Impaired flow-mediated dilation with age is not explained by L-arginine bioavailability or endothelial asymmetric dimethylarginine protein expression

Phillip E. Gates, Meghan L. Boucher, Annemarie E. Silver, Kevin D. Monahan, and Douglas R. Seals

Department of Integrative Physiology, University of Colorado, Boulder, Colorado

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Gates PE, Boucher ML, Silver AE, Monahan KD, Seals DR. Impaired flow-mediated dilation with age is not explained by L-arginine bioavailability or endothelial asymmetric dimethylarginine protein expression. J Appl Physiol 102: 63–71, 2007. First published August 31, 2006; doi:10.1152/japplphysiol.00660.2006.—Aging is associated with a decline in vascular endothelial function, manifesting in part as impaired flow-mediated arterial dilation (FMD), but the underlying mechanisms are uncertain. Impaired FMD may be mediated in part by a decrease in synthesis of nitric oxide by endothelial nitric oxide synthase, and in clinical populations this has been attributed to competitive inhibition of L-arginine binding sites by asymmetric dimethylarginine (ADMA). If this mechanism is involved in the age-associated decline in FMD, increasing L-arginine concentration may swing the competitive balance in favor of L-arginine binding, restoring nitric oxide synthesis, and enhancing FMD in older humans.

To test this hypothesis, we measured FMD (brachial ultrasound) in 10 younger (21 ± 1 yr) and 12 older healthy men and women (60 ± 2 yr) following infusion of vehicle or vehicle + L-arginine. Baseline FMD in the older subjects was only ~60% of that in the younger subjects (P = 0.002). L-Arginine did not significantly increase FMD in either group despite 23-fold (older) and 19-fold (younger) increases in plasma L-arginine concentrations (P < 0.0001 vs. control). Protein expression (immunofluorescence) in vascular endothelial cells showed that ADMA and the enzyme isoform that controls its degradation, dimethylarginine dimethylaminohydrolase II, were not different in the younger and older subjects. Endothelium-independent vasodilation (sublingual nitroglycerine) was not different between age groups or conditions. We conclude that acutely increasing plasma concentrations of L-arginine do not significantly improve brachial artery FMD in healthy older subjects and thus does not restore the age-associated loss of FMD. Together with the finding that endothelial cell ADMA protein expression was not increased in older adults, these findings suggest that competitive inhibition of L-arginine binding sites on endothelial nitric oxide synthase by ADMA is not an important mechanism contributing to impaired conduit artery endothelium-dependent dilation with aging in healthy humans.

dimethylarginine dimethylaminohydrolase II; L-arginine paradox

Several mechanisms are thought to contribute to impaired vascular endothelial function, but reduced bioavailability of nitric oxide (NO) has emerged as a primary factor in older adults (5, 35, 36). NO is synthesized in endothelial cells from the amino acid L-arginine by the enzyme endothelial NO synthase (eNOS). NO rapidly diffuses to vascular smooth muscle cells where it stimulates soluble guanylate cyclase, inducing cGMP-dependent vasodilation. Thus reduced NO bioavailability following physiological stimulation of arterial endothelial cells manifests as impaired arterial dilation (40).

A number of mechanisms may contribute to a reduced NO bioavailability (5, 30, 40), but those underlying age-associated impairments in vascular endothelial function are incompletely understood. One possibility is reduced cellular bioavailability of the NO precursor L-arginine. Acute infusion of L-arginine improves vascular endothelium-dependent dilation in several clinical populations (4, 13, 31). This occurs despite the fact that endothelial concentrations of L-arginine are thought to be greater than those that would be rate limiting for eNOS, giving rise to the notion of the “L-arginine paradox” (11). Increased endothelial production and/or expression of asymmetric dimethylarginine (ADMA) provides a compelling explanation of this paradox. ADMA reduces NO synthesis by competing with L-arginine for binding sites on eNOS, thereby inhibiting eNOS activity (11). If reduced vascular function results from elevated endothelial ADMA, presumably L-arginine administration would swing the competitive balance in favor of L-arginine binding, restoring NO synthesis and enhancing vascular endothelium-dependent dilation.

