomfort at the puncture site, occasional dizziness and nausea, and bruising. Thrombosis (blood clots attached to walls of a blood vessel), embolism (blood clot in the circulation), and infections are very rare but are also potential risks. The risks will be minimized or eliminated by having only trained medical personnel from the GCRC who use sterile techniques to draw blood. Additionally, trained assistants will monitor you while blood is being obtained in a lying down position. All blood samples will be taken in the laboratory or clinic under sterile conditions using "one-time use" needles and containers. Proper procedures will be followed for the protection and safety of those individuals taking blood, and for the disposal of biohazardous waste.

Vascular Profiling: The risks of this vascular stiffness test are similar to that of taking blood pressure and an ECG. There may be minor discomfort when the cuffs are inflated.
ECG: There is a minimal risk due to potential allergic reaction to adhesive on the ECG electrodes.
EMG: There is a minimal risk due to potential allergic reaction to adhesive on the electrodes.
Blood pressure: The inflated arm cuff and wrist sensor may cause temporary tingling and numbness (Colin).
Ultrasound Doppler: You may experience minor risk of redness at the point where the ultrasound probe is pressed against the skin.
Single-knee Kick Exercise: You may experience temporary muscle fatigue and shortness of breath. These feelings go away very quickly after exercise is stopped. It is possible that you may also experience cramping in the legs, muscle strain, and muscle soreness, which will be minimized by proper warm-up, familiarization procedures, and cool-down. At any point during the study, should you feel muscle and joint pain, please tell us and we will stop the study immediately.
Near Infrared Spectroscopy: There are no known risks associated with near infrared spectroscopy measurements.
Abnormal Test Results: In the event that abnormal test results are obtained, either during the initial screening tests or during the exercise test, you will be apprised of the results (within one week of obtaining the results) and recommended to contact your medical provider for follow-up.
Stopping the Test: If you should feel that you cannot continue a test, you may stop the experiment at any time. It will be considered an uncompleted test.

Injury Clause:
Medical care is available in the event of injury from the research but neither financial compensation nor free medical treatment is provided. You are not waiving any rights that you may have against Pennsylvania State University for injury from negligence of the University or investigators.

5. Benefits:

A. To You: There are no clinical (i.e. treatment) benefits associated with this protocol.
B. To Society: This study will help us determine whether changes in leg blood flow responses with age are due in part to greater leg muscle fatigue in older adults.

6. Statement of Confidentiality:

All records associated with your participation in the study will be subject to the usual confidentiality standards similar to medical records (e.g., such as records maintained by physicians, hospitals, etc.) and in the event of any publication resulting from the research, no personally identifiable information will be disclosed. The Office of Human Research Protections in the U.S. Department of Health and Human Services, the U.S. Food and Drug Administration (FDA), the Office for Research Protections at Penn State and the Biomedical Institutional Review Board (IRB) may review records related to this project. Throughout the study, a number randomly assigned to you will be used as an identifier for allforms, records and data compiled. Consistent with the conduct of human research studies, the data will not be available to anyone outside of the experimental research team.

7. Rights to Ask Questions:

Feel free to contact the person below at any time if you have questions regarding your participation in this study. You may ask any questions about the research procedures, and these questions will be answered. All questions should be directed to the person below. He may be contacted at any time or via phone about the nature, conduct, or a problem with the study. The Office for Research Protections can be contacted at 201 Kern Building, University Park, PA 16802 or by calling (814) - 865 - 1775 with regards to questions about the rights of research participants. In the event of a research-related emergency, you may contact the GCRC medical staff (7AM – 5PM) at (814) - 865 - 7103. In the event of a research-related injury, please call:

David N. Proctor, PhD
Department of Kinesiology and Physiology
105 Noll Physiological Research Center
Pennsylvania State University
University Park, PA 16802
Work: (814) - 863 – 0724
email: dnp3@psu.edu

I, _____ have been given an opportunity to ask any questions I may have, and all such questions or inquiries have been answered to my satisfaction.

