High-dose ascorbic acid infusion abolishes chronic vasoconstriction and restores resting leg blood flow in healthy older men

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Jablonski KL, Seals DR, Eskurza I, Monahan KD, Donato AJ.
High-dose ascorbic acid infusion abolishes chronic vasoconstriction and restores resting leg blood flow in healthy older men. J Appl Physiol 103: 1715–1721, 2007. First published August 23, 2007; doi:10.1152/japplphysiol.00553.2007.—Resting whole leg blood flow and vascular conductance decrease linearly with advancing age in healthy adult men. The potential role of age-related increases in oxidative stress in these changes is unknown. Resting leg blood flow during saline and ascorbic acid infusion was studied in 10 young (25 ± 1 yr) and 11 older (63 ± 2 yr) healthy normotensive men. Plasma oxidized LDL, a marker of oxidative stress, was greater in the older men (P < 0.05). Absolute resting femoral artery blood flow at baseline (iv saline control infusion) was 25% lower in the older men (238 ± 25 vs. 316 ± 38 ml/min; P < 0.05), and it was inversely related to plasma oxidized LDL (r = −0.56, P < 0.01) in all subjects. Infusion of supraphysiological concentrations of ascorbic acid increased femoral artery blood flow by 37% in the older men (to 327 ± 52 ml/min; P < 0.05), but not in the young men (352 ± 41 ml/min; P = 0.28), thus abolishing group differences (P = 0.72). Mean arterial blood pressure was greater in the older men at baseline (86 ± 4 vs. 78 ± 2 mmHg; P < 0.05), but it was unaffected by ascorbic acid infusion (P ≥ 0.70). As a result, the lower baseline femoral artery blood flow in the older men was mediated solely by a 32% lower femoral artery vascular conductance (P < 0.05). Baseline femoral vascular conductance also was inversely related to plasma oxidized LDL (r = −0.65, P < 0.01). Ascorbic acid increased femoral vascular conductance by 36% in the older men (P < 0.05) but not in the young men (P = 0.31). In conclusion, ascorbic acid infused at concentrations known to scavenge reactive oxygen species restores resting femoral artery blood flow in healthy older adult men by increasing vascular conductance. These results support the hypothesis that oxidative stress plays a major role in the reduced resting whole leg blood flow and increased leg vasoconstriction observed with aging in men.

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Methods
Subjects

A total of 21 healthy men, 10 young (aged 19–31 yr) and 11 older (59–78 yr), volunteered to participate and provided written informed consent.

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consent. All subjects had seated resting blood pressure <140/90 mmHg, had a body mass index <30 kg/m², and were free of clinical disorders as assessed by medical history, physical examination, blood chemistries, and hematologic evaluation. The older men also demonstrated normal electrocardiogram and blood pressure responses to incremental treadmill exercise performed to volitional exhaustion. Any candidate who demonstrated an ankle-brachial pressure index consistent with the possibility of peripheral vascular disease (i.e., <0.90) (39) was excluded. Subjects were nonsmokers, not taking medications or dietary supplements (including antioxidants), and not regularly exercising. All procedures were approved by the Human Research Committee and were performed in the General Clinical Research Center at the University of Colorado at Boulder.

Measurements

The methods have been described in detail previously (9–11, 17, 33, 34). All measurements were performed after a ≥4-h fast (12 h for determination of metabolic parameters) and abstinence from caffeine. During the main experimental sessions, subjects were instrumented with an intravenous catheter in the arm for infusion of saline and ascorbic acid and acquisition of blood.

Femoral artery ultrasonography. A duplex ultrasound machine (Toshiba Power Vision 6000) equipped with a high-resolution (7.5 MHz) linear-array transducer was used to measure blood velocity parameters and vessel diameter on the common femoral artery as described previously (9, 10). Blood flow in milliliters per minute was calculated by the following equation: velocity,

\[ \text{velocity}_{\text{mean}} \times \pi \times (\text{vessel diameter/2})^2 \times 60. \]

The data were analyzed by the same investigator (K. L. Jablonski), who was blinded to the group assignment and condition of the subject. Arterial blood pressure was measured over the brachial artery using the oscillometric technique (Dinamap, Critikon, FL) (9). Leg vascular conductance was calculated as femoral blood flow/mean arterial blood pressure, and leg vascular resistance as mean arterial blood pressure/femoral blood flow.