An important component of this cell-signaling pathway is the enzyme dimethylarginine dimethylaminohydrolase II (DDAH II), the predominant DDAH isoform in endothelial cells (11). DDAH II regulates the endothelial concentration of ADMA by metabolizing it to form citrulline and methylamines (11). However, reactive oxygen species, which are typically elevated in patients with CVD, inhibit DDAH II activity with a corresponding accumulation of ADMA (23). Because free radical bioavailability is elevated in older adults (14, 15, 36), it is plausible that this mechanism is partly responsible for the age-associated decline in vascular endothelial function. If so, acute administration of L-arginine should improve vascular endothelium-dependent dilation in older adults. Thus in the present study we hypothesized that acute intravenous infusion of L-arginine would improve flow-mediated dilation (FMD), an endothelium-dependent, NO-associated dilation stimulated by an increase in physiological shear stress (6), in healthy older but not younger adult humans. To gain mechanistic insight into

Address for reprint requests and other correspondence: D. R. Seals, Dept. of Integrative Physiology, 354 UCB, Univ. of Colorado, Boulder, CO 80309 (e-mail: seals@colorado.edu).

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the molecular mechanisms involved, we also measured markers of oxidative stress/antioxidant defenses and protein expression of ADMA and DDAH II in vascular endothelial cells.

In addition, because endothelium-derived molecules are involved in the regulation of arterial stiffness in animal models and because endothelium-denuded rat aorta exhibit increased arterial stiffness (43), we reasoned that restoring vascular endothelial function may also reverse some of the age-associated increase in arterial stiffness. Age-associated arterial stiffness is a key risk factor for systolic hypertension, unfavorable changes in ventricular-vascular coupling, and CVD, yet few studies have determined whether improvements in vascular endothelial function in conduit arteries have corresponding effects on the large elastic arteries. We therefore measured the distensibility of the carotid artery while plasma L-arginine concentrations were either normal or elevated.

METHODS

Subjects

Ten younger (5 men, 5 women; 18–26 yr) and 12 older healthy, sedentary men and women (6 men, 6 women; 52–71 yr) participated in the study. Screening measurements, baseline subject characteristics, and 3-day dietary analysis were obtained during three preliminary visits as described previously (19, 28). All subjects were normotensive [blood pressure < 140/90 mmHg (systolic/diastolic)], non-smokers, nonobese [body mass index (BMI) < 30 kg/m²], and free of cardiovascular disease as assessed by medical history, physical examination, blood chemistry, and resting and exercise electrocardiogram (older subjects only). Participants were not taking medications and were asked to abstain from taking dietary supplements 4 wk before the experimental sessions. The Human Research Committee of the University of Colorado at Boulder approved all procedures, and written informed consent was obtained from all participants. The investigation conformed to the principles outlined in the Declaration of Helsinki.

Experimental Procedures

Two experimental sessions were conducted on separate days in a quiet, semidarkened, temperature-controlled room with subjects in a fasted state (12 h). Subjects were instrumented and then rested supine for 20 min before initiation of the protocol. Either vehicle only (saline) or vehicle + L-arginine (Merck Biosciences; 30 g at 1 g/min) were infused through an intravenous catheter over a 30-min period. Infusions and all analyses were undertaken in a double-blind, randomized fashion. Baseline left brachial artery diameter and blood flow measurements were made 30 min after completion of the infusion, and the FMD procedure was initiated at 35 min postinfusion. The 35-min delay was necessary because L-arginine infusion is associated with a small reduction in blood pressure and release of insulin. Previous observations in our laboratory (unpublished data) showed that blood pressure and plasma insulin return to baseline concentrations after ~30 min. Following the FMD procedure, images of the left carotid artery were obtained with a separate ultrasound transducer over a 30-min period. After satisfactory carotid artery images had been recorded, the brachial artery image was displayed, and sublingual nitroglycerin (0.4 mg) was administered (~55 min postinfusion). Right brachial artery blood pressure (Dinamap Pro-110; Critikon, Tampa, FL) was measured before the infusion and at 0, 20, and 40 min following the infusion. Blood samples were drawn through the intravenous catheter before the infusion and at 40 min postinfusion.

