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Asymmetrical Dimethylarginine: A Novel Risk Factor for Coronary Artery Disease

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Summary

Background: Asymmetrical dimethylarginine (ADMA) is an endogenous competitive inhibitor of nitric oxide synthase and has been associated with systemic atherosclerosis; however, the role of ADMA in patients with coronary artery disease (CAD) has not been investigated.

Hypothesis: The present study was designed to determine whether the plasma ADMA level predicts the presence of CAD independently, and whether the plasma ADMA level correlates with the extent and severity of coronary atherosclerosis.

Methods: In all, 97 consecutive patients with angina and positive exercise stress test were enrolled prospectively for coronary angiography. According to the result of angiography, the subjects were divided into two groups: Group 1 (n = 46): patients with normal coronary artery or mild CAD (< 50% stenosis of major coronary arteries); Group 2 (n = 51): patients with significant CAD (≥ 50% stenosis of major coronary arteries). Plasma levels of ADMA and L-arginine were determined by high-performance liquid chromatography. In addition, we used coronary atherosclerotic score to assess the extent and severity of CAD.

Results: The plasma levels of ADMA in Group 2 patients were significantly higher than those in Group 1 patients (0.66 ± 0.17 μM vs. 0.44 ± 0.09 μM, $p < 0.001$); these were accompanied by significantly lower plasma L-arginine/ADMA ratio in patients with significant CAD (Group 1 vs. 2: 194.0 ± 55.3 vs. 136.7 ± 50.3, $p < 0.001$). In a multivariate stepwise logistic regression analysis, both plasma ADMA level and plasma L-arginine/ADMA ratio were identified as independent predictors for CAD. Moreover, there were significant positive and negative correlations between coronary atherosclerotic score and plasma ADMA level as well as plasma L-arginine/ADMA ratio, respectively (plasma ADMA level: $r = 0.518$, $p < 0.001$; L-arginine/ADMA ratio: $r = 2\ 0.430$, $p < 0.001$).

Conclusions: Both plasma ADMA level and plasma L-arginine/ADMA ratio were useful in predicting the presence of significant CAD and correlated significantly with the extent and severity of coronary atherosclerosis. Our findings suggest that plasma ADMA level may be a novel marker of CAD.

Key words: asymmetrical dimethylarginine, coronary artery disease, nitric oxide

Introduction

Endothelium-derived nitric oxide (NO) is synthesized from L-arginine by NO synthase and plays a pivotal role in the regulation of vascular tone.[1] In addition, NO inhibits platelet adhesion and aggregation and leukocyte adhesion, and suppresses smooth muscle cell proliferation.[2–4] Impaired NO bioavailability may contribute to the development of endothelial dysfunction, which is considered to be important in the initiation and progression of atherosclerosis and has been related to various risk factors of coronary artery disease (CAD), such as hypercholesterolemia,[5] essential hypertension,[6] diabetes, aging,[7] as well as active and passive smoking.[8] Nitric oxide also contributes importantly to resting vascular tone and acetylcholine-induced vasodilation in normal and atheromatous coronary arteries.[7, 9–12] However, the mechanism of derangement of L-arginine-NO pathway that leads to endothelial dysfunction is still not fully clear. Recently, asymmetrical dimethylarginine (ADMA) has been implicated as an important contributing factor.[13–15]

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Asymmetrical dimethylarginine is characterized as a circulating endogenous inhibitor of NO synthase.[16, 17] It may compete with L-arginine as the substrate for NO synthase, increase oxidative stress by uncoupling of electron transport between NO synthase and L-arginine, and hence decrease both the production and availability of endothelium-derived NO.[18] Elevations of ADMA level have been observed in urine from hypertensive rats,[19] in the regenerating endothelium of balloon-injured vessels,[20] and in plasma from patients with hypercholesterolemia,[13] congestive heart failure,[21] peripheral arterial occlusive disease,[14] essential hypertension,[22] and hypertriglyceridemia.[23] These reports suggested that elevation of ADMA might be involved in the pathogenesis of atherosclerosis. Moreover, the restoration of endothelial function by supplementation of L-arginine also suggested that competitive inhibition of NO synthase pathway might be one of the important mechanisms.[13, 24] However, the relation between ADMA and CAD has not been well investigated. Recently, two studies have shown that plasma ADMA level is both an independent risk factor of cardiovascular mortality in patients undergoing hemodialysis and a predictor of acute coronary events in middle-aged men.[25, 26] In this study, we aimed to determine whether the plasma ADMA level and L-arginine/ADMA ratio (as an index of the bioavailability of plasma L-arginine) predict the presence or absence of CAD independently, and whether both parameters correlate with the extent and severity of coronary atherosclerosis as assessed by coronary angiography.

