THESIS

ACUTE ASCORBIC ACID ADMINISTRATION IMPROVES EXERCISE HYPEREMIA DURING RHYTHMIC BUT NOT SINGLE CONTRACTIONS IN AGING HUMANS

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2009

COLORADO STATE UNIVERSITY

APRIL 29, 2009

WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY CARRIE BETH SIMPSON ENTITLED ACUTE ASCORBIC ACID ADMINISTRATION IMPROVES EXERCISE HYPEREMIA DURING RHYTHMIC BUT NOT SINGLE CONTRACTIONS IN AGING HUMANS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

ACUTE ASCORBIC ACID ADMINISTRATION IMPROVES ENDOTHELIAL FUNCTION DURING RHYTHMIC BUT NOT SINGLE CONTRACTIONS IN AGING HUMANS

Age-related increases in oxidative stress are known to impair endothelium dependent vasodilation in older healthy humans. As a result, many researchers have speculated that endothelial dysfunction contributes to impaired muscle blood flow and vascular control during exercise. Further, elevations in oxidative stress and subsequent endothelial dysfunction could possibly explain our recent observations of impaired contraction-induced rapid vasodilation in older adults. Therefore, we directly tested the hypothesis that acute ascorbic acid administration would augment (1) rapid vasodilation in response to single muscle contractions as well as (2) the hyperemic response to sustained rhythmic contractions in older healthy humans, and that this would be due to improved endothelium-dependent vasodilation.

In 14 young (22±1 yrs) and 14 healthy older men and women (65±2 yrs), we measured forearm blood flow (FBF; Doppler ultrasound) and calculated vascular conductance (FVC) responses to single, 1 second dynamic contractions at 10, 20, and 40% maximum voluntary contraction (MVC) before and after intra-arterial administration of ascorbic acid (AA). We also measured these variables during rhythmic handgrip exercise at 10% maximum voluntary contraction. After 5 minutes of steady-

state exercise with saline, ascorbic acid (AA) was infused via brachial artery catheter for 10 minutes during continued exercise.

For single contractions, prior to AA peak vasodilator responses to all contraction intensities were impaired ~35-50% in older adults ($P<0.05$), as were the immediate (1st cardiac cycle post contraction) vasodilator responses at 20 and 40% MVC (~50%; $P<0.05$). In contrast to our hypothesis, AA did not influence contraction-induced rapid vasodilation in either group (all NS). Regarding rhythmic handgrip exercise, FBF (~28%) and FVC (~31%) were lower in older vs young adults ($P=0.06$ and $P<0.05$) prior to AA. In young adults, AA administration did not significantly influence FBF and FVC, whereas FBF and FVC increased 30±4% in older adults at end exercise ($P<0.05$). AA did not influence vasodilator responses to sodium nitroprusside in either group, but significantly improved vasodilation to acetylcholine in older adults only ($P<0.05$).

We conclude that endothelial dysfunction is not the primary mechanism underlying impaired contraction-induced rapid vasodilation with human aging; however acute AA administration increases muscle blood flow during dynamic exercise in older adults, which is likely due to an improvement in endothelium dependent vasodilation.

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CHAPTER I

REVIEW OF THE LITERATURE

One of the hallmark changes associated with aging is a decline in endothelial vasodilator function, and this decline in function is believed to be due, in part, to an increase in oxidative stress (Taddei *et al.*, 2000; Jablonski *et al.*, 2007). Oxidative stress may stem from increased free radical production, reduced capacity of metabolic pathways that have antioxidant functions, or a combination of these. If the body's antioxidant capabilities become overwhelmed or impaired, as seen with aging and numerous diseases, the vasculature becomes subject to damage by the continued presence of elevated oxidative stress.

In addition to changes in the oxidation status of blood vessels, aging is also associated with changes in a person's physiological response to exercise. Exercise evokes an increase in blood flow to active skeletal muscle, but depending on the type of exercise stimulus employed (single or rhythmic contractions), different regulatory mechanisms are stimulated. During continuous rhythmic muscle contractions, numerous elements contribute to exercise hyperemia, including mechanical deformation of the blood vessels, the sympathetic nervous system, release of local metabolic factors, and substances produced by the endothelium in response to increased levels of shear stress (Saltin *et al.*, 1998). Following a brief, single muscle contraction, the hyperemic response occurs immediately (Corcondilas *et al.*, 1964), is graded with contraction intensity (Tschakovsky *et al.*, 2004), and is due to smooth muscle cell hyperpolarization (Hamann *et al.*, 2004).

Interestingly, exercise hyperemia in attenuated in older adults during both sustained exercise (Lawrenson *et al.*, 2003; Proctor & Parker, 2006) and single muscle contractions (Carlson *et al.*, 2008), and because age is also associated with endothelial dysfunction (Taddei *et al.*, 2001) researchers have been prompted to hypothesize that declines in exercise hyperemia in older populations may be linked to age-related impairments in vascular function. With these ideas in mind, the purpose of this review is to examine aging as it relates to vascular function and control during exercise as well as to establish a foundation for the present experiment.

Aging and the Endothelium

It is well known that the vascular endothelium is negatively affected by age (DeSouza *et al.*, 2000; Taddei *et al.*, 2000; Lawrenson *et al.*, 2003; Poole *et al.*, 2003; Eskurza *et al.*, 2004), and endothelial dysfunction is a notorious precursor to numerous clinical conditions and cardiovascular diseases. As the main interface between circulating blood and the vascular wall, the endothelium is an essential component of vascular control and blood flow regulation (Luscher & Vanhoutte, 1990) both at rest and during exercise. If endothelial cells are damaged by hypertension, hypercholesterolemia, or other age-associated vascular conditions, the ability of these cells to properly function becomes compromised and the blood vessels' capacity to maintain proper tone is impaired (Taddei *et al.*, 1998; Taddei *et al.*, 2001).

More specifically, the endothelium has a primary role in maintaining vascular tone through its regulation of the production and release of nitric oxide (NO) (Taddei *et al.*, 1998; Faraci & Didion, 2004). If the endothelium becomes damaged or impaired in such a way that it cannot produce sufficient amounts of bioavailable NO, vascular tone is

not properly maintained or controlled. Without proper regulation of vascular tone, blood vessels are unable to appropriately respond to stimuli requiring changes in blood pressure and blood flow, as is the case with exercise.

In addition to nitric oxide, prostaglandin-mediated vasodilation helps to maintain blood flow during exercise (Clifford & Hellsten, 2004; Schrage *et al.*, 2004). Vasodilating prostaglandins are produced by the endothelium as well as skeletal muscle (Clifford & Hellsten, 2004) and their production has been observed to decline with advancing age (Schrage *et al.*, 2007). Together, the reduction in both NO bioavailability and PG production with advancing age suggest that age-related declines in these substances may lead to impaired endothelium-dependent vasodilation in older populations. For this reason, the homeostatic level of nitric oxide and prostaglandins is an important element to take into account when addressing endothelial function and blood flow regulation in both young and older individuals during exercising conditions.

Additionally, the contribution of oxidative stress to age-related declines in endothelium-dependent vasodilation is noteworthy due to the consequential decrease in vasoactive substances that leads to impairments in blood flow. Elevations in oxidative stress not only damage blood vessel walls, but the free radicals responsible for causing such stress and damage also react readily with nitric oxide. In fact, the superoxide anion, which is commonly present at increased concentrations with age, reacts with NO to form peroxynitrate (ONOO-) at a rate approximately three times faster than it does with the enzyme superoxide dismutase (Faraci & Didion, 2004; Jackson *et al.*, 2007). This reaction not only perpetuates the continued production of free radicals, but it also consumes available nitric oxide (Gryglewski *et al.*, 1986), thereby reducing the net

bioavailability of NO and leading to impaired vasodilation in older individuals. Taken together, the combined effect of reduced production and/or increased breakdown of endothelial-derived vasodilatory substances can lead to impaired endothelium-dependent dilation, and the manner in which these processes may affect blood flow regulation during exercise can be considerable, especially in older individuals.

Aging and Oxidative Stress

Regarding age in particular, impairment of the endothelium is evidenced by progressively weakened responses to endothelium-dependent vasodilation in older individuals (Taddei *et al.*, 1995; DeSouza *et al.*, 2000; Taddei *et al.*, 2000; Taddei *et al.*, 2001). Advancing age has been associated with an increase in oxidative stress and subsequent endothelial dysfunction (Shigenaga *et al.*, 1994; Taddei *et al.*, 2001), and one of the primary putative contributors to this oxidative stress is increased free radical production (Jablonski *et al.*, 2007; Rizvi & Maurya, 2007), possibly combined with impaired functioning of antioxidant pathways (Rizvi & Maurya, 2007).

Free radicals are a form of reactive oxygen species (ROS) that have one unpaired electron and can react *in vivo* to cause damage to DNA, proteins, lipids, the vasculature, and more (Shigenaga *et al.*, 1994; Jackson *et al.*, 2007). Superoxide (O_2^-) is the initial product of a one-electron reduction of oxygen, and it is the precursor for most ROS, as well as an intermediate in the process of oxidative chain reactions that go on to generate more free radicals. As alluded to previously, increased levels of superoxide and other ROS have been shown to decrease NO bioavailability, contributing to enhanced vascular resistance (Ungvari *et al.*, 2003; Plantinga *et al.*, 2007) and the possibility for cellular damage. As relatively more free radicals are generated than broken down by antioxidant

defense mechanisms, the process of 'oxidative stress' begins, rendering the vasculature susceptible to harmful effects such as plaque formation, increased stiffness, and altered responsiveness to vasoactive substances (Luscher *et al.*, 1993b; Wu & Thiagarajan, 1996; Shimokawa, 1999; Rizvi & Maurya, 2007), all of which increase a person's risk of developing vascular disease.

Further, because free radicals are believed to be one of the main factors leading to a decline in NO bioavailability, they are also identified as having a role in reducing endothelium-dependent vasodilation (Shigenaga *et al.*, 1994; Taddei *et al.*, 2001; Rizvi & Maurya, 2007). To illustrate this concept, researchers commonly evaluate endothelial function of the vasculature using acetylcholine and sodium nitroprusside to assess endothelium-dependent and endothelium-independent vasodilation, respectively. Most often, vasodilation in response to acetylcholine has been observed to decline with age (DeSouza *et al.*, 2000; Taddei *et al.*, 2000; Taddei *et al.*, 2001), while the response sodium nitroprusside more commonly does not differ among age groups (DeSouza *et al.*, 2000; Taddei *et al.*, 2001). Because acetylcholine acts directly on the endothelium to cause dilation, whereas sodium nitroprusside acts via the surrounding smooth muscle, the impaired vasodilatory response traditionally points to endothelium-dependent dilation as the element that is impaired with age. It is important to note, however, that vasodilation induced by sodium nitroprusside has also been shown to decline with age (Taddei *et al.*, 2000; Newcomer *et al.*, 2005; Parker *et al.*, 2006), which means that changes in vascular smooth muscle cells cannot be completely ruled out as having no influence on vascular control with age.

Due to the increased presence of free radicals in the vasculature of older individuals, antioxidants are commonly used as a means of evaluating endothelial function and blood flow regulation with less influence from high levels of oxidative stress. Ascorbic acid is a common antioxidant used in the laboratory, and at supraphysiologic levels it has the ability to scavenge free radicals within the vasculature. By decreasing the concentration of free radicals, ascorbic acid restores the bioavailability of nitric oxide, allowing it to properly function as a vasodilator and augment regional blood flow (Eskurza *et al.*, 2004; Jablonski *et al.*, 2007). In older subjects, ascorbic acid supplementation has been shown to decrease the markers of oxidative stress, and improve both endothelium-dependent and flow mediated dilation in the brachial artery (Eskurza *et al.*, 2004). It has also been used to successfully restore resting blood flow to the femoral artery in older males, bringing resting blood flow values up to levels observed in young males (Jablonski *et al.*, 2007).