Measurements

Brachial artery FMD and vascular endothelium-independent dilation. Brachial artery endothelial function was assessed noninvasively by using reactive hyperemia and vascular endothelium-independent dilation (6) following established guidelines (12), as described previously by our laboratory (14–16). Briefly, the subject lay supine with the left arm supported at heart level, abducted, supinated, and fixed in position by using a positioning pillow (Versaform, Sammons Preston, Bolingbrook, IL) and metal frame. A forearm blood-pressure cuff (E20 and AG101 rapid cuff inflator, Hokanson, Bellevue, WA) was placed at least 2 cm below the antecubital crease. An adjustable-frequency transducer set at 15 MHz (Power Vision 6000, Toshiba, Tochigi, Japan) was used to obtain a two-dimensional ultrasound image of the brachial artery at least 3 cm above the antecubital crease. The transducer was then clamped (Flexlarm, Islandia, NY) in place to prevent movement. To facilitate accurate instrumentation and to ensure location of the same arterial segment for each visit, we measured anatomic landmarks and instrument positions, a digital photograph was taken, and the ultrasound image was printed and used for reference during the subsequent visit.

Baseline brachial diameter and blood flow were recorded for at least 60 cycles. To stimulate FMD, reactive hyperemia was induced by rapidly inflating the forearm blood-pressure cuff to 80 mmHg and increasing the pressure to 250 mmHg within 5 s to occlude blood flow (radial pulse was checked). Following 5 min of occlusion, the cuff was rapidly deflated. Blood flow was recorded during the first 12 arterial pulses following cuff deflation to establish the magnitude of the hyperemic response, after which the brachial artery was imaged continuously through 2 min postocclusion. To assess vascular endothelium-independent dilation, sublingual nitroglycerin (0.4 mg) was administered, and brachial artery images were recorded over the subsequent 10 min.

All ultrasound brachial images were recorded and analyzed by the same investigator with the use of purpose-written software (Vascular Imaging/Brachial Analyzer, Medical Imaging Applications, Coralville, IA). Blood flow-Doppler spectral envelopes were measured manually by using software integral to the ultrasound machine, and blood flow was calculated as \( (V - \pi r^2) \times (6 \times 10^{-1}) \), where \( V \) is mean blood flow velocity (cm/s), \( r \) is the radius of the brachial artery (cm), and \( 6 \times 10^{-1} \) converts blood flow to milliliters per minute. Although blood flow was recorded off-angle (to optimize the B-mode image), angle correction (always 68°) was used, and this error was systematic across trials. This angle gave good alignment with blood flow, and angles of 50–70° have been shown to be accurate compared with known flow rates in mechanical models (34).

Carotid artery distensibility. Images of the common carotid artery were obtained by using a 7.5-MHz ultrasound transducer as described previously (19). Images were recorded to videotape and later grabbed at ~15 frames/s to a personal computer. At least 30 cycles were analyzed by using dedicated software (Vascular Image/Carotid Analyzer, Medical Imaging Applications).

Blood viscosity and shear stress. Whole blood viscosity (dyn·s/cm², where 1 dyn = 10⁻⁵ N) was obtained from a sample drawn immediately following the FMD procedure. Viscosity was measured at incremental shear rates from 0.3 to 60 rpm at 37°C by using a cone and plate viscometer (DV-1+; Brookfield Engineering, Stoughton, MA). Shear stress (dyn/cm²) was calculated as \( \mu \cdot V/D \), where \( \mu \) is blood viscosity at 60 rpm, \( D \) is artery diameter (cm), and \( V \) is in centimeters per second.

Blood measurements. In addition to standard baseline metabolic screening variables, plasma samples were analyzed for concentrations of L-arginine, insulin, oxidized low-density lipoproteins (LDL), and superoxide dismutase by using conventional assays.

Vascular endothelial cell protein expression. The procedures used for collection of venous endothelial cells and measurement of vascular endothelial cell protein expression (VECPE) have been described in
detail (9, 10, 18) and more recently by our laboratory (14). Following instrumentation, but prior to any experimental procedures, endothelial cells were collected from an antecubital vein by using sterile J-wires briefly advanced and retracted through the right arm catheter. The wires were transferred to a dissociation buffer solution, and endothelial cells were recovered by washing and centrifugation. Cells were fixed with 3.7% formaldehyde and plated on poly-l-lysine-coated slides (Sigma Chemical, St. Louis, MO).

