Local ascorbate administration augments NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans

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Holowatz LA, Kenney WL. Local ascorbate administration augments NO- and non-NO-dependent reflex cutaneous vasodilation in hypertensive humans. Am J Physiol Heart Circ Physiol 293: H1090–H1096, 2007. First published May 4, 2007; doi:10.1152/ajpheart.00295.2007.—Full expression of reflex cutaneous vasodilation (VD) is dependent on nitric oxide (NO) and is attenuated with essential hypertension. Decreased NO-dependent VD may be due to 1) increased oxidant stress and/or 2) decreased L-arginine availability through upregulated arginase activity, potentially leading to increased superoxide production through uncoupled NO synthase (NOS). The purpose of this study was to determine the effect of antioxidant supplementation (alone and combined with arginase inhibition) on attenuated NO-dependent reflex cutaneous VD in hypertensive subjects. Nine unmedicated hypertensive [HT; mean arterial pressure (MAP) = 112 ± 1 mmHg] and nine age-matched normotensive (NT; MAP = 81 ± 10 mmHg) men and women were instrumented with four intradermal microdialysis (MD) fibers: control (Ringer), NOS inhibited (NOS-I; 10 mM Nω-nitro-L-arginine), L-ascorbate supplemented (Asc; 10 mM L-ascorbate), and Asc + arginase inhibited [Asc+A-I; 10 mM L-ascorbate + 5 mM (S)-(2-boronoethyl)-l-cysteine-HCl + 5 mM Nω-hydroxy-nor-l-arginine]. Oral temperature was increased by 0.8°C via a water-perfused suit. L-arginine availability through upregulated arginase activity decreases NO bioavailability by competing for the common NO synthase subunit. In vivo human studies examining vascular oxidant stress involved in active vasodilation include vasoactive intestinal peptide (3), histamine I receptor activation (38), cyclooxygenase-produced vasodilators (27), and substance P (37). Furthermore, activation of these pathways may contribute to active vasodilation in part through nitric oxide (NO)-dependent pathways, which are required for full expression of active vasodilation, contributing ~30–40% to the total vasodilatory response (20, 30).

Essential hypertension is associated with attenuated cutaneous vasodilation during local (6) and systemic thermal stress (16, 22). We (16) have recently shown that NO-dependent reflex cutaneous vasodilation is attenuated in hypertensive skin and that increasing L-arginine availability through acute arginase inhibition augments reflex vasodilation. Arginase catalyzes the conversion of L-arginine to L-ornithine and urea in the final step of the urea cycle and is capable of limiting NO synthesis (13). In addition to upregulated arginase contributing to decreased NO production, there are likely additional mechanisms impeding NO bioavailability in hypertensive cutaneous vasculature.

One potential mechanism contributing to the reduced NO bioavailability implicated in the pathogenesis of hypertensive vascular pathology is increased oxidative stress (35). In essential hypertensive vasculature, there is increased production of reactive oxygen species (ROS) coupled with decreased degradation of ROS through dysfunctional enzymatic antioxidant defense mechanisms (25). Furthermore, augmented ROS (superoxide) production reduces NO bioavailability by readily reacting with newly synthesized peroxynitrite, which further potentiates superoxide production by uncoupling endothelial NO synthase (eNOS) (10). eNOS uncoupling can result from peroxynitrite-mediated oxidation of the essential NOS cofactor tetrahydrobiopterin (BH4) and/or inadequate L-arginine availability (28). Together, these data suggest that there may be a mechanistic link between hypertension-associated increased arginase activity decreasing L-arginine availability and potentially contributing to increased oxidant stress through uncoupled eNOS.

In vivo human studies examining vascular oxidant stress mechanisms commonly utilize high-dose antioxidant supplementation for measurement of functional NO-dependent vasodilation. To this end, acute L-ascorbate (Asc) supplementation
in the forearm muscle vasculature in humans with essential hypertension increases endothelium-dependent vasodilation through NO-dependent mechanisms (31). In these studies, Asc may increase NO bioavailability directly through antioxidant capabilities and/or indirectly by stabilization of the essential NOS cofactor BH₄, enabling NOS to “recouple” and produce functional NO (15, 32). The purpose of the present study was to determine the relative role of oxidant stress and augmented arginase activity on attenuated NO-dependent vasodilation in human hypertensive cutaneous vasculature. We sought to control for age-related increases in oxidant stress and arginase activity (17) by matching subjects with unmedicated essential hypertension to age- and sex-matched healthy normotensive control subjects. We hypothesized that 1) local acute antioxidant (Asc) supplementation would modestly augment NO-dependent reflex cutaneous vasodilation and 2) arginase inhibition (to increase L-arginine availability, enabling NOS to recouple and produce functional NO) combined with Asc supplementation would further enhance NO-dependent reflex vasodilation over Asc supplementation alone.