Considering the negative effects of age and oxidative stress on the vasculature, it is possible that endothelial dysfunction, which has thus far been observed mainly in response to pharmacological stimuli, may also have an impact on a person's ability to regulate blood flow during exercise. In fact, while age-related reductions nitric oxide-mediated vasodilation have been linked with impaired endothelium-dependent vasodilation (Taddei *et al.*, 1995; Taddei *et al.*, 2000), there is no direct evidence to indicate whether this impairment extends to the vessel's ability to control blood flow during a hyperemic stimulus like exercise. Therefore, additional research is necessary to determine if there is in fact a link between age-associated endothelial dysfunction and blood flow regulation during exercise.

Aging and Exercise Capacity

In addition to changes in the oxidation status of blood vessels, aging is also associated with changes in a person's physiological response to exercise and a corresponding decline in exercise capacity (Holloszy & Kohrt, 1995). Exercise capacity is multidimensional, relating to one's aerobic capacity (VO_2 max), cardiopulmonary endurance, muscular strength, and physical ability. As such, it is a useful tool for evaluating the overall health of an individual and is related to one's risk for future disease development (Myers *et al.*, 2002). Peak exercise capacity has been determined to be a stronger predictor of increased risk of death than clinical symptoms in both healthy individuals and those with cardiovascular disease (Myers *et al.*, 2002). Additionally, reductions in exercise capacity frequently lead to declines in functional capacity, loss of independence, and an overall reduction in quality of life for older individuals (Holloszy & Kohrt, 1995).

Given the well known decline in endothelial function with age, it is plausible to speculate that this impairment of the vasculature relates to age-associated declines in exercise capacity as well, since the endothelium is a primary regulator of blood flow and vascular conductance and exercise requires an increase in both. However, despite the reasoning of this explanation, it remains unclear whether these changes occur independently or jointly. In fact, there is no direct evidence to indicate that exercise capacity and endothelial function develop independent of one another; nor is there clear evidence directly linking endothelial dysfunction to reductions in exercise capacity. In this context, further investigations are required to determine if any link exists between these two elements and to what degree they may or may not influence one another.

Aging and Exercising Blood Flow and Vascular Control

As mentioned previously, age-related declines in endothelial function (DeSouza *et al.*, 2000; Taddei *et al.*, 2000; Lawrenson *et al.*, 2003; Poole *et al.*, 2003) have important implications for blood flow regulation to skeletal muscle both at rest and during exercise. Specifically in reference to exercise, older adults display attenuated blood flow responses during both small (Lawrenson *et al.*, 2003) and large (Poole *et al.*, 2003; Proctor *et al.*, 2003) muscle mass exercise. Given that blood flow responses to each type of exercise are lower with age, it appears as though age-associated declines occur on many levels and are not necessarily limited to the size of the muscle.

Additionally, during exercising conditions adequate blood flow is required in order to sustain continuous contractions (Clifford & Hellsten, 2004; Proctor & Parker, 2006). If blood flow is limited during exercise, the active muscle is faced with oxygen and nutrient deprivations that in turn lead to fatigue, subsequent cessation of exercise, and eventual declines in exercise capacity. Because exercise capacity generally declines with age (Holloszy & Kohrt, 1995) along with endothelial vascular function (DeSouza *et al.*, 2000; Taddei *et al.*, 2000; Lawrenson *et al.*, 2003; Poole *et al.*, 2003; Eskurza *et al.*, 2004), researchers have been prompted to speculate that age-related declines in muscle blood flow and aerobic capacity potentially result from impairments in endothelial control of vascular tone during exercise. Suitably, elevated oxidative stress is a common explanation for the attenuated blood flow responses seen in older subjects (relative to young adults) during exercise. Recalling the previous discussion, it has been demonstrated that high levels of oxidative stress impair endothelium-dependent dilation by interfering with the bioavailability of nitric oxide (Ungvari *et al.*, 2003; Plantinga *et*

al., 2007), thereby reducing the blood vessel's ability to relax and subsequently allow for increased blood flow to active skeletal muscle (Shigenaga *et al.*, 1994; Taddei *et al.*, 2001; Rizvi & Maurya, 2007). By interfering with the production of nitric oxide, free radicals essentially lessen the vasodilator's influence on the vasculature and nullify its vasodilatory effects, therefore contributing to impaired blood flow responses during exercise.

Of further note on this topic is the fact that there appears to be some form of redundancy acting on the vessels to maintain exercising blood flow in individuals who are at risk for endothelial dysfunction, such as older adults. In particular, when both nitric oxide and vasodilating prostaglandins (PGs), are inhibited during exercise (Boushel *et al.*, 2002; Schrage *et al.*, 2004), there is a resulting decrease in exercising blood flow to the active muscle. A similar reduction in exercising blood flow is seen with single inhibition of nitric oxide; however, the same response is not observed when PGs alone are inhibited. Instead, there is almost no effect on exercising muscle blood flow with single blockade of PGs (Schrage *et al.*, 2004), which implies the existence of some type of complementary mechanism acting to restore or maintain blood flow when PG formation is inhibited during exercise. Such redundancy may have important implications for populations displaying endothelial dysfunction, such as older individuals, who may not have the ability to regulate the production and release of these substances as well as young healthy individuals during an exercise stimulus.

Despite the stimulus for vasodilation and the interaction of these pathways during exercise, local vasodilation is simultaneously balanced by a certain degree of vasoconstriction in active muscles with the purpose of maintaining systemic blood

pressure (Buckwalter *et al.*, 1997; O'Leary *et al.*, 1997; Proctor & Parker, 2006). As a means of shunting blood to active skeletal muscle, exercise brings about vasoconstriction in inactive tissue along with local vasodilation in active regions of the body (Proctor & Parker, 2006). However, during large muscle mass exercise, when substantial vasodilation is necessary to meet the metabolic demand of the tissue, the vasodilatory stimulus in the active muscle must be simultaneously limited by some level of vasoconstriction in order to prevent steep declines in systemic blood pressure (Proctor & Parker, 2006). Interestingly, the level of tonic sympathetic nerve activity present in older individuals is commonly elevated (Sundlof & Wallin, 1978; Ng *et al.*, 1993), leading to enhanced vasoconstriction in this population. This elevated sympathetic nerve activity has been associated with decreased exercising blood flow and, in fact, during both submaximal and maximal leg exercise, attenuated blood flow has been observed in older adults relative to their younger counterparts (Proctor & Parker, 2006). Such reductions in blood flow are coupled with decreased vascular conductance, which may potentially result from reduced vasodilator production (as a result of endothelial impairment), augmented sympathetic constriction, or a combination of both that interact to limit exercising blood flow.

Nonetheless, there appears to be significant alterations in the regulation of the hyperemic response to exercise in older compared to young adults. While there are numerous elements interacting to maintain the appropriate level of blood flow and vascular tone during exercise, including mechanical deformation of the blood vessels, the sympathetic nervous system, release of local metabolic factors (K+, adenosine, ATP, and NO), and substances produced by the endothelium in response to increased shear stress

(Saltin *et al.*, 1998; Clifford & Hellsten, 2004; Proctor & Parker, 2006), it seems that these pathways and the ensuing responses may be linked at some level to the endothelium and how well it is able to respond to the stress of exercise. Also, as a consequence of healthy human aging, the contribution of both nitric oxide and vasodilating prostaglandins to exercise hyperemia is significantly reduced, indicating that these pathways are not augmenting exercise hyperemia to the same degree as they are in younger individuals (Schrage *et al.*, 2004; Schrage *et al.*, 2007). Specifically, when compared to young adults, nitric oxide-mediated vasodilation is reduced by approximately 40% and prostaglandin-mediated vasodilation appears to be completely abolished in older subjects (Schrage *et al.*, 2004). Taken together, it is apparent that blood flow regulation during exercise is negatively influenced by age, and altered vasodilatory pathways may contribute to both reductions in exercise hyperemia as well as increased sympathetic constriction leading to reduced blood flow during exercise (Schrage *et al.*, 2004).

Aging and Contraction-Induced Rapid Vasodilation

In response to single, brief muscle contractions, the hyperemic response in healthy humans peaks within 4-6 cardiac cycles post-contraction and is graded with contraction intensity (Tschakovsky *et al.*, 2004; Carlson *et al.*, 2008). Additionally, by clamping smooth muscle cell membrane potential within the skeletal muscle of dogs, Hamman and colleagues successfully demonstrated that the hyperemic response to a contraction was virtually abolished upon inhibition of hyperpolarization (Hamann *et al.*, 2004). These results led to the conclusion that rapid vasodilation results from smooth muscle cell hyperpolarization rather than activation of the skeletal muscle pump, and this rapid

vasodilation is now recognized as obligatory in the process of increased muscle blood flow upon contraction (Hamann *et al.*, 2004). Along with hyperpolarization, the endothelium has also been determined to be an important component to vasodilation. As shown by Clifford and colleagues, removal of the vascular endothelium in isolated blood vessels leads to a significant reduction (~30%) in the degree of dilation observed in response to a mechanical stimulus (Clifford *et al.*, 2006). While dilation was not completely abolished upon removal of the endothelium, the attenuated response to the stimulus does provide strong evidence to support the role of the endothelium in the process of vasodilation.

Commonly, when blood flow regulation and vasodilatory responses have been assessed during exercise, they have been paired with rhythmic contractions that are maintained for a period of time. More recently, there has been a shift towards understanding the regulation of blood flow in response to a single muscle contraction because single contractions afford researchers the ability to examine vascular tone independent of the effects of subsequent contractions (Hamann *et al.*, 2004; Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007) and sympathetic nervous system influences (Corcondilas *et al.*, 1964; Buckwalter & Clifford, 1999). Small muscle mass exercise in particular is useful when evaluating this response because it does not elicit changes in mean arterial pressure or heart rate (Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007; Carlson *et al.*, 2008), which may influence the interpretation of the cause of hyperemia.

Applying these concepts to human aging, recent data from our laboratory indicate that contraction-induced rapid vasodilation is significantly impaired in older compared with young healthy adults (Carlson *et al.*, 2008), and to the best of our knowledge, this

issue has not been previously investigated in relation to the potential influences of oxidative stress and endothelial dysfunction. However, because the endothelium has an essential role in vasodilation (Clifford & Hellsten, 2004), and it is well established that endothelial function is impaired with age, it is possible that the hyperemic response to single contraction may be impaired in older adults due to age-associated elevations in oxidative stress. In this context, further investigations are necessary to determine if the effect of age and any associated declines in endothelial function influence contraction-induced hyperemia in the same manner as steady state exercise.

Statement of the Problem

Whether impaired endothelial function is mechanistically-linked to the observed blunted contraction-induced rapid vasodilation in older humans is unknown. Also, there is no definitive evidence directly linking impaired endothelial function to attenuated vascular control during dynamic exercise in older populations, and it is not known whether improvements in endothelial function lead to enhanced exercise hyperemia in older adults.

Hypothesis

Therefore, we sought to test the hypothesis that acute ascorbic acid administration would augment (1) rapid vasodilation in response to single muscle contractions as well as (2) the hyperemic response to sustained contractions in older healthy humans, and that this would be due to improved endothelium-dependent vasodilation.