For immunofluorescence staining, cells were rehydrated with PBS and rendered permeable by using 0.1% Triton X-100 (Alfa Aesar, Ward Hill, MA). After blocking nonspecific binding sites with 5% donkey serum (Jackson Immunoresearch, West Grove, PA), cells were incubated with a primary antibody to ADMA (UpState, Charlottesville, VA) or DDAH II (Abcam, Cambridge, MA) followed by an anti-rabbit (Research Diagnostics, Concord, MA) or anti-goat (Jackson Immunoresearch) secondary CY3 antibody, respectively. After blocking nonspecific binding sites with 5% horse serum, cells were fixed with 3.7% formaldehyde and plated on poly-L-lysine-coated slides (Sigma Chemical, St. Louis, MO). Endothelial cells were identified by the presence of von Willebrand factor staining, and nuclear integrity was confirmed by using 4′,6′-diamidino-2-phenylindole hydrochloride staining. Once endothelial cells with intact nuclei were identified, images were captured and analyzed by using Metamorph Software (Universal Imaging, Downingtown, PA) to quantify the intensity of CY3 staining, hence quantifying VECPE. Values are reported as ratios of VECPE to human umbilical vein endothelial cell (HUVEC; control cells) VECPE. Reporting ratios minimizes the possible confound of differences in intensity of staining among different staining sessions.

Statistics

Statistical analyses were conducted by using SPSS for Macintosh version 11.0 (SPSS, Chicago, IL). One-tailed t-tests were used to compare baseline subject characteristics, dietary composition, and endothelial cell protein expression between younger and older groups. Two-way ANOVA was used to analyze carotid artery distensibility and brachial artery change in diameter as a percentage of baseline, relative to blood flow and relative to shear stress. Analysis of covariance was used to covary for differences in baseline brachial artery diameter. Repeated-measures ANOVA was used to analyze brachial artery blood flow, brachial artery diameter, and oxidative stress markers from pre- to postinfusion and to analyze blood pressure measurements. Condition (L-arginine or control) and age group were entered as factors in all ANOVA models. Bivariate relations were determined by using Pearson zero-order correlation coefficients. Significance was set at \( P < 0.05 \).

RESULTS

Subject Characteristics and Dietary Composition

Subject characteristics are presented in Table 1. The older subjects had higher body fat and BMI and lower maximal aerobic capacity (all \( P < 0.05 \)). Diastolic arterial blood pressure was higher in the older subjects, but all subjects were normotensive. The fasting metabolic blood profile is shown in Table 2. Plasma low-density lipoprotein cholesterol was \( \sim 30\% \) higher in the older subjects (\( P < 0.05 \)) but within a normal adult range. Plasma total and high-density lipoprotein cholesterol and other metabolic markers were not significantly different between groups. Caloric intake from fat was lower in the older compared with the younger subjects (Table 3; both \( P < 0.05 \), but carbohydrate and protein consumption was not different. Older subjects consumed more beta-carotene, but intake of other antioxidant nutrients was similar between groups (Table 3).

During the FMD procedure, plasma arginine was \( \sim 19 \) and \( \sim 23\%-fold higher, respectively, following L-arginine infusion compared with baseline concentrations in the younger and older groups (both \( P < 0.05 \), Fig. 1A). L-Arginine infusion was associated with a temporary increase in plasma insulin concentrations in both groups that resolved before measurement of FMD (~40 min after infusion) in both the younger and the older subjects (Fig. 1B). L-Arginine also was associated with a temporary decrease in arterial blood pressure, particularly in the younger subjects (Fig. 2, A–C). However, blood pressure had returned to baseline levels before the FMD measurements and did not differ from levels during the control FMD condition. Heart rate was not affected by L-arginine infusion in either group (Fig. 2D).

Cardiovascular Response to L-Arginine Infusion

The FMD vascular occlusion procedure induced an approximately fivefold increase in brachial blood flow (Fig. 3A). The hyperemic increase in shear stress (Fig. 3B) was similar between conditions and groups, indicating that the physiological stimulus for FMD was consistent between younger and older subjects and trials. The hyperemic shear stress stimulus increased brachial artery diameter in both the older and the younger subjects (both \( P < 0.0001 \); Fig. 3C), demonstrating that the physiological stimulus was sufficient to induce FMD in both groups. FMD in the older subjects was \( \sim 60\% \) of that in the younger subjects under baseline control conditions (\( P = 0.002 \); Fig. 3D). Following L-arginine infusion, peak brachial artery diameter during FMD was not significantly different from the control condition in either group. Therefore, FMD was not significantly improved in the older adults and, thus, was not restored to levels seen in the younger subjects (Fig. 3, C and D). The same was true when the change in brachial diameter was expressed relative to the change in the shear stress stimulus (Fig. 3E). Statistical outcomes were not different when the change in brachial artery diameter in response to FMD was covaried for the difference (~0.02 cm) in baseline brachial artery diameter between groups.

