Endogenous Nitric Oxide Synthase Inhibitor
A Novel Marker of Atherosclerosis

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Background—Exposure to risk factors such as hypertension or hypercholesterolemia decreases the bioavailability of endothelium-derived nitric oxide (NO) and impairs endothelium-dependent vasodilation. Recently, a circulating endogenous NO synthase inhibitor, asymmetric dimethylarginine (ADMA), has been detected in human plasma. The purpose of this study was to examine the relationship between plasma ADMA and atherosclerosis in humans.

Methods and Results—Subjects (n=116; age, 52±1 years; male:female ratio, 100:16) underwent a complete history and physical examination, determination of serum chemistries and ADMA levels, and duplex scanning of the carotid arteries. These individuals had no symptoms of coronary or peripheral artery disease and were taking no medications. Univariate and multivariate analyses revealed that plasma levels of ADMA were positively correlated with age (P<0.0001), mean arterial pressure (P<0.0001), and Δ glucose (an index of glucose tolerance) (P=0.0006). Most intriguingly, stepwise regression analysis revealed that plasma ADMA levels were significantly correlated to the intima-media thickness of the carotid artery (as measured by high-resolution ultrasonography).

Conclusions—This study reveals that plasma ADMA levels are positively correlated with risk factors for atherosclerosis. Furthermore, plasma ADMA level is significantly correlated with carotid intima-media thickness. Our results suggest that this endogenous antagonist of NO synthase may be a marker of atherosclerosis. (Circulation. 1999;99:1141-1146.)

Key Words: aging ■ risk factors ■ diabetes mellitus ■ dimethylarginine ■ hypertension ■ smoking

Endothelial vasodilator dysfunction precedes the clinical development of atherosclerosis1–5 and may contribute to the progression of disease.6 Risk factors for the development of atherosclerosis, such as hypertension, hypercholesterolemia, and smoking, have been shown to cause endothelial vasodilator dysfunction and, when coexisting, have an additive effect.7–10 Treatment of these factors restores endothelial function4,5,11 and decreases cardiovascular mortality.12–15 These observations suggest a relationship between endothelial vasodilator dysfunction and risk factors for atherosclerosis, although the pathophysiological mechanisms for endothelial dysfunction are not fully elucidated.16

The endothelium plays a pivotal role in control of vascular tone by releasing several vasoactive substances, such as nitric oxide (NO). In addition to its action as a vasodilator, NO inhibits platelet aggregation, leukocyte adhesion, and smooth muscle cell proliferation.17–22 A decrease in the bioavailability of endothelium-derived NO has been demonstrated in patients with risk factors.10 The reduction in NO bioavailability may due in part to the action of a circulating endogenous NO synthase inhibitor, N6,N7-dimethylarginine (asymmetric dimethylarginine; ADMA).23–26 Intra-arterial administration of ADMA causes vasoconstriction in forearm vessels27 via inhibition of endothelium-derived NO synthesis. We and others have demonstrated high levels of ADMA in urine from hypertensive rats,28 in plasma from hypercholesterolemic rabbits,29 in patients with peripheral arterial occlusive disease,30 and in the regenerating endothelium of balloon-injured vessels.31 These reports indicate that ADMA may be involved in vascular disease.

It is well known that L-arginine supplementation enhances the synthesis of endothelium-derived NO,30 restores endothelial vasodilator function,32–34 inhibits platelet aggregation35,36 and cell adhesion,20,37–40 and attenuates atherosclerosis41–45 in hypercholesterolemic animals and in humans. It is possible that L-arginine exerts these beneficial effects by reversing the action of the competitive inhibition by ADMA.46 Accumulating evidence suggests that a derangement of the NO synthase pathway plays a critical role in atherogenesis and that ADMA may participate in this endothelial dysfunction. Accordingly, the present study was designed to determine the association of the circulating endogenous NO synthase inhibitor with coronary risk factors and/or with atherosclerosis in humans.