Body composition and leg fat-free mass. Total fat mass and fat-free mass were determined using dual-energy X-ray absorptiometry (DPX-IQ, Lunar). Regional analysis of the right leg was determined from the whole body scans (Lunar software version 3.1) as previously described (9, 10). Waist circumference and waist-to-hip ratio (WHR) were measured according to previously published guidelines (30) and were used as estimates of total abdominal fat (40).

Plasma measures. Venous blood samples were immediately centrifuged, and plasma or serum samples were placed in aliquots and stored at −80°C until analysis. Oxidized LDL, a measure of oxidative modification of lipids and marker of systemic oxidative stress, was determined with an ELISA plate assay (Alpco Diagnostics, Windham, NH). Plasma concentrations of glucose and total (Roche Diagnostic Systems, Indianapolis, IN) and HDL (Diagnostic Chemicals, Oxford, CT) cholesterol were determined by enzymatic and colorimetric methods, and LDL cholesterol was determined with the Friedewald equation (20). Plasma concentrations of insulin and endothelin-1 were measured using conventional radioimmunoassays, and plasma norepinephrine and epinephrine were determined by high-performance liquid chromatography (Dionex, Sunnyvale, CA). The homeostasis model assessment (fasting glucose × fasting insulin/22.5; HOMA) was used as an index of insulin sensitivity (23). Plasma concentrations of ascorbic acid were analyzed as described previously (17).

Habitual physical activity. Habitual physical activity was estimated from an activity questionnaire as described previously (14).

Protocol

Measurements of femoral blood flow and arterial blood pressure were obtained during saline infusion and during acute administration of a pharmacological dose of ascorbic acid (American Regent Laboratories, Shirley, NY) as described recently (16, 17, 33). First, a vehicle control was administered that consisted of a priming bolus of saline (20 min) followed by a drip infusion of saline (60 min). Next, a priming bolus of ascorbic acid was administered (0.06 g/kg fat-free mass dissolved in 100 ml of saline infused at 5 ml/min for 20 min) followed by a drip infusion (0.02 g/kg fat-free mass dissolved in 30 ml of saline administered over 60 min at 0.5 ml/min). The total volume infused for both saline and ascorbic acid was 130 ml. Venous blood samples were obtained during the drip infusions of saline and ascorbic acid from which plasma catecholamines and endothelin-1 were measured to provide insight into potential mechanisms of oxidative stress suppression of leg blood flow.

A saline (120 min) time and volume control study was conducted in four men to determine whether infusion of this quantity of fluid would cause an upward drift in leg blood flow with time that could confound interpretation of the responses to ascorbic acid. Leg blood flow did not increase above preinfusion control levels at any time during the 120-min saline infusion (% change in leg blood flow compared with preinfusion: 90 min −8 ± 6%; 120 min −7 ± 9%).

Ascorbic acid was chosen because it is a potent water-soluble antioxidant that, when infused at supraphysiological concentrations, scavenges and markedly reduces the bioavailability of superoxide anions (27) and acutely improves vascular function in several groups with risk factors for cardiovascular disease or clinical cardiovascular disease (12, 17, 22, 32, 35–37, 45). Previously, our laboratory has demonstrated that the present protocol for ascorbic acid infusion decreases plasma concentrations of oxidized LDL and isoprostanes in healthy middle-aged and older adults (3, 4). Therefore, the protocol used in the present study is a proven method to acutely reduce oxidative stress to determine its tonic influence on vascular function in humans.