Endothelium-independent vasodilation in response to sublingual nitroglycerin was not different between younger and older subjects and was similar between control and L-arginine conditions (Fig. 3F). Carotid artery distensibility in the older

### Table 1. General subject characteristics at baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>Younger (( n = 10 ))</th>
<th>Older (( n = 13 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>21±1</td>
<td>60±2*</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171±3</td>
<td>171±3</td>
</tr>
<tr>
<td>Body mass, kg</td>
<td>65±3</td>
<td>74±4</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22±1</td>
<td>25±1*</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>23±3</td>
<td>30±2*</td>
</tr>
<tr>
<td>Waist:hip ratio</td>
<td>0.78±0.02</td>
<td>0.84±0.03</td>
</tr>
<tr>
<td>Resting heart rate, beats/min</td>
<td>59±3</td>
<td>58±2</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>105±2</td>
<td>114±5</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>60±2</td>
<td>70±3*</td>
</tr>
<tr>
<td>( V˙O_{2\text{max}} ), ml·kg⁻¹·min⁻¹</td>
<td>47±2</td>
<td>29±1*</td>
</tr>
</tbody>
</table>

Data are means ± SE. SBP, systolic arterial blood pressure; DBP, diastolic arterial blood pressure; \( V˙O_{2\text{max}} \), maximal O₂ consumption. *\( P < 0.05 \) vs. younger group.
Table 2. Subject hematology and baseline fasting metabolic profile

<table>
<thead>
<tr>
<th>Variable</th>
<th>Younger</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol mmol/l</td>
<td>4.36±0.29</td>
<td>5.01±0.26</td>
</tr>
<tr>
<td>mg/dl</td>
<td>169±11</td>
<td>194±10</td>
</tr>
<tr>
<td>Low-density lipoprotein mmol/l</td>
<td>2.44±0.27</td>
<td>3.20±0.12*</td>
</tr>
<tr>
<td>mg/dl</td>
<td>94±11</td>
<td>124±5*</td>
</tr>
<tr>
<td>High-density lipoprotein mmol/l</td>
<td>1.38±0.07</td>
<td>1.41±0.07</td>
</tr>
<tr>
<td>mg/dl</td>
<td>53±3</td>
<td>54±6</td>
</tr>
<tr>
<td>Triglycerides mmol/l</td>
<td>1.20±0.18</td>
<td>1.32±0.17</td>
</tr>
<tr>
<td>mg/dl</td>
<td>106±16</td>
<td>116±15</td>
</tr>
<tr>
<td>Plasma insulin pmol/l</td>
<td>49±6</td>
<td>33±9</td>
</tr>
<tr>
<td>μU/ml</td>
<td>7.1±2.8</td>
<td>4.7±1.3</td>
</tr>
<tr>
<td>Plasma glucagon, ng/l</td>
<td>59±7</td>
<td>55±6</td>
</tr>
<tr>
<td>Plasma glucose mmol/l</td>
<td>5.0±0.1</td>
<td>5.2±0.3</td>
</tr>
<tr>
<td>mg/dl</td>
<td>90±2</td>
<td>95±5</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>46±2</td>
<td>43±1</td>
</tr>
<tr>
<td>Blood viscosity, dyn/cm²</td>
<td>0.035±0.002</td>
<td>0.036±0.002</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>0.039±0.002</td>
<td>0.038±0.002</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. younger group.

subjects at baseline was ~50% of that observed in the younger controls (P < 0.001; Fig. 4). L-Arginine infusion did not change carotid distensibility in either older or younger subjects.

Plasma Oxidative Stress and Antioxidant Markers

Plasma oxidized LDL was ~40% higher (P = 0.002, Fig. 5A), whereas plasma superoxide dismutase was not different (Fig. 5B), in the older compared with the younger subjects at baseline. Plasma concentrations were not consistently influenced by infusion of L-arginine. In the pooled subjects, FMD (%baseline) during both conditions was inversely related to baseline plasma oxidized LDL (control r = −0.53, P = 0.02; L-arginine r = −0.49, P = 0.04), and this relation tended to be stronger in men (r = −0.72, P = 0.02) than in women (r = −0.53, P = 0.06). Baseline plasma superoxide dismutase did not correlate with baseline FMD or the change in FMD in response to L-arginine infusion.

