Acute ascorbate supplementation alone or combined with arginase inhibition augments reflex cutaneous vasodilation in aged human skin

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SKIN BLOOD FLOW IS CONTROLLED by dual sympathetic innervation comprising an adrenergic vasoconstrictor system and active vasodilator system (9). With increasing body core temperature, skin blood flow is reflexly increased by an initial withdrawal of tonic adrenergic vasoconstriction and on reaching a specific temperature threshold is further increased by the active vasodilator system (30). Cutaneous active vasodilation is purportedly mediated by the cotransmission of acetylcholine and an unknown neurotransmitter(s) from the sympathetic vasodilator system (16). Furthermore, nitric oxide (NO) is required for full expression of cutaneous active vasodilation and contributes ~30% of the total vasodilatory response, where histamine and vasoactive intestinal peptide contribute to active vasodilation through NO-dependent mechanisms (4, 15, 26, 37, 38).

Human aging in the absence of overt pathology is associated with attenuated reflex cutaneous vasodilation (17, 18). Aged humans have a reduced functional cotransmitter(s) contribution to the increase in skin blood flow during hyperthermia and rely predominantly on NO-dependent mechanisms (13), despite the fact that cutaneous NO-dependent vasodilation is compromised with advancing age (28). Arginase is upregulated with advanced age and preferentially metabolizes the common substrate L-arginine in the final step of the urea cycle, resulting in reciprocal regulation of endothelial NO synthase (eNOS) (5). Furthermore, restoring L-arginine availability for NO synthesis through eNOS by acute arginase inhibition augments reflex vasodilation in aged human skin (14).

In addition to arginase upregulation, other age-related alterations in the mechanisms affecting NO bioavailability may also contribute to attenuated reflex cutaneous vasodilation. One alternative mechanism potentially contributing to impaired NO bioavailability in skin involves an age-related increase in oxidative stress (24). Reactive oxygen species (ROS), including superoxide, increase in the skin with advancing age via both an increase in production and a decrease in degradation by a reduction in superoxide scavenging enzymes (19, 24). In vascular endothelial cells, superoxide can react with NO to form peroxynitrite up to four times faster than what can be metabolized, resulting in decreased NO bioavailability and therefore reduced NO-dependent vasodilation (3). Additionally, eNOS itself can uncouple and become a source of ROS in the presence of inadequate substrate and/or essential cofactor availability (29).

Acute ascorbate (Asc) supplementation in aged human forearm vasculature restores attenuated NO-dependent vasodilation through direct superoxide scavenging and by stabilizing the essential NOS cofactor tetrahydrobiopterin (BH4) without affecting NOS activity (11, 34). Moreover, in human skin, topical administration of 3–8% Asc solutions reduces oxidant stress associated with aging (19, 31) and induced by exposure to ultraviolet irradiation (7, 22). Therefore, the aim of this study was to determine the effect of acute Asc administration in attenuated reflex cutaneous vasodilation in aged skin. We hypothesized that Asc supplementation would augment reflex cutaneous vasodilation in aged skin. We further hypothesized that increasing L-arginine availability for NO synthesis through NOS by inhibiting arginase in combination with Asc supplementation would enhance reflex vasodilation over Asc supplementation alone.

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METHODS

Subjects. Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University and conformed to the guidelines set forth by the Declaration of Helsinki. Verbal and written consent was voluntarily obtained from all subjects before participation. Studies were performed on 11 young (18–27 yr old) and 10 older (65–72 yr old) men and women. Each subject underwent a complete medical screening, including blood chemistry, lipid profile evaluation (Quest Diagnostics Nichol Institute, Chantilly, VA), physical examination, and an assessment of maximal oxygen uptake (SensorMedics, Yorba Linda, CA). All subjects were screened for the presence of cardiovascular, dermatological, renal, and neurological disease. Subjects were healthy, normally active, not endurance trained [30–70th percentile values for maximal aerobic power normalized for age (1)], normotensive, nondiabetic, healthy nonsmokers who were not taking medications, including aspirin therapy, hormone replacement therapy, or oral contraceptives. All young female subjects were studied on days 2–7 of the early follicular phase of their menstrual cycle. Experimental testing was conducted between September and February.

