Impaired L-Arginine Transport and Endothelial Function in Hypertensive and Genetically Predisposed Normotensive Subjects

Markus P. Schlaich, MD; Melinda M. Parnell, PhD; Belinda A. Ahlers, PhD; Samara Finch, BSc; Tanneale Marshall, BSc; Wei-Zheng Zhang, PhD; David M. Kaye, MD, PhD

Background—Impaired endothelium-dependent NO-mediated vasodilation is a key feature of essential hypertension and may precede the increase in blood pressure. We investigated whether transport of the NO precursor L-arginine is related to decreased endothelial function.

Methods and Results—Radiotracer kinetics ([3H]L-arginine) were used to measure forearm and peripheral blood mononuclear cell arginine uptake in hypertensive subjects (n=12) and in 2 groups of healthy volunteers with (n=15) and without (n=15) a family history of hypertension. In conjunction, forearm blood flow responses to acetylcholine and sodium nitroprusside were measured before and after a supplemental intra-arterial infusion of L-arginine. In vivo and in vitro measures of L-arginine transport were substantially reduced in the essential hypertension and positive family history groups compared with the negative family history group; however, no difference was detected in peripheral blood mononuclear cell mRNA or protein expression levels for the cationic amino acid transporter CAT-1. Plasma concentrations of L-arginine and N\textsubscript{G},N\textsubscript{G}-dimethylarginine (ADMA) did not differ between groups. L-Arginine supplementation improved the response to acetylcholine only in subjects with essential hypertension and positive family history.

Conclusions—Similar to their hypertensive counterparts, normotensive individuals at high risk for the development of hypertension are characterized by impaired L-arginine transport, which may represent the link between a defective L-arginine/NO pathway and the onset of essential hypertension. The observed transport defect is not due to apparent alterations in CAT-1 expression or elevated endogenous ADMA.

Key Words: hypertension ■ endothelium ■ nitric oxide ■ amino acids ■ arginine

The endothelium plays a pivotal role in the regulation of vascular tone.\textsuperscript{1} Endothelium-derived NO is generated from its precursor L-arginine via the catalytic action of endothelial NO synthase (eNOS). Formation of NO in endothelial cells depends on an adequate and continuing supply of L-arginine and several cofactors.\textsuperscript{2,3}

Endothelium-dependent NO formation has been demonstrated repeatedly to be reduced in patients with essential hypertension compared with normotensive control subjects.\textsuperscript{4,5} Furthermore, endothelium-dependent vasodilation is impaired in normotensive subjects with a family history of essential hypertension, suggesting that impaired endothelial function may not occur simply as a consequence of increased blood pressure but may rather be a cause of the condition.\textsuperscript{6,7} Interestingly, supplementation of L-arginine has been demonstrated to improve endothelium-dependent vasodilation to acetylcholine in genetically predisposed subjects. Increased availability of L-arginine has also been shown to augment endothelial function in other cardiovascular diseases, including hypercholesterolemia,\textsuperscript{2,8} coronary heart disease,\textsuperscript{9,10} and congestive heart failure.\textsuperscript{11} Using newly developed techniques, we have recently identified an impairment of L-arginine transport in congestive heart failure patients with endothelial dysfunction, providing a potential mechanistic explanation for their markedly reduced endothelial properties.\textsuperscript{12}

In the present study we sought to test the hypothesis that impaired endothelium-dependent vasodilation in essential hypertension is due to impaired transport of the NO precursor L-arginine across the cell membrane, thereby potentially leading to a relative intracellular substrate deficiency for eNOS and reduced NO formation. We investigated young hypertensive subjects and normotensive subjects with and without a genetic background of essential hypertension to further address the possibility that a genetically linked defect of the L-arginine/NO pathway may be involved in the development of essential hypertension.

