THE EFFECTS OF ASCORBIC ACID ON SKELETAL MUSCLE BLOOD FLOW IN AGED RATS

by

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Abstract

During exercise aged individuals exhibit endothelial dysfunction and decreased levels of whole-limb blood flow (BF), both of which may be linked mechanistically to age-related increases in reactive oxygen species (ROS). Ascorbic acid (AA) reduces levels of ROS and has been shown to alleviate vascular and hyperemic dysfunction at rest (Jablonski et al., 2007) and during small muscle mass exercise in humans (Kirby et al., 2009). However, the effect of AA on vascular function and BF to individual muscles during whole-body exercise is not known.

**PURPOSE:** To test the hypothesis that a single high-dose infusion of AA would increase BF to the hindlimb musculature of old rats at rest and during treadmill running. **METHODS:** 18 old (~28 months) Fischer 344 x Brown Norway rats were randomized into rest (n=9) and exercise (n=9) groups. BF to the total hindlimb and individual muscles (28 individual muscles and muscle parts) was evaluated via radiolabeled microspheres before and after intra-arterial AA administration (76 mg/kg in 3 ml heparinized saline, 30 minute infusion) at rest and during submaximal treadmill running (20m/min, 5% grade). Total antioxidant capacity (TAC) and thiobarbituric acid reactive species (TBARS) were measured before and after AA to determine the ability of this specific dose of AA to increase levels of plasma antioxidants and decrease levels of ROS, respectively. **RESULTS:** At rest: AA increased TAC (~37%, P<0.05) but did not change TBARS (Pre: 6.8±0.7 vs Post: 7.0±1.0 µM, P>0.05). AA decreased total hindlimb BF (Pre: 25±3 vs Post: 16±2 ml/min/100g, P<0.05) and BF to 8 of the 28 muscles that were evaluated. During exercise: TAC was increased (~35%, P<0.05) and TBARS were decreased (Pre: 9.8±2.0 vs Post: 7.0±1.0 µM, P<0.05). However, there was no effect on either total hindlimb BF (Pre: 154±14 vs Post: 162±13, P>0.05) or BF to any of the individual muscles evaluated. **CONCLUSIONS:** Increased TAC via AA infusion reduces hindlimb muscle BF at rest but had no effect on BF during whole-body dynamic exercise. Thus, even though TBARS decreased, there was no evidence that AA supplementation increases blood flow to the locomotor muscles of old rats during whole-body exercise.
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Local blood flow regulation is altered with advancing age. Specifically, it has been shown that aged humans exhibit decreased levels of whole-limb blood flow during both rest (Dinenno, Jones, Seals, & Tanaka, 1999; Jablonski et al., 2007) and exercise (Proctor et al., 1998) (Kirby et al., 2009). Aged rats exhibit reductions in functional hyperemia (Hammer & Boegehold, 2005) and altered hindlimb blood flow distributions compared to their young counterparts (Musch, Eklund, Hageman, & Poole, 2004), and both humans and rats evidence impaired endothelial function (Egashira et al., 1993) (Taddei et al., 2000; Eskurza, Monahan, Robinson, & Seals, 2004). One common mechanism that has been proposed to contribute to these local blood flow alterations is oxidative stress, a condition in which reactive oxygen species (ROS) overwhelm the body’s antioxidant defenses. Although it is likely that ROS affect the vasculature through a variety of mechanisms, the primary pathway is thought to be through the interaction of ROS with the potent signaling molecule nitric oxide (Cai & Harrison, 2000). Specifically, ROS decrease NO bioavailability by directly scavenging free NO (Cai & Harrison, 2000) (Darley-Usmar, Wiseman, & Halliwell, 1995) and by reducing tetrahydrobiopterin (BH4) content (Bevers et al., 2006) (Crabtree et al., 2009), which is a necessary cofactor for NO production.

The implications of reduced NO bioavailability are profound. In regards to muscle blood flow, NO clearly plays a role in maintaining blood flow during rest and recovery from exercise, as blockade of nitric oxide synthase (NOS) results in decreases in flow (Brock et al., 1998) (Duffy, New, Tran, Harper, & Meredith, 1999). Although there is some evidence linking NO to the maintenance of blood flow during steady state exercise in humans (Schrage, Joyner, & Dinenno, 2004) (Schrage, Eisenach, & Joyner, 2007), the majority of studies have found no effect of NOS blockade on blood flow during exercise (Endo et al., 1994) (Brock et al., 1998) (Duffy, New, Tran, Harper, & Meredith, 1999). In contrast to this, rats that are given a NOS inhibitor both before and during treadmill running evidence profound decreases in blood flow to not only the working muscles, but also to the kidneys and splanchnic organs (Hirai, Visneski, Kearns, Zelis, & Musch, 1994) (Copp, Hirai, Hageman, Poole, & Musch, 2010).

In an effort to eliminate the age-related excess of ROS and restore NO bioavailability, and therefore endothelial function, a variety of studies have been performed that utilize the
potent antioxidant ascorbic acid (Eskurza et al., 2004) (Jablonski et al., 2007) (Kirby et al., 2009) (Wray et al., 2009). This therapeutic strategy has strong support, as acute infusions of ascorbic acid have been shown to decrease levels of superoxide, a specific ROS that reacts with NO (Jackson, Xu, Vita, & Keaney, 1998). Ascorbic acid is also able to increase resting whole-leg blood flow in aged subjects such that their blood flow is similar to that found in young healthy individuals (Jablonski et al., 2007). Further research has demonstrated that this effect of ascorbic acid also occurs during small muscle mass exercise in the forearm (Kirby et al., 2009). Taken together, this evidence strongly supports a role of ROS in altering blood flow in the aged population and also highlights the effectiveness of ascorbic acid supplementation to alleviate this problem.

