Acute Reversal of Endothelial Dysfunction in the Elderly After Antioxidant Consumption

D. Walter Wray, Steven K. Nishiyama, Ryan A. Harris, Jia Zhao, John McDaniel, Anette S. Fjeldstad, Melissa A.H. Witman, Stephen J. Ives, Zachary Barrett-O’Keefe, Russell S. Richardson

Abstract—Aging is associated with a pro-oxidant state and a decline in endothelial function. Whether acute, enteral antioxidant treatment can reverse this decrement in vascular function is not well known. Flow-mediated vasodilation and reactive hyperemia were evaluated after consumption of either placebo or an oral antioxidant cocktail (vitamin C, 1000 mg; vitamin E, 600 IU; α-lipoic acid, 600 mg) in 87 healthy volunteers (42 young: 25 ± 1 years; 45 older: 71 ± 1 years) using a double-blind, crossover design. Blood velocity and brachial artery diameter (ultrasound Doppler) were assessed before and after 5-minute forearm circulatory arrest. Serum markers of lipid peroxidation, total antioxidant capacity, endogenous antioxidant activity, and vitamin C were assayed, and plasma nitrate, nitrite, and 3-nitrotyrosine were determined. In the placebo trial, an age-related reduction in brachial artery vasodilation was evident (young: 7.4 ± 0.6%; older: 5.2 ± 0.4%). After antioxidant consumption, flow-mediated vasodilation improved in older subjects (placebo: 5.2 ± 0.4%; antioxidant: 8.2 ± 0.6%) but declined in the young (placebo: 7.4 ± 0.6%; antioxidant: 5.8 ± 0.6%). Reactive hyperemia was reduced with age, but antioxidant administration did not alter the response in either group. Together, these data demonstrate that antioxidant consumption acutely restores endothelial function in the elderly while disrupting normal endothelium-dependent vasodilation in the young and suggest that this age-related impairment is attributed, at least in part, to free radicals. 

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Key Words: aging ■ endothelium ■ free radicals ■ NO ■ vascular function

In recent years, considerable effort has been expended to evaluate the relationship among age, oxidative stress, and vascular health, with largely equivocal results. However, there is convincing evidence of an overall increase in plasma free radical concentration with advancing age1-12 and a subsequent reduction in NO bioavailability, which has been implicated in the age-related decline in endothelial function.3-7 As endothelial dysfunction and increased vascular oxidative stress have been identified as predictors for risk of cardiovascular events,8 antioxidant (AO) supplementation as a noninvasive approach to further understand vascular function, and the potential of this approach in the prevention and treatment of vascular dysfunction, is particularly attractive in the elderly population.

Despite the accepted association between oxidative stress and vascular health, chronic, large scale (>1000 subjects) clinical trials have failed to demonstrate a beneficial effect of long-term AO consumption on cardiovascular disease morbidity and mortality.9 However, smaller, interventional studies focused on acute AO-mediated changes in vascular reactivity have provided more promising results. Infused, supraphysiologic doses of vitamin C have been documented to transiently restore endothelium-dependent vasodilation10,11 and skeletal muscle blood flow12-14 in the elderly, and BH4 administration (a cofactor for endothelial NO production) acutely improves endothelial function.15 However, studies using more practical interventions, such as oral vitamin C at over-the-counter doses, have failed to demonstrate an improvement in age-related endothelial dysfunction.10 Moreover, the effect of combined AOs administered concomitantly on vascular function in the elderly remains largely unknown. Thus, despite the myriad studies that have examined the role of oxidative stress on cardiovascular health with healthy aging, mechanistic studies designed to abruptly reduce plasma oxidative stress and to subsequently determine the acute effects of this intervention on AO capacity, oxidative stress, and vascular function have not been undertaken.