Methods

Subjects
We studied 30 young (aged 18 to 35 years), male, healthy, normotensive subjects with (positive family history group; n=15) and without (negative family history group; n=15) a family history of
essential hypertension and 12 untreated young, male, hypertensive subjects (essential hypertension group) who also had a family history of essential hypertension. Hypertension was defined as blood pressure >140 mm Hg systolic or >90 mm Hg diastolic at repeated sphygmonanometric measurements. Participants were classified as normotensive if blood pressure was <130 mm Hg systolic and <80 mm Hg diastolic. Subjects presenting with a systolic blood pressure between 130 and 139 mm Hg and/or a diastolic blood pressure between 80 and 89 mm Hg were classified as high normal or borderline hypertensive subjects and excluded from the study. The casual blood pressure readings on the first 2 screening occasions were confirmed by intra-arterial blood pressure measurement during the forearm blood flow study.

None of the participants had diabetes or hypercholesterolemia or was on any concurrent medication or dietary supplementation (including antioxidants) or smoked. Written informed consent was obtained from all participants. The Alfred Hospital Ethics Review Committee approved the study protocol.

In Vivo [3H]-L-Arginine Kinetics
Forearm [3H]-arginine kinetics and assessment of endothelial function were performed on the same day. The study commenced at 9 AM after an overnight fasting period of at least 12 hours. A 3F cannula was inserted into the brachial artery for the infusion of radiolabeled L-arginine, as indicated below. A 5F cannula was inserted percutaneously into a deep antecubital forearm vein for venous blood sampling, as previously described.1 After a stabilization period of 20 minutes, the resting forearm blood flow was measured, and the intra-arterial infusion of radiolabeled arginine commenced. After an initial priming bolus of 1 μCi of [4,5-3H]-arginine (ICN Pharmaceuticals; specific activity 98 to 106 Ci/mmol) in 2 mL of 0.9% NaCl, a continuous intra-arterial infusion of 100 nCi/min of [4,5-3H]-arginine commenced. Deep venous blood samples were drawn after a steady state was reached, as demonstrated in previous studies,12 and immediately transferred to ice-chilled tubes containing EGTA and stored on ice until the completion of the study.

Blood samples were centrifuged at 4°C, and plasma was stored at −70°C. The plasma concentration of [3H]-arginine was as previously described,14 and an index of arginine uptake in the forearm, the rate of [3H]-arginine uptake, was calculated as described previously.12

[3H]-L-Arginine Transport in Peripheral Blood Mononuclear Cells
[3H]-arginine transport by peripheral blood mononuclear cells (PBMCs) was assessed as described previously.13 In brief, 30 mL of peripheral blood was collected, and PBMCs were then isolated by Ficoll-Paque (Pharmacia) density gradient centrifugation according to the manufacturer’s instructions. For uptake studies, PBMCs were incubated in balanced salt solution containing L-arginine in concentrations ranging from 1 to 300 μmol/L, which included 100 μmol/L [3H]-arginine, for a period of 5 minutes at 37°C. Uptake studies were performed in duplicate. Nonspecific uptake was determined in the presence of 10 mmol/L L-lysine. Uptake studies were performed in duplicate. Nonspecific uptake was determined in the presence of 10 mmol/L L-lysine.

Determination of L-Arginine and Dimethylarginines
Plasma concentrations of L-arginine, N(0),N(0)-dimethylarginine (ADMA), and N(0),N(0)-dimethylarginine (SDMA) were measured by reverse-phase liquid chromatography with a time-controlled orthophthalaldehyde precolumn derivatization, as previously described.17

Pharmacological Appraisal of Endothelial Function and Effect of Supplemental L-Arginine
Forearm blood flow was assessed by venous-occlusion plethysmography (ECSR Plethysmograph, Hokanson), as described previously.18 Acetylcholine (BDH Chemicals) was infused at sequential doses of 9.25 and 37 μg/min. Sodium nitroprusside (BDH Chemicals) was administered at sequential doses of 200 and 800 ng/min. Each dose was infused for 5 minutes. Forearm blood flow was measured at the end of each infusion period as the average of 3 consecutive steady state measurements. Forearm vascular resistance was calculated as the mean arterial pressure divided by forearm blood flow.

To evaluate the effect of increased L-arginine availability on endothelial function, infusion of acetylcholine and sodium nitroprusside was repeated during concomitant intra-arterial infusion of L-arginine hydrochloride (Clinalfa AG) at 10 μmol/min, a dose that does not alter forearm blood flow or elicit any systemic effects.7 To exclude an effect of L-arginine, forearm blood flow was measured again after 10 minutes. L-Arginine hydrochloride infusion was continued, and acetylcholine and sodium nitroprusside infusion was repeated as described above. The sequence of the drugs infused was randomized to exclude any drug order effects.