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Methods

Subjects
Between September 1996 and December 1997, 118 people, 26 to 77 years old, volunteered for this study. These subjects were undergoing a routine medical evaluation that included a complete history and physical examination, serum chemistries, electrocardiography, and chest radiography. They were not taking any medication and had no symptoms of coronary or peripheral arterial disease. Two subjects were excluded because they had renal dysfunction (serum creatinine ≥1.5 mg/dL). Consequently, 116 subjects (100 male and 16 female; age, 52 ± 1 years) were enrolled. Informed consent was obtained, and the study protocol was approved by the Institutional Ethics Committee of Kurume University School of Medicine.

Study Design
In the morning, after subjects had fasted overnight, blood pressure was measured in the right arm at least twice with a mercury sphygmomanometer after subjects had rested in the supine position for ≥5 minutes. Systolic and diastolic pressures were determined as the first and fifth phases of the Korotkoff sounds, respectively, and a mean arterial pressure was calculated. After fasting blood samples were obtained for plasma ADMA and lipid profile measurements, a 75-g oral glucose tolerance test was performed in each subject. Blood samples were taken at 0, 60, and 120 minutes after glucose loading to measure plasma glucose levels. The incremental area of plasma glucose level over time (Σ glucose) was calculated as an index of glucose tolerance. Cigarette smokers were defined as subjects who were current smokers or who had ceased tobacco use within 3 months of entry into the study. Family history was considered positive if a first-degree relative had clinical evidence of coronary artery disease (angina pectoris or myocardial infarction) at ≤60 years of age. Diabetes mellitus was diagnosed according to the criteria of the World Health Organization, defined as a fasting glucose level of ≥140 mg/dL and/or plasma glucose level of ≥200 mg/dL 2 hours after glucose administration.47 Hypertension was defined as systolic blood pressure ≥140 mm Hg or diastolic blood pressure ≥90 mm Hg. Gender was not considered a risk factor because all female subjects were postmenopausal.

Intima-Media Thickness
The intima-media thickness (IMT) of the common carotid artery was determined by duplex ultrasonography (SSA-380A, Toshiba) with a 10-MHz transducer. Longitudinal B-mode images at the diastolic phase of the cardiac cycle were recorded by a single trained technician who was blinded as to the subject’s background. The images were magnified and printed with a high-resolution line recorder (LSR-100A, Toshiba). Measurements of IMT were made by the same technician using fine slide calipers at 3 levels of the lateral and medial walls 1 to 3 cm proximal to the carotid bifurcation. The mean of these 6 measurements was taken as the value for the IMT. Interobserver and intraobserver variations were 3.8% and 4.2%, respectively.

Chemical Analysis
Plasma concentrations of ADMA were determined by high-performance liquid chromatography as previously described.24 Serum total and HDL cholesterol, triglyceride, and creatinine levels were determined enzymatically with commercial kits (Boehringer Diagnostica and Wako Chemicals). Creatinine clearance was calculated with the following formula: [(140 – age) × weight (kg)] / 72 × serum creatinine (×0.85 for women). LDL cholesterol was calculated by the Friedewald formula. Plasma glucose was measured by the glucose dehydrogenase ultraviolet test (Merck Liquid Glu, Kanto Chemical Co).

Statistical Analysis
Results were expressed as mean ± SEM. Univariate analysis of the effects of each potential risk factor on ADMA was performed with linear regression for continuous variables (age; systolic, diastolic, and mean blood pressure; total cholesterol; and Σ glucose) and with 1-way ANOVA for categorical variables (smoking and family history). The interaction among risk factors, creatinine clearance, and ADMA was examined by multiple stepwise regression analysis. Univariate analyses of the effects of each potential risk factor or ADMA on IMT were performed with linear regression for continuous variables (age; systolic, diastolic, and mean blood pressure; total cholesterol; Σ glucose; and ADMA) and with 1-way ANOVA for categorical variables (smoking and family history). The interaction between risk factors, ADMA, and IMT was then examined by multiple stepwise regression analysis. A probability of <0.05 was accepted as the level of statistical significance.