CHAPTER II

MANUSCRIPT

Endothelium-dependent Vasodilatation and Exercise Hyperaemia in Ageing Humans: Impact of Acute Ascorbic Acid Administration

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Running Title: Ageing, Endothelial Dysfunction, and Exercise Hyperaemia

Key Words: muscle blood flow, vitamin c, vascular function, age

Word Count: 5,902

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Abstract

Age-related increases in oxidative stress impair endothelium-dependent vasodilatation in humans, leading to the speculation that endothelial dysfunction contributes to impaired muscle blood flow and vascular control during exercise in older adults. We directly tested this hypothesis in 14 young (22±1 yrs) and 14 healthy older men and women (65±2 yrs). We measured forearm blood flow (FBF; Doppler ultrasound) and calculated vascular conductance (FVC) responses to single muscle contractions at 10, 20, and 40% maximum voluntary contraction (MVC) before and during ascorbic acid (AA) infusion, and we also determined the effects of AA on muscle blood flow during mild (10% MVC) continuous rhythmic handgrip exercise. For single contractions, the peak rapid hyperaemic responses to all contraction intensities were impaired ~45% in the older adults (all $P < 0.05$), and AA infusion did not impact the responses in either age group. For the rhythmic exercise trial, FBF (~28%) and FVC (~31%) were lower ($P = 0.06$ and 0.05) in older versus young adults after 5 minutes of steady-state exercise with saline. Subsequently, AA was infused via brachial artery catheter for 10 minutes during continued exercise. AA administration did not significantly influence FBF or FVC in young adults (1-3%; $P = 0.24$-0.59), whereas FBF increased 34±7% in older adults at end exercise, and this was due to an increase in FVC (32±7%; both $P < 0.05$). This increase in FBF and FVC during exercise in older adults was associated with improvements in vasodilator responses to acetylcholine (ACH; endothelium-dependent) but not sodium nitroprusside (SNP; endothelium-independent). AA had no effect on ACH or SNP responses in the young. We conclude that acute AA administration does not impact the observed age-related impairment in the rapid hyperaemic response to brief muscle

contractions in humans, however it does significantly increase muscle blood flow during continuous dynamic exercise in older adults, and this is likely due (in part) to an improvement in endothelium-dependent vasodilatation.

Introduction

Blood flow and oxygen delivery increase to contracting muscle, a complex response involving mechanical factors, the sympathetic nervous system, as well as local metabolic and endothelium-derived substances that influence vascular tone (Saltin *et al.*, 1998). With respect to the latter, a variety of local endothelium-dependent vasodilators are recognized to increase during exercise and partially control blood flow and vascular tone in contracting human skeletal muscle (Clifford & Hellsten, 2004; Saltin, 2007). For example, local inhibition of nitric oxide and vasodilating prostaglandins during rhythmic handgrip exercise independently reduces forearm hyperaemia in young healthy humans (Schrage *et al.*, 2004), and combined inhibition of these substances has been documented to reduce muscle blood flow during knee extensor exercise (Boushel *et al.*, 2002; Mortensen *et al.*, 2007). Additionally, recent work by Hillig and colleagues demonstrated redundancy between nitric oxide and the cytochrome P450 pathway, leading to the speculation that an endothelium-derived hyperpolarizing factor increases during exercise and is also involved in local vascular control (Hillig *et al.*, 2003). Together, these observations indicate a significant role of the endothelium in regulating exercise hyperaemia in healthy humans.

Human ageing is associated with a progressive decline in endothelial function that predisposes older adults to increased risk for thrombosis, atherosclerotic vascular disease, as well as ischemic heart and cerebrovascular disease (Luscher *et al.*, 1993b; Wu & Thiagarajan, 1996; Shimokawa, 1999). Ageing is also associated with reductions in exercise capacity (Holloszy & Kohrt, 1995), and this may be due in part to impaired blood flow and oxygen delivery to contracting muscles (Lawrenson *et al.*, 2003; Poole *et*

al., 2003; Proctor *et al.*, 2003). Collectively, these findings have lead to the hypothesis that impaired endothelial vasodilator function contributes to exercise intolerance with age and other disease states involving endothelial dysfunction via impaired vascular control during exercise (Drexler & Hornig, 1996; Proctor & Parker, 2006). Consonant with this concept, recent work by Schrage and colleagues indicates that the normal contributions of endothelium-derived nitric oxide and vasodilating prostaglandins to exercise hyperaemia are significantly reduced with age in older healthy humans (Schrage *et al.*, 2007). Although the mechanisms underlying these changes are unclear, age-related increases in oxidative stress are suggested to impair endothelium-dependent vasodilatation in humans, and accumulating data indicates that acute antioxidant administration (e.g., ascorbic acid) can reverse endothelial dysfunction as evidenced by the restoration of acetylcholine- and flow-mediated vasodilatation in older adults (Taddei *et al.*, 2001; Eskurza *et al.*, 2004). Despite these observations, it is presently unknown whether acutely improving endothelial function augments muscle blood flow via local vasodilatation during dynamic exercise in older humans.

To date, the majority of studies designed to understand muscle blood flow and vascular control in exercising young and older adults have been performed during steady-state exercise. However, in recent years, particular attention has been given to the rapid hyperaemic response following a single muscle contraction, as this allows for the determination of contraction-induced vasodilatation without the impeding effects of subsequent contractions on vascular tone (Hamann *et al.*, 2004; Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007). Additionally, the mechanisms influencing vascular tone at exercise onset appear to differ from those involved in the control during steady-state exercise

(Clifford & Hellsten, 2004). Upon the release of a single contraction, the typical rapid hyperaemic response peaks within 3-6 cardiac cycles and the magnitude is graded with contraction intensity (Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007). Importantly, this response is independent of sympathetic neural influences (Corcondilas *et al.*, 1964; Buckwalter & Clifford, 1999), and during small muscle mass exercise (e.g., isolated handgrip), can also occur without significant changes in heart rate and arterial pressure (Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007; Carlson *et al.*, 2008). Thus, under specific experimental conditions, this rapid hyperaemic response reflects a local vasodilator response within the vascular bed of the contracting muscle. With respect to ageing, we recently documented that skeletal muscle contraction-induced rapid vasodilation is significantly impaired in the forearm vasculature of older healthy adults (Carlson *et al.*, 2008). It is currently unknown whether impaired endothelial vasodilator function is mechanistically-linked with this blunted rapid vasodilatory pattern following a single brief muscle contraction. Further, whether improving endothelium-dependent vasodilatation restores the blunted rapid hyperaemia in older adults has not been investigated.

Accordingly, the purpose of the present study was to directly test the hypothesis that acute improvements in endothelium-dependent vasodilator function via brachial artery infusion of ascorbic acid augments blood flow to contracting muscle of older adults via local vasodilatation. To do so, we determined the effects of ascorbic acid in young and older adults on (1) forearm haemodynamic responses to single, brief muscle contractions, (2) forearm haemodynamics during dynamic (continuous) contractions of the forearm muscles, and (3) the vasodilator responses to intra-arterial infusions of

endothelium-dependent (acetylcholine) and endothelium-independent (sodium nitroprusside) agonists.

Methods

Subjects

With Institutional Review Board approval and after written informed consent, a total of 14 young and 14 older healthy adult men and women participated in the present study. All subjects were normotensive and free from overt cardiovascular disease as assessed from casual blood pressure measurements and a medical history. Older subjects were further evaluated for clinical evidence of cardiopulmonary disease with a physical examination and resting and maximal exercise electrocardiograms. All subjects were sedentary to moderately active, non-smokers, not taking any medications including antioxidants, and studies were performed after a minimum of a 4-hour fast. Subjects provided written, informed consent after all potential risks and procedures were explained. This study was approved by the Human Research Committee of Colorado State University and was performed according to the Declaration of Helsinki.

Arterial Catheterization

A 20-gauge, 7.6-cm catheter was placed in the brachial artery of the non-dominant arm under aseptic conditions after local anesthesia (2% lidocaine) for local administration of study drugs. The catheter was connected to a 3-port connector as well as a pressure transducer for mean arterial pressure (MAP) measurement and continuously

flushed at 3 ml h^{-1} with heparinized saline (Dinenno *et al.*, 2003; Dinenno & Joyner, 2004; Kirby *et al.*, 2008). The two side ports were used for infusions of vasoactive drugs.

Blood Samples

Measures of total cholesterol, low- and high-density lipoproteins (LDL and HDL), and triglycerides were performed via conventional methods by the clinical laboratory of the Poudre Valley Hospital (Fort Collins, CO). Oxidized-LDL was measured via standard Elisa assay (Mercodia, Inc., Uppsala, Sweden) as a marker of circulating oxidative stress via the General Clinical Research Center of the Milton S. Hershey Medical Center (Hershey, PA).

Body Composition and Forearm Volume

Body composition was determined by dual-energy X-ray absorptiometry (DEXA; DPX-IQ, Lunar Radiation). Total forearm volume and fat-free mass (FFM) were calculated from regional analysis of the experimental forearm (from the proximal to distal radioulnar joint) from whole-body DEXA scans with Lunar software version 4.7e for normalization of individual drug doses (Dinenno *et al.*, 2002; Carlson *et al.*, 2008). Body mass index was calculated as bodyweight (kg) divided by height (meters) squared.

Forearm Blood Flow and Vascular Conductance

A 4 MHz pulsed Doppler probe (Model 500V, Multigon Industries, Mt. Vernon, NY, USA) was used to measure brachial artery mean blood velocity (MBV) with the probe securely fixed to the skin over the brachial artery proximal to the catheter insertion

site as previously described by our laboratory (Dinenno & Joyner, 2003; Kirby *et al.*, 2007; Carlson *et al.*, 2008). The probe insonation angle relative to the skin was 45 degrees. A linear 12 MHz echo Doppler ultrasound probe (GE Vingmed Ultrasound Vivid7, Horten, Norway) was placed in a holder securely fixed to the skin immediately proximal to the velocity probe to measure brachial artery diameter. For the single contraction trials, brachial artery diameter was measured in triplicate prior to any contractions, as we and others have shown that brachial diameter does not change in response to this stimulus (Tschakovsky *et al.*, 2004; Carlson *et al.*, 2008). For the rhythmic handgrip exercise trials, brachial diameter was measured in triplicate at rest and at each minute of exercise. For the pharmacological tests, brachial diameter was measured in triplicate at rest and after 5 minutes of drug infusion (see below for details). Forearm blood flow was calculated as:

FBF = MBV (cm s^{-1}) * π (brachial artery diameter/2)^2 * 60, where the FBF is in ml min^{-1}, the MBV is in cm s^{-1}, the brachial diameter is in cm, and 60 is used to convert from ml s^{-1} to ml min^{-1}. Forearm vascular conductance (FVC) was calculated as (FBF/MAP) * 100, and expressed as ml min^{-1} 100 $mmHg^{-1}$.

Single Dynamic Forearm Contractions

Maximum voluntary contraction (MVC) was determined for the experimental arm as the average of three maximal squeezes of a handgrip dynamometer (Stoelting, Chicago, IL) that were within 3% of each other. Brief, dynamic forearm contractions were performed at 10, 20, and 40% of the subject's MVC using a handgrip pulley system attached to weights corresponding to each workload. The weight was lifted 4-5 cm over

the pulley for a single, 1-second dynamic contraction as previously described (Carlson *et al.*, 2008). These mild-to-moderate contraction intensities were chosen to limit the contribution of systemic haemodynamics to forearm vasodilator responses and to eliminate reflex increases in sympathetic nervous system activity, and thus isolate the local effects of muscle contraction on vascular tone (Carlson *et al.*, 2008). Two minutes of relaxation were given between each contraction to allow continuous measures of forearm haemodynamics post-contraction, as well as ample time for haemodynamics to return to baseline values (Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007; Carlson *et al.*, 2008). Workload intensity was randomized and counterbalanced across subjects to eliminate any order effect and trials were performed in triplicate to calculate an average response for each subject.

Rhythmic Handgrip Exercise

Using the same pulley system, subjects performed rhythmic, dynamic handgrip exercise at 10% MVC with a duty cycle of 1s contraction - 2s relaxation (20 contractions per minute) using audio and visual signals to ensure the correct timing (Dinenno & Joyner, 2003; Kirby *et al.*, 2008). Similar to the rationale provide above, this mild intensity rhythmic handgrip exercise was chosen to (a) limit the contribution of systemic haemodynamics to forearm hyperaemic responses and (b) eliminate reflex activation of the sympathetic nervous system (Seals & Victor, 1991; Carlson *et al.*, 2008). Further, mild contractions can be performed for a significant amount of time without evoking progressive increases in heart rate and arterial pressure (Schrage *et al.*, 2004; Schrage *et al.*, 2007). Thus, our experimental model aims to isolate the local effects of muscle

contractions on forearm hyperaemia without engaging potentially confounding systemic influences on vascular tone.