Statistical Analysis
Vascular reactivity data are presented as mean±SEM; all other values are presented as mean±SD. A probability value <0.05 was considered to indicate statistical significance. Multigroup comparisons of variables were performed by 1-way ANOVA followed by the Bonferroni correction. Comparisons of dose-response curves to vasoactive substances were analyzed by ANOVA for repeated measures. Relations between variables were determined by linear regression analysis. The data were processed with the use of the software package SigmaStat for Windows 2.03 (SPSS Inc).

Results
Baseline characteristics of the study cohort are summarized in Table 1. The 3 study groups were well matched with regard to parameters potentially influencing endothelium-dependent vasodilation. Hypertensive subjects had higher systolic and diastolic blood pressure values and a higher body mass index than both groups of normotensive subjects (all P<0.05). The latter 2 groups had similar systolic and diastolic blood pressure levels and body mass index. Forearm blood flow at baseline did not differ between the 3 groups. There was a trend toward an increased forearm vascular resistance in hypertensive subjects compared with normotensive control subjects with no family history (P=0.06).

Forearm 1-Arginine Transport
There was a progressive increase in the deep venous concentration of the radiotracer in response to a continuous intra-ar-
terial infusion of [3H]-arginine, which reaches steady state levels in plasma after ≈20 to 30 minutes.14 Forearm blood flow was not altered by infusion of [3H]-arginine and was similar in all 3 groups when [3H]-arginine uptake was assessed. At steady state,12 uptake of the radiotracer across the forearm was substantially blunted in both essential hypertension and positive family history groups (Figure 1).

Forearm [3H]-arginine uptake correlated with the response of forearm blood flow to both doses of acetylcholine (acetylcholine 9.25 μg/min: r=0.64, P=0.004; acetylcholine 37 μg/min: r=0.52, P=0.02). There was a significant negative correlation between forearm [3H]-arginine uptake and the degree of the potentiating effect of L-arginine on vasodilation in response to acetylcholine for the dose of 37.5 μg/min (r=-0.67; P=0.001), suggesting that the lower the forearm [3H]-arginine uptake was at baseline, the higher was the augmenting effect of L-arginine on acetylcholine-induced vasodilatation.

**TABLE 1. Clinical Characteristics of the Study Cohort**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Negative Family History Group (n=15)</th>
<th>Positive Family History Group (n=15)</th>
<th>Essential Hypertension Group (n=12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>25.3±5.8</td>
<td>24.8±4.3</td>
<td>25.4±4.5</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.4±2.5</td>
<td>23.3±2.4</td>
<td>25.1±3.9*</td>
</tr>
<tr>
<td>Systolic blood pressure, mm Hg</td>
<td>122.1±5.9</td>
<td>120.4±7.3</td>
<td>153.9±11.2‡</td>
</tr>
<tr>
<td>Diastolic blood pressure, mm Hg</td>
<td>72.9±5.1</td>
<td>74.2±5.2</td>
<td>88.0±9.4†</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>69.8±7.2</td>
<td>72.3±9.2</td>
<td>75.2±8.9</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.15±0.74</td>
<td>4.30±0.61</td>
<td>4.44±0.65</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>2.36±0.70</td>
<td>2.53±0.54</td>
<td>2.69±0.34</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.17±0.32</td>
<td>1.13±0.35</td>
<td>1.18±0.32</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.35±0.67</td>
<td>1.25±0.72</td>
<td>1.24±0.44</td>
</tr>
<tr>
<td>Plasma glucose, mmol/L</td>
<td>4.52±0.42</td>
<td>4.80±0.40</td>
<td>4.48±0.53</td>
</tr>
<tr>
<td>Plasma sodium, mmol/L</td>
<td>142.6±1.2</td>
<td>142.1±2.0</td>
<td>141.7±1.7</td>
</tr>
<tr>
<td>Plasma potassium, mmol/L</td>
<td>4.27±0.35</td>
<td>4.26±0.29</td>
<td>4.23±0.41</td>
</tr>
<tr>
<td>Plasma norepinephrine, pg/mL</td>
<td>261.8±121.9</td>
<td>221.9±165.0</td>
<td>201.3±74.8</td>
</tr>
<tr>
<td>Plasma epinephrine, pg/mL</td>
<td>31.8±23.1</td>
<td>30.1±43.8</td>
<td>59.7±57.7</td>
</tr>
<tr>
<td>Forearm blood flow (rest), mL/(min · 100 mL of tissue)</td>
<td>3.72±1.41</td>
<td>3.86±1.49</td>
<td>3.88±1.19</td>
</tr>
<tr>
<td>Forearm vascular resistance, mm Hg/(mL · min · 100 mL of tissue)</td>
<td>24.5±6.4</td>
<td>27.2±11.3</td>
<td>31.9±8.9</td>
</tr>
</tbody>
</table>