Determination of endothelial function after an acute AO intervention is most easily achieved by assessing the degree of brachial artery vasodilation in response to an acute increase in shear stress. This flow-mediated vasodilation (FMD) of the brachial artery subsequent to a period of vascular occlusion provides a relevant index of endothelium-dependent vasodilation that reflects NO bioavailability15 and

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correlates well with coronary artery reactivity. Importantly, both coronary and peripheral vasodilator function have been convincingly documented to predict future cardiovascular events, and, as such, assessment of endothelium-dependent vasodilation may provide valuable prognostic and diagnostic patient information. Likewise, the magnitude of reactive hyperemia relates inversely to traditional cardiovascular disease risk factors and has also emerged as a clinically relevant tool in predicting future cardiovascular events. However, the role of NO in this response is minimal, and, thus, reactive hyperemia remains a clinically relevant, although somewhat nonspecific, indicator of microvascular reactivity. Together, FMD and reactive hyperemia may, therefore, be seen as complimentary measurements that allow an opportunity to compare endothelial and microvascular function, as well as determination of the specific effect of oxidative stress and AO administration on these distinct regions of the vasculature.

In the current study, we sought to fill a void in the clinical literature by administering an oral AO cocktail (vitamin C, E, and α-lipoic acid) to abruptly reduce plasma free radical concentration and to assess the acute vascular responses to this reduction in oxidative stress in young and older subjects. Two hypotheses were tested: we hypothesized that AO consumption would improve FMD in the elderly but decrease FMD in their young, healthy counterparts, and we hypothesized that reactive hyperemia would not be affected by AO consumption in either young or older subjects.

Methods

Subjects and General Procedures

Forty-two young (25±1 years) and 45 older (71±1 years) healthy volunteers participated in the current study. All of the subjects were nonsmokers, normotensive (<140/90 mm Hg), and free of overt cardiovascular disease, as determined by health history questionnaire and physical examination. Exclusion criteria included subjects with diagnosed cardiovascular disease, diabetes mellitus, hypercholesterolemia, hypertension, and women who were pregnant. Subjects were not taking any prescription medication and were asked to abstain from vitamin supplements for 10 days before enrollment and during the course of the study. Subjects arrived at the laboratory at 8:00 AM in a fasted state, having abstained from exercise and caffeine for 12 previous hours, in accordance with recently published guidelines. Premenopausal women were studied within the first 5 days of their menstrual cycle, as FMD measurements of the brachial artery have been documented to fluctuate with menstrual phase. Protocol approval and written informed consent were obtained according to the University of Utah and Veteran Affairs Institutional Review Board requirements. All of the data collection took place with subjects supine, in a thermoneutral environment.

AO Supplementation

All of the subjects reported to the laboratory twice within 1 week (>48 hours apart) and received the AO cocktail or placebo (PL) in a balanced, double-blind, crossover design. Supplements were taken in 2 doses, separated by 30 minutes to improve absorption, consumed 90 and 60 minutes before the FMD protocol. The first dose consisted of 300 mg of α-lipoic acid, 500 mg of vitamin C, and 200 IU of vitamin E, and the second dose was 300 mg of α-lipoic acid, 500 mg of vitamin C, and 400 IU of vitamin E. PL microcrystalline cellulose capsules of similar taste, color, and appearance were likewise consumed in 2 doses within the same time frame. We have reported previously on the efficacy of this AO cocktail to reduce carbon- and O2-centered free radical levels, as measured by electron paramagnetic resonance spectroscopy, in both young and older subject populations.

Measurements

Details of the FMD procedure have been described previously and were performed in accordance with current recommendations. Briefly, a blood pressure cuff was placed on the right arm proximal to the elbow and distal to the placement of the ultrasound Doppler probe on the brachial artery (BA). The BA was imaged approximately midway between the antecubital and axillary regions, and measurements of BA diameter and blood velocity (Vmean) were obtained continuously at rest and for 2 minutes after cuff deflation (Logiq 7, GE Medical Systems, Milwaukee, WI).