Vasoactive Drug Administration

Endothelium-dependent vasodilatation was determined by intra-arterial infusion of acetylcholine (ACH; Miochol-E, Novartis Inc.) at 16 μg $100ml^{-1}$ forearm volume min^{-1} for 5 minutes and endothelium-independent vasodilatation was assessed via intra-arterial infusion of sodium nitroprusside (SNP; Nitropress, Hospira Inc.) at 4 μg 100ml forearm $volume^{-1}$ min^{-1} for 5 minutes (DeSouza *et al.*, 2000; DeSouza *et al.*, 2002). As a method of acutely improving endothelium-dependent vasodilatation (Taddei *et al.*, 2000; Taddei *et al.*, 2001), the potent antioxidant ascorbic acid (Vitamin C, American Regent Inc.) was infused at 8 mg 100 ml forearm $volume^{-1}$ min^{-1} for 10 minutes during handgrip exercise as a loading dose (see *Experimental Protocol* below), and at 40% of this loading dose for maintenance infusion throughout the remainder of the experiment.

Experimental Protocol

Subjects were studied in the supine position with the experimental arm extended 90º laterally at heart level. The experimental timeline is depicted in Figure 1. Two minutes of resting data were acquired prior to all experimental trials. To establish endothelium-dependent and -independent vasodilator responsiveness, ACH and SNP (respectively) were individually infused via brachial artery catheter for 5 minutes. The order of ACH and SNP was counterbalanced across subjects and 15 minutes of rest was allowed following each drug infusion. Next, subjects performed single brief forearm

contractions at 10, 20, and 40% MVC for 1-second with 2 minutes of rest in between contractions. After 10 minutes of rest, rhythmic handgrip exercise was performed at 10% MVC with saline for 5 minutes to achieve steady-state haemodynamics, and ascorbic acid was then infused during continued exercise for 10 more minutes equaling a total time of 15 minutes of handgrip exercise (Trial 1). The dose of ascorbic acid was reduced to 40% of the original dose for the remainder of the experiment. Following 15 minutes of rest, ACH and SNP infusions were repeated during maintenance ascorbic acid administration to determine the influence of ascorbic acid on endothelium-dependent and -independent vasodilatation. Single forearm contractions were again performed to determine the impact of ascorbic acid on contraction-induced rapid vasodilatation. Lastly, a second trial (Trial 2; n = 13 for young, n = 14 for older) of rhythmic handgrip exercise was performed for 5 minutes to determine forearm haemodynamics during the transition from rest to steady-state exercise with ascorbic acid already present and to confirm that any changes in forearm haemodynamics during Trial 1 were not simply due to a 'time effect' of continued exercise.

Data Acquisition and Analysis

Data was collected and stored on computer at 250 Hz and analyzed off-line with signal-processing software (WinDaq, DATAQ Instruments, Akron, OH, USA). Mean arterial pressure (MAP) was determined from the arterial pressure waveform. Baseline FBF, HR, and MAP represent an average of the last minute of the resting time period prior to all exercise trials and pharmacological vasodilatory tests. The data presented for the ACH and SNP trials represent the final 30-seconds of drug infusion. For the single

contraction trials, the post-contraction data represent the first unimpeded cardiac cycle immediately after release of the contraction, and this beat-by-beat analysis was performed for a total of 30 cardiac cycles (Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007; Carlson *et al.*, 2008). For the rhythmic exercise trials, the minute-by-minute hyperaemic (FBF) and vasodilatory values (FVC) represent the last 30 seconds of that minute at rest and during exercise. The % change in FBF during drug infusions and exercise was calculated as:

((FBF drug or exercise - FBF baseline)/(FBF baseline)) x 100.

Changes in FVC were calculated in a similar fashion.

Statistics

All values are reported as means ± S.E.M. Comparison of subject characteristics and the haemodynamic values at specific time points between groups for the ACH, SNP, and exercise conditions were made with unpaired t-tests, and the within group values for each hyperaemic condition with paired t-tests. Specific hypothesis testing within exercise trials was performed to assess mean group differences between young and older adults using two-way repeated measures analysis of variance. *Post-hoc* analysis was performed using the Tukey's test when significance was observed. Significance was set at $P < 0.05$.

Results

Subject Characteristics

The mean age difference between the young and older adults was 43 yrs. There were no significant age-group differences in BMI, whole-body FFM, forearm volume, forearm FFM, MVC, or HDL-cholesterol. Older individuals had a greater body fat

percentage and total and LDL-cholesterol ($P < 0.05$; Table 1), although these values were within normal levels. Baseline haemodynamics for all trials were not different between young and older adults.

Effect of Ascorbic Acid on Forearm Haemodynamic Responses to Single Muscle Contractions

Dynamic blood flow responses following single forearm muscle contractions at 10, 20 and 40% MVC are shown in Figures 2A-C. At all exercise intensities, both young and older groups demonstrated the typical temporal hyperaemic pattern, with peak hyperaemia occurring ~3-4 cardiac cycles post-contraction. Consistent with previous findings from our laboratory (Carlson *et al.*, 2008), older adults had a blunted immediate (first beat post-contraction; ~35-50%) and peak hyperaemic response (~45%) compared to young adults at all exercise intensities (Figures 2A-C; $P < 0.05$). This impairment was observed for a total of 4, 11, and 13 beats post-contraction for contraction intensities of 10, 20, and 40% MVC, respectively. This rapid hyperaemic response was unaffected by infusion of ascorbic acid in young adults. In contrast to our hypothesis, ascorbic acid administration in older adults also did not augment the rapid hyperaemia seen following a brief single muscle contraction at any exercise intensity (Figures 2A-C). Data were identical when analyzed as percentage increases in FVC (not shown).

Effect of Ascorbic Acid on Forearm Haemodynamics during Rhythmic Handgrip Exercise

Rhythmic dynamic handgrip exercise performed at 10% MVC significantly increased FBF and FVC from baseline within the first minute and throughout the duration of the exercise trial in both young and older adults ($P < 0.05$; Table 2, Figure 3). Absolute FBF and FVC tended to be lower in older compared with young adults when expressed as absolute blood flow (Table 2, Figure 3A; $P = 0.06 – 0.09$ for minutes 1-6), and was significantly lower when expressed as the percent increase in FBF and FVC from baseline (Table 2, Figure 3B; $P < 0.05$ for minutes 1-10). Ascorbic acid infusion had no effect on FBF or FVC in young adults throughout the entire exercise bout (Table 2, Figures 3A-B; $P > 0.05$), but significantly and progressively increased FBF and FVC from steady-state in older adults beginning at minute 7 (2^{nd} minute of ascorbic acid infusion) until the end of exercise (minute 15) (Table 2, Figures 2A-B; $P < 0.05$).

The peak effect of ascorbic acid infusion on FBF and FVC during steady-state exercise was calculated as the percent increase from minute 5 of exercise (end saline) to the final minute of exercise (end ascorbic acid; Figure 4A-B). Ascorbic acid infusion increased FBF (34±7%) and FVC (32±7%) from steady-state exercise in older adults (both $P < 0.05$), whereas forearm haemodynamics were unchanged in young adults (ΔFBF = 3±2%; ΔFVC = 1±2%; *NS*). MAP and HR were not significantly different between young and older adults at baseline and were not significantly altered throughout the experimental trial (Table 2). Brachial artery diameter at rest was not different in young and older adults (0.43±0.02 vs 0.42±0.02 cm; $P = 0.55$) and was similar after 5

minutes exercise with saline (0.42±0.02 vs 0.41± 0.02 cm) and at the end of ascorbic acid infusion (0.42±0.02 vs 0.41±0.02 cm).

Effect of Prior Ascorbic Acid on Forearm Haemodynamics from Rest to Steady-State Exercise

Ascorbic acid had no impact on resting forearm blood flow in either young or older adults (Figure 5A and B). In contrast to the observed trend for an age-associated impairment in the absolute hyperaemic and vasodilatory responses seen during the transition from rest to steady-state exercise in trial 1, FBF in older adults during ascorbic acid infusion increased to similar values as young adults within the 1st minute of exercise (Figure 5A). This pattern was similar throughout all 5 minutes of exercise (Figure 5A), and the FBF values at rest and after 5 minutes of steady-state exercise before and during concurrent ascorbic acid administration are shown in Figure 5b. Percentage increases in FBF also were not significantly different at minute 1 of exercise in young and older adults (316 ± 40% vs 255±30%; $P = 0.23$), and were similar at end exercise (329±45% vs 308±40%; $P = 0.73$). MAP and HR were again not significantly different between young and older adults, thus FVC values were similar to FBF (data not shown).

Effect of Ascorbic Acid on Endothelium-Dependent and -Independent Vasodilatation

Under control conditions, the increase in FBF in response to acetylcholine was blunted in older (Δ FBF = 420 ± 67%) compared with young adults (Δ FBF = 706 ± 100%; $P < 0.05$), and this impairment was no longer observed during ascorbic acid administration (older Δ FBF = 744 ± 83%; young = 709 ± 44%; *NS*; Figure 6A). The

increase in FBF to sodium nitroprusside was also attenuated in older compared with young adults under control conditions (Δ FBF = 385 ± 43% vs 671 ± 113%, respectively; $P < 0.05$); however these responses were unaffected by ascorbic acid infusion (older ΔFBF = 365 ± 32%; young = 662 ± 93%; Figure 6B). No significant differences in HR or MAP were observed between or within groups ($P > 0.05$), thus changes in FVC were similar as FBF (not shown).

Plasma Markers of Oxidative Stress

At baseline, plasma oxidized-LDL was greater in the older compared with young subjects (36.5±1.7 vs 28.8±2.4 U L^{-1}; $P < 0.05$). Infusion of ascorbic acid did not affect these plasma levels in either young (29.8.7±2.2 U L^{-1}) or older adults (37.5±2.7 U L^{-1}), which most likely reflects that the ascorbic acid was administered locally via brachial artery catheter and was dose-adjusted to forearm volume. Importantly, the improvements in acetylcholine-mediated vasodilatation (see above) in older subjects is consistent with prior studies and provides evidence that ascorbic acid was effective at the level of the forearm vasculature (Taddei *et al.*, 2000; Taddei *et al.*, 2001).

Discussion

In the present study, we directly determined whether acutely improving endothelial vasodilator function would augment blood flow responses to contracting muscle in ageing humans. The primary new findings of the present study are as follows. First, acute infusion of ascorbic acid does not impact the rapid hyperaemic responses to single, brief mild-to-moderate muscle contractions in young or older adults. Second,

local ascorbic acid infusion during rhythmic handgrip exercise increased forearm blood flow by ~30% in older adults during continuous exercise, and this was due to significant increases in forearm vascular conductance (vasodilatation). In contrast, ascorbic acid did not influence forearm haemodynamics in the young subjects. Third, when older adults transitioned from rest to steady-state exercise with concurrent ascorbic acid infusion, steady-state blood flow was significantly improved and the impaired responses under control conditions were no longer observed compared with young adults. Finally, age-related impairments in the vasodilator responses to the endothelium-dependent agonist acetylcholine were no longer evident during ascorbic acid infusion. To the best of our knowledge, these data are the first to demonstrate that acute improvements in endothelium-dependent vasodilatation are associated with augmented blood flow responses to dynamically contracting skeletal muscles of ageing humans.

