Asymmetric Dimethylarginine (ADMA): A Novel Risk Factor for Endothelial Dysfunction
Its Role in Hypercholesterolemia

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Background—Asymmetric dimethylarginine (ADMA) is an endogenous competitive inhibitor of nitric oxide (NO) synthase. Because endothelial NO elaboration is impaired in hypercholesterolemia, we investigated whether plasma concentrations of ADMA are elevated in young, clinically asymptomatic hypercholesterolemic adults. We further studied whether such elevation of ADMA levels was correlated with impaired endothelium-dependent, NO-mediated vasodilation and urinary nitrate excretion. In a randomized, double-blind, placebo-controlled study, we investigated whether these changes could be reversed with exogenous L-arginine.

Methods and Results—We measured plasma levels of L-arginine, ADMA, and symmetrical dimethylarginine (SDMA) by high-performance liquid chromatography in 49 hypercholesterolemic (HC) and 31 normocholesterolemic (NC) humans. In 8 HC subjects, endothelium-dependent forearm vasodilation was assessed before and after an intravenous infusion of L-arginine or placebo and compared with 8 NC control subjects. ADMA levels were significantly elevated by 100% (2.17 ± 0.15 versus 1.03 ± 0.09 μmol/L; P < 0.05) in HC subjects compared with NC adults. L-Arginine levels were similar, resulting in a significantly decreased L-arginine/ADMA ratio in HC subjects (27.7 ± 2.4 versus 55.7 ± 5.4; P < 0.05). In 8 HC subjects, intravenous infusion of L-arginine significantly increased the L-arginine/ADMA ratio and normalized endothelium-dependent vasodilation and urinary nitrate excretion. ADMA levels were inversely correlated with endothelium-mediated vasodilation (R = 0.762, P < 0.01) and urinary nitrate excretion rates (R = 0.534, P < 0.01).

Conclusions—We find that ADMA is elevated in young HC individuals. Elevation of ADMA is associated with impaired endothelium-dependent vasodilation and reduced urinary nitrate excretion. This abnormality is reversed by administration of L-arginine. ADMA may be a novel risk factor for endothelial dysfunction in humans. (Circulation. 1998;98:1842-1847.)

Key Words: asymmetric dimethylarginine ■ atherosclerosis ■ nitric oxide ■ endothelium-derived factors ■ vasodilation
control subjects. We also determined the functional significance of elevated ADMA levels in terms of indexes of endothelial function. Finally, we performed a randomized, double-blind, placebo-controlled study to determine if L-arginine could reverse the perturbation of the NO synthase pathway in these patients.

Methods

Subjects

HC subjects (n=49) with no symptoms or signs of atherosclerotic vascular disease (by a complete cardiovascular history and examination, including an ankle-brachial index and an ECG) were recruited from the Stanford University preventive medicine and vascular medicine clinics. Hypercholesterolemia was defined as the presence of fasting total cholesterol levels >220 mg/dL or LDL concentrations >160 mg/dL. HC subjects were compared with a group of age-matched NC control subjects (n=31). None of the subjects were taking lipid-lowering medication. Subjects with a previous history of liver or renal disease or with diabetes mellitus were excluded; all subjects were nonsmokers. Their characteristics and fasting plasma lipid levels are given in the Table. The study protocol was approved by the Stanford University Review Board for Human Studies, and each subject gave written informed consent.