Statistical Analysis

An unpaired r-test was used to assess group differences in subject characteristics and baseline circulating humoral factors and cardiovascular function. To determine the effect of acute ascorbic acid infusion on femoral blood flow, on vascular conductance and vascular resistance, and on mean arterial pressure, repeated-measures ANOVA was used. If significant differences were observed, post hoc analyses were performed using paired t-tests with the Bonferroni correction to identify significant differences among the mean values. Potential bivariate relations of interest between variables were assessed using Pearson product-moment correlation analyses. Data analysis was performed with SPSS software (version 12.0). Differences were considered significant at P < 0.05.

RESULTS

Subject Characteristics

Subject characteristics are presented in Table 1. There were no significant group differences in body mass, resting diastolic blood pressure, plasma HDL cholesterol, plasma insulin, HOMA, or physical activity. Body mass index, body fat percentage, waist circumference, WHR, resting systolic blood pressure, plasma total and LDL cholesterol, and fasting glucose were higher in the older compared with the young men (all \(P < 0.05\)). Plasma oxidized LDL concentrations were greater in the older men (84.3 ± 9.2 vs. 64.0 ± 4.7 U/l; \(P < 0.05\)). Leg fat-free mass was similar in the young (10.4 ± 0.5 kg) and older (10.1 ± 0.4 kg) men.

Ascorbic Acid Concentrations

Plasma ascorbic acid concentrations did not differ in the young (64 ± 6 μmol/l) and older (65 ± 8 μmol/l) men during baseline saline control conditions (\(P = 0.99\)). The infusion of ascorbic acid increased plasma ascorbic acid concentrations...
20-fold or more above baseline levels in both groups (young: +1,278 ± 67 μmol/l; older: +1,021 ± 81 μmol/l; both P < 0.001 vs. baseline).

Resting Leg Blood Flow

Values for femoral artery mean diameter and blood flow velocity are presented in Table 2, and femoral artery blood flow is shown in Fig. 1, top. Absolute resting femoral artery blood flow during saline control was 25% lower in the older compared with the young men (238 ± 25 vs. 316 ± 38 ml/min; P < 0.05). In all subjects, baseline femoral blood flow was inversely related to plasma concentrations of oxidized LDL (r = −0.56, P < 0.01; Fig. 2).

Compared with saline control, during ascorbic acid infusion femoral artery blood flow was increased by 37% in the older (saline: 86 ± 4 mmHg; ascorbic acid: 85 ± 3 mmHg; P = 0.70) men. Heart rate was similar during the saline control and ascorbic acid infusions in the young (saline: 57 ± 3 beats/min; ascorbic acid: 56 ± 2 beats/min; P = 0.35) and older (saline: 54 ± 2 beats/min; ascorbic acid: 54 ± 2 beats/min; P = 0.85) men.

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Age, yr</td>
<td>25±1</td>
<td>63±2*</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>77±3</td>
<td>81±3</td>
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<tr>
<td>BMI, kg/m²</td>
<td>22.6±0.8</td>
<td>25.2±1*</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>16±2</td>
<td>26±2*</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>78±2</td>
<td>93±2*</td>
</tr>
<tr>
<td>WHR</td>
<td>0.81±0.01</td>
<td>0.92±0.02*</td>
</tr>
<tr>
<td>Systolic BP, mmHg</td>
<td>113±4</td>
<td>124±5*</td>
</tr>
<tr>
<td>Diastolic BP, mmHg</td>
<td>65±3</td>
<td>74±4</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>153±7</td>
<td>198±8*</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>84±5</td>
<td>120±8*</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>51±2</td>
<td>50±5</td>
</tr>
<tr>
<td>Fasting glucose, mg/dl</td>
<td>84±3</td>
<td>94±3*</td>
</tr>
<tr>
<td>Plasma insulin, μU/ml</td>
<td>5.2±0.8</td>
<td>5.0±2.0</td>
</tr>
<tr>
<td>HOMA</td>
<td>1.13±0.08</td>
<td>1.23±0.08</td>
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<tr>
<td>Physical activity, METs</td>
<td>45±12</td>
<td>48±17</td>
</tr>
</tbody>
</table>

Values are means ± SE, n, no. of subjects; BMI, body mass index; WHR, waist-to-hip ratio; BP, resting arterial blood pressure; HOMA, insulin sensitivity index (homeostasis model assessment); physical activity, average daily leisure and occupational activity; METs, metabolic equivalents. *P < 0.05 vs. young.