Results

Subjects
There were 74 nonsmokers and 42 smokers. Average systolic and diastolic blood pressures were 129 ± 2 and 78 ± 1 mm Hg, respectively, and 40 subjects had hypertension. A family history of coronary artery disease was present in 7 subjects. Average total, LDL, and HDL cholesterol, triglyceride, and creatinine levels and creatinine clearance were 193 ± 3 mg/dL, 110 ± 3 mg/dL, 53 ± 1 mg/dL, 151 ± 9 mg/dL, 0.96 ± 0.01 mg/dL, and 81 ± 2 mL/min, respectively; 18 subjects had total cholesterol levels >220 mg/dL. Mean fasting plasma glucose level and Σ glucose were 99 ± 2 mg/dL and 398 ± 10 mg · dL⁻¹ · h, respectively; 26 subjects had diabetes.

Intra–Risk Factor Correlations
There were weak but significant correlations between age and arterial pressure (age versus systolic, diastolic, and mean arterial pressure: r = 0.38, P < 0.0001; r = 0.35, P < 0.0001; and r = 0.38, P = 0.0001, respectively) and between age and Σ glucose (r = 0.22, P = 0.04). There were no other intra–risk factor correlations.

Plasma ADMA and Risk Factors
Mean plasma ADMA was 0.51 ± 0.01 μmol/L (range, 0.30 to 0.82 μmol/L). Univariate analysis revealed a significant correlation between plasma ADMA and age (r = 0.54, P < 0.0001), arterial blood pressure (systolic, diastolic, or mean arterial pressure: r = 0.45, P < 0.0001; r = 0.41 P < 0.0001; and r = 0.46, P < 0.0001, respectively) and Σ glucose (r = 0.31, P = 0.0006) (Figure 1). Plasma ADMA was not correlated with tobacco use, cholesterol (total, LDL, HDL, or triglycerides), or creatinine, but it was inversely correlated with creatinine clearance (r = −0.36, P = 0.001). By stepwise multiple regression analysis (Table 1; r² = 0.41), plasma ADMA was significantly correlated with age (F = 21.6, P = 0.006), mean arterial pressure (F = 11.8, P = 0.007), and Σ glucose (F = 5.1, P = 0.01).

Plasma ADMA and IMT
Mean IMT was 0.59 ± 0.01 mm (range, 0.36 to 0.94 mm). By univariate analysis, IMT was positively correlated with plasma levels of ADMA (r = 0.51, P < 0.00001), age (r = 0.36, P < 0.00001), arterial pressure (systolic, diastolic, and mean arterial pressure: r = 0.36, P < 0.0001; r = 0.41, P < 0.0001; and r = 0.41, P < 0.0001, respectively), and Σ glucose (r = 0.30, P = 0.01). By stepwise multiple regression analysis (Table 2; r² = 0.41), IMT was significantly correlated with age (F = 23.8, P = 0.0001) and plasma ADMA (F = 11.1, P = 0.03) only. The correlation between plasma ADMA and IMT was
still significant even after adjusting for age ($r=0.33$, $P=0.0003$) (Figure 2).

**Discussion**

The salient findings of this study are that plasma concentration of ADMA was significantly correlated with the risk factors of aging, hypertension, and diabetes and that plasma levels of ADMA were positively correlated with the IMT of the carotid artery. Our findings suggest that the circulating endogenous NO synthase inhibitor may be an early marker of atherosclerosis in humans.