Instrumentation and measurements. All protocols were performed in a thermoneutral laboratory with the subject in the supine position with the experimental arm at heart level. On arrival to the laboratory between the hours of 0700–0900, subjects were instrumented with four intradermal microdialysis fibers (10 mm, 20-kDa cutoff membrane, MD 2000, Bioanalytical Systems) in the skin on the right ventral forearm. Microdialysis sites were at least 4.0 cm apart to ensure no cross-reactivity of pharmacological agents between sites. Microdialysis fibers were placed at each site by first inserting a 25-gauge needle through unanesthetized skin by using sterile technique. The entry and exit points were ~2.5 cm apart. The microdialysis fiber was then threaded through the internal lumen of the needle, and the needle was withdrawn, leaving the fiber in place. The microdialysis fibers were taped in place and perfused with lactated Ringer solution during the insertion trauma resolution protocol at a rate of 2.0 μl/min (Bee Hive controller and Baby Bee microinfusion pumps, Bioanalytical Systems) for 60–90 min until resolution of the insertion trauma.

To obtain an index of skin blood flow, cutaneous red blood cell flux was measured with an integrated laser-Doppler flowmeter probe placed in a local heater (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK) on the skin directly above each microdialysis membrane. All laser-Doppler probes were calibrated using Brownian standard solution. Cutaneous vascular conductance (CVC) was calculated as red blood cell flux divided by mean arterial pressure.

To control whole body temperature, subjects wore a water-perfused suit that covered the entire body except head, hands, and experimental arm and a water-impermeable rain suit to minimize evaporative heat loss. Electrocardiogram output was monitored throughout the protocol, and blood pressure was measured via brachial auscultation every 5 min. Oral temperature (Ton) was continuously monitored during baseline and throughout whole body heating with a thermistor placed in the sublingual sulcus as an index of body core temperature. The subjects were instructed to keep the thermistor in the same location in the sublingual sulcus and not to open their mouths or speak during the protocol. Mean skin temperature was calculated as the unweighted average of six copper-constantan thermocouples placed on the chest, middle back, abdomen, upper arm, thigh, and calf. During the insertion trauma resolution and baseline periods, thermoneutral water (34°C) was perfused through the suit to clamp whole body temperature. During whole body heating, 50°C water was perfused through the suit to raise Ton 0.8°C above baseline body temperature. On reaching a plateau in skin blood flow, the temperature of the water perfusing the suit was decreased to 46°C to clamp skin temperature for an additional 10 min, which corresponded with a ΔTon of 0.8°C.

Experimental protocol. Red blood cell flux over each microdialysis site was monitored during the insertion trauma resolution period (60–90 min). After this period, microdialysis sites were randomly assigned to receive 1) lactated Ringer solution to serve as control; 2) 10.0 mM Nω-nitro-l-arginine (l-NNAME; Calbiochem, San Diego, CA) to competitively inhibit NO production by NOS; 3) 10 mM l-ascorbate (Sigma) to supplement antioxidants; and 4) the combination of 10 mM l-ascorbate and 5.0 mM (s)-2-boronoethyl-l-cysteine-HCl (BEC) and 5.0 mM Nω-hydroxy-nor-l-arginine (nor-NOHA) to supplement antioxidants and to inhibit arginase (Calbiochem). All pharmacological agents were dissolved in lactated Ringer solution.

Our laboratory previously showed that a 10.0 mM dose of l-NNAME and a dose of 5 mM BEC + 5 mM nor-NOHA was sufficient to maximally inhibit NOS and arginase in both subject groups, respectively (14, 28). Extensive pilot work was conducted to determine the final concentration of Asc used in the protocol. In three middle age (40–50 yr old) pilot subjects, varying concentrations (1, 5, 7.5, 10.0, and 20.0 mM) of Asc were delivered at a rate of 2 μl/min to different skin microdialysis sites for 90 min before and then throughout a standardized local heating protocol described elsewhere (27). Although the mechanisms for the local heating-induced rise in skin blood flow are different from reflex cutaneous vasodilation, the local heating protocol was chosen because the plateau phase of the local heating response is largely mediated by NO-dependent mechanisms. After the established plateau in skin blood flow during local heating, 10 mM l-NNAME was infused until a skin blood flow decreased to a stable plateau. Concentrations >7.5 mM Asc did not further increase the NO-dependent plateau phase of the local heating response. Furthermore, the Δ-value from the established plateau in skin blood flow before l-NNAME infusion and the post-l-NNAME plateau was significantly increased by Asc supplementation at doses >5 mM [Asc: 82 ± 2 vs. control: 60 ± 2% maximal CVC (CVCmax); P < 0.001].