Ageing and Contraction-Induced Rapid Vasodilatation: effect of ascorbic acid

In young healthy adults, single (brief) muscle contractions evoke a rapid hyperaemic response that is graded with contraction intensity (Corcondilas *et al.*, 1964; Tschakovsky *et al.*, 2004; Kirby *et al.*, 2007). Although the exact mechanisms underlying this phenomenon are unclear, the impact of a "muscle pump" effect appears negligible and thus vascular smooth muscle cell hyperpolarization and subsequent vasodilatation is obligatory to observe this response (Hamann *et al.*, 2004). Recent studies implicate a role for several potential contributors to this rapid vasodilatation including mechanical effects on the vasculature (which is in part dependent on an intact endothelium) (Clifford *et al.*, 2006; Kirby *et al.*, 2007), K^+ released during muscle

activation (Armstrong *et al.*, 2007), and acetylcholine spillover from motor nerves (VanTeeffelen & Segal, 2006). Further, data from Duza & Sarelius (2004) obtained from skeletal muscle arterioles of mice indicate that an intact endothelium is obligatory for the initiation of contraction-induced vasodilatation. In agreement with recent findings from our laboratory (Carlson *et al.*, 2008), the data from the present study indicate that the rapid hyperaemic responses to single, brief muscle contractions of mild-to-moderate intensities are significantly impaired in older compared with young healthy adults. Further, our data indicate that improving acetylcholine-mediated (endothelium-dependent) vasodilatation via ascorbic acid infusion does not impact on the rapid hyperaemic responses in young, and more importantly, older humans. Together, these observations might provide further support for the hypothesis that the "normal" rapid vasodilator responses are not mediated via acetylcholine in humans (Brock *et al.*, 1998; Dyke *et al.*, 1998; Naik *et al.*, 1999). Although we cannot completely rule out a possible role of impaired endothelial function in this blunted rapid vasodilatory response with age, our data indicate that this is insensitive to the improvements in vascular function mediated via ascorbic acid. We speculate that this age-related impairment may be due to impaired K^+ signaling (either release of or responsiveness to) or impaired mechanically-induced vasodilatation, but future investigations will be required to elucidate the specific underlying mechanisms.

Ageing and Exercise Hyperaemia During Rhythmic Handgrip Exercise: effect of ascorbic acid

In the present study, we infused ascorbic acid into the brachial artery during rhythmic handgrip exercise to determine whether muscle blood flow would increase during sustained (continuous) exercise in young and older healthy adults. Our findings clearly indicate that blood flow increased ~30% in older adults, whereas ascorbic acid was without effect in young adults (Figure 2). Further, when the subjects transitioned from rest to exercise with concurrent ascorbic acid infusion (Figure 5), similar results were obtained, indicating that this effect of ascorbic acid on muscle blood flow in older subjects cannot be attributed to a drift in haemodynamics over the course of the original fifteen minute exercise bout. Given that there were no changes in heart rate or mean arterial pressure throughout the exercise trials, the increases in muscle blood flow were due to a corresponding increase in vascular conductance (i.e. vasodilatation). Further, we demonstrated that the age-related decrease in endothelium-dependent vasodilatation (via acetylcholine) is abolished after infusion of ascorbic acid. Taken together, these data are the first to demonstrate that acute improvements in endothelial function increases muscle blood flow during continuous exercise via local vasodilatation in older healthy humans.

Endothelial dysfunction is one hallmark of vascular ageing, and represents a significant risk for cardiovascular disease risk and progression (Luscher *et al.*, 1993b; Shimokawa, 1999). In addition to its role in maintaining vascular health, the endothelium produces vasodilator (e.g., nitric oxide, prostacyclin, endothelium-derived hyperpolarizing factor) and vasoconstrictor (e.g., endothelin-1, thromboxane) substances that significantly influence vascular tone (Luscher *et al.*, 1993a). Indeed, recent evidence

implicates a significant role for endothelium-derived substances in controlling muscle blood flow and vascular tone in contracting muscle of young humans (Hillig *et al.*, 2003; Clifford & Hellsten, 2004; Saltin, 2007). As such, there has been much speculation that age-related reductions in endothelium-dependent vasodilatation play a role in the impaired skeletal muscle perfusion often observed during exercise in older healthy and diseased adults (Drexler & Hornig, 1996; Proctor & Parker, 2006). Consistent with this, work by Schrage and colleagues indicate that the normal contributions of nitric oxide and vasodilating prostaglandins to exercise hyperaemia are impaired with age (Schrage *et al.*, 2007). In the present study, we extend these observations by demonstrating that acute improvements of endothelial vasodilator function (achieved via ascorbic acid administration) is associated with significant improvements in muscle blood flow during exercise in older but not young adults. These collective observations provide support for the hypothesis that endothelial dysfunction is mechanistically-linked with altered vascular control during dynamic forearm exercise in ageing humans.

Potential Mechanisms

Although we clearly demonstrate that ascorbic acid significantly improves muscle blood flow to contracting muscle during continuous exercise via vasodilatation in older adults, the signaling mechanism(s) underlying this improvement remains unclear. Consistent with previous data (Taddei *et al.* 2000, Taddei *et al.* 2001), ascorbic acid did not influence resting forearm haemodynamics, indicating that the improvements in blood flow during exercise (and acetylcholine infusion) in older adults is only observed during stimulation of the endothelium. It is possible that ascorbic acid is acting to increase nitric

oxide bioavailability, an event that could be due to direct scavenging of free radicals (e.g, O_2^-) (Nishikimi, 1975), or via the stabilization for nitric oxide synthesis tetrahydrobiopterin (BH_4), an important cofactor for nitric oxide synthesis (Heller *et al.*, 2001). In this context, recent work in older experimental animals and humans support the hypothesis that increasing BH_4 concentrations significantly improve endothelium-dependent vasodilatation (Eskurza *et al.*, 2005; Delp *et al.*, 2008). Whether this translates to improved vascular control in contracting muscle of ageing humans is unknown. Another possibility is that age-associated oxidative stress increases endothelin-mediated vasoconstriction, and that ascorbic acid acutely reverses this detrimental effect of endothelin on local vasodilator function, thereby allowing for greater hyperaemic responses during exercise in older adults (Bohm *et al.*, 2007; Van Guilder *et al.*, 2007). Clearly, future studies will be needed to determine the specific mechanisms involved in the ascorbic acid-mediated improvement in muscle blood flow and vascular control during exercise of ageing humans.

If the ascorbic acid in the present study is working specifically to reduce the accumulation of reactive oxygen species during exercise and this in turn improves vascular control in older adults, we do not know whether these species are being generated within the blood vessels or from the contracting skeletal muscle. Our finding that acetylcholine-mediated vasodilatation was restored in older adults during ascorbic acid does provide indirect evidence that at least part of this stress is present at rest and most likely within the endothelial cells (Donato *et al.*, 2007). However, it is well known that muscle contractions can increase reactive oxygen species production (Bailey *et al.*, 2007), and this coupled with evidence of reduced antioxidant defense systems in skeletal

muscle of ageing humans (Pansarasa *et al.*, 1999), clearly suggests a possible interaction between ascorbic acid and free radicals generated from the muscle tissue. Again, future investigations will be required to determine the potential sources of free radical generation during muscle contractions in ageing humans.

Experimental Considerations

Several experimental considerations exist for the present study. First, it is possible that the absolute forearm blood flow and vascular conductance values during steady-state exercise in Trial 1 (prior to ascorbic acid) tended to be lower in older adults due to the trend for an age-related decline in MVC (and thus exercise workload) compared with young subjects. Although this was not statistically significant (~18%; P = 0.1), this could partially explain why steady-state haemodynamics were lower in our older subjects, especially when viewed in light of a recent study showing no difference in forearm blood flow during handgrip exercise with age (Donato *et al.*, 2006). With respect to our single contraction data, we have previously demonstrated that the immediate hyperaemic response is independent of any age-related difference in workload (Carlson *et al.*, 2008), so we don't believe this influences the interpretation of our single contraction data. Irrespective of the potential discrepancy in forearm haemodynamics during steady-state exercise between studies, we would like to emphasize that this should not influence the interpretation of our data as it relates to the ascorbic acid-mediated improvement in forearm blood flow and vascular conductance of ascorbic acid during rhythmic handgrip exercise.

Second, in regards to our pharmacological tests, we found that the vasodilator responses to the endothelial agonist acetylcholine were significantly impaired with age, an observation that is very consistent with previous studies on this topic (DeSouza *et al.*, 2000; Taddei *et al.*, 2000; Taddei *et al.*, 2001; DeSouza *et al.*, 2002). We also found that the responses to sodium nitroprusside (endothelium-independent vasodilator) were reduced with age, and these data are in contrast to some (DeSouza *et al.*, 2000; Taddei *et al.*, 2001), but not all (Taddei *et al.*, 2000; Newcomer *et al.*, 2005; Parker *et al.*, 2006) previous studies showing that vascular smooth muscle cell responsiveness is preserved with age in humans. The reasons for this discrepancy are unclear, but could suggest that preserved smooth muscle cell function is simply not a universal finding with healthy ageing. However, it is important to note that in the present study, the age-associated differences in vasodilatation to acetylcholine were abolished during ascorbic acid infusion, whereas the responses to sodium nitroprusside were unaffected. Thus, the ascorbic acid-mediated increases in forearm blood flow we observed during rhythmic exercise were associated specifically with improved endothelium-dependent vasodilator function, and were not related to changes in smooth muscle cell responsiveness.

Finally, we employed mild-to-moderate intensity exercise of a small muscle mass (forearm) to isolate the local effects of muscle contraction on vascular tone, and limit the potential modulatory influences of cardiac output (systemic arterial flow) and the sympathetic nervous system on muscle blood flow responses (Dinenno *et al.*, 2005; Koch *et al.*, 2005). Further, the forearm vasculature is not under greater tonic sympathetic vasoconstriction at rest (Dinenno *et al.*, 2002), whereas the leg circulation is characterized by augmented basal α-adrenergic vasoconstrictor tone (Dinenno *et al.*,

2001). Future studies will be required to determine whether ascorbic acid is capable of improving vascular control in ageing humans during higher intensity exercise or larger muscle mass exercise (e.g., cycling) where cardiac output and sympathetic neural influences are involved in the integrative control of skeletal muscle blood flow.

Conclusions

The collective findings from the present investigation indicate that acute improvements in endothelial vasodilator function are associated with augmented blood flow responses to contracting muscles of aging humans. However, this improved hyperaemia was only observed during continuous dynamic exercise, as the responses to single, brief contractions were not influenced by ascorbic acid and thus still significantly impaired with age. The exact signaling mechanisms underlying this improved exercise hyperaemia during continuous exercise need to be elucidated, and whether other longer-term interventions that improve endothelial vasodilator function in humans (e.g. aerobic exercise training) translate to improved muscle blood flow during exercise in older adults remains to be determined. Finally, the divergent effects of ascorbic acid on contraction-induced rapid vasodilatation versus sustained exercise highlight the complex alterations in vascular physiology with human ageing.

Acknowledgements

We would like to thank Whitney Lewis for her assistance in these experiments, as well as the subjects who volunteered to participate. This research was supported by National Institutes of Health awards AG022337, AG027150, and HL087952 (F.A. Dinenno).