Analyses

Vmean was automatically calculated using commercially available software (Logiq 7). End-diastolic, ECG R-wave gated images were collected via video output from the Logiq 7 for offline analysis of BA vasodilation using automated edge-detection software (Medical Imaging Applications, Coralville, IA). FMD was quantified as the maximal percentage of change in BA diameter after cuff release. Shear rate was calculated as follows: shear rate (s⁻¹)=Vmean/8/π [vessel diameter]. Blood flow was calculated as follows: Blood flow (mL · min⁻¹)=Vmean · Vmean/(vessel diameter)² · 60. For both shear rate and blood flow, cumulative area under the curve values were integrated with the trapezoidal rule and calculated as follows: Σ yi[(yi+yi−1)+1/2] (yi+yi−1−yi)(yi+yi−1−yi). Reactive hyperemia was quantified as cumulative BA blood flow for 2 minutes (area under the curve) after cuff occlusion. Normalized FMD was calculated by dividing FMD (percentage) by the cumulative shear rate area under the curve at the time of peak BA vasodilation.

Assays

In a subset of subjects (n=18 young; n=30 older), blood samples were obtained from the antecubital vein immediately before FMD testing on both PL and AO visits. Total AO capacity was assessed by the ferric reducing ability of plasma assay, and endogenous AO activity was assessed by determining superoxide dismutase (Cayman Chemical Company, Ann Arbor, MI). Plasma ascorbate concentration was also determined (Cosmo Bio, Carlsbad, CA). Quantitative determination of thiobarbituric acid reactive substances was performed to assess lipid peroxidation, a marker of oxidative stress (Bioassay Systems, Hayward, CA). Total nitrate and nitrite were measured using a standard colorimetric nitrate reductase/Griess reaction assay (Cayman Chemical Company). Measurement of nitrotyrosine was performed using competitive ELISA (Cell Biolabs, San Diego, CA). A lipid panel was obtained for all of the subjects by standard techniques.

Statistics

Statistics were performed with the use of commercially available software (SigmaPlot 11, Systat Software Inc, Point Richmond, CA). Repeated measures 2-way ANOVA was used to identify significant changes in measured variables between conditions and groups, with the Bonferroni test used for post hoc analysis when a significant main effect was found. All of the group data are expressed as mean±SE. Significance was established at P<0.05.

Results

Subject characteristics are presented in Table 1, and assay results are documented in Table 2. In the PL trial, FMD was significantly attenuated in older subjects compared with their younger counterparts (older PL: 5.2±0.4%; younger PL: 7.4±0.6%; Figure 1). In the older group, AO administration significantly improved FMD (5.2±0.4% to 8.2±0.6%, PL versus AO). In contrast, AO administration in the young group had a detrimental effect on FMD (7.4±0.6% to 5.8±0.6%, PL versus AO; Figure 1). When normalized for...
The present study sought to evaluate the efficacy of acute AO administration to abruptly reduce plasma oxidative stress and identify the efficacy of AOs available over the counter as capable of acutely improving endothelial function in the elderly.

**Table 1. Subject Characteristics**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Young (n=42)</th>
<th>Older (n=45)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>25±1</td>
<td>71±1*</td>
</tr>
<tr>
<td>Height, cm</td>
<td>172±2</td>
<td>168±1</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>64±2</td>
<td>73±2*</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22±1</td>
<td>25±1*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>157±6</td>
<td>182±8*</td>
</tr>
<tr>
<td>High-density lipids, mg/dL</td>
<td>55±3</td>
<td>52±3</td>
</tr>
<tr>
<td>Low-density lipids, mg/dL</td>
<td>93±4</td>
<td>114±7*</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>69±6</td>
<td>100±8</td>
</tr>
<tr>
<td>BA diameter, mm</td>
<td>3.7±0.1</td>
<td>4.0±0.2*</td>
</tr>
<tr>
<td>BA blood flow, mL/min</td>
<td>44±3</td>
<td>73±6*</td>
</tr>
</tbody>
</table>

BA indicates brachial artery.
*Data show the significant difference between young and older groups, P<0.05.