However, there are two key limitations to these previous ascorbic acid studies. The first limitation is that these studies have only been done in individuals who are either resting or performing small muscle mass exercise. It is therefore unknown whether or not ascorbic acid has any effect on blood flow during whole-body, dynamic exercise. This is of prime importance due to the functional implications regarding exercise performance. Secondly, the human studies involving ascorbic acid only document an effect in the large conduit arteries and are unable to evaluate any changes in blood flow and its distribution among the individual muscles. This may be of extreme importance due to the fact that Musch and colleagues documented a clear redistribution of blood flow in aged rats such that blood flow was reduced to several highly oxidative muscles and increased to several highly glycolytic muscles even though skeletal muscle bulk blood flow remained unaltered (Musch et al., 2004). Therefore, this study has been designed to test the hypothesis that a single high-dose infusion of ascorbic acid would not only increase blood flow to the hindlimb musculature of old rats at rest and during treadmill running, but would also preferentially redistribute that flow to the highly oxidative muscles.
CHAPTER 2 - Review of the Literature

It is clear that maximal exercise capacity declines with age as a result of decreased cardiac output and/or reduced oxygen extraction (Ogawa et al., 1992). Exercise performance is also compromised in aging as a result of changes to local blood flow regulation that disrupt the normal matching of blood flow to skeletal muscle metabolism. For example, it has been shown that whole-leg blood flow is reduced in aged humans during both rest (Dinenno et al., 1999) (Jablonski et al., 2007) and exercise (Proctor et al., 1998; Beere, Russell, Morey, Kitzman, & Higginbotham, 1999) (Poole, Lawrenson, Kim, Brown, & Richardson, 2003) (Koch, Newcomer, & Proctor, 2005). Aged rats exhibit altered hindlimb blood flow distributions compared to their young counterparts (Musch et al., 2004), and both humans and rats evidence impaired endothelial function (Egashira et al., 1993) (Taddei et al., 2000) (Eskurza et al., 2004). Finally, Behnke and colleagues demonstrated that the O$_2$ delivery to O$_2$ uptake ratio is reduced at rest and during the rest-contraction transition in aged rats, a condition that would be expected to increase the O$_2$ deficit and contribute to premature fatigue (Behnke, Delp, Dougherty, Musch, & Poole, 2005). Due to the clear implications that these age-related changes have on lowering exercise performance, and therefore activities of daily living, it is important to explore both the underlying mechanisms responsible for these changes as well as potential therapeutic interventions that may improve normal function.

Effect of Aging on Peripheral Blood Flow

Dinenno and colleagues measured whole-leg blood flow in young and aged subjects and determined that femoral artery blood flow was 26% lower and femoral artery vascular conductance was 32% lower in older men while they were at rest (Dinenno et al., 1999). These changes were attributed to a large (75%) increase in sympathetic nerve activity (SNA) and a lower leg oxygen demand in the aged males. Using femoral vein thermodilution, Proctor and colleagues also demonstrated that older endurance trained men evidence ~20-30% decreases in leg blood flow during large-muscle-mass exercise (Proctor et al., 1998). These subjects also demonstrated a reduction in vascular conductance, indicating a decrease in either the production of local vasodilators or the responsiveness of the vasculature to these compounds.
Aged rats also show alterations in peripheral blood flow. Hammer and colleagues demonstrated that arteriolar diameters and blood flow in the resting spinotrapezius muscle of old rats is similar to young rats, but that increases in diameter and flow during muscle contractions that occurred in response to muscle contractions were significantly lower in the aged rats (Hammer & Boegehold, 2005). A study conducted by Musch and colleagues compared regional blood flow responses in young and old Fischer 344 x Brown Norway rats both at rest and during whole-body submaximal exercise (Musch et al., 2004). It was found that although aged rats exhibited the same levels of total hindlimb blood flow as their younger counterparts, that flow was redistributed in such a way that blood flow was decreased to 6 highly oxidative muscles but increased to 8 low oxidative fibers during submaximal treadmill running. Importantly, this study clearly highlighted the fact that aging has profound effects on functional blood flow and vascular control at the level of the microcirculation.

Mechanisms of Blood Flow Alterations

Increases in Sympathetic Nerve Activity

Although there are several physiological mechanisms that may potentially contribute to these age-related blood flow alterations, it appears as though increases in both sympathetic nerve activity (SNA) and reactive oxygen species (ROS) that are seen with normal healthy aging play key roles. The increases in sympathetic nerve activity provide a relatively clear means of reducing blood flow by providing a direct vasoconstrictor stimulus to the peripheral vasculature. Indeed, initial evidence for this effect was provided by Dinenno and colleagues who showed that while at rest aged men exhibit approximately 30% lower vascular conductance and 45% higher vascular resistance versus their young counterparts, indicating a significant chronic vasoconstriction in the aged men (Dinenno et al., 1999). Additionally, these aged men evidenced 75% higher leg SNA, which further supported this potential mechanism.

To test the hypothesis that increased SNA was the cause of the decreases in whole-limb blood flow, Dinenno and colleagues conducted an experiment in which femoral blood flow was measured before and after alpha-adrenergic receptor blockade while the subjects were at rest (Dinenno, Tanaka, Stauffer, & Seals, 2001). The main finding was that alpha-adrenergic receptor blockade restored absolute femoral blood flow, vascular conductance, and vascular
resistance of the aged men to levels seen in the young controls. This data clearly confirms that increases in SNA are involved in the reductions in blood flow that are seen during aging.