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Table 1: Subject Characteristics and Baseline Haemodynamics

Variable	Young	Older
Male:Female	9:5	9:5
Age (years)	22 ± 1	65 ± 2*
Body mass index (kg m^{-2})	24.5 ± 0.8	24.1 ± 0.8
Body fat (%)	19.5 ± 2.62	29.04 ± 2.45*
Whole-Body FFM (kg)	58.68 ± 3.76	48.59 ± 3.48
Forearm FFM (g)	842 ± 75	700 ± 36
Forearm volume (ml)	968 ± 75	830 ± 68
MVC (kg)	44 ± 3	36 ± 3
10% MVC (kg)	4.4 ± 0.3	3.6 ± 0.3
Total cholesterol (mmol l^{-1})	4.0 ± 0.3	5.1 ± 0.4*
LDL cholesterol (mmol l^{-1})	2.6 ± 0.2	3.4 ± 0.3*
HDL cholesterol (mmol l^{-1})	1.1 ± 0.1	1.2 ± 0.1
Triglycerides (mmol l^{-1})	0.7 ± 0.1	1.2 ± 0.3
Mean arterial pressure (mmHg)	94 ± 3	99 ± 2
Heart Rate (beats min^{-1})	55 ± 2	59 ± 2
Forearm blood flow (ml min^{-1})	35 ± 6	34 ± 3
Forearm blood flow (ml $100g^{-1}$ min^{-1})	3.4 ± 0.4	4.1 ± 0.3
Forearm VC (ml min^{-1} 100 mmHg $^{-1}$)	36 ± 5	34 ± 3

Data presented as mean ± SEM. FFM = fat free mass; MVC = maximal voluntary contraction; LDL = low density lipoprotein; HDL = high density lipoprotein; MAP = mean arterial pressure; HR = heart rate; VC = vascular conductance. *$P < 0.05$ *versus* younger.

Table 2: Forearm and Systemic Haemodynamics Values during Trial 1 Rhythmic Exercise

		Saline						*Ascorbic Acid*									
Time (min)	**Age**	**Rest**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**
HR (beats min^{-1})	**Young**	55±2	59±2	57±2	57±2	58±2	58±2	58±2	58±2	58±2	58±2	57±2	57±2	58±2	58±3	58±2	58±2
	Older	59±2	63±2	63±2	62±2	63±2	62±2	62±2	62±2	63±2	62±2	62±2	62±2	62±2	62±2	63±2	62±2
MAP (mmHg)	**Young**	94±3	98±3	99±3	99±3	98±3	97±3	98±3	98±3	98±3	98±3	96±3	98±3	98±3	98±3	97±3	98±3
	Older	99±2	103±2	103±2	104±2	104±2	103±2	103±2	104±2	104±2	104±2	103±2	104±2	105±2	103±3	102±2	103±2
FVC (ml min^{-1} mmHg^{-1})	**Young**	36±5	132±14	135±13	135±12	136±13	141±13	141±13	140±13	141±13	146±14	145±15	145±14	144±14	142±14	145±15	144±14
	Older	34±3	94±13*	100±13*	100±14*	99±14*	100±15*	104±14*	110±15	113±15	112±14	117±16	124±18	122±19	126±21	128±21	132±21
Δ FVC (%)	**Young**	-	335±48	346±47	355±52	358±53	378±58	380±61	372±57	368±52	383±52	377±54	383±58	379±59	374±59	378±55	375±54
	Older	-	177±26*	198±28*	195±30*	191±30*	194±30*	204±29*	220±29* †	229±29* †	226±27* †	241±31* †	259±35 †	253±37 †	262±40 †	270±42 †	283±41 †

Data are mean ± SEM. HR = heart rate; MAP = mean arterial pressure; FVC = forearm vascular conductance; * P < 0.05 vs young; † P <0.05 vs end control saline (minute 5 of exercise)

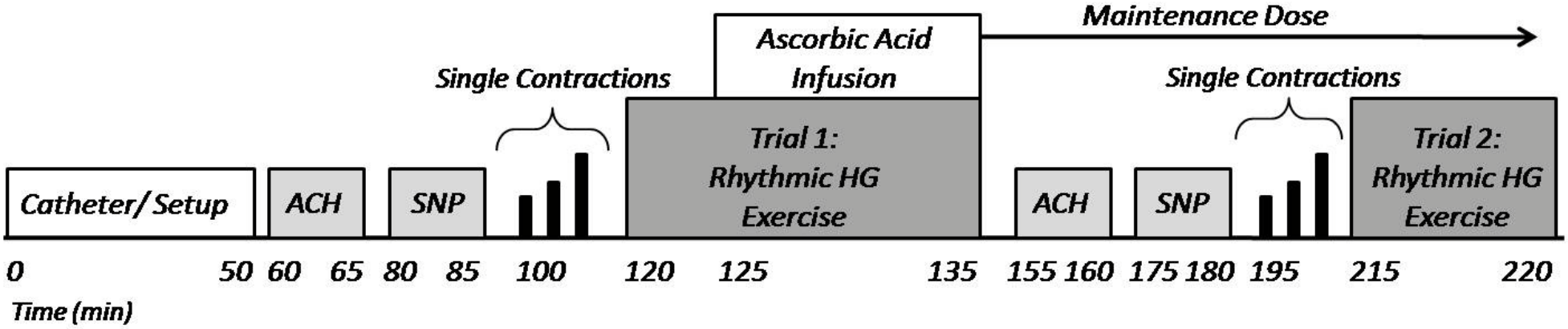

Figure 1: Experimental Timeline. Following placement of the brachial artery catheter and general setup, subjects received a 5 minute intra-arterial infusion of either acetylcholine or sodium nitroprusside to assess endothelium-dependent or endothelium-independent vasodilatation, respectively. Next, single 1-s dynamic forearm muscle contractions were performed at 10, 20, and 40% MVC in triplicate and in random order. Noted as Trial 1, rhythmic handgrip exercise was performed at 10% MVC for 5 minutes with saline to achieve "steady-state" haemodynamics, and the next 10 minutes consisted of continued exercise with concurrent ascorbic acid infusion equaling a total of 15 minutes. The dose of ascorbic acid was then reduced to 40% of the original dose and infused for the remainder of the experiment. During maintenance ascorbic acid administration, endothelium-dependent and -independent vasodilatation was again tested, followed by single contractions, and then a second 5-minute rhythmic handgrip exercise trial (Trial 2). HG = Handgrip. See text for further details.

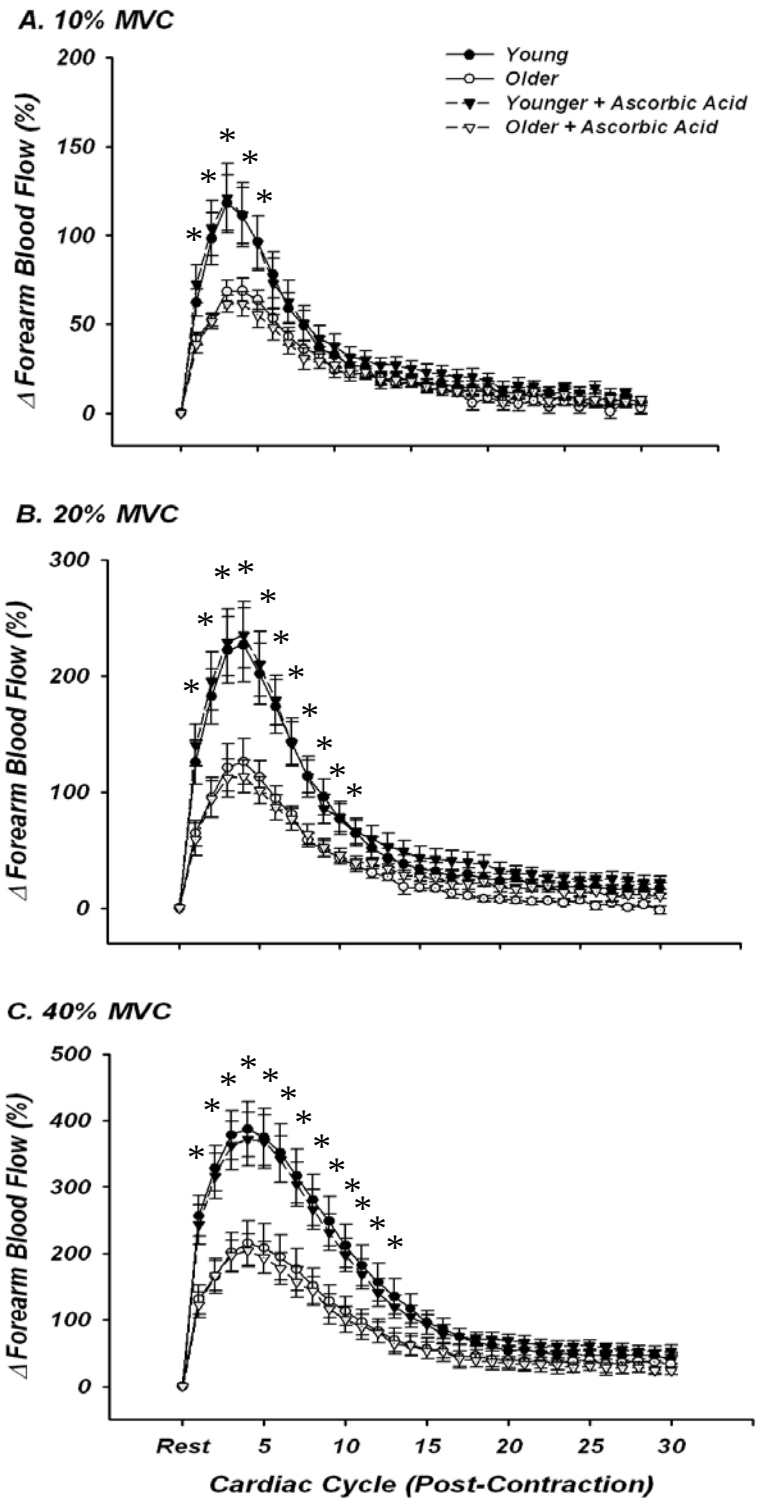

Figure 2: Dynamic rapid hyperaemic responses to single forearm muscle contractions at 10, 20, and 40% MVC. The peak hyperaemic response occurred at 3-4 cardiac cycles post-contraction for all exercise intensities regardless of age or ascorbic acid administration. There were significant age group differences in contraction-induced rapid vasodilatation for all intensities, and ascorbic acid did not impact this response in either young or older adults. * P <0.05 vs older.