METHODS

Subjects. Experimental protocols were approved by the Institutional Review Board at The Pennsylvania State University. Verbal and written consents were voluntarily obtained from all subjects before their participation. Studies were performed on nine unmedicated subjects with essential hypertension (57 ± 4 yr, 6 men and 3 women) and nine normotensive age- and sex-matched control subjects (57 ± 3 yr, 6 men and 3 women). Each subject reported to the laboratory on three separate occasions to determine their blood pressure status in accordance with the guidelines set forth by the American Heart Association (1). Subjects then underwent a complete medical screening, including blood chemistry, lipid profile evaluation (Quest Diagnostics Nichol Institute, Chantilly, VA), resting ECG, and physical examination. All subjects were screened for the presence of cardiovascular disease other than hypertension, dermatological, and neurological diseases. Subjects were normally active, nondiabetic, healthy nonsmokers who were not taking medications, including aspirin therapy, hormone replacement therapy, or oral contraceptives. All premenopausal women (4 women) were studied on days 2–7 of the follicular phase of their menstrual cycle. Seventeen of the eighteen subjects also participated in a previous study in our laboratory on a separate day (16).

Instrumentation and measurements. All protocols were performed in a thermoneutral laboratory with the subject in the semisupine position with the experimental arm at heart level. After arrival to the laboratory, subjects were instrumented with four intradermal microdialysis fibers as previously described (16–19) (MD 2000, Bioanalytical Systems) (10-mm, 30-kDa cutoff membrane) in the skin on the right ventral forearm. The microdialysis fibers were taped in place and perfused with lactated Ringer solution to serve as control, 2) 10.0 mM N⁵-nitro-L-arginine (L-NAME; Calbiochem, San Diego, CA) to competitively inhibit NO production by NOS, 3) 10 mM Asc (Sigma, St. Louis, MO) to supplement antioxidants (17), or 4) the combination of 10 mM Asc and 5.0 mM (S)-(2-boronoethyl)-L-cysteine-HCl and 5.0 mM N⁵-nitro-L-arginine to supplement antioxidants and to inhibit arginase (Asc+A-NAME; Calbiochem) (16, 18). Our group (16–18) has previously used these doses of Asc and arginase inhibitors in conjunction with intradermal microdialysis in an aged population with known endothelial dysfunction. All pharmacological solutions were mixed just before usage, dissolved in lactated Ringer solution, sterilized with the use of syringe microfilters (Acrodisc, Pall, Ann Arbor, MI), and wrapped in foil.

All microdialysis sites were perfused continuously with assigned pharmacological agents for at least 60 min before the start of the baseline and throughout baseline (20 min) and whole body heating at a rate of 2.0 µl/min. After the baseline data collection period, whole body heating was conducted to raise Tₑₑₑ by 0.8°C. After a 0.8°C rise in Tₑₑₑ, body temperature was clamped for 30–40 min. After 10 min of stable laser-Doppler flux values, 10 mM L-NAME was perfused through all microdialysis fibers at a rate of 2.0 µl/min to inhibit NOS within each treatment site. L-NAME infusions were discontinued after laser-Doppler flux decreased to a stable plateau (Fig. 1). At the end of the whole body heating protocol, each microdialysis site was perfused with 28.0 mM sodium nitroprusside (SNP; Nitropress, Abbott 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 SNP infusion to ensure that maximal CVC had been obtained.

![Figure 1](http://ajpheart.physiology.org/)

**Fig. 1.** Representative tracings of cutaneous vascular conductance (CVC) as a percentage of maximal vs. time from the control site in a normotensive (●) and a hypertensive (○) subject. Whole body heating started at minute 10, and N⁵-nitro-L-arginine (L-NAME) was perfused through the microdialysis sites after oral temperature increased 0.8°C (minute 65 in this example).
Data acquisition and analysis. Data were acquired at 40 Hz using Labview software and a National Instruments data-acquisition system (Austin, TX). Data were 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 Tor and are presented as a percentage of maximal CVC (%CVCmax). Δ%CVCmax in the control, Asc, and Asc+A-I sites is the difference between the plateau values in %CVCmax before and after NOS inhibition. Maximal CVC was calculated as the average of the stable 10-min plateau in laser-Doppler flux values divided by MAP during SNP and local heating.