Data are mean±SD.

*P<0.05, †P<0.01 vs both negative family history and positive family history groups.

**Figure 1.** Comparison of forearm [3H]-arginine uptake at steady state conditions expressed as disintegrations per minute in normotensive subjects without (NFH) and with (PFH) a positive family history of hypertension and hypertensive subjects (EH). *P<0.01 vs NFH.

**CAT-1 mRNA and Protein Expression in PBMCs**

To examine potential molecular mechanisms for the observed defect in transmembrane L-arginine uptake in hypertensive and genetically predisposed normotensive subjects, we used...
real-time PCR and Western analysis to measure the abundance of mRNA and protein for CAT-1, the predominant cationic amino acid transporter in PBMCs. As shown in Figure 3, mRNA and protein expression of CAT-1 did not differ across the study groups.

Baseline Plasma L-Arginine and Dimethylarginine Concentrations

Plasma L-arginine and SDMA concentrations were similar in all 3 groups (Table 2). Plasma ADMA concentrations tended to be elevated in hypertensive subjects (P=0.067). There was a tendency toward an inverse relation between ADMA plasma concentrations and endothelium-dependent vasodilation in response to acetylcholine at a dose of 37 μg/min for all subjects (r=-0.31, P=0.063). No significant relation was evident between ADMA concentrations and forearm [3H]L-arginine uptake.

Vascular Response to Acetylcholine and Sodium Nitroprusside

No significant changes in blood pressure or heart rate were observed during drug administration, verifying the local application of each drug and excluding systemic effects of vasoactive substances. Before administration of acetylcholine and sodium nitroprusside, forearm blood flow was similar in the 3 groups (Figures 4 and 5). The intra-arterial infusion of acetylcholine increased forearm blood flow in a dose-dependent manner in all groups (P<0.01). However, the response of forearm blood flow to acetylcholine infusion was substantially reduced in both the essential hypertension and positive family history groups compared with the negative family history group for both doses tested (P<0.05). No difference was observed in acetylcholine-induced vasodilation between the essential hypertension and positive family history groups (Figure 4). Intra-arterial administration of sodium nitroprusside also increased forearm blood flow in a dose-dependent manner in all groups, but the response of forearm blood flow did not differ between the 3 groups (Figure 5). Blood pressure and heart rate did not change in either group during infusion of either acetylcholine or sodium nitroprusside.

Effects of Supplemental L-Arginine Infusion on Vascular Response to Acetylcholine and Sodium Nitroprusside

Infusion of L-arginine at a dose of 10 μmol/min did not produce any significant changes in forearm blood flow in either group. Immediately before and 10 minutes after L-arginine infusion, forearm blood flow was 4.1±0.6 versus 4.5±0.5 mL/min per 100 mL (P=NS) in subjects with negative family history, 4.2±0.7 versus 4.7±0.5 mL/min per 100 mL (P=NS) in subjects with positive family history, and 4.3±0.5 versus 4.8±0.4 mL/min per 100 mL (P=NS) in subjects with essential hypertension, respectively. L-Arginine infusion had no effect on blood pressure or heart rate in any group.