Determination of L-Arginine and Dimethylarginines

Plasma concentrations of L-arginine, N\(^\circ\),N\(^\circ\)-dimethylarginine (ADMA), and N\(^\circ\),N\(^\circ\)-dimethylarginine (SDMA) were measured by high-performance liquid chromatography (HPLC) and precolumn derivatization with o-phthalaldehyde (OPA) by a modification of a previously published method.\(^{\text{13}}\) L-Homoarginine (10 μmol/L) was added to 0.5 mL of plasma as an internal standard. Plasma samples and standards were extracted on solid-phase extraction cartridges (CBA Bond Elut, Varian). Recovery rates were 82.9±3.8%. Eluates were dried over nitrogen and resuspended in double-distilled water for HPLC analysis. HPLC was performed on a computer-controlled Varian Star chromatography system consisting of a ternary gradient HPLC pump (Varian 9010), an automatic injector with automated sample-reagent mixing capabilities (Varian 9090), and a fluorescence detector (Varian Fluorichrom III). Samples and standards were incubated for exactly 1 minute with OPA reagent (5.4 mg/mL OPA in borate buffer, pH 8.4, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC. The OPA derivatives of L-arginine, ADMA, and SDMA were separated on a 250×4.5-mm-ID 7-μm Nucleosil phenyl column (Supelco) with the fluorescence detector set at λ\(^{\text{exc}}\)=340 nm and λ\(^{\text{em}}\)=450 nm. Samples were eluted from the column with 0.96% citric acid/methanol (70:30), pH 6.8, at a flow rate of 1 mL/min. Variability of the method was <7%, and the detection limit of the assay was 0.15 μmol/L.

Biochemical Analyses

Urinary nitrate excretion was determined by use of a commercially available chemiluminescence apparatus (Dasibi Corp) as previously described.\(^{\text{13}}\) Fasting lipid levels and creatinine concentrations were determined in plasma samples drawn into sodium EDTA by the Stanford University Hospital Laboratory using standard laboratory methods. Creatinine clearance was calculated either from plasma and urinary creatinine concentrations or according to the method of Cockroft and Gault\(^{\text{26}}\) when urine samples were not available. Total cholesterol, HDL, and triglyceride concentrations were measured with an Abbott Spectrum II autoanalyzer. LDL concentrations were calculated according to the Friedewald formula.\(^{\text{27}}\)

L-Arginine Infusion and Forearm Blood Flow Measurement

In 8 HC subjects (4 men, 4 women), the effects of acute intravenous infusion of L-arginine or vehicle on the plasma L-arginine/ADMA ratio, urinary nitrate excretion, and endothelium-dependent, NO-mediated vasodilation were studied in a double-blind, randomized, placebo-controlled crossover study. The subjects received, in a randomized sequence on 2 experimental days separated by 1 week,
a single intravenous infusion of 14 g of L-arginine (≈10 mg/kg of body weight) or the corresponding placebo (0.9% saline) over 20 minutes. Flow-induced vasodilation was assessed as the increase in brachial artery diameter in response to augmented flow elicited by 3 minutes of vascular occlusion. It has been previously shown that flow-induced vasodilation in the human brachial artery is largely due to the endothelial elaboration of NO. Brachial artery blood flow and diameter were measured by duplex ultrasonography (Hewlett-Packard 2500, 7.5-MHz transducer with 5.5-MHz pulsed Doppler) before and immediately after infusion. Venous plasma samples were drawn before and 2 minutes after the infusion for measurement of L-arginine and dimethylarginine concentrations. Urine samples were collected in the period 1 hour before and the period 1 hour after the start of the infusion to assess urinary nitrate excretion as an index of systemic NO production. Urinary dimethylarginine clearances were calculated from plasma and urinary concentrations of ADMA and SDMA. Basal measurements of flow-dependent brachial artery vasodilation and urinary nitrate excretion were also made in a group of 8 NC control subjects (4 men, 4 women).

Calculations and Statistical Analyses

All data are given as mean ± SEM. Differences between HC and NC subjects as well as the effects of L-arginine versus vehicle administration were tested for statistical significance by ANOVA followed by Fisher’s protected least significant difference test. Linear regression curves and correlation coefficients were calculated according to the least squares method. Statistical significance was assumed for P<0.05.