Table 3. Subject energy intake, dietary composition, and nutrition at baseline

<table>
<thead>
<tr>
<th>Variable</th>
<th>Younger</th>
<th>Older</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total energy intake kJ</td>
<td>12,438±1,396</td>
<td>8,721±573*</td>
</tr>
<tr>
<td>cal</td>
<td>2,971±333</td>
<td>1,953±136*</td>
</tr>
<tr>
<td>Carbohydrate, g</td>
<td>330±31</td>
<td>261±18</td>
</tr>
<tr>
<td>Fat, g</td>
<td>128±17</td>
<td>82±8*</td>
</tr>
<tr>
<td>Protein, g</td>
<td>118±17</td>
<td>84±8</td>
</tr>
<tr>
<td>Vitamin A, IU</td>
<td>12,360±2,121</td>
<td>16,525±2,324</td>
</tr>
<tr>
<td>Vitamins B1 and B2, mg</td>
<td>2.5±0.4</td>
<td>1.7±0.1</td>
</tr>
<tr>
<td>Vitamin B6, mg</td>
<td>3.0±0.5</td>
<td>2.2±0.2</td>
</tr>
<tr>
<td>Vitamin C, mg</td>
<td>157±30</td>
<td>185±23</td>
</tr>
<tr>
<td>Vitamin E, IU</td>
<td>383±65</td>
<td>301±38</td>
</tr>
<tr>
<td>Beta-carotene, μg</td>
<td>5,500±1,031</td>
<td>8,997±1,376*</td>
</tr>
<tr>
<td>Folate, μg</td>
<td>664±106</td>
<td>523±40</td>
</tr>
<tr>
<td>Niacin, mg</td>
<td>39±8</td>
<td>25±3</td>
</tr>
<tr>
<td>Potassium, mg</td>
<td>4,167±684</td>
<td>3,099±246</td>
</tr>
<tr>
<td>Sodium, mg</td>
<td>4,749±854</td>
<td>3,194±319</td>
</tr>
<tr>
<td>Zinc, mg</td>
<td>23±7</td>
<td>16±2.9</td>
</tr>
</tbody>
</table>

Data are means ± SE. *P < 0.05 vs. younger group.

DISCUSSION

In the present study we hypothesized that the impaired peripheral conduit artery endothelium-dependent dilation in older subjects would be at least partially restored by increasing plasma concentrations of the NO precursor L-arginine. Contrary to our hypothesis, we found that a >20-fold increase in plasma L-arginine concentration did not significantly improve brachial artery FMD in older adults. This lack of effect of L-arginine was associated with an absence of age group differences in VECPE of ADMA, which was, in turn, associated...
with similar endothelial DDAH II protein levels in the younger and older subjects. Finally, carotid artery distensibility was unaltered in the presence of higher plasma L-arginine concentrations. Taken together, these findings are consistent with the concept that the age-associated decreases in peripheral conduit artery endothelium-dependent dilation and large elastic artery compliance are not primarily mediated by inhibition of L-arginine bioactivity via accumulation of endothelial cell ADMA. The absence of an age-associated increase in endothelial cell ADMA concentration is perhaps, in turn, explained by preserved concentrations of endothelial DDAH II.

Our findings are comparable with data from a study of medically managed patients with stable coronary artery disease (1). In this study oral L-arginine administration almost doubled plasma L-arginine concentration, but there was no improvement in FMD. One possibility is that lipid-lowering therapy in these patients resulted in a cardiovascular risk phenotype more comparable to that in our healthy population and that L-arginine supplementation is ineffective in individuals with this phenotype. However, our data are inconsistent with a small but significant increase in FMD with 14 days of oral supplementation of L-arginine in adults over 70 yr of age (3). This may reflect an effect of L-arginine in adults older than those studied in the present investigation and/or the influence of more prolonged L-arginine administration. However, other factors such as the effects of circulating insulin concentrations also could explain these differences. Plasma insulin concentrations are elevated following L-arginine administration and may account in part for improvements in FMD reported following L-arginine supplementation (20, 24). In contrast to this earlier study (3), which did not report insulin concentrations, in the present investigation we timed the FMD measurement to isolate the effect of L-arginine from that of insulin. Our data may therefore reflect an effect of L-arginine that is independent of an effect of insulin.