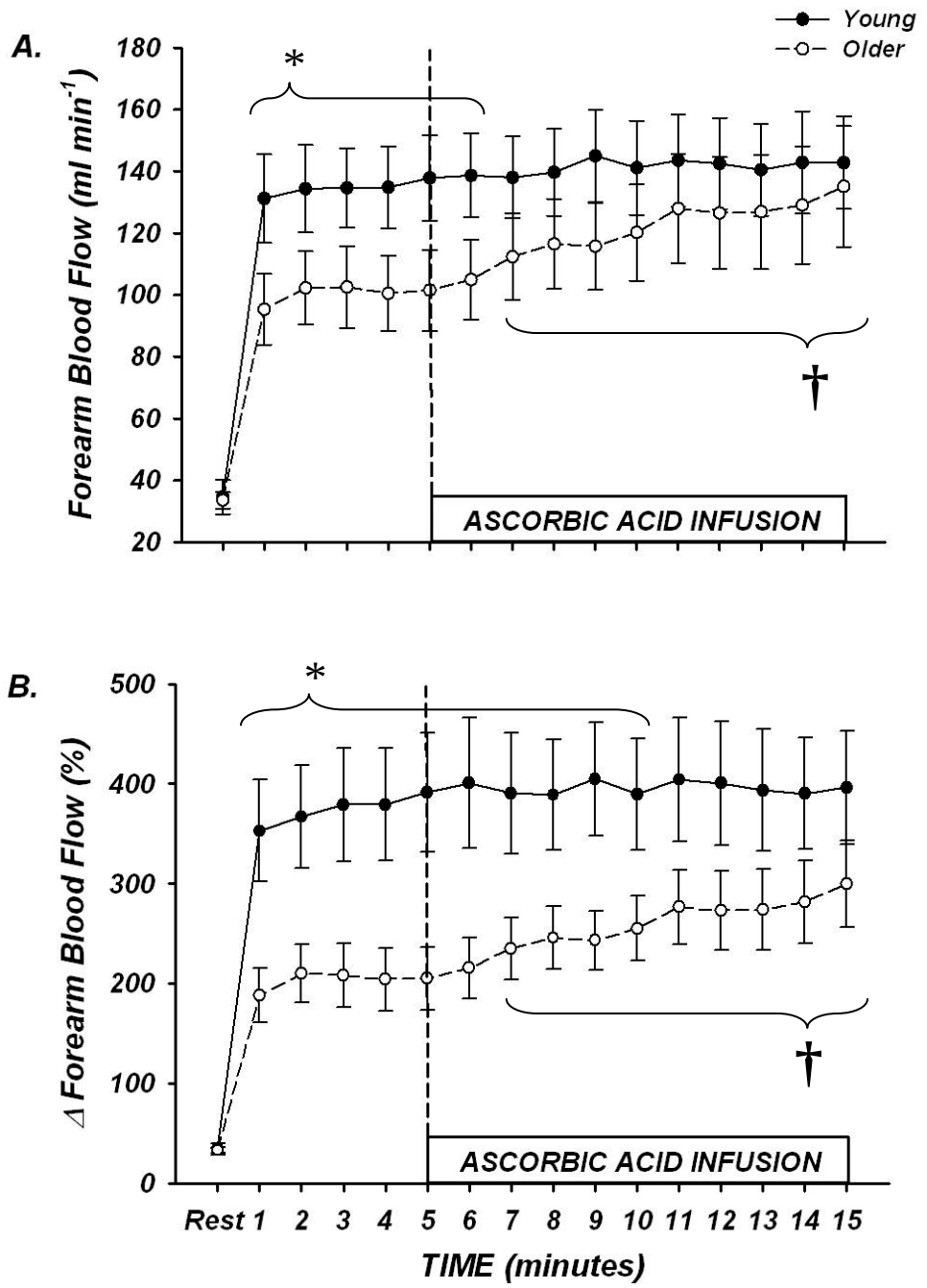

Figure 3: Forearm hyperaemic responses to mild rhythmic handgrip exercise before and during ascorbic acid infusion. Prior to ascorbic acid, absolute forearm blood flow tended to be lower in older vs young adults during mild intensity steady-state exercise (A; * P = 0.06 – 0.09 for minutes 1-6). When expressed as percentage increases from baseline, forearm hyperaemic responses were significantly reduced in older adults (B; * $P < 0.05$ for minutes 1-10). Infusion of ascorbic acid significantly increased forearm blood flow in older, but not young adults during continued exercise. † P <0.05 vs steady state exercise within older group for minutes 7-15.

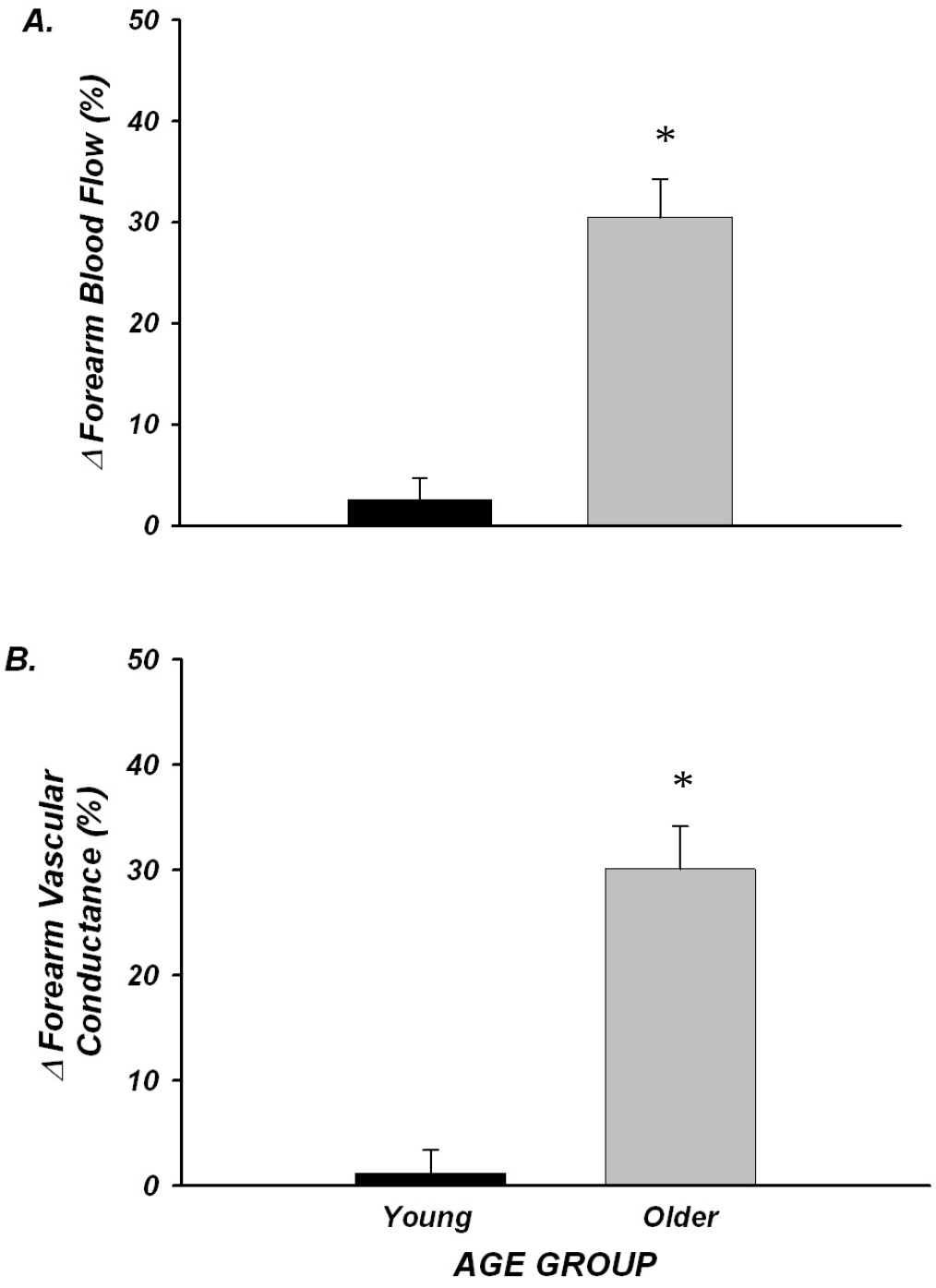

Figure 4: Peak effect of ascorbic acid on forearm blood flow and vascular conductance during steady-state exercise. Acute infusion of ascorbic acid increased forearm blood flow by ~30% in older adults, whereas the increase was minimal and non-significant in young adults (A). Similar responses were observed when quantified as changes in vascular conductance, indicating that the increase in blood flow was due to local vasodilatation (B). * P <0.05 vs young.

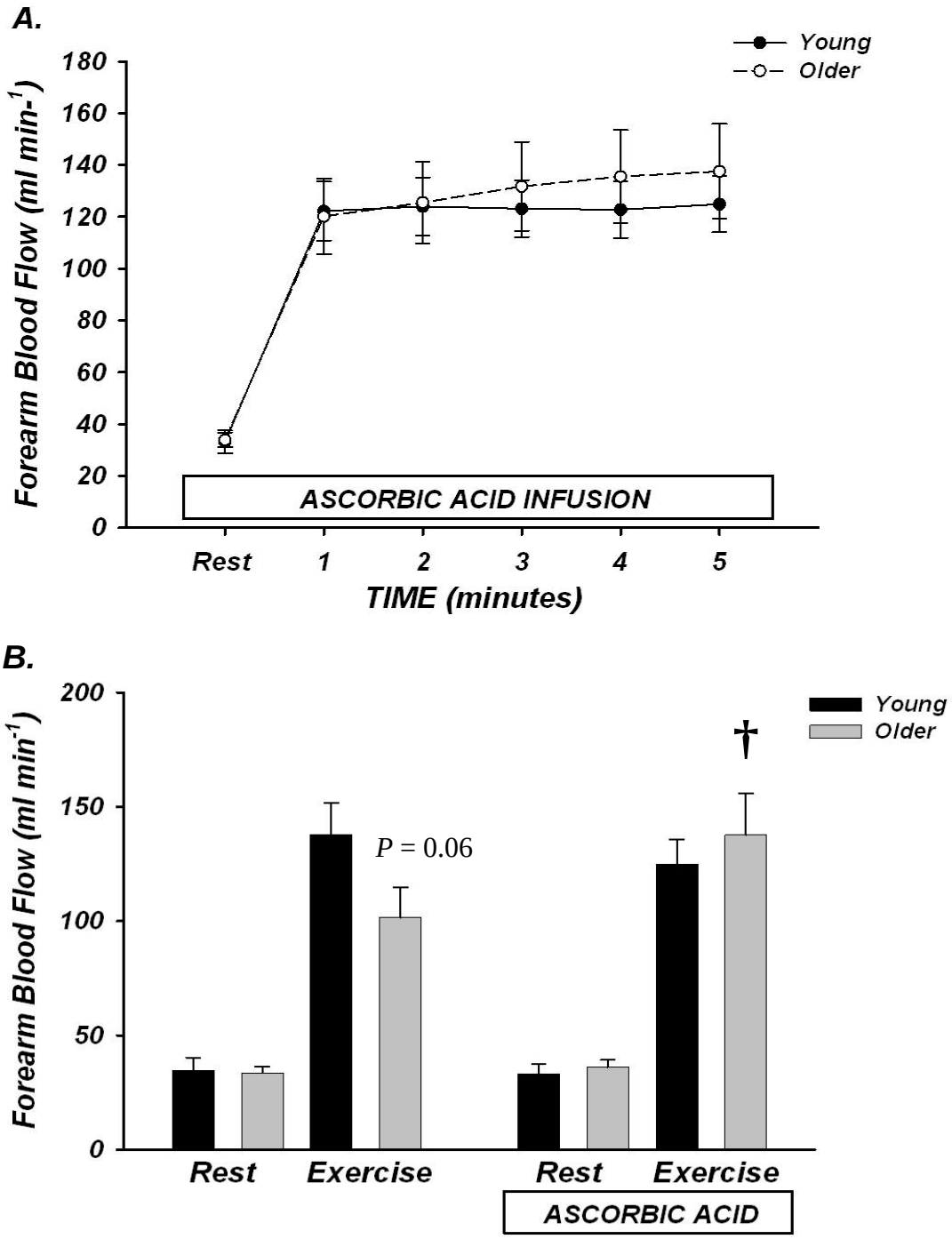

Figure 5: Effect of ascorbic acid on forearm blood flow from rest to steady-state handgrip exercise. When ascorbic acid was administered prior to the onset of exercise, forearm blood flow increased to similar levels within 1 minute of exercise in young and older adults and this persisted throughout the exercise trial (A). In Panel B, forearm blood flow at rest and after 5 minutes of steady-state exercise from Trial 1 (no ascorbic acid; control) and Trial 2 (concurrent ascorbic acid infusion) are shown.† $P < 0.05$ vs without ascorbic acid within age group.