Results

Baseline Plasma L-Arginine and Dimethylarginine Concentrations

Plasma L-arginine concentrations were 52.4±5.4 μmol/L in HC subjects and 49.8±4.2 μmol/L in NC control subjects (P=NS). HC subjects had significantly higher ADMA plasma levels than NC control subjects (2.17±0.15 and 1.03±0.09 μmol/L, respectively; P<0.05). More than half of the HC subjects studied had ADMA levels >2 SDs above the mean value in the NC control group (Figure 1A). SDMA plasma concentrations were not different in HC than in NC subjects (0.73±0.10 and 0.60±0.09 μmol/L, respectively; P=NS). Elevation of ADMA plasma concentrations resulted in a lower mean L-arginine/ADMA ratio in HC subjects than in NC subjects (27.7±2.4 versus 55.7±5.4; P<0.05; Figure 1B).

Lipid profiles and creatinine clearances for both groups are given in the Table. There was a positive correlation between plasma LDL cholesterol and ADMA concentrations (R=0.421, P<0.01; Figure 2) and between plasma total cholesterol and ADMA concentrations (R=0.372, P<0.01) but not between creatinine clearances and ADMA concentrations (R=0.113, P=0.402).

Perturbation of the NO Synthase Pathway in Hypercholesterolemia

Baseline flow-induced brachial artery vasodilation was 2.7±1.8% in 8 HC subjects compared with 9.8±1.0% in NC control subjects (P<0.01). Plasma ADMA levels were significantly higher in these HC subjects than in NC control subjects (2.2±0.2 and 0.9±0.1 μmol/L, respectively; P<0.05), whereas L-arginine concentrations did not differ significantly (36.8±3.0 and 34.8±3.6 μmol/L, respectively;
Urinary nitrate excretion was lower in HC than in NC subjects at baseline (124.0±14.1 versus 205.8±12.1 μmol/mmol creatinine; P<0.05). In multiple regression analysis, baseline flow-induced vasodilation was inversely correlated with plasma ADMA levels (R=0.762, P<0.01; Figure 3A), whereas total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, age, and blood pressure were not independent predictors of endothelium-dependent, NO-mediated vasodilation. Urinary nitrate excretion was also inversely correlated with plasma ADMA levels (R=0.534, P<0.01; Figure 3B).

Significant amounts of ADMA and SDMA were found in urine. In HC subjects, renal ADMA and SDMA clearances were 14.6±2.7 and 48.0±6.5 mL/min, respectively, compared with 18.4±2.9 and 51.7±7.7 mL/min, respectively, in NC control subjects (P=NS).

Effects of Intravenous L-Arginine Infusion in HC Subjects

L-Arginine infusion increased plasma L-arginine concentrations to 4843.9±233.5 μmol/L without significantly changing ADMA levels (2.2±0.2 μmol/L). This resulted in improved flow-induced vasodilation in each of the 8 subjects (mean, 8.6±2.2%; Figure 4A). In contrast, placebo infusion did not affect plasma L-arginine or ADMA concentrations (36.3±3.1 and 2.1±0.1 μmol/L, respectively; each P=NS versus baseline), nor did it improve flow-induced vasodilation (mean, 3.5±2.5%; P=NS versus baseline; Figure 4B). Urinary nitrate excretion increased after L-arginine infusion but not after placebo infusion (P<0.05; Figure 5). Infusion of L-arginine or placebo did not affect renal clearances of ADMA (L-arginine, 17.2±3.4 mL/min; placebo, 15.8±7.3 mL/min) or SDMA (L-arginine, 57.5±8.8 mL/min; placebo, 49.9±8.5 mL/min), nor did the infusions influence creatinine clearance.

Discussion

Our study shows for the first time that plasma concentrations of ADMA, an endogenous competitive inhibitor of NO synthesis, are elevated in asymptomatic HC humans compared with NC control subjects. This is associated with impaired endothelium-dependent, NO-mediated vasodilation in the brachial artery and reduced urinary nitrate excretion. Intravenous infusion of L-arginine reverses the endothelial dysfunction. The improvement in endothelial vasodilator function is likely due to the metabolism of L-arginine to NO, as reflected by an increase in urinary nitrate excretion.