Reduced bioactivity of DDAH II and associated accumulation of ADMA have been postulated to be responsible for reduced FMD in clinical populations and to explain the L-arginine paradox (11). In our older subjects, VECPE of ADMA and DDAH II was not significantly different in the younger and older subjects and did not correlate with either baseline FMD or the FMD response to L-arginine. Acutely increasing plasma L-arginine concentration may not have improved FMD because there was no baseline imbalance between L-arginine and ADMA. Therefore, the results of our study suggest that augmented endothelial ADMA-linked reductions in L-arginine bioavailability are not mechanistically involved in the age-associated reduction in peripheral conduit artery endothelium-dependent dilation in healthy adults. Although oxidized LDL was elevated in our healthy older subjects, this may not have been sufficient [i.e., compared with clinical disease states (11)] to disrupt ADMA metabolism by DDAH II in endothelial cells in vivo.

In the present study, within the pooled subject sample, baseline plasma oxidized LDL concentration was negatively associated with FMD. This relation was apparent in both sexes but tended to be stronger in men than in women. Higher concentrations of oxidized LDL indicate that the older subjects in this study were in a state of oxidative stress relative to the younger controls, as reported previously (36). Oxidative stress is associated with degradation and reduced half-life of NO (22) and is thought to be one of the primary mechanisms by which vascular endothelial function is impaired in older adults (36, 38). Consistent with this, we and others have shown that infusion of the potent antioxidant ascorbic acid improves endothelium-dependent dilation in sedentary older but not younger subjects (15, 36, 38). It is possible that the L-arginine infusion did augment NO synthesis but failed to increase NO bioavailability in vascular smooth muscle cells (and, thus, FMD) in the older adults because of increased free radical formation.
scavenging of NO. However, this would seem unlikely given that baseline oxidative stress in patients with CVD and angina pectoris is greater than in healthy older adults, yet acute L-arginine infusion nevertheless improves endothelium-dependent dilation in these patients (31).

Improved coronary artery dilation in response to pharmacological stimulation (acetylcholine) has been reported following L-arginine infusion in older patients (7). However, in contrast to our healthy sample of older adults, this subject cohort included smokers and men and women with blood pressure up to 150/95 mmHg (systolic/diastolic). Compared with normotensive subjects, hypertensive subjects have worse endothelial function and may be more responsive to L-arginine infusion (37). Finally, Taddei et al. (37) showed that forearm resistance vessel dilation was partially restored after L-arginine infusion in normotensive older adults at the two highest concentrations of acetylcholine. In this study, plasma concentrations of L-arginine and insulin were not reported. Comparison with our results also is made difficult because large conduit artery FMD and forearm resistance vessel dilation in response to acetylcholine do not correlate and, therefore, are believed to reflect different properties of the vascular endothelium (17).

Recent data provide a compelling mechanistic explanation for the lack of effect of L-arginine infusion in our subject cohort. Consistent with our findings in humans, addition of extracellular L-arginine to aortic rings from older rats failed to restore vascular endothelial function (42). However, when the enzyme arginase was inhibited or knocked down, eNOS signaling and L-arginine responsiveness were restored, suggesting

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**Fig. 3.** The FMD procedure resulted in a ~5-fold increase in brachial artery blood flow (A). Brachial artery shear stress during reactive hyperemia was similar between groups and trials (B). Baseline brachial artery FMD was significantly lower in older compared with younger subjects (C–E). Infusion of L-arginine did not restore endothelial function in the older subjects to levels seen in younger subjects, regardless of the expression of FMD (C–E). The vasodilatory response to nitroglycerin was not different between younger and older subjects (F). *Significantly greater than corresponding baseline measurement ($P < 0.01$); †significantly lower than equivalent measurement in younger subjects ($P < 0.01$).

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**Fig. 4.** Carotid artery distensibility measured at isobaric blood pressure was reduced in the older subjects and was not improved with L-arginine infusion. *Significantly lower than corresponding measurement in the younger group ($P < 0.0001$).
a modulatory role of arginase in the bioavailability of L-arginine (42). These findings imply that L-arginine transport into vascular endothelial cells was intact in the older rats but was ineffective in stimulating increases in NO in the presence of elevated concentrations of arginase. In our experiment, it seems reasonable to speculate that intravenous L-arginine infusion may not have improved FMD because of modulation of L-arginine bioavailability by arginase. That is, arginase may be a more important contributor than ADMA to impaired vascular endothelial function in healthy older adults. Further experimentation in humans is warranted to determine the role of arginase in accelerated vascular aging and the efficacy of combined L-arginine supplementation and arginase inhibition.