FMD, Age, and AOs

BA FMD testing has emerged as a simple, noninvasive means of evaluating endothelial function in a variety of healthy and disease populations. Vasodilation of the BA subsequent to a period of vascular occlusion provides an index of endothelium-dependent vasodilation that reflects NO bioavailability and is thought to be predictive of future cardiovascular events. These previous studies have led to the concept that results of endothelial function testing may serve as a “barometer for cardiovascular health” and promoted

**Table 2. Serum Measurements of Antioxidant Capacity, Oxidative Stress, and Antioxidant Activity**

<table>
<thead>
<tr>
<th>Variable and Condition</th>
<th>Young (n=18)</th>
<th>Older (n=30)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C, µg/mL</td>
<td>1.4±0.2</td>
<td>2.2±0.2†</td>
</tr>
<tr>
<td>Thio-lead nitric acid reactive substances, µM</td>
<td>1.51±0.19</td>
<td>1.70±0.16</td>
</tr>
<tr>
<td>Ferric-reducing AO power, µM/L</td>
<td>803±32</td>
<td>947±41†</td>
</tr>
<tr>
<td>Superoxide dismutase, U/ml</td>
<td>9.4±0.8</td>
<td>10.6±0.8†</td>
</tr>
<tr>
<td>3-Nitrotyrosine, nM</td>
<td>242±21</td>
<td>275±32</td>
</tr>
<tr>
<td>Nitrite, µM</td>
<td>8.0±0.7</td>
<td>8.0±0.7</td>
</tr>
<tr>
<td>Nitrite:nitrate</td>
<td>0.09±0.01</td>
<td>0.11±0.01</td>
</tr>
</tbody>
</table>

PL indicates placebo; AO, antioxidant.
*Data show the significant difference between young and older groups, P<0.05.
†Data show the significant difference between PL and AO, P<0.05.
interest in interventions with the prospect of improving endothelial health and thereby limiting cardiovascular risk.

We observed an age-related decline in FMD in the elderly (Figure 1), which is in agreement with the majority of studies that have collectively identified a decline in endothelium-dependent vasodilation with advancing age, often as early as the fourth decade. However, this age-related decrement appeared readily reversible, as evidenced by improved FMD after AO administration. Indeed, AO consumption acutely improved BA FMD in the elderly to that of their younger counterparts (Figure 1), suggesting that an AO cocktail at modest, enteral doses is sufficient to acutely reverse age-related endothelial dysfunction. This profound improvement in endothelial function extends former studies demonstrating the capacity of high-dose, infused AOs to acutely improve endothelium-dependent vasodilation in the elderly, now revealing a similar beneficial effect using a typical, over-the-counter combination of AOs available to the general public.

As discussed above, FMD is generally thought to provide a bioassay of NO, and as such it is likely that this AO-mediated improvement in FMD in the elderly was achieved through an acute increase in NO bioavailability.

In contrast to the positive outcome in the elderly, the AO intervention proved detrimental to endothelium-dependent vasodilation in young, healthy individuals (Figure 1). Previous studies from our group have identified a similar pattern of “paradoxical” age-specific AO effects, which appears to be related to the underlying level of oxidative stress. Specifically, during handgrip exercise, BA vasodilation is impaired in the elderly, but this age effect is restored to that of the young after AO consumption; however, AO administration had an opposite effect in the young, resulting in an impaired vasodilatory capacity during exercise. Findings from the present study thus complement these previous exercise studies through the use of BA FMD measurements, a widely accepted method for examining endothelial function.

Evidence of negative responses to AO in the young appears to support the emerging concept of a beneficial role for free radicals in the peripheral circulation. Indeed, others have documented that free radicals, such as H₂O₂ and reactive oxygen species (ie, ONOO⁻) may also act as potent vasodilators and, thus, may, in fact, contribute to FMD. In addition to direct vasodilatory properties, free radicals may also play an important role in the upregulation of endogenous AO capacity through increased expression of several enzymes known to “detoxify” free radicals. Considering these former studies and the present data, we speculate that, in the young, where pro-oxidant and AO forces are somewhat balanced, the aggressive reduction in free radical concentration after AO administration may have removed or suppressed certain oxidative species that possess some beneficial properties in terms of vascular regulation, resulting in the observed decrement in FMD.