Previous studies have shown that endothelial vasodilator function is impaired in HC humans, even in the absence of overt atherosclerotic vascular disease. The maximal increase in forearm blood flow in response to intra-arterial acetylcholine is reduced in young HC humans, as is flow-induced vasodilation during the hyperemic response after transient arterial occlusion.35 Joannides and coworkers36 have previously shown that flow-induced vasodilation in the human brachial artery is blocked by intra-arterial infusion of N\textsuperscript{\textgamma}-monomethyl-L-arginine (L-NMMA), indicating that flow-induced vasodilation is NO dependent. The degree of impair-
ment of endothelium-dependent vasodilator function is related to the number of cardiovascular risk factors present, among which the plasma LDL cholesterol level may be one of the most important. The mechanism(s) leading to this defect may include an increased degradation and/or reduced synthesis of NO.

We found that plasma ADMA levels are doubled in HC humans in association with evidence of reduced NO synthesis. ADMA has been shown to be an endogenous inhibitor of NO synthesis. Studies of isolated vessels and cultured endothelial cells suggest that ADMA concentrations between 1 and 10 μm/L inhibit endothelium-dependent vasodilation and vascular NO synthase activity. This is further corroborated by the present study, which shows an inverse correlation between the L-arginine/ADMA ratio and NO-dependent vasodilation as well as between this ratio and urinary nitrate excretion. The doubling of ADMA plasma concentrations in HC subjects may reflect an even greater level of this endogenous NO synthase inhibitor within endothelial cells. In experimental models of vascular injury, there is attenuated endothelium-dependent vasodilation even after the intimal lining has been fully regenerated. Recently, it has been shown that in regenerated endothelial cells, levels of ADMA (as well as another NO synthase inhibitor, L-monomethyl-arginine) are elevated 3-fold compared with normal cells.

The origin of ADMA in hypercholesterolemia is currently unclear. Data from metabolism studies in animals suggest that dimethylarginines derive from the degradation of methylated proteins. Increased endogenous formation, impaired metabolic degradation, or reduced clearance of ADMA may cause its increased plasma levels. Dimethylarginines have been shown to be excreted via the kidneys and to accumulate in chronic renal failure. In the present study, however, there was no evidence of impaired renal function in our subjects. Moreover, direct measurement of renal ADMA and SDMA clearances revealed no significant difference compared with NC control subjects. ADMA is metabolized to citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH). Inhibition of DDAH causes a gradual vasoconstriction of vascular segments, which is reversed by L-arginine. This latter finding suggests that ADMA may be an endogenous modulator of NO synthase activity. Whether hypercholesterolemia affects this metabolic pathway or whether another pathway is involved in the hypercholesterolemia-induced accumulation of ADMA remains undetermined.

Administration of L-arginine has been shown to reverse endothelial dysfunction in HC rabbits. In HC humans, intravenous or oral administration of L-arginine improves endothelium-dependent, acetylcholine-induced vasodilation. A vasodilator effect of intravenous L-arginine has also been observed in patients with severe peripheral arterial occlusive disease. Taken together with these previous studies, our observations indicate that exogenous L-arginine may compete with the endogenous NO synthase inhibitor ADMA to restore NO synthesis. This interaction between L-arginine and ADMA is likely a direct competition for the NO synthase enzyme, because L-arginine infusion did not affect renal ADMA clearance.

We speculate that ADMA may represent a novel risk factor for atherosclerosis. In HC rabbits, long-term oral administration of L-arginine restores endothelial elaboration of NO, reduces endothelial adhesiveness for monocytes, inhibits platelet aggregation, retards atherogenesis, and even induces regression of preexisting lesions. By contrast, long-term antagonism of NO synthase accelerates atherogenesis in animal models. Recently, it has been observed that ADMA levels are correlated with the severity of disease in patients with peripheral arterial disease.

In conclusion, the present study shows that the plasma concentration of ADMA, an endogenous competitive inhibitor of NO synthase, is elevated in HC humans. Elevated ADMA concentrations are associated with impaired endothelium-dependent, NO-mediated vasodilation and reduced urinary nitrate excretion. These effects of ADMA are counteracted by administration of the NO precursor, L-arginine. ADMA may be a determinant of endothelial vasodilator dysfunction and a novel risk factor for atherosclerosis.

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