All microdialysis sites were perfused with assigned pharmacological agents continuously for at least 60 min before the start of the baseline and during the baseline and heating periods at a rate of 2.0 μl/min. Baseline data were collected for 20 min before the start of whole body heating. After the baseline data collection period, whole body heating was conducted to raise Ton by 0.8°C. Following a 0.8°C rise in Ton, body temperature was clamped for 10 min. At the end of the heating protocol, each microdialysis site was perfused with 28.0 mM sodium nitroprusside (Nitropress, Abbot Laboratories, Chicago, IL) at a rate of 4.0 μl/min to achieve maximal CVC. Local heating of the skin to 43°C was conducted simultaneously with sodium nitroprusside infusion to ensure that maximal CVC had been obtained.

Data acquisition and analysis. Data were acquired with the use of Labview software and National Instruments data acquisition system (Austin, TX). The data were collected at 40 Hz, digitized, recorded, and stored on a personal computer for further analysis. CVC data were averaged over 3-min periods for baseline and every 0.1°C rise in Taw and are presented as a percentage of CVCmax.

Student’s t-tests were used to determine significant differences between the young and older groups for physical characteristics and for baseline absolute Taw. A three-way repeated-measures mixed model analysis of variance was conducted to detect differences between subject groups at the pharmacological treatment sites over the rise in Taw (SAS, version 9.1). Post hoc comparisons with Bonferroni corrections were performed when appropriate to determine where differences between groups and drug treatments occurred. The level of significance was set at α = 0.05 for main effects and α = 0.016 after Bonferroni correction. Values are presented as means ± SE.

RESULTS

Subject physical characteristics are presented in Table 1. There was no significant difference between subject groups for body mass index, systolic blood pressure, diastolic blood
pressure, or mean arterial pressure. The older subjects group had significantly lower maximal aerobic capacity and higher total cholesterol and low-density lipoproteins. There was no significant difference between the groups for high-density lipoproteins. Baseline Tor was not different between the subject groups (P = 0.43).

Table 1. Subject characteristics

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Older</th>
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<tbody>
<tr>
<td>Sex (M, F)</td>
<td>7, 4</td>
<td>5, 5</td>
</tr>
<tr>
<td>Age, yr</td>
<td>22 ± 1</td>
<td>68 ± 1*</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>23 ± 1</td>
<td>25 ± 1</td>
</tr>
<tr>
<td>VO₂max, ml·kg⁻¹·min⁻¹</td>
<td>46 ± 3</td>
<td>29 ± 3*</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>150 ± 7</td>
<td>205 ± 10*</td>
</tr>
<tr>
<td>HDL, mg/dl</td>
<td>51 ± 3</td>
<td>57 ± 5</td>
</tr>
<tr>
<td>LDL, mg/dl</td>
<td>83 ± 8</td>
<td>127 ± 7*</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>114 ± 3</td>
<td>120 ± 4</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>73 ± 2</td>
<td>77 ± 2</td>
</tr>
<tr>
<td>MAP, mmHg</td>
<td>87 ± 2</td>
<td>91 ± 2</td>
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<tr>
<td>Baseline Tor, °C</td>
<td>36.40 ± 0.08</td>
<td>36.32 ± 0.08</td>
</tr>
</tbody>
</table>

Values are means ± SE. HDL, high-density lipoprotein; LDL, low-density lipoprotein; MAP, mean arterial pressure; Tor, oral temperature; SBP, systolic blood pressure; DBP, diastolic blood pressure; VO₂max, maximal oxygen uptake; BMI, body mass index. *Significant difference vs. young subject group (P < 0.05).

Figure 1 shows the CVC response as a function of the rise in body core temperature for all of the treatment sites. The control site was significantly attenuated beginning at a ΔTor of 0.3°C in the older subject group compared with the young subject group (Fig. 1A). NOS inhibition significantly decreased CVC compared with the control site in both subject groups (P < 0.001). Additionally, when compared with the young subject group, CVC was attenuated in the older subject group in the NOS-inhibited site at ΔTor ≈ 0.6°C (P < 0.001). There was no significant difference between the Asc site and the control site for the young subject group. However, the aged subjects had significantly higher CVC with Asc compared with the control site at ΔTor > 0.7°C (P < 0.05) (Fig. 1B). There were no significant differences between subject groups for the Asc site. Similarly, combined arginase inhibition and Asc augmented CVC above the level of the control site in the older subject group at ΔTor > 0.6°C (P < 0.001), such that CVC was not significantly different from the control site in the young group (Fig. 1C). Combined Asc + arginase inhibition did not significantly change CVC compared with the control site in the young subjects.