Together, these data in young and older individuals demonstrate a dichotomous effect of AO consumption on endothelial function with age. Consumption of an AO cocktail at over-the-counter doses acutely improved endothelial function in the elderly while disrupting normal endothelium-dependent vasodilation in the young.

**Reactive Hyperemia, Age, and AOs**

After circulatory occlusion, resistance vessel vasodilation provokes an increase in arm vascular conductance and, thus, acts as the downstream stimulus for upstream increases in BA...
shear rate and subsequent BA vasodilation. However, the factors that contribute to reactive hyperemia, per se, are thought to be somewhat more complex than BA FMD. Indeed, this response may be attributed to a combination of endothelial-dependent dilation, inherent myogenic action, and vasodilatory metabolites produced in response to tissue ischemia, including adenosine and nonendothelium-dependent vasodilators.

Like FMD, the magnitude of reactive hyperemia relates inversely to traditional cardiovascular disease risk factors and has, thus, emerged as a clinically relevant tool in predicting future cardiovascular events. However, the role of NO in this response appears modest, and, thus, reactive hyperemia remains a clinically relevant, although somewhat nonspecific, indicator of microvascular reactivity. In the present study, these complimentary measurements allowed for intersubject and intrasubject comparison of the effects of AO administration on both endothelial and microvascular functions, as well as an opportunity to further define the specific effect of acute AO consumptions on NO bioavailability. As illustrated in Figure 2, reactive hyperemia was significantly reduced in older subjects in the PL trial. The mechanisms for this age-related difference remain unknown, but it is speculated that an age-related decline in sensitivity to ischemia or production of vasodilatory metabolites may at least partially explain these intriguing findings. This suggestion is supported by the recent observation that the muscle metaboreflex becomes less sensitive with advancing age. AO administration did not alter reactive hyperemia in either group, which was anticipated based on former work from our group and others, which failed to identify NO as a significant contributor to postocclusion reactive hyperemia. These findings are in line with other AO studies that have been unable to document a change in reactive hyperemia after intra-arterial ascorbic acid or BH4 in elderly subjects. When viewed in conjunction with the marked AO effect on FMD, the lack of a treatment effect in the microvasculature further supports the assertion that the AO cocktail consumed in the present study likely improved endothelial function in the elderly through a reduction in oxidative stress and subsequent improvement in conduit artery NO bioavailability. It is also tempting to speculate that free radicals may play a proportionally bigger role than NO in governing reactive hyperemia through vascular smooth muscle hyperpolarization or the oxidative activation of the cGMP-dependent kinase via disulfide dimerization, although discerning these mechanistic pathways is beyond the scope of the present study.

Quantitative Assessment of AO Efficacy
One essential component of any study examining the efficacy of an AO intervention is detecting increases in circulating AO concentrations and documenting a subsequent reduction in free radical concentration and NO production. In terms of AOs, we determined that vitamin C concentration and markers of AO capacity increased in both young and older groups (Table 2), confirming the equally efficacious effect of the AO treatment between groups. Thus, it is apparent that AO-mediated changes in endothelial function cannot be attributed to differences in absorption of the AO cocktail. It is also noteworthy that administration of exogenous AOs increased superoxide dismutase (superoxide dismutase) activity (Table 2), one of the most ubiquitous endogenous AOs. Thus, it seems that AO consumption resulted in a 2-tier effect, including both direct scavenging effects of free radicals and a secondary decrease in oxidative stress via “sparing” of endogenous AOs, which collectively improved endothelial function in the elderly.