**Reactive Oxygen Species**

Unlike sympathetic nerve activity, ROS, which are a group of free radicals that includes superoxide, hydrogen peroxide, hydroxyl, and peroxynitrite, present themselves as a much more complex mechanism that may be reducing peripheral blood flow through a variety of possible physiological pathways. However, it is thought that the primary pathway through which ROS exert their effect upon the vasculature is through their interaction with the potent signaling molecule nitric oxide (NO). Normally NO, along with relatively small amounts of superoxide ($O_2^-$), is produced by the conversion of L-arginine to L-citrulline by the enzyme nitric oxide synthase (NOS). The requisite cofactor BH4 is also required for proper conversion. However, during aging elevated levels of superoxide react directly with NO to form peroxynitrite, thereby reducing the amount of free NO (Darley-Usmar et al., 1995; Cai & Harrison, 2000). In addition to this direct effect, ROS also have the ability to reduce BH4 to the inactive trihydrobiopterin, which uncouples the NOS enzyme and results in a reduction in NO production along with an increase in $O_2^-$ production (Bevers et al., 2006; Crabtree et al., 2009). Clearly, this “double-sided attack” of ROS has the ability to profoundly reduce NO bioavailability in the body.

**Implications of Reduced NO Bioavailability**

**Muscle Blood Flow**

There is some controversy in the literature regarding the role of NO in muscle blood flow control, particularly regarding whether or not NO helps develop and maintain blood flow during steady-state exercise. The most common way to explore this issue has been to compare blood flow both before and after infusion of a NOS inhibitor (L-NAME or L-NMMA). Data that supports the role of NO in maintaining exercise hyperemia has been produced by Schrage and colleagues, who have shown that forearm blood flow is decreased by approximately 20% after NOS inhibition during rhythmic handgrip exercise (Schrage et al., 2004; Schrage et al., 2007). In opposition to this, Saltin and colleagues have shown no effect of NOS inhibition on blood flow during knee-extensor exercise (Frandsen et al., 2001). It must be noted however, that the apparent lack of effect of NOS inhibition in the latter study may be due to either low doses of the
antagonist or to the fact that other vasoactive substances may have been increased. This last hypothesis emphasizes the inherent redundancy that is built into normal blood flow regulation and is supported by studies that show significant reductions in blood flow when other blockers are used in conjunction with a NOS blocker (Hillig et al., 2003). Unfortunately, this creates uncertainty regarding the role of NO in maintaining exercise hyperemia and could potentially mean that an infusion of ascorbic acid, which is thought to act via an NO-dependent pathway, may not have an effect on blood flow during steady-state exercise in humans.

Contrary to the human data, it does appear that NO plays a clear role in exercise-induced hyperemia in rats. Hammer and Boegehold demonstrated that functional hyperemia is reduced in the contracting spinotrapezius of aged rats (Hammer & Boegehold, 2005). Hirai and colleagues demonstrated that an acute infusion of L-NAME before treadmill running in healthy young rats decreased blood flow to 16 of the 28 individual muscles that were evaluated in the study (Hirai et al., 1994). Importantly, the vasodilatory effect of acetylcholine (Ach) was restored after infusion of L-arginine, indicating that it was indeed an NO-dependant response. Copp and colleagues have confirmed that blood flow decreases during treadmill running in rats even when the L-NAME is infused during exercise (Copp et al., 2010). Therefore this data supports the role of NO in regulating blood flow in the exercising rat and supports the hypothesis that ascorbic acid may augment the blood flow response to exercise in aged rats.

It does appear, however, that NO has a very clear role in regulating blood flow during rest. Radegran and Saltin decreased blood flow by ~55% after infusion of L-NMMA at rest (Radegran & Saltin, 1999). Other studies have shown reductions in resting blood flow in the 25 – 70% range after NOS blockade (Endo et al., 1994; Brock et al., 1998; Duffy et al., 1999). Despite the variability regarding the magnitude of these effects, the consistent direction of these responses indicates clearly the negative implications of reduced levels of NO.

Kidney and Splanchnic Organ Blood Flow

Data from Hirai and colleagues show that L-NAME infusion decreases blood flow to both the kidneys and to all of the splanchnic organs (Hirai et al., 1994). This is in agreement with other studies that have shown an approximately 26% decreases in total renal blood flow after L-NAME infusion in anesthetized dogs (Gomez, Strick, & Romero, 2008) and to Sigmon
and Beierwaltes who showed that L-NAME infusion decreased blood flow to the visceral organs (Sigmon & Beierwaltes, 1993).

**Sympathetic Nerve Activity**

Interestingly, there is also evidence to support the hypothesis that NO has the ability to attenuate alpha-adrenergic responsiveness. Although there is little research regarding this potential mechanism at rest, there is a significant amount of related data during exercise which supports a potential role for ROS to affect blood flow at rest and during exercise. The well-established response to exercise is an increase in sympathetic nerve activity, the main function of which is to redistribute the blood to the active muscles in an attempt to maintain adequate perfusion and meet the metabolic demands of the muscle. This increased level of sympathetic nerve activity is targeted to both resting and exercising skeletal muscle. However, the vasoconstrictor response to this increased SNA appears to be attenuated in the contracting skeletal muscle, a phenomenon that has been termed functional sympatholysis (Thomas & Victor, 1998). It has been demonstrated that the increased production of NO during skeletal muscle contractions appears to play a key role in this response, potentially via an interaction with K\textsubscript{ATP} channels (Thomas & Victor, 1998). Regardless of the mechanism, the apparent ability of NO to attenuate increased sympathetic stimulation during exercise may have some crossover significance in conditions in which NO bioavailability is limited. Evidence of this possibility exists in studies that discovered that NO-mediated attenuation of sympathetic vasoconstriction in contracting skeletal muscle is impaired in conditions that are associated with a decrease in NO bioavailability, specifically chronic myocardial infarctions and aging (Thomas, Zhang, & Victor, 2001) (Dinenno, Masuki, & Joyner, 2005). Therefore, restoration of NO bioavailability by ascorbic acid may improve muscle blood flow by attenuating the increased levels of SNA seen during aging.