Figure 2 summarizes the CVC responses in all of the treatment sites during the plateau in skin blood flow with a 0.8°C rise in body core temperature. CVC at baseline is...
Because of a functional reduction in cotransmitter(s)-mediated vasodilation, older subjects rely predominantly on NO-dependent vasodilation to increase skin blood flow during heat stress, with cotransmitter(s) contributing only modestly with significant increases in body core temperature (13, 28). Attenuated reflex vasodilation in aged humans is likely due, in part, to an age-related increase in oxidative stress, supported by the finding that acute administration of supraphysiological doses of Asc was able to augment reflex cutaneous vasodilation.

Asc alone and combined with arginase inhibition only augmented skin blood flow in our aged subject group with significant increases in body core temperature, whereas NOS inhibition resulted in an attenuated skin blood flow response in both subject groups much earlier in whole body heating. Although we cannot discount the fact that Asc and the arginase inhibitors may have had other nonspecific effects on the aged vasculature and may have augmented vasodilation through other non-NO-dependent pathways, given the mechanism of action of Asc and the arginase inhibitors, it is likely that these treatments increased skin blood flow through NO-dependent mechanisms. Since we did not observe an increase in skin blood flow in the young subjects group, these findings suggest that potential age-related changes in either eNOS protein concentrations or activity may be present. However, evidence from human cell culture studies and animal models evaluating age-associated changes in eNOS expression, absolute concentration, and activity are divergent (6, 25), and there has not been a direct assessment of age-related changes in eNOS protein concentration and activity in human skin. Another likely explanation for our findings is that there are different mechanisms mediating the early and late phase of reflex vasodilation and that our interventions were selective for the later phase. Although NO is involved in both the early and the late phase of active vasodilation, the upstream pathways mediating NO release may be different, with acetylcholine and VIP contributing to the early phase and H1 receptor activation contributing to the late phase through NO-dependent mechanisms (26, 33, 37, 38).

NO bioavailability in the vasculature is dictated by the balance of NO production and degradation. In aged human skin, there is an increase in superoxide production and a decrease in degradation through attenuated superoxide dismutase and glutathione peroxidase activity, leading to overall increased oxidative stress (24). Superoxide directly decreases NO bioavailability by reacting with NO, forming peroxynitrite at a rate up to four times faster than superoxide metabolism by superoxide dismutase (3). Furthermore, peroxynitrite is capable of oxidizing critical cofactors for NOS, including BH4, leading to NOS uncoupling. During NOS uncoupling, NOS itself becomes dysregulated, and electron flow from the reductase domain to the oxidase domain of the enzyme is directed toward molecular oxygen instead of the NO substrate L-arginine, resulting in superoxide production instead of NO (10, 20, 21, 35). NOS uncoupling also occurs from compromised L-arginine availability (36). In the context of the present study, since acute Asc supplementation was able to augment reflex vasodilation in the aged, these data suggest that overall oxidative stress is increased in aged human skin and impairs NO bioavailability through an increase in free radical production and aberrant metabolism, which may include NOS uncoupling.

DISCUSSION

The principal finding of the present study was that Asc alone selectively augmented reflex cutaneous vasodilation in aged subjects. Moreover, Asc combined with arginase inhibition (to increase L-arginine availability for NO synthesis through NOS) resulted in an additional increase in cutaneous vasodilation compared with sites treated with Asc alone. These treatments were effective at increasing reflex vasodilation only in the aged subject group with significant increases in body core temperature at $\Delta T_{cr} > 0.7^\circ C$ and $\Delta T_{cr} > 0.6^\circ C$ for Asc supplementation and Asc + arginase inhibition, respectively. Collectively, these data suggest 1) that age-associated increases in oxidative stress and arginase activity both limit cutaneous vasodilation in aged skin; and 2) that reflex vasodilation can be augmented with antioxidant supplementation and acute arginase inhibition.