Oxidative stress was significantly reduced after AO administration in the elderly, as documented by a reduction in lipid peroxidation end products (thiobarbituric acid reactive substances; Table 2). Interestingly, the same cannot be said for the young, in whom thiobarbituric acid reactive substances did not decrease as a consequence of the AO cocktail. These findings, in combination with our previous reports identifying the efficacy of this AO cocktail to reduce carbon- and O2-centered free radical levels utilizing EPR spectroscopy in both young and older subject populations, confirm the ability of this AO treatment to acutely reduce oxidative stress when baseline levels are elevated, as in the elderly. Surprisingly, AO consumption did not affect 3-nitrotyrosine in either group and was reduced in the elderly compared with young in the PL condition (Table 2). This was an unexpected finding, considering the age-related increase in thiobarbituric acid reactive substances and the subsequent reduction after AO consumption in the older group but is in agreement with previous work reporting similar tissue 3-nitrotyrosine levels in both young and older rats before and after chronic AO supplementation.

Plasma nitrite and nitrite:nitrate were assessed as a surrogate measure of vascular NO bioavailability (Table 2), as it has been demonstrated that circulating nitrite sensitively reflects acute changes in regional endothelial NO synthase activity. For both nitrite and nitrite:nitrate, we observed an age-related decline that is consistent with previous studies and the functional measurements of FMD (Figure 2). However, we were unable to detect a significant decrease in either nitrite or nitrite:nitrate after AO consumption. As with the 3-nitrotyrosine, this lack of an AO effect on plasma nitrite and nitrite:nitrate may reflect the somewhat subtle nature of the oral AO treatment used in the present study, an intervention that appears to provoke functional improvements in endothelium-dependent vasodilation without detectable increases in circulating nitrite levels.

Perspectives
In contrast to the largely disappointing findings from clinical trials concerning the beneficial effect of chronic AO administration on cardiovascular health, the present study has identified a marked and acute improvement in endothelial-dependent vasodilation in the elderly after consumption of an oral AO cocktail. However, the difference in design between acute and long-term interventional studies precludes a direct comparison with data from these previous studies. Indeed, the present study administered a single AO load to provoke an abrupt reduction in plasma oxidative stress and to subsequently determine the acute effects of this intervention on AO capacity, oxidative stress, and vascular function in young and older individuals. Using this short-term, interventional design has
allowed us to demonstrate the ability of an oral AO cocktail to reverse the age-associated impairment in endothelial function in the elderly without the additional confounding variables associated with longer-term treatment studies. These findings provide important mechanistic insight regarding the link between endothelial dysfunction and oxidative stress in the elderly and demonstrate a striking similarity in endothelial function in response to a simple, oral AO intervention.

Experimental Considerations
It should be noted that the 3-nitrotyrosine assay reflects peroxynitrite from multiple upstream sources and is the combined consequence of basal NO bioavailability, superoxide levels, and superoxide dismutase activity. Thus, the reduction in 3-nitrotyrosine in the elderly, and lack of an effect after AO administration, is somewhat difficult to interpret due to this assay’s lack of specificity. We also acknowledge that the present data do not offer insight concerning the potential vascular benefits of a long-term AO effect after AO administration, is somewhat difficult to interpret due to this assay’s lack of specificity. We also acknowledge that the present data do not offer insight concerning the potential vascular benefits of a long-term AO treatment. It is anticipated that follow-up studies involving chronic administration of this oral AO cocktail will build on these acute vascular measurements, allowing a more appropriate comparison with existing, larger-scale AO studies.

Summary
Administration of an AO cocktail documented to reduce plasma oxidative stress significantly improved endothelial function in the elderly, reversing the age-related decrement in FMD. In contrast, the same AO intervention proved detrimental to endothelium-dependent vasodilation in young, healthy individuals. AO administration did not alter reactive hyperemia in either group, supportive of an endothelium-dependent, NO-specific effect. These findings reveal a pivotal role of oxidative stress in age-associated endothelial dysfunction and support the efficacy of oral AO administration in physiological doses as a means to acutely improve conduit artery endothelial function in the elderly.

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Disclosures
None.

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