8. Compensation:

The cost of all tests and procedures directly related to participation in this study will be paid for by the study. You will be paid $40 dollars for completing this study. If you live out of town (>30 miles driving distance from State College), we will pay for any travel expense incurred or provide transportation if needed. Total payments within one calendar year will be treated as taxable income and therefore taxes will be taken from the total amount. This may require you to claim the compensation that you receive for participation in this study as taxable income.

9. Voluntary Participation:

I, _____ understand that my participation in this study is voluntary, and that I may withdraw from this study at any time by notifying the investigator. My withdrawal from this study or my refusal to participate will in no way affect my care or access to medical services. I can decline to answer specific questions.

You must be 18 years of age or older to consent to participate in this research study. If you consent to participate in this research study and to the terms above, please sign your name and indicate the date below. You will be given a copy of this consent form to keep for your records.

This is to certify that I, _____, consent to give permission for my participation as a volunteer in this program of investigation. I understand that I will receive a signed and dated copy of this consent form. I have read this form, and understand the content of this consent form.

I, _____, have defined and explained the procedures and protocols of the study to the above volunteer.

Volunteer: _____ Date: _____

Investigator: _____ Date: _____

VITA

Beth A. Parker

Education

May 2008	Ph.D., Kinesiology, The Pennsylvania State University
May 2000	B.S., Nutrition, Cornell University

Fellowships

2005-2008	National Institute on Aging (NIA) Gerontology Fellow
2003-2004	University Fellow, Pennsylvania State University

Awards

2006-2007	Kinesiology Dissertation Award
2005-2006	American College of Sports Medicine Foundation Doctoral Grant

Publications

1). **Parker BA**, Proctor DN (2005). Flow-mediated dilation. *J Appl Physiol*. Oct; 99(4):1620.

2). Ridout, SJ, **Parker, BA**, and Proctor, DN (2005). Age and regional specificity of peak limb vascular conductance in women. *J Appl Physiol*. Dec; 99(6):2067-74.

3).Proctor, DN and **Parker, BA** (2006). Vasodilation and Vascular Control in Contracting Muscle of the Aging Human. *Microcirculation*. June; 13(4): 315-27.

4) **Parker, BA**, Ridout, SJ, and Proctor, DN (2006). Age and Flow-mediated Dilation:A Comparison of Dilatory Responsiveness in the Brachial and Popliteal Arteries. *Am J Physiol Heart Circ Physiol*. Dec; 291(6): H3043-9.

5) **Parker, BA**, Smithmyer, SL, Jarvis, SS, Ridout, SJ, Pawelczyk, JA, and Proctor, DN (2007). Evidence for Reduced Sympatholysis in the Leg Resistance Vasculature of Healthy Older Women. *Am J Physiol Heart Circ Physiol*. Feb; 292(2): H1148-56.

6) **Parker BA**, Smithmyer SL, Pelberg JA, Mishkin AD, Herr MD, Proctor DN (2007). Sex Differences in Leg Vasodilation during Graded Knee Extensor Exercise in Young Adults. *J Appl Physiol*. Nov; 103(5):1583-91.

7) **Parker BA**, Smithmyer SL, Proctor DN (2007). Hormone Therapy is Associated with Preserved Smooth Muscle Structure and Dilation in the Arterial Vasculature of the Leg in Older Women. *Maturitas*. Jan; 59(1):46-54.

8) **Parker BA**, Smithmyer SL, Pelberg JA, Mishkin AD, Proctor DN (2007). Sex-specific Influence of Aging on Exercising Leg Blood Flow. *J Appl Physiol*. December 27; Epub ahead of print.

9) **Parker BA**, Smithmyer SL, Ridout SJ, Ray, CA, Proctor DN (2008). Age and Microvascular Responses to Knee Extensor Exercise in Women. In review; *Eur J Appl Physiol*.