**Effect of ROS on the Vasculature**

In addition to their effect on NO bioavailability, it also appears ROS themselves exhibit certain vasoactive properties. However, these effects are currently not completely understood and remain somewhat contradictory. For example, several studies have shown that vascular conductance is increased and endothelial dysfunction is reversed after infusion of an antioxidant (Eskurza et al., 2004) (Jablonski et al., 2007; Kirby et al., 2009). In opposition to this, recent
work has also shown that hydrogen peroxide, a specific ROS, can be either a vasoconstrictor or a vasodilator depending on the exact conditions (Lucchesi, Belmadani, & Matrougui, 2005). As a result of this apparent dual-natured effect, reductions of ROS by ascorbic acid could mean that either powerful vasoconstrictor or vasodilator substances are being eliminated. To further complicate this matter, very recent work has shown that ascorbic acid actually generates hydrogen peroxide, and that it is through this ROS that ascorbic acid actually produces part of its beneficial effect on the vasculature (Garry, Edwards, Fallis, Jenkins, & Griffith, 2009). Clearly, more work must be done in this area in order to determine first whether or not different ROS exert disparate effects on the vasculature and secondly if there is a threshold of ROS species needed for them to act as vasoconstrictors.

**Ascorbic Acid**

Supplementation with the antioxidant ascorbic acid has been proposed as a potential therapeutic intervention to reduce levels of ROS, particularly superoxide, and restore NO bioavailability. Jackson and colleagues demonstrated this ability by showing that ascorbate, the reduced form of ascorbic acid, prevents the interaction of superoxide and nitric oxide when it is administered at supraphysiological (i.e. by direct infusions) concentrations (Jackson et al., 1998). Ezkurza and colleagues used this as a biochemical basis to test whether or not ascorbic acid could improve flow-mediated dilation, an endothelium-dependant response that is impaired with age, in both sedentary and physically active aged adults (Eskurza et al., 2004). The results of this study showed that the acute infusions of ascorbic acid significantly increased flow-mediated dilation in the sedentary aged, but not the young and exercise-trained aged, men. This data indicated that only individuals who are subjected to increased levels of oxidative stress (i.e. sedentary aged males) show improved outcomes from ascorbic acid infusion.

In a more direct assessment of its effect on blood flow, Jablonski and colleagues examined the effect of ascorbic acid infusions on resting whole-leg blood flow (Jablonski et al., 2007). It was first reported that aged men had increased levels of oxidative stress and ~25% lower levels of whole-leg blood flow compared to their younger counterparts. Infusion of ascorbic acid increased femoral artery blood flow by 37% in the aged men while there was no effect on blood flow in the young men, thus abolishing the age-related impairment in resting blood flow.
There has also been some evidence of a positive effect of ascorbic acid on blood flow during exercise. Kirby and colleagues (Kirby et al., 2009) examined the effect of ascorbic acid infusions on forearm blood flow during rhythmic handgrip exercise and found that ascorbic acid was able to increase forearm blood flow by approximately 30% in the aged, but not the young, subjects. Although this study was not designed to determine the exact mechanisms responsible for this beneficial effect, the authors postulated that it might be due to a reduction of ROS levels and subsequent improvement in NO bioavailability and BH₄ stabilization. Another potential explanation was that the ROS that were being produced during exercise were acting as vasoconstrictors and their reduction led to an improved vasodilatory response.

**Conclusion**

Alterations in the local control of blood flow that occur during aging are likely the result of numerous factors. However, the increased levels of ROS that are seen during aging appear to be at least partially responsible for these perturbations. The primary means through which ROS exert their effect on the vasculature is thought to be through their ability to limit NO bioavailability. NO is clearly involved in blood flow control through both its direct function as a vasodilator and on its ability to reduce sympathetic nerve activity during exercise (i.e. functional sympatholysis). Ascorbic acid has been proposed as a novel way to combat increases in ROS and has shown the ability to increase blood flow during rest and small muscle mass exercise. The effects of ascorbic acid supplementation on blood flow to individual muscles are unknown, as is its ability to either increase or decrease blood flow during whole-body exercise. If improvements in blood flow are seen during exercise, ascorbic acid supplementation could provide a means to improve exercise performance in this activity-challenged population.
CHAPTER 3 - Methods

Animal Selection and Care

Nineteen old (O, ~28 mo old) Fischer 344 x Brown Norway rats were utilized in this study. These rats were chosen because they represent O rats according to the life span of the Fischer 344 x Brown Norway strain (Larkin, Halter, & Supiano, 1996) and are subject to fewer of the age-related pathologies that can be seen in other highly inbred strains such as the Fischer 344 (Lipman, Chrisp, Hazzard, & Bronson, 1996). Upon arrival, the rats were divided into exercise (E, n=9) and resting (R, n = 10) groups. Rats were maintained on a 12:12 hour light-dark cycle and received food and water ad libitum. All experiments were conducted under the guidelines established by the National Institutes of Health and Kansas State University’s Animal Care and Use Committee.

Familiarization

All E rats were first familiarized with running on a motor-driven treadmill over a period of 8 weeks. The familiarization protocol consisted of running 3-5 days/week for 5 min/day at 20m/min on a 5% grade. This familiarization phase, although extended over a period of time, should not have provided a sufficient stimulus to produce a physiological exercise training effect (Dudley, Abraham, & Terjung, 1982).