Coinfusion of L-arginine did not alter endothelium-dependent vasodilation in response to acetylcholine in the negative family history group (Figure 6A). However, concomitant L-arginine administration improved the response of forearm blood flow to acetylcholine infusion in both the

**TABLE 2. L-Arginine and Metabolites**

<table>
<thead>
<tr>
<th></th>
<th>Negative Family History Group (n=14)</th>
<th>Positive Family History Group (n=13)</th>
<th>Essential Hypertension Group (n=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Arginine, μmol/L</td>
<td>100.64±57.63</td>
<td>129.51±57.43</td>
<td>122.75±29.52</td>
</tr>
<tr>
<td>L-Citrulline, μmol/L</td>
<td>39.45±9.24</td>
<td>45.37±14.55</td>
<td>32.69±12.78</td>
</tr>
<tr>
<td>ADMA, μmol/L</td>
<td>1.11±0.6</td>
<td>0.87±0.77</td>
<td>1.61±0.63</td>
</tr>
<tr>
<td>SDMA, μmol/L</td>
<td>0.37±0.21</td>
<td>0.55±0.18</td>
<td>0.59±0.58</td>
</tr>
</tbody>
</table>

Data are mean±SD.
positive family history and essential hypertension groups ($P<0.05$) (Figure 6B and 6C). In the presence of L-arginine, the dose-response curve to acetylcholine was no longer statistically significant different between the 3 groups.

To exclude a nonspecific effect of L-arginine on acetylcholine-induced vasodilation, sodium nitroprusside was also infused in the presence of L-arginine. The response of forearm blood flow to sodium nitroprusside was similar both before and during concomitant L-arginine infusion in all groups (sodium nitroprusside 800 ng/min: negative family history group, 7.2±0.6 versus 6.8±0.7 mL/min per 100 mL; positive family history group, 7.2±0.5 versus 7.4±0.7 mL/min per 100 mL; essential hypertension group, 6.8±0.6 versus 7.4±0.9 mL/min per 100 mL; $P=NS$ for all comparisons). Again, no differences in the dose-response curves were observed between the 3 groups (data not shown).

**Discussion**

Impaired endothelial function has been demonstrated to be an early marker of atherosclerotic vascular disease in that it is present even before structural alterations of the arterial wall are detectable. The importance of endothelium-derived NO formation is further substantiated by the demonstration of impaired endothelial function in patients with risk factors for atherosclerosis, such as hypercholesterolemia and arterial hypertension.
It seems plausible to argue that these risk factors play a causative role with regard to the development of endothelial dysfunction, possibly triggered by the “endothelial injury” hypothesis. However, at least with regard to hypertension, there is a substantial body of evidence indicating that endothelial dysfunction is already present in normotensive offspring of hypertensive families, suggesting that impaired endothelium-dependent vasodilation might precede the onset of essential hypertension and not occur simply as a consequence of the condition.6,7,20

The observation that supplementation of the NO precursor L-arginine has the potential to restore impaired endothelial function in several conditions may provide some insight into the underlying mechanisms. One possible explanation is a relative intracellular substrate deficiency for endothelial NO synthase, thereby leading to a decreased NO bioavailability. In the present study we therefore tested the hypothesis that defective cellular L-arginine transport is associated with essential hypertension and may already exist in normotensive subjects with a strong genetic predisposition to essential hypertension. In agreement with previous reports,6,7 we demonstrated that endothelium-dependent vasodilation is impaired in hypertensive subjects and in normotensive subjects with a positive family history and that L-arginine can improve the attenuated response to acetylcholine. By means of a complementary in vivo and in vitro approach, we observed for the first time that an impairment of L-arginine transport exists in both hypertensive subjects and normotensive subjects with a positive family history of essential hypertension. Furthermore, we demonstrated that mRNA and protein expression of CAT-1, the predominant transporter for L-arginine, does not differ between the groups and that impaired L-arginine uptake is not due to increased plasma concentrations of the endogenous NO synthase inhibitor ADMA.