In the present study, we enrolled asymptomatic subjects who had no clinical evidence of coronary or peripheral arterial diseases or renal dysfunction. These inclusion criteria obviated the influence of medications and excluded an effect of renal clearance or overt atherosclerosis on plasma ADMA levels. Among the remaining 116 subjects, we found no correlation between plasma levels of ADMA and creatinine clearance by stepwise multiple regression analysis, which indicates that the increases in plasma ADMA in subjects with risk factors were not due to the decrease in renal clearance of ADMA. There were 16 female subjects in our study. We did not include gender as a risk factor because all 16 subjects were postmenopausal females, in whom the degree of endothelial dysfunction as well as the incidence of cardiovascular events is increased.

We measured plasma ADMA concentration by derivatizing it with ortho-phthalaldehyde and measuring the derivative with high-performance liquid chromatography and a fluorescence detector, as previously described. When we apply this method to human plasma samples, the recoveries of ADMA are $\geq 80\%$. This method permits quantitative determination of ADMA at concentrations as low as 0.1 $\mu$mol/L in human plasma. With this assay, the ranges of plasma ADMA in subjects without risk factors were similar to those of control subjects in previous studies.

**Table 1. Stepwise Multiple Regression Analysis for Determinant of Plasma ADMA**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.004</td>
<td>21.6</td>
<td>0.006</td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>0.0027</td>
<td>11.8</td>
<td>0.007</td>
</tr>
<tr>
<td>$\Sigma$ glucose</td>
<td>0.0019</td>
<td>5.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>-0.18</td>
<td>3.7</td>
<td>0.15</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.14</td>
<td>2.2</td>
<td>0.18</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>-0.14</td>
<td>2.1</td>
<td>0.24</td>
</tr>
<tr>
<td>Positive family history</td>
<td>0.07</td>
<td>0.5</td>
<td>0.25</td>
</tr>
</tbody>
</table>

$r^2=0.41$.

**Table 2. Stepwise Multiple Regression Analysis for Determinant of IMT**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.005</td>
<td>23.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>Plasma ADMA</td>
<td>0.29</td>
<td>11.1</td>
<td>0.03</td>
</tr>
<tr>
<td>Mean arterial pressure</td>
<td>0.16</td>
<td>3.0</td>
<td>0.10</td>
</tr>
<tr>
<td>$\Sigma$ glucose</td>
<td>0.14</td>
<td>2.1</td>
<td>0.16</td>
</tr>
<tr>
<td>Smoking</td>
<td>-0.05</td>
<td>0.33</td>
<td>0.56</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>0.02</td>
<td>0.05</td>
<td>0.81</td>
</tr>
<tr>
<td>Positive family history</td>
<td>-0.02</td>
<td>0.03</td>
<td>0.94</td>
</tr>
</tbody>
</table>

$r^2=0.41$.
Plasma ADMA and Atherosclerosis
The endothelial vasodilator dysfunction observed in the coronary or forearm vascular beds of patients with atherosclerosis is reversed by L-arginine supplementation. Since L-arginine is abundant in endothelial cells, depletion of this substrate is not likely to account for the endothelial dysfunction. It is possible that endogenous NO synthase inhibitors may contribute to endothelial dysfunction; indeed, after balloon injury of the rabbit iliac artery, the regenerated endothelium manifests a vasodilator dysfunction, associated with markedly elevated levels of intracellular ADMA. L-Arginine supplementation may overcome this competitive inhibition to restore endothelium-mediated vasodilation. In humans, Vallance and colleagues demonstrated that intrarterial infusion of ADMA at concentrations much higher than plasma levels causes vasoconstriction in normal subjects. A recent study by Böger et al showed that modest increases in ADMA (≥2 μmol/L) were associated with hypercholesterolemia and had physiological effects in humans. Thus, it remains possible that the plasma concentrations achieved in the present study could be biologically effective, because ADMA is concentrated in endothelial cells. However, the proof that the ADMA concentrations used in the present study are sufficiently high to achieve a physiological effect in humans is still lacking.