Table 2. Femoral artery parameters

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young Saline</th>
<th>Ascorbic acid Saline</th>
<th>Older Saline</th>
<th>Ascorbic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Velocity, cm/s</td>
<td>6.8±0.01</td>
<td>7.5±0.7</td>
<td>5.0±0.4†</td>
<td>6.6±0.9*</td>
</tr>
<tr>
<td>Diameter, cm</td>
<td>0.99±0.01</td>
<td>0.99±0.02</td>
<td>1.0±0.03</td>
<td>1.0±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. *P < 0.05 vs. older saline control. †P < 0.05 vs. young saline control.

Mean Arterial Blood Pressure

Mean arterial blood pressure was higher in the older men (P < 0.05), but it did not change from saline baseline control levels during ascorbic acid infusion in either the young (saline: 78 ± 2 mmHg; ascorbic acid: 78 ± 2 mmHg; P = 0.82) or the older (saline: 86 ± 4 mmHg; ascorbic acid: 85 ± 3 mmHg; P = 0.70) men. Heart rate was similar during the saline control and ascorbic acid infusions in the young (saline: 57 ± 3 beats/min; ascorbic acid: 56 ± 2 beats/min; P = 0.35) and older (saline: 54 ± 2 beats/min; ascorbic acid: 54 ± 2 beats/min; P = 0.85) men.

Leg Vascular Conductance and Resistance

Results for femoral artery vascular conductance are presented in Fig. 1, bottom. The lower baseline (saline control) femoral artery blood flow in the older compared with the young men was solely mediated by a 32% lower femoral artery vascular conductance (P < 0.05; Fig. 1). In all subjects, baseline femoral vascular conductance was inversely related to plasma oxidized LDL (r = −0.65, P < 0.01; Fig. 2).
In the older men, the increase in absolute femoral blood flow during ascorbic acid infusion was associated with a 36% increase in femoral vascular conductance compared with saline control \((P < 0.05)\). In contrast, femoral vascular conductance was not significantly different during ascorbic acid infusion compared with saline control in the young men \((P = 0.31)\).

The same age-associated differences in baseline vasoconstrictor state and responsiveness to ascorbic acid infusion were observed when values were expressed as leg vascular resistance instead of vascular conductance.

**Circulating Neurohumoral Factors**

Values for the circulating neurohumoral factors are presented in Table 3. Baseline plasma concentrations of norepinephrine and endothelin-1 were greater in the older men (both \(P < 0.05\)), whereas there was no group difference in plasma epinephrine. Plasma concentrations of these neurohumoral factors were not different during the saline and ascorbic acid infusion conditions.

**DISCUSSION**

The key novel finding of the present study is that ascorbic acid infused at concentrations known to scavenge reactive oxygen species restores resting femoral artery blood flow in healthy older adult males to levels observed in young men by increasing vascular conductance (reducing vascular resistance). This finding provides strong experimental support for the hypothesis that oxidative stress plays a major role in the reduced resting whole leg blood flow and increased leg vasoconstriction observed with aging in men. Based on the recent findings of Moreau and colleagues (33), the results of the present study also suggest that this tonic vasoconstrictor effect of oxidative stress may be much greater in middle-aged and older men than in postmenopausal women.