Our results are in agreement with the concept that L-arginine availability can be a rate-limiting step for NO synthesis despite previous observations that the intracellular concentration of L-arginine is in excess of the \( K_m \) value for NOS activity.16 Potential mechanisms for this apparent paradox include sequestration of L-arginine in intracellular pools that are poorly accessible to eNOS and compartmentalization of eNOS within plasma membrane caveolae colocalized with a plasma membrane arginine transporter.16 Alternatively, an exchange of intracellular inhibitors of NO synthesis, such as asymmetrical ADMA against extracellular L-arginine, has been suggested.21

Our findings are indicative of an impairment of L-arginine transport in both hypertensive and genetically predisposed normotensive subjects. Given that the normal plasma concentration of L-arginine is well below the saturating range for cationic amino acid transporter–mediated transport,22 supplementation of L-arginine would be expected to result in an increased rate of transport and a subsequent increase in substrate availability intracellularly. Indeed, in those subjects with impaired L-arginine transport, substrate infusion restored impaired endothelium-dependent vasodilation, whereas no such effect was seen in control subjects with unaltered L-arginine transport capacity. A reduction in L-arginine transport would interfere with the preferential delivery of extracellular L-arginine to eNOS16 and with the exchange of intracellular inhibitors of NO synthesis against extracellular L-arginine,21 thereby leading to decreased NO synthesis.

Arginine transport by endothelial cells is predominantly mediated by the system y\(^+\) carrier. At normal physiological concentrations of arginine, CAT-1 is the predominant transport system in endothelial cells, accounting for 60% to 80% of the total carrier-mediated uptake activity.23 To further examine the mechanisms responsible for the observed reduction in transport activity on a molecular level, we used real-time PCR and Western analysis to measure levels of CAT-1 mRNA and protein in PBMCs isolated from the same volunteers. We were encouraged in this line of thinking by previous results from our laboratory in heart failure patients demonstrating a significant reduction in the expression of CAT-1 mRNA, possibly related to circulating cytokines known to be present in heart failure.11 However, in contrast to the findings in heart failure patients, we did not detect differences in CAT-1 mRNA or protein levels in the present study, making it less likely that changes in expression of this arginine transport system are responsible for our findings. However, in this study we cannot rule out differences in the subcellular localization of CAT-1 or possibly interactions with other proteins as a potential causative mechanism for our findings.

We also tested the hypothesis that an impaired L-arginine uptake in the essential hypertension and positive family history groups may be influenced by endogenous ADMA, as suggested previously by other investigators.24 Despite a tendency toward higher plasma concentrations of ADMA in hypertensive subjects and a tendency toward an inverse relation between ADMA plasma concentrations and endothelium-dependent vasodilation, ADMA levels were not related to forearm \(^{[3H]}\)L-arginine uptake, making a significant effect of ADMA on L-arginine transport unlikely. The lack of statistical significance in ADMA levels between the groups may be due to the small sample size.

Whether decreased availability of L-arginine contributes to impaired endothelial function remains controversial. Consistent with the present study, Taddei and colleagues7 demonstrated that the intra-arterial administration of L-arginine in normotensive offspring of hypertensive parents increased vasodilation in response to acetylcholine. However, this observation is in contrast to that by Panza et al,5 who reported that intra-arterial infusion of L-arginine had no effect on acetylcholine-induced vasodilation in middle-aged essential hypertensive patients (aged \( 48.4 \pm 7 \) years), whereas it improved endothelium-dependent vasodilation in age-matched normotensive control subjects. It has previously been shown that increasing age is associated with declining endothelial function,25 perhaps explaining some of the differences between these studies. However, we recently demonstrated that this phenomenon is unlikely to be due to an age-related decline in arginine transport.26 Of relevance to the present study, it has been proposed that the endothelial dysfunction that occurs in hypertension seems to represent an accelerated form of dysfunction that occurs in aging.27 Other potential mechanisms have also been proposed for the age-related
decline in endothelial function, including an increase in vascular oxidant stress.\textsuperscript{24}

In conclusion, we have demonstrated for the first time that L-arginine transport is impaired in hypertensive and normotensive subjects with a genetic background of essential hypertension. Further studies on the precise mechanism for our observations and into the potential therapeutic value of manipulation of the L-arginine/NO pathway in hypertensives and those genetically at risk are warranted.

Acknowledgments

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