Methods

Study Patients

We prospectively enrolled 97 consecutive patients (81 men, 16 women, mean age 68.9 ± 8.2 years) with angina symptoms and positive exercise stress test, who were referred to this institute for coronary angiography. Patients with renal dysfunction (serum creatinine > 1.5 mg/dl), hepatic or thyroid disease, chronic or acute inflammation, cerebrovascular disease, or symptoms/signs suggestive of congestive heart failure were excluded. Patients with myocardial infarction or unstable angina in the previous 1 month were also excluded. The associated cardiovascular risk factors included age, systemic hypertension, hypercholesterolemia, smoking, and diabetes mellitus. Systemic hypertension was diagnosed if blood pressure was > 140/90 mmHg on two occasions or if the patients were taking antihypertensive drugs. Hypercholesterolemia was defined as fasting total cholesterol level > 200 mg/dl or if the patients were already taking lipid-lowering agents. Diabetes mellitus was diagnosed if there was a fasting glucose level > 126 mg/dl and/or plasma glucose level of > 200 mg/dl 2 h after glucose administration, or if the patient was taking oral hypoglycemic agents or receiving insulin injection therapy for blood glucose control at the present time. Since all female patients were post menopause without receiving hormone replacement therapy, gender was not considered to be a major risk factor.

Study Protocol

All medications were withdrawn for 12 h. Cigarette smoking and beverages containing alcohol or caffeine were avoided for at least 12 h. After 12 h overnight fasting, blood samples were collected for measurement of plasma ADMA, L-arginine, lipid profile, creatinine, and fasting blood glucose. Symmetrical dimethylarginine (SDMA), the biologically inactive stereoisomer of ADMA, was also determined. Informed consent was obtained from all patients. Coronary angiography was performed by standard procedure. According to the results of coronary angiography, the study population was divided into two groups: Group 1 consisted of patients with smooth coronary arteries (22 patients) and mild CAD (< 50% stenosis in major coronary arteries, 24 patients). Group 2 consisted of patients with significant stenosis of coronary artery (≥ 50% stenosis in at least one major coronary artery, 51 patients).

Determination of Plasma L-Arginine and Asymmetrical Dimethylarginine Concentrations

The blood samples were centrifuged at 3000 rpm for 10 min at 4˚C immediately after collection. The plasma samples were then kept frozen at 2 70˚C until analysis. Plasma L-arginine and ADMA concentrations were determined by high-performance liquid chromatography (HPLC) using precolumn derivatization with o-phthaldialdehyde (OPA).[14] Briefly, plasma samples and standards were extracted on solid-phase extraction cartridges (Sep-Pak, Accell Plus CM, Waters, Milford, Mass., USA). High-performance liquid chromatography was carried out on a liquid chromatography system (Model 470, Waters). Samples and standards were incubated for exactly 3 min with the OPA reagent (5.4 mg/ml OPA in 0.4 M borate buffer, pH 10.0, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC system. Samples were eluted from the column with 0.96% citric acid/methanol 68.5/31.5 (v/v), pH 6.8, at flow rate of 1 ml/min. The OPA derivatives of L-arginine, ADMA, and SDMA were separated on a C_6H_5 column (Microsorb-MV™, Varian, Walnut Creek, Calif., USA), and the fluorescence detector was set for an excitation wavelength of 340 nm and an emission wavelength of 455 nm. The recovery rate for ADMA was > 90%, and the within- and between-assay variation coefficients were not more than 7 and 8%, respectively. Fasting serum creatinine, total and high-density lipoprotein (HDL) cholesterol, triglycerides, and blood sugar levels were determined by an autoanalyzer (Model 7600-310, Hitachi, Tokyo, Japan). Low-density lipoprotein (LDL) cholesterol level was calculated according to the Friedewald formula.