Our results show that acute Asc supplementation increases reflex cutaneous vasodilation in aged human skin. Given the nonspecific antioxidant mechanism of action of Asc, it is likely that the increase in skin blood flow in the aged subject group was due to an increase in NO. NO is required for full expression of cutaneous active vasodilation in young skin and contributes $\sim 30\%$ to the total skin blood flow response (15, 32).
We were able to augment cutaneous vasodilation with acute antioxidant supplementation using the potent nonspecific antioxidant L-ascorbate. These findings are consistent with the forearm muscle vascular bed, where NO-dependent vasodilation during infusions of endothelium-dependent agonists is restored in the presence of supraphysiological levels of Asc (8, 34). Interestingly, Asc is capable of increasing NO via several different mechanisms, including 1) directly scavenging ROS and 2) stabilizing the essential NOS cofactor BH4 by recycling oxidized BH3 to BH4, thereby inhibiting the production of superoxide through uncoupled NOS without directly altering NOS activity (11, 20). In the aged human forearm muscle vasculature, BH4 deficiency contributes to attenuated endothelium-dependent vasodilation, whereas acute BH4 supplementation restores the vasodilatory responses to intra-arterial infusions of acetylcholine through NO-dependent mechanisms (12). In relation to the current data, increased reflex vasodilation in aged skin during Asc supplementation is likely due to a combination of direct ROS scavenging effects and stabilization of BH4. However, direct supplementation with BH4 in aged human skin is necessary to more fully elucidate its role in NOS uncoupling and oxidative stress in aged human skin.

In addition to BH4 deficiency, NOS can also uncouple from inadequate L-arginine availability (29, 36). We have recently shown that L-arginine availability for NO synthesis is compromised in aged skin and that restoring the available pool of L-arginine for NO synthesis through arginase inhibition and/or L-arginine supplementation augments reflex cutaneous vasodilation (14). Arginase competes for the common NOS substrate L-arginine and is upregulated with advanced age (5). In the present study, combined Asc + arginase inhibition significantly increased CVC greater than the Asc-supplemented site alone in aged skin. These findings suggest that both oxidative stress and limited L-arginine bioavailability independently contribute to attenuated reflex cutaneous vasodilation. Alternatively, these mechanisms may be linked, and upregulated arginase activity may directly contribute to age-related increases in oxidative stress through NOS uncoupling due to limited L-arginine availability.

If oxidative stress and upregulated arginase activity independently contribute to attenuated cutaneous vasodilation, the resulting cutaneous vasodilation during combined antioxidant supplementation and arginase inhibition should be greater than the vasodilation during the individual treatments. However, if the current data are compared with an arginase-inhibited site from our previous study that used many of the same subjects and utilized the same protocol, there is no difference between the arginase-inhibited site and combined Asc + arginase-inhibited site (arginase inhibited: 40 ± 5 vs. Asc + arginase inhibited: 41 ± 3% CVC max, P = 0.71). It is possible that the arginase inhibition alone maximized the capacity of the cutaneous vessels to vasodilate at this level of hyperthermia (+0.8°C), approaching a “ceiling effect” of the vessel, and a greater vasodilatory stimulus may unmask differences between the drug treatment sites. Thus it is difficult to tease out the precise contributions and interactions of oxidative stress and upregulated arginase activity to decreased NO-dependent vasodilation with our model. The use of other cutaneous vasodilator stimuli, such as prolonged local heating which induces NO-dependent vasodilation, may provide further insight into these questions.

Limitations. We did not directly show that the dose of Asc delivered to the cutaneous vasculature through microdialysis reduced oxidative stress in aged skin. However, the dose of Asc utilized in this study was more than double compared with studies where topical administration of an 8.0% Asc solution decreased markers of oxidative stress in human skin (22, 23). Moreover, topical administration of this dose reached peak concentration and demonstrated effective antioxidant capabilities within 1 h of administration. We delivered an 18% (10 mM) Asc solution directly to the cutaneous vasculature through intradermal microdialysis and let this solution perfuse the microdialysis fiber for at least 1 h before the start of baseline measurements.

We cannot discount the possibility that augmented reflex cutaneous vasodilation in the aged subjects may have been the result of nonspecific effects of Asc on non-NO-dependent pathways. ROS act as signaling molecules to mediate cold-induced constriction of the vascular smooth muscle (2) and may also mediate vasoconstriction through a variety of pathways. Non-NO-dependent effects of antioxidant treatment on the cutaneous vasculature may have been revealed if we had simultaneously inhibited NOS and supplemented with antioxidants.

Summary. In summary, acute Asc supplementation increased reflex cutaneous vasodilation in aged skin. Asc supplementation combined with arginase inhibition, to increase L-arginine availability for NO synthesis through NOS, resulted in a greater increase in skin blood flow during hyperthermia. These treatments did not alter reflex cutaneous vasodilation in young subjects. Collectively, these data indicate that age-related increases in oxidative stress and upregulated arginase activity may contribute to attenuated reflex cutaneous vasodilation.

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REFERENCES

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ASCORBATE AND CUTANEOUS VASODILATION