Student’s paired t-tests were used to determine significant differences between the groups for physical characteristics. Two-way repeated-measures ANOVA was conducted to detect differences from blood pressure and pharmacological treatment on maximal CVC and on the within-site Δ%CVCmax. A mixed model, three-way repeated-measures ANOVA was conducted to detect differences between subject groups at the pharmacological treatment sites over the rise in Tor (SAS, version 8.01). Tukey’s post hoc tests were performed when appropriate to determine where differences between groups and drug treatments occurred. The level of significance was set at α = 0.05. Values are presented as means ± SE.

RESULTS

Subject characteristics are presented in Table 1. The subjects were well matched for age, sex, body-mass index, total cholesterol, and high-density and low-density lipoproteins. The hypertensive subjects had significantly higher resting systolic and diastolic pressures and MAP (all P < 0.001).

Figure 1 illustrates the timeline of l-NAME administration with representative tracings in control microdialysis sites from a normotensive subject and a subject with essential hypertension.

Figure 2 shows the %CVCmax responses for all sites to the rise in body core temperature in both subject groups. %CVCmax in the control site was attenuated in the hypertensive subjects starting with change in Tor (ΔTor) ≥0.6°C compared with the normotensive group (P < 0.05). NOS inhibition throughout whole body heating attenuated %CVCmax in both subject groups starting at ΔTor ≥0.3°C in the hypertensive group and ΔTor ≥0.2°C in the normotensive group (both P < 0.05). Asc and Asc+A-I significantly increased %CVCmax in the hypertensive group at ΔTor ≥0.4°C (P < 0.05 vs. control) but did not alter %CVCmax in the normotensive group (P > 0.05 vs. control). There was not a significant difference between the Asc- and Asc+A-I-treated sites in either subject group (P > 0.05).

Figure 2 also illustrates the mean %CVCmax after NOS inhibition within the control, Asc, and Asc+A-I sites at ΔTor = 0.8°C. NOS inhibition within the control site decreased %CVCmax in both subject groups (P < 0.05). Furthermore, there was not a significant difference between the groups in %CVCmax in the control site after NOS inhibition (%CVCmax: 29 ± 4 for hypertensive vs. 34 ± 4 for normotensive; P > 0.05). Asc and Asc+A-I increased %CVCmax after NOS inhibition in the hypertensive group (%CVCmax: 41 ± 4 Asc and 40 ± 4 for Asc+A-I; P < 0.05 vs. control) but not in the normotensive group (%CVCmax: 29 ± 3 for Asc, 30 ± 4 for Asc+A-I; P > 0.05 vs. control).

Figure 3 summarizes the %CVCmax responses in all of the treatment sites during the plateau in skin blood flow with a 0.8°C rise in body core temperature before and after within-site NOS inhibition. Compared with the normotensive group, vasodilation at the control site was attenuated in the hypertensive group (P < 0.001), and there was a blunted decrease with NOS inhibition (hypertensive: −8 ± 1 vs. normotensive: −14 ± 2 Δ%CVCmax; P < 0.001). Compared with their respective control sites, NOS inhibition in the Asc and combined Asc+A-I sites caused a greater Δ%CVCmax in the hypertensive group (Asc: −19 ± 4, Asc+A-I: −17 ± 4; P < 0.05 vs. control) than in the normotensive subject group (Asc: −11 ± 3, Asc+A-I: −15 ± 3; P > 0.05 vs. control) (Fig. 3, inset).

Maximal absolute CVC was attenuated in the hypertensive group compared with the normotensive group (hypertensive: 1.32 ± 0.11 vs. normotensive: 1.78 ± 0.20 flux/mmHg; P = 0.001). Within each subject group, there was no effect of pharmacological treatment on maximal absolute CVC (P > 0.05).

DISCUSSION

The primary findings of this study were that humans with essential hypertension have 1) attenuated reflex cutaneous vasodilation that can be augmented with acute Asc supplementation alone or combined with arginase inhibition and 2) the increase in cutaneous vasodilation with these treatments is mediated by both NO- and non-NO-dependent mechanisms (Figs. 2 and 3). These data suggest that increased ROS associated with hypertension directly decrease NO-dependent vasodilation. Contrary to our hypothesis, concurrent Asc supplementation and arginase inhibition did not result in a greater increase in vasodilation than Asc alone, suggesting that 1) there is redundancy in the underlying cellular mechanisms that these treatments affect (ROS and uncoupled NOS) and/or 2) that Asc supplementation alone maximized the capacity of the cutaneous vessels to vasodilate at this level of hyperthermia.