Evaluation of the Extent and Severity of Coronary Atherosclerosis

The extent and severity of coronary atherosclerosis were assessed by a "coronary atherosclerotic score" developed by Azar *et al.*[27] In brief, the coronary artery tree was divided into nine segments: the left main coronary artery; the proximal,

mid, and distal left anterior descending artery; the proximal and distal circumflex artery; the proximal and mid-right coronary artery; and the posterior descending artery. Each of these segments was scored from 0 to 3 depending on the most severe diameter stenosis according to the following system: 0 = normal, 1 = stenosis between 1 and 49%, 2 = stenosis between 50 and 99%, 3 = total stenosis, with each of the segments distal to the occlusion arbitrarily given a score of 1. The "coronary atherosclerotic score" was generated as the sum of the scores in all segments. Moreover, the numbers of coronary arteries with stenosis ≥ 50% were also calculated in each patient with significant CAD as "coronary vessel score." All angiograms were reviewed by experienced cardiologists blinded to the levels of L-arginine and dimethylarginine.

Statistical Analysis

All parametric values were presented as mean ± standard deviation. Univariate analysis was performed using Student's *t*-test for parametric continuous data and chi-square test or Fisher's exact test for categorical data. Parameters with p values < 0.25 were entered into multivariate stepwise logistic regression analysis to evaluate their independent effect on the risk of CAD. The odds ratios (OR) and 95% confidence intervals (CI) were calculated. Receiver operating characteristics (ROC) analysis was performed to estimate the potential of plasma ADMA and L-arginine/ADMA ratio to discriminate between patients with and without significant CAD. The predictive accuracy of each parameter was calculated as the area under the ROC curve. Linear regression analysis was used for correlation between the extent of coronary atherosclerosis and levels of ADMA as well as plasma L-arginine/ADMA ratio, respectively. A p value of < 0.05 was considered to be statistically significant. The Statistical Package for Social Sciences 10.0 (SPSS Inc., Chicago, Ill., USA) software was used for statistical analysis.

Results

Patient Characteristics

The clinical characteristics of study patients are presented in Table I. The mean ages and gender of both groups were similar; and the distributions of systemic hypertension, diabetes mellitus, current smoking status, hypercholesterolemia, and use of medications showed no difference between both groups.

Plasma L-Arginine and Asymmetrical Dimethylarginine Level

The plasma concentrations of L-arginine in both groups were similar (82.3 ± 15.9 vs. 83.8 ± 17.6 μM, p = 0.65). The plasma concentrations of ADMA in Group 2 patients were significantly higher than those of Group 1 patients (0.66 ± 0.17 vs. 0.44 ± 0.09 μM, p < 0.001) (Fig. 1A). We used plasma L-arginine/ADMA ratio as an index of the bioavailability of L-arginine in the plasma,[13, 14] and elevation of plasma ADMA level resulted in a significant lower plasma L-arginine/ADMA ratio in patients with significant CAD (Group 1 vs. Group 2: 194.0 ± 55.3 vs. 136.7 ± 50.3, p < 0.001) (Fig. 1B). Moreover, in patients of Group 1, the plasma ADMA level in those with mild CAD were also significantly higher than in those with normal coronary artery (0.47 ± 0.09 vs. 0.41 ± 0.09 μM, p = 0.02). On the other hand, there was no significant difference in plasma SDMA level between both groups (Group 1 vs. Group 2: 0.68 ± 0.32 vs. 0.62 ± 0.31 μM, p = 0.32). The plasma ADMA level did not correlate significantly with total plasma cholesterol/HDL cholesterol/LDL