Surgical Procedures

On the day of the experiment, each animal was weighed and anesthetized with a 5% isoflurane-oxygen mixture. While anesthesia was being maintained with a 2% isoflurane-oxygen mixture, one catheter (PE-50 connected to PE-10) was introduced into the ascending aorta via the right carotid artery and another was placed into the caudal (tail) artery as previously described (Musch & Terrell, 1992). Both catheters were then tunneled subcutaneously and exposed through a small incision in the dorsal region of the neck. The two incisions used to introduce both of the catheters were then sutured closed and anesthesia was subsequently terminated. The rat was then given ≥ 1 hour to recover from anesthesia and surgery, a period of
time that has been shown to be sufficient to allow all hemodynamic parameters to stabilize (Flaim et al., 1984).

**Blood Flow Measurements**

*At Rest*

After recovery, the rat was placed on the treadmill belt and the carotid catheter was attached to a pressure transducer (Gould Statham P23ID, Valley View, OH, USA) which was connected to a recorder in order to directly record heart rate (HR) and blood pressure (BP) while the caudal catheter was connected to a 1-ml plastic syringe that was attached to a Harvard infusion/withdrawal pump (model 907, Cambridge, MA, USA). After establishing baseline HR and BP measurements, the carotid catheter was disconnected from the pressure transducer and attached to a 1-ml plastic syringe containing approximately 0.5-0.6 x 10^6 15 µm microspheres (^46^Sc or ^85^Sr, injected in random order). At this point, withdrawal of blood from the caudal artery was initiated at a rate of 0.25 ml/min, after which time the microspheres were infused into the aortic arch via the carotid catheter. The carotid catheter was then reattached to the pressure transducer to confirm that the microsphere infusion did not obviously alter HR or BP. Blood withdrawal from the caudal artery was then terminated, with the resulting blood being used as the reference sample.

Upon completion of the first microsphere infusion, the rat remained at rest and ascorbic acid (76 mg ascorbic acid/kg body weight dissolved in 3 ml heparanized saline) was infused over a 30 minute period. This particular dose of ascorbic acid was utilized because it has shown beneficial effects in certain disease states (sepsis) in rats (Armour, Tyml, Lidington, & Wilson, 2001; Tyml, Li, & Wilson, 2005) and has been used previously in our lab in conjunction with the antioxidant tempol (Herspring et al., 2008; Copp et al., 2009). After infusion was completed, a second microsphere infusion was performed with the other labeled microsphere using the same procedures listed above.

*Exercise*

Upon recovery from anesthesia each rat was placed on the treadmill belt and the carotid catheter was attached to the pressure transducer and recorder while the tail artery was connected to a 1-ml syringe that was attached to the Harvard infusion/withdrawal pump. After resting BP
and HR measurements were taken, treadmill exercise was initiated progressively and reached a speed of 20 meters/min on a 5% grade within 30 seconds. Between 2.5 and 3 minutes of total exercise time, the carotid artery catheter was disconnected from the pressure transducer and attached to a 1-ml plastic syringe containing approximately 0.5-0.6 x 10^6 15 µm microspheres (^{46}Sc or ^{85}Sr, injected in random order). Between 3 to 3.5 minutes of total exercise time withdrawal of blood from the caudal artery was initiated at a rate of 0.25 ml/min, after which time the microspheres were infused into the ascending aorta via the carotid catheter. This catheter was then reattached to the pressure transducer while the rat was still exercising to confirm that the microsphere infusion did not obviously alter HR or BP. At this point (~4 – 4.5 minutes of total exercise time) exercise and withdrawal from the caudal artery was terminated.

Upon completion of the first exercise session, the rat was allowed to recover for 30 minutes, after which ascorbic acid (76 mg ascorbic acid/kg body weight dissolved in 3 ml heparanized saline) was infused over an additional 30 minutes. After infusion was completed a second microsphere infusion was performed with the other radioactive isotope using the same exercise and injection protocol listed above.

**Determination of Blood Flow**

Following the second microsphere infusion, each animal was anesthetized with a 5% isoflurane-oxygen mixture and given an overdose of pentobarbital administered via the carotid catheter. The thorax was opened, and placement of the carotid catheter into the aortic arch was confirmed visually. Identification and removal of the kidneys, visceral organs, and the muscles of both hindlimbs were performed by anatomical dissection. The specific muscles examined in this study included 1) ankle extensors: soleus (S), plantaris (P), red portion of the gastrocnemius (G_R), white portion of the gastrocnemius (G_W), middle portion of the gastrocnemius (G_M), tibialis posterior (TP), flexor digitorum longus (FDL), flexor hallicus longus (FHL); 2) ankle flexors: red portion of the tibialis anterior (TA_R), white portion of the tibialis anterior (TA_W), extensor digitorum longus (EDL), peroneals (Per); 3) knee extensors; vastus intermedius (VI), vastus medialis (VM), red portion of the vastus lateralis (VL_R), white portion of the vastus lateralis (VL_W) middle portion of the vastus lateralis (VL_M), red portion of the rectus femoris (RF_R), white portion of the rectus femoris (RF_W); 4) knee flexors: anterior portion of the biceps femoris (BF_A), posterior portion of the biceps femoris (BF_P), semitendinosus (ST), red portion of the
semimembranosus (SM_R), white portion of the semimembranosus (SM_W); 5) thigh adductors: adductor longus (AL), adductor magnus and brevis (AMB), gracilis (GR), pectineus (Pec). Upon removal all tissues were immediately weighed and placed into counting vials.