Long-term administration of L-arginine causes a sustained enhancement of NO synthesis in the hypercholesterolemic rabbit, which is associated with reduced progression and even regression of intimal lesions. By contrast, long-term administration of NO synthase antagonists increases endothelial adhesiveness for monocytes and accelerates lesion formation in this model. The antiatherogenic action of NO is mediated in part by its effect to suppress an oxidant-sensitive transcriptional cascade leading to the expression of adhesion molecules (eg, vascular cell adhesion molecule) and chemokines (eg, monocyte chemotactic protein) that mediate monocyte adherence to the endothelium.

Evidence that a derangement of the NO synthase pathway may contribute to atherogenesis in humans has recently been provided by Böger and colleagues. They reported that plasma levels of ADMA in patients with peripheral arterial disease correlated with urinary nitrate production (a reflection of systemic NO production) and with the clinical severity of the occlusive disease. It was not clear from their study whether the increase in plasma ADMA levels was the cause or consequence of atherosclerosis, because their patients had advanced disease. However, in the present study, we observed that the plasma level of ADMA correlated with several risk factors in the absence of clinical disease. More intriguingly, plasma ADMA levels were significantly and quantitatively correlated with the IMT of the carotid artery, a noninvasive measure of atherosclerosis. Taken together with the findings by Böger et al and the preceding animal studies, our results raise the provocative concept that plasma ADMA may be a novel marker of atherosclerosis, although the causal role remains unknown.

An alternative interpretation is that the elevation of plasma ADMA is an epiphenomenon of vascular injury. Because plasma ADMA had the strongest correlation with age by stepwise multiple regression analysis (F=21.6, P=0.006), it could be argued that ADMA reflects a vascular degenerative process associated with aging. Indeed, an endothelial vasodilator dysfunction is observed with aging that is reversible with L-arginine. However, plasma ADMA remained a predictor for IMT after we adjusted for age (Figure 2). Although there was an inverse correlation between plasma ADMA and creatinine clearance by univariate analysis, the possibility that the increase in ADMA could be due to reduced creatinine clearance is less likely, because this association was lost by multivariate analysis for plasma ADMA (Table 1).

Metabolism of ADMA
McDermott demonstrated that plasma dimethylarginines arise mainly from degradation of intracellular methylated proteins and are eliminated via urinary excretion. ADMA is metabolized to citrulline by the intracellular enzyme dimethylarginine dimethylaminohydrolase (DDAH). Antagonists of DDAH block ADMA degradation and cause a slowly developing contraction in isolated vascular rings; the contraction is reversed by addition of L-arginine to the medium. This observation is consistent with the hypothesis that ADMA produced by vascular cells modulates the synthesis of endothelium-derived NO. An elevation of plasma and/or vascular ADMA could therefore promote vasoconstriction, as well as activate key processes in atherogenesis. At least 3 possibilities exist for an elevation of plasma and/or vascular ADMA: a decrease in renal filtration, a decreased activity of DDAH, or an increased hydrolysis of methylated protein.

Study Limitations
Although we demonstrated no significant correlation between plasma ADMA and several other risk factors (hypercholesterolemia, smoking, and family history), this may be due to the size or demographics of our study population. Indeed, an association between ADMA and hypercholesterolemia has been demonstrated in a series of studies. Another limitation is that the present study focused on carotid IMT as an indicator of vascular disease. Although this parameter is frequently taken as an index of atherosclerosis, an increase in this parameter may be due to medial hypertrophy (as with longstanding hypertension) or intimal thickening (as with atherosclerosis). The present study did not determine whether ADMA correlates with clinically significant vascular events or whether ADMA plays a causal role in the pathogenesis of human atherosclerosis.

Clinical Implications
The present study demonstrated a strong relationship of plasma ADMA with other risk factors and with carotid artery thickening in subjects without overt cardiovascular disease. This study raises the provocative concept that ADMA may be a novel marker of atherosclerosis.

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References


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