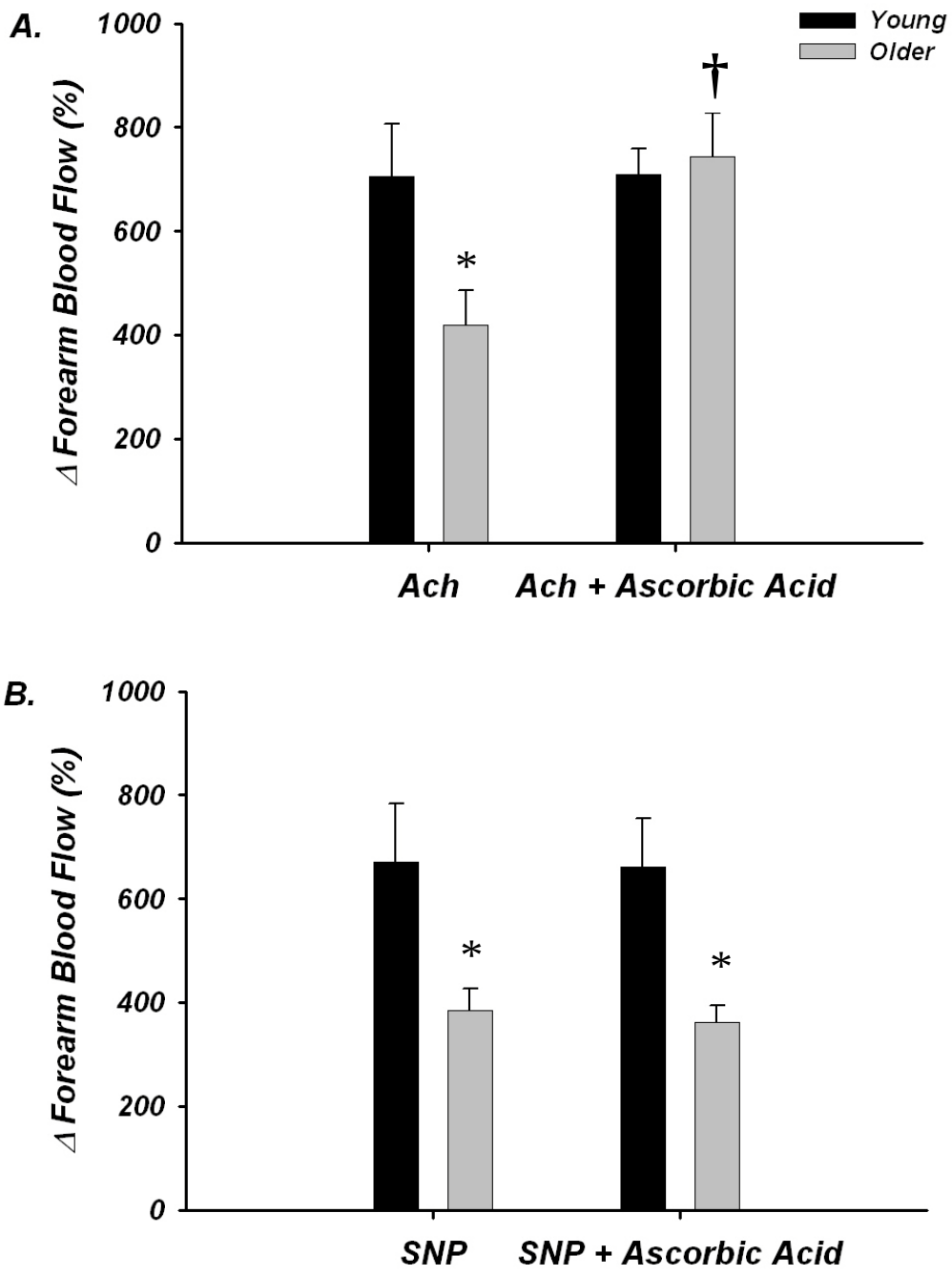

Figure 6: Forearm vasodilatation to intra-arterial infusion of acetylcholine and sodium nitroprusside in young and older adults. At baseline, percentage increases in forearm blood flow to acetylcholine (endothelium-dependent) were significantly impaired in older compared with young adults, and this age-associated impairment was abolished during ascorbic acid infusion (A). Older adults also had an impaired forearm blood flow response to sodium nitroprusside (endothelium-independent) at baseline, and this was unaffected during ascorbic acid infusion (B). * P <0.05 vs young; † P <0.05 vs without ascorbic acid within age group.

Colorado State University

Office of Regulatory Compliance
Office of Vice President for Research
Fort Collins, CO 80523-2011
(970) 491-1553
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Notice of Human Research Amendment Approval

Principal Investigator:	Frank Dinenno, HES, 1582
Title:	Regional Blood Flow Control and Vascular Function: Effects of Aging and Regular Physical Activity
Protocol #:	04-151H
Committee Action:	**Amendment Approved:** December 7, 2006
HRC Administrator:	Janell Meldrem

The Human Research Committee reviewed and approved your request to amend the above-referenced project. The approved amendments are below.

Amendment(s):

- to increase the number of particpants by 60 - accepted.
- to add the grant title: Aging, Endothelial Dysfunction, and ATP-mediated Vasodilation
- to change the title on the consent form to reflect both grants to: Regional Blood Flow Control and Vascular Function: Effects of Aging and Regular Physical Activity

Investigator Responsibilities:

- It is the responsibility of the PI to immediately inform the Committee of any serious complications, unexpected risks, or injuries resulting from this research.
- It is also the PI's responsibility to notify the Committee of any changes in experimental design, participant population, consent procedures or documents. This can be done with a memo describing the changes and submitting any altered documents.
- Students serving as Co-Principal Investigators may not alter projects without first obtaining PI approval. The PI is ultimately responsible for the conduct of the project.

This approval is issued under Colorado State University's OHRP Federal Wide Assurance 00000647.

If you have questions, please contact me at 1-1655 or janell.meldrem@colostate.edu.

attachment Date of Correspondence 12/8/06

Consent to Participate in a Research Study
Colorado State University

TITLE OF STUDY: Aging, Endothelial Dysfunction, and Impaired Vascular Control During Exercise
PRINCIPAL INVESTIGATOR: *Frank A. Dinenno, Ph.D. 491-3203*
CO-PRINCIPAL INVESTIGATOR: *Wyatt Voyles, M.D. 663-3107*

WHY AM I BEING INVITED TO TAKE PART IN THIS RESEARCH? *You are a man or woman between the ages of 18-35 or 55-90 years. You are either not regularly exercising or are moderately active. Our research is looking at the effect of aging on muscle blood flow control during exercise.*

WHO IS DOING THE STUDY? *This research is being performed by Frank Dinenno, Ph.D., of the Department of Health and Exercise Science, and Wyatt Voyles, M.D., of the Heart Center of the Rockies. Trained graduate students, undergraduate students, research assistants, or research associates are assisting with the research. These studies are paid for by the National Institute on Aging (a part of the US Government).*

WHAT IS THE PURPOSE OF THIS STUDY? *The way in which blood flow (and oxygen delivery) and vascular function are regulated by local factors during exercise is being studied. Importantly, cardiovascular regulation under these conditions might change in older people and might be different between men and women. The purpose of the research is to understand how age-related changes in blood vessel function might affect blood flow to muscle during exercise.*

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST?
This whole research project will take place over a period of approximately two years. However, your part of this study will be either:
_____ *1) one or two visits over a several day period, or* _____ ***(your initials)***
_____ *2) several visits over a few to several weeks.* _____ ***(your initials)***

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

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

_____ ***(your initials)***

_____ ***Pregnancy Test.*** *If you are female you will be required to have a sample of your urine tested for the presence of human chorionic gonadotropin (HCG), a hormone which indicates whether you may be pregnant. This will require approximately 1 cup of your urine. If you are*

pregnant or the test indicates that you are pregnant you will not be able to participate in this study. (~10 minutes)

_____ ***(your initials)***

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

_____ **(*your initials)***

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

_____ ***(your initials)***

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

_____ ***(your initials)***

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

_____ ***(your initials)***

_____ ***Forearm Exercise.*** *You will lay flat on a bed and squeeze your hand and forearm muscles using a handgrip device while your hand and arm are comfortably secured. The intensity of the exercise will range from very easy to moderately difficult, and you will be asked to perform this exercise for ~20 minutes a couple of times throughout the study with plenty of rest in between exercise trials. (1 – 2 hours)*

_____ ***(your initials)***

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

_____ ***(your initials)***

____ ***Doppler Ultrasound.*** *The blood flow in your arm will be measured using an ultrasound machine which produces sound waves to measure your blood vessel size and the speed of your blood. (2-3 hours)*

_____ ***(your initials)***

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

_____ ***(your initials)***

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

_____ ***(your initials)***

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

_____ ***(your initials)***

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

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

_____ ***(your initials)***

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

_____ ***(your initials)***

____ ***Drug Administration (~ 2 - 4 hours):***

Vasoconstrictors – cause temporary narrowing of the blood vessels (minutes)
___ L-NAME
___ Ketorolac

Vasodilators – temporarily relax the blood vessels (minutes)
___ Acetylcholine
___ Sodium Nitroprusside

No major effects
___ Ascorbic Acid (Vitamin C)

_____ ***(your initials)***

FUTURE USE OF BLOOD SAMPLES

It is possible that we may want to use any extra blood for future research not described in this consent form. For example, this may include determination of certain gene expressions that

relate to various measures of cardiovascular function measured as part of this study. This information will remain private as will all of the data collected from the study.

Only choose one of the following:

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

_____ ***(your initials)***

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

_____ ***(your initials)***

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

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

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

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

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

  _____ ***(your initials)***

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

  _____ ***(your initials)***

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

  _____ ***(your initials)***

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

  _____ ***(your initials)***

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

  _____ ***(your initials)***

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

  _____ ***(your initials)***

- _____ *Venous Catheterization- The risk of allergic reaction to lidocaine is extremely low. There is a risk of bruising, **clotting**, slight risk of infection, local soreness, and fainting.*

  _____ ***(your initials)***

- _____ *Arterial Catheterization – The risk of allergic reaction to lidocaine is extremely low. There is a risk that pain or discomfort may be experienced when the catheter is inserted in the artery, and local soreness after the study. In about 1 in 10 cases a small amount of bleeding under the skin will cause a bruise. There is about a 1 in 1,000 risk of infection or significant blood loss. In about 1 in 4,000 damage may occur to the artery requiring surgery in order to prevent loss of limb and/or digit (finger) function.*

  _____ ***(your initials)***

- _____ *Drug Administration - The risks associated with drug administration include temporary increases or decreases in blood pressure and heart rate. Any changes in blood pressure and heart rate during this part of the study are modest and less severe than experienced during the treadmill test. These symptoms should resolve when the drug stops. With any of the vasoconstrictor drugs, there is a slight risk that ischemia (lack of blood to the tissues) could occur (e.g. heart attack). Risks of these effects are minimized by calculating the amount of drug given relative to the size of your forearm, and not the entire body. Finally, there is a potential risk of an allergic reaction to vasoactive drug administration. If you are allergic to aspirin, you should not participate.*

  _____ ***(your initials)***

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

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

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

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

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

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

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

WILL I RECEIVE ANY COMPENSATION FOR TAKING PART IN THIS STUDY? *For experiments that involve the blood sample, venous or arterial catheterization, you will be paid $25/hour.*

WHAT HAPPENS IF I AM INJURED BECAUSE OF THE RESEARCH? *Please be aware that for this study the University has made special arrangements to provide initial medical coverage for any injuries that are **directly related** to your participation in this research project. The research project will provide for the coverage of reasonable expenses for emergency medical care related to the treatment of research-related injuries, if necessary.*

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

In light of these laws, you are encouraged to evaluate your own health and disability insurance to determine whether you are covered for any physical injuries or emotional distresses you might sustain by participating in this research, since it may be necessary for you to rely on your

individual coverage for any such injuries. Some health care coverages will not cover research-related expenses. If you sustain injuries, which you believe were caused by Colorado State University or its employees, we advise you to consult an attorney.

Questions concerning treatment of subjects' rights may be directed to Celia S. Walker at (970) 491-1563.

WHAT IF I HAVE QUESTIONS? *Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions about the study, you can contact the principal investigator, Frank Dinenno, Ph.D., at (970)491-3203, or via email at fdinenno@cahs.colostate.edu. If you would like to ask a medical doctor about your participation in the study, you may contact Wyatt Voyles, M.D. at 663-3107. If you have any questions about your rights as a volunteer in this research, contact Celia Walker, Director of Regulatory Compliance, at 970-491-1553. We will give you a copy of this consent form to take with you.*

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

_____ _____

Signature of person agreeing to take part in the study Date

_____

Printed name of person agreeing to take part in the study

_____ _____

Name of person providing information to participant Date

_____

Signature of Research Staff

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

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