**Role of Oxidative Stress in Tonic Leg Vasoconstriction With Aging in Healthy Men**

Our finding that resting whole leg blood flow was 25% lower in middle-aged and older compared with young healthy men, and that this was mediated solely by reduced vascular conductance (increased vascular resistance), is consistent with previous observations from our laboratory (9–11). The results of the present study extend these earlier findings by providing the first evidence that oxidative stress is a key mechanism involved in the leg vasoconstriction observed with aging in healthy men. That plasma oxidized LDL concentrations, a marker of oxidative stress, were increased in the older group and were inversely related to leg blood flow and vascular conductance in the overall subject sample is consistent with this conclusion. However, the strongest, most direct experimental evidence supporting the involvement of oxidative stress is that infusion of supraphysiological concentrations of ascorbic acid, an intervention known to reduce the bioavailability of reactive oxygen species and thus decrease oxidative stress (3, 4, 27), selectively increased leg blood flow in the older men, restoring flow to levels observed in young adult men. This effect was consistent, occurring in 9 of the 11 older men, and was mediated solely by an increase in leg vascular conductance because mean arterial blood pressure did not change during ascorbic acid infusion. The improvements in femoral blood flow with ascorbic acid in the older men were not related to their lower blood flows under saline control conditions, and therefore they were not simply a function of a lower baseline flow. Taken together, our results strongly support the idea that oxidative stress is a key mechanism involved in the increased leg vasoconstriction observed in older healthy men.

We wish to point out that although the older men were healthy and free of known clinical diseases, they did differ from the young controls in several cardiovascular risk factors, many of which have been linked to oxidative stress. As such, it is possible that the effects of oxidative stress on leg blood flow and vascular conductance in the older men may be, at
least in part, the result of one or more of these factors rather than the direct effects of older age.

Oxidative Stress-Related Suppression of Leg Blood Flow With Aging in Men and Women

Using the same methods as the present investigation, Moreau and colleagues (33) recently found that ascorbic acid increased leg blood flow only in premenopausal women, reducing the pre- to postmenopausal group difference by ~30%. In contrast, in the present study, ascorbic acid increased leg blood flow in older men to levels not significantly different from those of young men. Although sex differences were not directly investigated in the present study, one interpretation of these observations is that oxidative stress exerts a greater tonic suppressive influence on leg blood flow and vascular conductance with aging in male than in female healthy adults. If so, it would be consistent with sex differences in markers of systemic oxidative stress such as plasma oxidized LDL (15, 17, 18) and plasma thiobarbituric acid-reacting substances and urinary levels of 8-isoprostaglandin F2α (26).

The physiological mechanisms underlying these apparent sex-related differences in baseline oxidative stress have not been determined. However, the presence of the potent antioxidant properties of circulating estrogen in women has been advanced as one possible explanation (5).

Mechanisms of Oxidative Stress-Associated Suppression of Leg Blood Flow With Aging in Men

The mechanisms by which oxidative stress is related to leg vasoconstriction with aging in healthy men remain to be determined. Our laboratory previously demonstrated that the reductions in leg blood flow and vascular conductance in older compared with young men are associated with increases in leg muscle sympathetic nerve activity (9) and enhanced tonic α-adrenergic vasoconstriction (11). As such, one possibility is that oxidative stress contributes to leg vasoconstriction with aging in men via stimulation of α-adrenergic signaling. In a previous study (4), our laboratory showed that the ascorbic acid infusion used in the present study does not reduce leg muscle sympathetic nerve activity. This suggests that if oxidative stress is modulating leg blood flow through α-adrenergic signaling, it is not affecting the sympathetic stimulus but rather some aspect(s) of vascular α-adrenergic responsiveness. This idea is consistent with the fact that plasma norepinephrine concentrations, an indirect measure of sympathetic nerve activity, were not affected by ascorbic acid infusion in the present study. Moreover, several experimental observations in animal models indicate that increased bioavailability of reactive oxygen species (oxidative stress) can modulate sympathetic α-adrenergic vasoconstriction via multiple cellular pathways (21, 24), including the interruption of nitric oxide signaling (46). Circulating epinephrine and endothelin-1 did not change with ascorbic acid infusion in the present study and, therefore, do not appear to have been involved in the increase in leg blood flow in the older men.