TABLE I Clinical characteristics of study subjects

	Group 1 (n = 46)	Group 2 (n = 51)	p Values
Age (years)	68 ± 8	70 ± 9	0.14
Gender (M/F)	36/10	45/6	0.27
Systemic hypertension (%)	32 (69.6)	34 (66.7)	0.83
Hypercholesterolemia (%)	20 (43.5)	24 (47.1)	0.84
Current smoker (%)	13 (28.3)	19 (19.6)	0.39
Diabetes mellitus (%)	8 (17.4)	17 (33.3)	0.10
Total cholesterol (mg/dl)	194 ± 34	184 ± 30	0.12
HDL-cholesterol (mg/dl)	42 ± 10	38 ± 10	0.06
LDL-cholesterol (mg/dl)	121 ± 27	115 ± 26	0.33
Triglyceride (mg/dl)	154 ± 117	156 ± 93	0.93
Medications			
Aspirin (%)	29 (63.0)	41 (80.4)	0.07
Calcium antagonists (%)	22 (47.8)	26 (51.0)	0.84
ACE inhibitors (%)	16 (34.8)	21 (41.2)	0.54
Beta blockers (%)	15 (32.6)	17 (33.3)	1.00
Lipid-lowering agents (%)	3 (6.5)	7 (13.7)	0.32
Coronary atherosclerotic score	1.0 ± 1.3	6.4 ± 2.4	
Coronary vessel score	0	1.7 ± 0.7	

Abbreviations: M = male, F = female, HDL = high-density lipoprotein cholesterol, LDL = low-density lipoprotein cholesterol, ACE = angiotensin-converting enzyme.

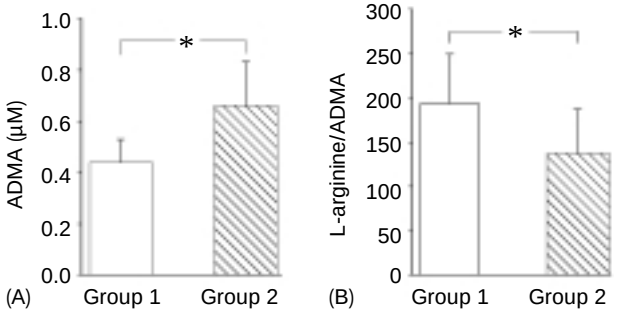

FIG. 1 (A) Plasma levels of asymmetrical dimethylarginine (ADMA) in Group 1 and 2 patients. Data are mean ± standard deviation (SD). * = p < 0.001. (B) Plasma L-arginine/ADMA ratio in Group 1 and 2 patients. Data are mean ± SD. * = p < 0.001.

cholesterol as well as triglyceride levels; neither did the plasma L-arginine/ADMA ratio.

Multivariate Analysis of Risk Factors for Coronary Artery Disease

In a multivariate stepwise logistic regression analysis of potential risk factors for CAD, plasma ADMA and HDL cholesterol levels were identified as significant independent predictors for CAD, and so was the plasma L-arginine/ADMA ratio (plasma ADMA level: $p < 0.001$; plasma L-arginine/ADMA ratio: $p < 0.001$). To test the predictive discrimination of plasma ADMA level and the ratio of plasma L-arginine/ADMA in patients with and without significant CAD, ROC curve analysis was used. The plasma ADMA level and plasma L-arginine/ADMA ratio yielded an area under curve of 0.87 ± 0.04 (mean ± standard error) and 0.81 ± 0.05, respectively, for separation of patients with and without significant CAD (Fig. 2). Both curves shown in Figure 2 were significantly different from a random distribution, that is, an area under curve of 0.5. By using a plasma ADMA level of 0.54 μM as the cutpoint, the analysis would yield a 78.4% sensitivity and 89.1% specificity; and the positive and negative predictive values would be 88.9 and 78.8%, respectively. Moreover, the OR of risk for CAD was 36.8 ($p < 0.001$) for patients with plasma ADMA level > 0.54 μM compared with those with lower levels (Table II). Similarly, using a plasma L-arginine/ADMA ratio of 140 as the cutpoint, lower L-arginine/ADMA ratio was significantly associated with increased risk of CAD (OR = 22.3, $p < 0.001$; Table II), with sensitivity 66.7%, specificity 89.1%, positive predictive value 87.2%, and negative predictive value 70.7%.