Blood flow to the tissues was determined by first measuring the radioactivity of each tissue sample on a gamma scintillation counter (Packard Auto Gamma Spectrometer, model 5230, Downers Grove, IL). Each individual sample’s level of radioactivity was then converted to an absolute blood flow measurement (ml/min) by using the reference sample method (Musch & Terrell, 1992). These absolute flows were then converted to relative measurements and expressed as ml/min/100 g of tissue. A difference of blood flow ≤15% between right and left kidneys and/or hindlimbs was used to confirm adequate mixing of the microspheres.

**Exclusion Criteria**

Of the 19 rats that were utilized initially for this study, three were excluded. One rat was excluded for inadequate microsphere mixing, another due to the development of a hindlimb lesion, and a third died during surgery. The remaining 16 rats were divided equally between the E (n=8) and R (n=8) groups.

**Blood Gases**

Arterial blood samples of approximately 0.25 ml were taken via the tail artery catheter from all E rats at rest and approximately 1.5 – 2 minutes after the termination of exercise both before and after ascorbic acid infusion. Samples were collected in 1-ml plastic syringes, immediately sealed and stored on ice, and delivered to the veterinary medicine diagnostics lab. Samples were analyzed for pH, partial pressure of CO₂ (PCO₂), partial pressure of O₂ (PO₂), oxygen saturation (O₂sat), and lactate. This was performed to determine any direct effects of ascorbic acid infusion on blood gases as well as to document complete recovery from exercise.

**Measurement of Oxidative Stress**

Arterial blood samples were taken at rest and within 1 -2 minutes after exercise both before and after ascorbic acid infusion. All blood samples were centrifuged immediately, with the resultant plasma being pipetted off and stored at -80° until analysis.
**Total Antioxidant Capacity**

Total antioxidant capacity was determined using a commercially available kit. Briefly, Trolox was used to standardize all antioxidants, and antioxidant capacity was then measured in Trolox equivalents. Absorbance of samples and standards were analyzed at 570 nm (Bio-Tek, Winooski, VT). A standard curve was prepared that spanned the range of measurements, and antioxidant capacity was calculated from the curve.

**Thiobarbituric Reactive Species (TBARS)**

Lipid peroxidation, a major indicator of oxidative stress (Armstrong & Browne, 1994; Yagi, 1998; Lefevre et al., 1998), in the plasma was assessed by using the TBARS assay (TBARS Assay Kit, ZeptoMetrix Corporation, Buffalo, New York) to estimate malondialdehyde (MDA) equivalents. Briefly, this was performed by combining the plasma samples with the buffer provided and then incubating them at 95 degrees for 60 minutes. The samples were allowed to cool and were subsequently centrifuged at 3000 rpm for 15 minutes. Finally, the supernatant from the samples was removed and were analyzed at 532 nm. Data were compared to standards and were expressed as concentrations of MDA equivalents in µM.

**Statistical Analysis**

All data are shown as mean ± SE. Data were compared using paired and unpaired Student’s t-tests. The level of significance was set at P ≤ 0.05. Pearson product moment correlations were performed to determine if the absolute changes in resting blood flow were correlated to estimated per cent sum of Type I and Type IIa muscle fibers. These estimations of fiber type were based upon measurements made by Delp and Dunn on the percentage of Type I and Type II muscle fibers in the individual muscles of the rat hindlimb (Delp & Dunn, 1996).
CHAPTER 4 - Results

Effect of Ascorbic Acid on TAC and TBARS

Infusion of ascorbic acid increased TAC both at rest (Pre: 51.4 ± 2.9 vs. Post: 70.5 ± 9.3 Trolox equivalents, P < 0.05) and during exercise (Pre: 48.8 ± 1.2 vs. 66.1 ± 4.8 Trolox equivalents, P < 0.05) (Table 4.1 and Figure 4.1). Despite these similar increases in TAC, TBARS were not different at rest (Pre: 6.8 ± 0.7 vs. Post: 7.0 ± 1.0 µM, P > 0.05) but were decreased during exercise (Pre: 9.8 ± 2.0 vs. Post: 6.1 ± 0.5 µM, P < 0.05) (Table 4.1 and Figure 4.2).

Effect of Ascorbic Acid on Muscle Blood Flow

Data for all muscle parts that were evaluated are presented in Table 4.2. At rest, ascorbic acid infusion significantly decreased total hindlimb BF (Pre: 25 ± 3 vs. Post: 16 ± 2 ml/min/100g, P < 0.05, Figure 4.3) and BF to 8 of the 28 individual muscles that were evaluated (Table 4.2). The absolute changes in BF to all of the individual muscles at rest showed no correlation to the per cent sum of Type I and Type IIa muscle fibers (Figure 4.4). However, when the soleus muscle was removed from the analysis a significant correlation emerged between these two variables, indicating that blood flow is reduced to a greater extent in the highly oxidative muscles than in the highly glycolytic muscles (Figure 4.5). The soleus muscle was removed from the second correlation analysis due to the fact that the soleus is a postural muscle that is tonically active even when the animal is at rest. The resulting correlation should therefore be more truly representative of the effect of ascorbic acid on skeletal muscle blood flow at rest. During exercise, ascorbic acid infusion had no effect on either total hindlimb BF (Pre: 154 ± 14 vs. Post: 162 ± 13 ml/min/100g, P > 0.05, Figure 4.3) or BF to any of the individual muscles that were evaluated (Table 4.2).