It also is possible that oxidative stress influences leg vasoconstrictor tone with aging in men through nonadrenergic mechanisms involving the synthesis and release of vasodilatory factors from the vascular endothelium. For example, oxidative stress is associated with reduced nitric oxide bioavailability (19, 47), and experimentally reducing nitric oxide bioavailability via inhibition of nitric oxide synthesis decreases limb blood flow and vascular conductance in humans (6, 13, 42). Aging is associated with oxidative stress and reduced tonic nitric oxide bioavailability (44, 45). Thus oxidative stress-dependent reductions in nitric oxide bioavailability could contribute to the lower leg blood flow and vascular conductance observed in older men. Vascular endothelial production of prostaglandins, endothelin-1, angiotensin II, or other locally produced vasodilator factors also could play a role in oxidative stress-associated reductions resting leg blood flow with aging.

Finally, although the ascorbic acid infusion increased leg blood flow and vascular conductance in the older men in the present study, there were no obvious reductions in systemic arterial blood pressure or changes in heart rate. We can only speculate that the increases in leg vascular conductance were not great enough to lower systemic vascular resistance and arterial pressure and/or evoke a baroreflex increase in heart rate. We are unaware of any data on the effects of a supra-physiological intravenous infusion of ascorbic acid on blood flow and vascular conductance in other regional circulations. It is possible that ascorbic acid does not produce vasodilation in other vascular beds in older men or that it evokes a constriction in some regional circulations that would act to offset the increase in leg vascular conductance.

Physiological and Clinical Significance

As noted previously (9 –11, 34, 35), our findings have potentially important implications for human aging as it relates to both physiological function and disease risk. With regard to physiological function, an augmented baseline leg vasoconstrictor state may act to inhibit hyperemic responses to large-muscle dynamic exercise, ambient heat stress, or other states associated with increased functional demand (28, 41). In addition, cardiovascular disease-related morbidity and mortality increase markedly with age, in part because of worsening of key risk factors (38). It has been postulated that tonic peripheral vasoconstriction and reduced blood flow are mechanistically involved in the development and/or maintenance of insulin resistance and the metabolic syndrome (29), key risk factors for cardiovascular disease, perhaps by contributing to impaired postprandial glucose disposal and/or clearance of atherogenic lipids (2). Oxidative stress appears to be linked to increased risk of cardiovascular disease via multiple mechanisms, including these metabolic abnormalities (7, 25, 43). The present results suggest the possibility that oxidative stress may contribute to a decline in physiological function and the development of metabolic disorders in older men by producing vasoconstriction and reducing peripheral blood flow under baseline conditions.

Although it was not an experimental goal of the present study to determine the efficacy of oral ascorbic acid administration for restoring resting leg blood flow in older men, a few comments regarding interventions may be helpful. We have shown that oral administration of ascorbic acid does not reverse other expressions of vascular aging (17), even in settings in which acute supraphysiological administration of this compound restores function (17). As discussed previously (17), we believe that this is because orally administered ascorbic acid does not reach plasma concentrations sufficient to effectively
scavenge superoxide anions, certainly not in a sustained manner that would be required to chronically improve vascular function. Preliminary observations on a small group of older healthy sedentary men indicate that this also is the case for resting leg blood flow (unpublished observations). Similarly, habitual aerobic exercise, also thought to exert antioxidant effects that can preserve some vascular functions with age (17), has no obvious influence on the decline in resting leg blood flow with aging in healthy men (10). However, recent findings indicate that resting leg blood flow is preserved in middle-aged men who regularly perform resistance exercise (31), and that resistance exercise training increases resting leg blood flow in previously sedentary middle-aged and older men and women, independent of changes in leg fat-free mass (1). Thus resistance exercise training is the only intervention that has been shown to improve resting leg blood flow in older adults.

Conclusions

In conclusion, the results of the present study provide the first direct evidence that oxidative stress is a key mechanism mediating the tonic leg vasoconstriction that develops with aging in healthy men. Our results also suggest that oxidative stress may play a greater role in the age-associated reductions in leg blood flow and vascular conductance in men than in women. These findings have implications for the mechanisms by which oxidative stress contributes to age-associated reductions in physiological function and risk of chronic disease.

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REFERENCES