Our data confirm earlier reports (22) that reflex cutaneous vasodilation is attenuated in subjects with essential hypertension. In the present study, cutaneous vasodilation at the control site was only modestly reduced when expressed as a percentage of maximal vasodilation; however, absolute maximal CVC was significantly attenuated. We chose to represent the data as a %CVCmax to account for variability in capillary density under each laser-Doppler flowmeter, allowing for comparisons between microdialysis treatment sites (4). Others (6) have consistently observed a reduction in maximal CVC using both venous occlusion plethysmography to measure absolute and laser-Doppler flowmetry to measure relative changes in skin.

Table 1. Subject characteristics

<table>
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<tr>
<th>Subject Characteristics</th>
<th>Normotensive</th>
<th>Hypertensive</th>
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<td>6, 3</td>
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<td>Age, yr</td>
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<td>57±4</td>
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<td>Body-mass index, kg/m²</td>
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<td>Total cholesterol, mg/dl</td>
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<td>189±16</td>
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<td>HDL, mg/dl</td>
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<td>LDL, mg/dl</td>
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<td>SBP, mmHg</td>
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</tr>
<tr>
<td>MAP, mmHg</td>
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Values are means ± SE. HDL, high-density lipoprotein; LDL, low-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure; MAP, mean arterial pressure. *Significant difference vs. normotensive subject group (P < 0.05).
blood flow during whole arm local heating. Together, these data suggest that reflex cutaneous vasodilation is significantly attenuated with hypertension with the reduction in maximal CVC, suggesting structural maladaptations of the cutaneous vessels that limit absolute vasodilator capacity. However, we also observed a clear reduction in NO-dependent vasodilation and alterations in vascular signaling that contribute to attenuated reflex cutaneous vasodilation in humans with essential hypertension.

We found that localized Asc administration in humans with essential hypertension augmented reflex cutaneous vasodilation through NO- and non-NO-dependent mechanisms, independent of arginase inhibition. In sites treated with Asc, there was a greater decrease in skin blood flow (Δ%CVCmax) with NOS inhibition during established hyperthermia, and the resulting post-L-NAME plateau (%CVCmax) was increased compared with control sites (Figs. 2 and 3), demonstrating an increase in NO- and non-NO-dependent cutaneous vasodilation, respectively.

A prooxidant vascular environment and resulting decreased NO bioavailability are implicated in the pathogenesis of hypertensive vascular disease (35). NO bioavailability is dictated

Fig. 2. Group means ± SE of CVC as a percentage of maximal response during passive whole body heating. Age-matched normotensive subject responses (left) and hypertensive subject responses (right) are shown. A: nitric oxide synthase (NOS)-inhibited site (○) throughout heating and during the plateau with change in oral temperature (ΔTor) = 0.8°C. B: ascorbate (Asc)-supplemented supplemented site (gray squares) throughout heating and the mean for the stable plateau after NOS inhibition. C: combined Asc + arginase-inhibited site (gray diamonds) throughout heating and the mean for the stable plateau after NOS inhibition. The control site (●) results throughout heating and means for the stable plateau after NOS inhibition are also illustrated in A–C for comparison. Asc and Asc + arginase inhibition augmented cutaneous vasodilation during heating and after within-site NOS inhibition in the hypertensive subject group. *P < 0.05 significant difference vs. the control site within subject groups.
by the balance of NO and oxidant production and clearance. With essential hypertension oxidant production, particularly superoxide, in the vasculature increases predominantly through NAD(P)H oxidase, xanthine oxidase, and uncoupled eNOS (35). In this deleterious cycle, superoxide decreases functional NO by reacting to form peroxinitrite, which then oxidizes the critical NOS cofactor BH4. BH4 deficiency can then ultimately decrease functional NO production through uncoupled NOS (14, 23, 24, 33). In the present study, we chose to directly supplement antioxidants to the cutaneous vasculature to examine a functional link between hypertension-associated increased oxidant stress and reduced NO-dependent vasodilation. However, Asc is a nonspecific antioxidant and may have increased functional NO bioavailability through a variety of mechanisms, including 1) direct scavenging of superoxide and 2) stabilization of the essential NOS cofactor BH4, enabling recoupling of NOS and resulting in functional NO production (15, 32). However, with the present data, we are unable to delineate which of these mechanisms led to the increase in NO-dependent vasodilation.