Effect of Ascorbic Acid on Kidney and Splanchnic Organ Blood Flow

At rest blood flow to the right kidney was equal to the left kidney and the infusion of ascorbic acid produced no significant decrease in total kidney flow (Table 4.3, P = 0.054). There was a significant (P < 0.05) decrease in blood flow to the large intestine, but blood flow to the small intestine, stomach, pancreas, spleen, or adrenal glands remained unchanged following
ascorbic acid infusion. During exercise there was also a decrease in blood flow to the large intestine but again blood flow to the kidneys or any other splanchnic organ remained unchanged after infusion of ascorbic acid.

**Blood Gases and Acid-Base Parameters**

There were no significant changes in pH, PCO$_2$, PO$_2$, O$_2$ sat, hematocrit, or lactate before and after ascorbic acid infusion while the rats were at rest. The post-exercise blood samples, however, indicated a significant decrease in PCO$_2$ with no change in any of the other measured variables.
Figure 4.1 Effect of ascorbic acid supplementation on total antioxidant capacity (TAC) during rest and exercise. AA = Ascorbic acid supplementation. * P < 0.05 vs control.
Figure 4.2 Effect of ascorbic acid supplementation on levels of MDA as assessed by the TBARS assay during rest and exercise. AA = ascorbic acid supplementation* P < 0.05 vs. Pre ascorbic acid supplementation
Figure 4.3 Effect of ascorbic acid on total hindlimb blood flow during rest and exercise. AA = pre ascorbic acid supplementation. * P < 0.05 vs. Pre ascorbic acid supplementation.
Figure 4.4 Plot showing the absolute change in blood flow (Δ BF) to all of the individual muscles and muscle parts at rest vs. the per cent sum of Type I and Type IIa fibers.
Figure 4.5 Correlation between the absolute change in blood flow (Δ BF) to all of the individual muscles and muscle parts except the soleus at rest and the per cent sum of Type I and Type IIa fibers.
Table 4.1 Average heart rate, mean arterial pressure (MAP), and blood gas analysis values in all experimental conditions. * P < 0.05 vs. pre ascorbic acid supplementation.
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Ascorbic Acid</th>
<th>Control</th>
<th>Ascorbic Acid</th>
</tr>
</thead>
<tbody>
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<td></td>
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<tr>
<td>Flexor halicus longus</td>
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<tr>
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<td>117±15</td>
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Table 4.2 Effect of ascorbic acid on blood flow at rest and during exercise. Blood flow is measured in ml/min/100 g of tissue. * P < 0.05 vs control.
<table>
<thead>
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<tr>
<td>Large intestine</td>
<td>243±17</td>
<td>194±22*</td>
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</tbody>
</table>

Table 4.3 Blood flow measured to the kidneys and splanchnic organs during all experimental conditions. Blood flow measured in ml/min/100g of tissue. * P < 0.05 vs. control
CHAPTER 5 - Discussion

The most important new findings of this study were that an acute infusion of ascorbic acid decreased muscle blood flow at rest and had no effect on muscle blood flow during steady state, whole-body exercise. These differing outcomes occurred despite the ability of ascorbic acid to produce similar increases in TAC during both rest and exercise and reduce levels of TBARS during exercise.

Potential Mechanisms

There is a very delicate balance between ROS and antioxidants in the body. Traditionally, most ROS have been viewed in a purely negative light due to their abilities to produce cellular damage (Nakamoto et al., 2007) and reduce NO bioavailability (Ogita & Liao, 2004). This view is particularly true in populations such as the aged that are subjected to increased levels of ROS. However, recent evidence has shown that ROS are necessary signaling molecules in the body and may also have several other beneficial effects. In regards to this investigation, there is evidence that some ROS, particularly hydrogen peroxide, can be potent vasodilators as well (Lucchesi et al., 2005; Marvar, Hammer, & Boegehold, 2007). This widely unforeseen role of ROS calls into question the effectiveness of using antioxidant supplementation as a means to improve blood flow, as any improvement in NO bioavailability may be greatly offset by reductions in vasodilatory ROS. It is possible that this may at least partially explain the reductions in blood flow that were found at rest due to the fact that fewer ROS are produced at rest as compared to exercise and their reduction by ascorbic acid may have removed an important vasodilator influence. However, this explanation may not be the whole story, as levels of TBARS did not decrease at rest after supplementation of ascorbic acid. Therefore it is also possible that ascorbic acid was able to decrease blood flow at rest due to the effect of NO on O2 consumption. Indeed Shen and colleagues demonstrated that reductions in NO bioavailability actually increase O2 consumption in dogs while they are either standing or walking (Shen et al., 2000). Therefore when NO bioavailability is increased, as is presumed to have happened after ascorbic acid infusion, it is likely that O2 consumption is decreased. Due to the fact that blood flow (and therefore O2 supply) is normally very closely matched to O2 consumption, it is possible that it was the drop in O2 consumption that was really driving the
reductions in blood flow that were observed at rest after infusion of ascorbic acid. This potential explanation is strengthened by the correlational analysis that indicates that the greatest reductions in blood flow at rest (with the exception of the soleus) were to the most highly oxidative muscle fibers, which would be the fibers that would primarily drive $O_2$ consumption.