Although plasma L-arginine concentrations were increased after infusion, it is possible that L-arginine was not transported into the endothelial cell by the cationic amino acid transporter 1 protein. In addition, two pools of L-arginine have been identified in vascular endothelial cells (8), and these may have been fully functional in our subjects so that L-arginine transport into the endothelial cell and/or augmentation of the L-arginine pool/s occurs if L-arginine bioavailability is a limiting factor for NO synthesis. It is not possible in humans to determine acute changes in arterial endothelial cell L-arginine concentration with L-arginine infusion. Nevertheless, on the basis of data from clinical populations using an experimental approach similar to the current study (2, 39), it seems reasonable to suggest that if L-arginine bioavailability were a primary mechanism in the age-related decline in vascular endothelial function, we would have seen an improvement in FMD consistent with that observed in patients with cardiovascular disease and/or risk factors (2, 39).

We wish to emphasize several experimental considerations in the present study. First, the L-arginine infusion was associated with an increase in circulating insulin and a corresponding small reduction in mean arterial pressure, both of which returned to baseline levels before the FMD procedure. This “correction” of blood pressure may have been caused by a baroreflex-mediated increase in sympathetic nerve activity and vasoconstriction, masking an effect of L-arginine on FMD. However, the absence of any increase in heart rate at the time of FMD measurement indicates that no baroreflex activation was present at that time. We also believe that this is an unlikely alternative explanation for our findings given that L-arginine restores endothelium-dependent dilation in a variety of groups with risk factors for CVD or with clinical CVD despite any such theoretical blood pressure-related vasoconstrictor influence. Similarly, the alteration and restoration of baseline blood pressure and insulin concentration may be indicative of tachyphylaxis of the endothelium in response to the L-arginine infusion. However, we are unaware of any evidence of such an effect of L-arginine, and this would be inconsistent with studies in healthy (3) and clinical populations (4, 13, 31) where prolonged L-arginine supplementation has improved endothelial function.

We used hyperemic flow as a stimulus for brachial artery dilatation in accordance with guidelines that were current at the initiation of the study (12). Recent debate has focused on the best method to characterize the hyperemic stimulus more precisely (29, 32, 33), and these should be considered in future investigations.
We hypothesized, but did not find, that infusion of L-arginine would restore FMD in the older adults to levels seen in younger subjects. One possibility is that with a greater number of older subjects the slightly higher mean FMD after L-arginine infusion in that group might reach statistical significance. However, the cohort size was adequate to establish highly significant age-group differences in FMD at baseline, and even after L-arginine infusion, FMD remained significantly lower in the older vs. younger subjects. In addition, a standard power analysis based on the small effect of L-arginine in the present study revealed that 172 and 294 subjects, respectively, would be needed for 60% and 80% probabilities of detecting a treatment difference on FMD between the two groups. Taken together, these observations suggest that L-arginine administration does not have a significant effect on vascular endothelial function in healthy older adults. Moreover, any such small influence of L-arginine on FMD is unlikely to be of clinical significance in the context of restoring vascular endothelial in older adults.

In the present investigation, we studied a combined group of men and women, and the mechanisms modulating vascular endothelial function may be different between sexes. However, there was no increase in FMD in either older men or postmenopausal (estrogen deficient) women following plasma L-arginine infusion. Thus the lack of effect of L-arginine was observed in both sexes.

Finally, we recognize that our measurements of endothelial protein expression were performed on cells obtained from veins as opposed to arteries as described previously (9). Clearly, cell samples from arteries would have been optimal for the FMD response reflects endothelium-dependent dilation of a conduit artery. However, because L-arginine infusion did not require an arterial catheter, the potential benefit of obtaining arterial cells did not justify the additional risk, particularly to our older subjects. Most importantly, although absolute levels of endothelial protein expression may differ in cells obtained from arteries vs. veins, we (unpublished observations) and others (9, 21) have established that venous cells consistently depict interindividual or intergroup differences in protein expression.

In conclusion, in the present investigation, we isolated the effect of increased plasma L-arginine concentration from an acute increase in insulin concentration during physiologically stimulated vascular endothelium-mediated dilation. We conclude that increasing the bioavailability of L-arginine does not significantly improve brachial artery FMD in healthy older subjects and, thus, does not restore the age-associated loss of FMD. Together with the finding that endothelial cell ADMA protein expression is not increased in older adults, these findings suggest that competitive inhibition of L-arginine binding sites on eNOS by ADMA is not an important mechanism contributing to impaired conduit artery endothelium-dependent dilation with aging in healthy humans.

GRANTS

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