Other mechanisms, including inadequate L-arginine availability, can also lead to eNOS uncoupling (34). We have recently shown that acute inhibition of arginase augments vasodilation in hypertensive skin (16), suggesting that arginase activity is upregulated in hypertensive cutaneous vasculature. Together, these data suggest that hypertension-associated upregulated arginase activity may cause decreased NO production and may contribute to increased superoxide synthesis through uncoupled eNOS. We hypothesized that concurrent inhibition of arginase and Asc supplementation would result in a greater increase in NO-dependent vasodilation than Asc alone; however, our data indicate that the resulting cutaneous vasodilation from the combined treatments was not additive. One possible explanation for this finding is that there is redundancy in the underlying cellular mechanisms of action of these treatments, where both treatments likely facilitated recoupling of eNOS, thereby increasing functional NO synthesis and decreasing superoxide production. Alternatively, the individual treatments (Asc or Asc + A-I) may have maximized the capacity of the hypertensive cutaneous vessels to vasodilate reaching a “ceiling” effect with this given hyperthermic stimulus (ΔT or = 0.8°C). It is possible, but not likely, that a further increase in cutaneous vasodilation with the combined treatments could be unmasked with a more significant hyperthermic stimulus.

An unexpected finding from the present study was that Asc supplementation alone and combined with arginase inhibition increased the resulting %CVCmax after NOS inhibition in hypertensive skin (Fig. 3). These data suggest that Asc supplementation augmented cutaneous vasodilation in hypertensive vasculature by non-NO-dependent as well as NO-dependent mechanisms. The results may be due to 1) hypertension-associated increased ROS augmenting vasoconstrictor tone or 2) possible nonspecific effects of Asc on the putative neurotransmitter pathways and/or 3) potential synergistic interactions between NO and these pathways involved in reflex cutaneous vasodilation.

In human cutaneous vascular smooth muscle cells, ROS act as signaling molecules to mediate cold-induced vasoconstriction through Rho kinase-dependent mechanisms (2). Because upregulated Rho kinase signaling is implicated in hypertensive vascular pathology (26), it is possible that ROS mediate enhanced vasoconstriction in the absence of a cold stimulus through Rho kinase-dependent mechanisms. Thus providing antioxidants would inhibit the ROS-mediated enhanced vasoconstriction unmasking augmented non-NO-dependent vasodilation.
Asc may also have mediated the increase in non-NO-dependent vasodilation through nonspecific effects on the putative neurotransmitter pathways implicated in reflex vasodilation. In vitro studies suggest that Asc enhances histamine 1 receptor sensitivity (7) and presynaptically increase VIP synthesis and release (5). Alternatively, increased NO itself may have augmented non-NO-dependent vasodilation through potential synergistic effects. In human skin, NO is capable of mediating cutaneous vasodilation synergistically with sympathetic neurotransmitters, resulting in a combined vasodilation that is greater than the sum of the individual contributions (36). The synergistic effect may occur downstream through cross-talk between NO-mediated cGMP-dependent and sympathetic neurotransmitter(s)-mediated cAMP-dependent mechanisms (8, 9), or NO may prejunctionally enhance the release of sympathetic neurotransmitter(s) (12).

The precise effects of Asc on the mechanisms and potential interactions between NO and sympathetic neurotransmitter(s) involved in active cutaneous vasodilation remains elusive. However, our data suggest that augmenting NO bioavailability in a clinical population with compromised endothelial function can also increase non-NO-dependent vasodilation.

In conclusion, NO-dependent reflex cutaneous vasodilation is attenuated in humans with essential hypertension, and antioxidation supplementation alone or combined with arginase inhibition augments cutaneous vasodilation during hyperthermia through NO- and non-NO dependent mechanisms. These data suggest that oxidative stress is increased in hypertensive cutaneous vasculature, which decreases functional NO bioavailability. Finally, in subjects with hypertension, the resulting vasodilation with concurrent Asc supplementation and arginase inhibition was not greater than the vasodilation with Asc alone, suggesting redundancy in underlying cellular mechanisms through uncoupled eNOS and/or that Asc maximized the cutaneous vasodilatory capacity as this level of hyperthermia.

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REFERENCES

28. Munzel T, Daiber A, Ulrich V, Mulsch A. Vascular consequences of endothelial nitric oxide synthase uncoupling for the activity and expres-