During exercise ROS production can increase greatly thereby producing a situation where there may be an overabundance of ROS. Under these circumstances antioxidant supplementation may prove to be beneficial. In this experiment, however, supplementation with ascorbic acid had no effect on blood flow to any of the locomotor muscles that were evaluated despite decreasing levels of TBARS. This could be due to a variety of reasons. First of all, a dramatic reduction in ROS may have eliminated a potent vasodilatory signal as postulated above. This reduction may have been enough to offset any increases in NO bioavailability that were created through supplementation, resulting in no net change in blood flow. In addition to the elimination of vasodilatory ROS, it is also possible that ascorbic acid supplementation did not improve blood flow in this experiment due to fact that ascorbic acid appears to manifest its NO-sparing properties through an endothelial NOS (eNOS) dependent pathway (Tyml, Li, & Wilson, 2008). eNOS is one of the three major NOS isoforms that include eNOS, neuronal NOS (nNOS), and inducible NOS (iNOS). It has been shown that, during sedentary aging, eNOS expression is either reduced or maintained with age, depending upon the specific muscle being evaluated, and also that nNOS expression is increased (Capanni et al., 1998) (Song, Kwak, Kim, & Lawler, 2009). If this is the case, it is possible that the lower levels of eNOS existing in aged rats simply present a diminished potential for ascorbic acid mediated increases in NO and subsequent vasodilation. Finally, these results must be viewed in terms of the exercise intensity that was utilized. The specific exercise protocol that was used in this study (20 m/min up a 5% grade) was chosen to represent a submaximal work rate. However, there is evidence that this pace, which roughly corresponds to a fast walk, may have actually been a very strenuous work rate for the aged rats. Indeed, not only did the average lactate concentrations in both the control and ascorbic acid trials approach 9 mmol/L (Table 4.1), but there was also very high amounts of blood flow being directed to the highly non-oxidative muscle fibers. As a result of this high work rate, it is possible that blood flow values were already so near to their maximum flow rates that there was simply no additional capacity left for ascorbic acid to improve blood flow with.
Comparison to the Literature

The present results seem to contradict the long-standing assumption that ascorbic acid improves blood flow in aged subjects. At rest, the aged rats in this investigation actually manifested an ascorbic acid-induced decrease in blood flow (25 ± 3 vs. 16 ± 2 ml/kg/min), as opposed the approximately 37% increase in blood flow reported in the study by Jablonski and colleagues (Jablonski et al., 2007). Other studies using disease models other than aging have also found positive vascular effects such as improved endothelial function after infusion of ascorbic acid (Tyml et al., 2005). However, there is some evidence to indicate that ascorbic acid may not always promote vasodilation. Sindler and colleagues have recently shown that the scavenging of ROS with the antioxidant Tempol actually reduced flow-induced vasodilation in aged soleus muscle arterioles (Sindler, Delp, Reyes, Wu, & Muller-Delp, 2009). Furthermore, the addition of catalase, an enzyme that scavenges hydrogen peroxide, to Tempol-treated arterioles completely abolished flow-induced vasodilation. Wray and colleagues also showed that improvements in flow-mediated dilation that occurred in aged subjects after exercise training were abolished after antioxidant supplementation (Wray, Uberoi, Lawrenson, Bailey, & Richardson, 2009). These data support the theory that some ROS appear to be necessary for vasodilation to occur and may explain why blood flow was not improved after ascorbic acid supplementation.

To our knowledge, this is the first study to actually measure blood flow to individual muscles or muscle parts during whole-body exercise after ascorbic acid infusion. The results from our study indicate that ascorbic acid actually had no effect on muscle blood flow during treadmill running. Although this is in contrast to data presented by Kirby and colleagues (Kirby et al., 2009), which showed a 34% increase in blood flow to the exercising forearm after ascorbic acid infusion, it is difficult to make direct comparisons between the two investigations due to the difference in exercise mode (treadmill running as opposed to handgrip exercise), blood flow measurements (individual muscle blood flow as opposed to femoral artery blood flow), and species that was being studied.

Experimental Considerations

There are some potential experimental limitations of this study. First of all, this study is based upon the assumption that this particular dose of ascorbic acid is able to effectively
decrease levels of ROS in the body. The primary measure that was used to assess this was the TBARS assay. This particular assay has been criticized for its lack of specificity towards its target molecule, malondialdehyde (MDA), which may lead to inaccurate results (Chirico, 1994). However, this assay has been widely used and has remained the most commonly employed assay to determine lipid peroxidation (Moore & Roberts, 1998). Therefore, although the TBARS assay may not be an ideal choice for measuring oxidative stress, it should still provide at least some indication of whether or not ascorbic acid was able to reduce levels of ROS in the body.

Additionally, there are other studies that have utilized an identical dosing regimen and have documented large increases in plasma ascorbic acid concentrations and improved outcomes in animals that are subjected to increased levels of ROS (Tyml et al., 2005; Tyml et al., 2008).

Another limitation of this study is that the radioactive microsphere technique does not allow for continuous measurement of blood flow. Therefore it was only possible to obtain steady-state blood flow responses and not the effect that ascorbic acid may have created immediately following the onset of exercise and during recovery from exercise as well. These are topics that should be addressed in the future, as there is generally much more evidence for a role of NO in controlling blood flow, and therefore a greater potential effect from ascorbic acid supplementation, during these transition periods (Brock et al., 1998).

**Summary and Conclusions**

The major conclusion of this study is that a high-dose infusion of ascorbic acid failed to increase hindlimb blood flow in aged rats during treadmill running but decreased blood flow while at rest. These responses occurred despite similar increases in TAC during both rest and exercise and a decrease in TBARS during exercise. These data indicate that high doses of ascorbic acid actually impair blood flow at rest, potentially via a reduction in vasoactive ROS. The lack of a blood flow response to ascorbic acid infusion during exercise may be due to either no net effect of increases in NO bioavailability in the face of decreases in vasoactive ROS or a lack of effect of ROS or NO bioavailability on the blood flow response to exercise. A variety of future studies that are able to directly assess the concentrations of NO and individual ROS after antioxidant supplementation may be able to help elucidate the mechanisms of these responses. It would be beneficial if these studies could occur under a variety of time frames and exercise intensities.
References