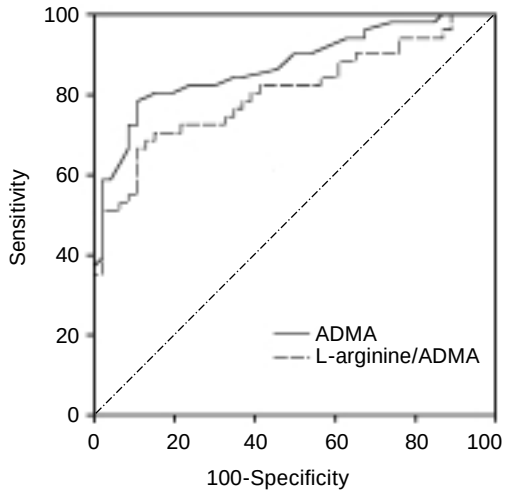

FIG. 2 Receiver operating characteristic curve (ROC) analysis for separation of patients with and without significant coronary artery disease. The areas under the ROC curve was 0.87 ± 0.04 (mean ± standard error) for plasma asymmetrical dimethylarginine (ADMA) level and 0.81 ± 0.05 for plasma L-arginine/ADMA ratio. Each was significantly different from a random distribution (-·-·-·-).

Correlation of Severity of Coronary Atherosclerosis with Plasma Asymmetrical Dimethylarginine (ADMA) Level and L-Arginine/ADMA Ratio

The mean coronary atherosclerotic scores in Group 2 patients were higher than those in Group 1 patients (6.4 ± 2.4 vs. 1.0 ± 1.3, $p < 0.001$; Table I). There was significant positive and negative correlation between coronary atherosclerotic score and plasma ADMA level and plasma L-arginine/ADMA ratio, respectively (plasma ADMA level: $r = 0.518$, $p < 0.001$; plasma L-arginine/ADMA ratio: $r = 2\ 0.430$, $p < 0.001$) (Fig. 3 A, B). On the other hand, the mean coronary vessel score was 1.7 ± 0.7 in Group 2 patients, with 23 patients having single-vessel disease, 22 patients having double-vessel disease, and the remaining 6 patients having triple-vessel disease. Similarly, there was significant positive and negative correlation between coronary vessel score and plasma ADMA level and plasma L-arginine/ADMA ratio, respectively (plasma ADMA level: $r = 0.527$, $p < 0.001$; plasma L-arginine/ADMA ratio: $r = -0.445$, $p < 0.001$) (Fig. 3 C, D).

Discussion

In the present study, the plasma level of circulating endogenous NO synthase inhibitor, ADMA, as well as the ratio of plasma L-arginine/ADMA, were helpful in predicting the presence or absence of significant coronary artery stenosis assessed by coronary angiography. In addition, plasma ADMA level and plasma L-arginine/ADMA ratio correlated significantly with the extent and severity of coronary atherosclerosis. Our findings suggest that plasma ADMA level may be a novel marker of significant CAD.

Endothelium-derived NO is synthesized from L-arginine by NO synthase and plays a central role in normal cardiovascular homeostasis. Impaired NO bioavailability causes endothelial dysfunction and not only contributes to the initiation and progression of atherosclerosis but is also associated with long-term risk of cardiovascular events in patients with CAD.[28, 29] The underlying mechanism of derangement of the L-arginine-NO pathway is still not clear, and reduced availability of L-arginine as a result of the elevation of endogenous NO synthase inhibitor may be one of the mechanisms, since several studies reported the restoration of endothelial dysfunc-

TABLE II Odds ratio (95% confidence interval) for the association between plasma asymmetrical dimethylarginine (ADMA) level,[a] plasma L-arginine/ADMA ratio,[b] and risk for coronary artery disease

	Odds ratio (95% CI)	p Values
ADMA, > 0.54 μM	36.8 (10.1–134.5)	< 0.001
HDL, continuous [a]	0.93 (0.88–0.99)	0.02
L-arginine/ADMA ratio, < 140	22.3 (6.2–79.7)	< 0.001
HDL, continuous [b]	0.93 (0.88–0.98)	0.01

Abbreviations: CI = confidence interval, HDL = high-density lipoprotein cholesterol.

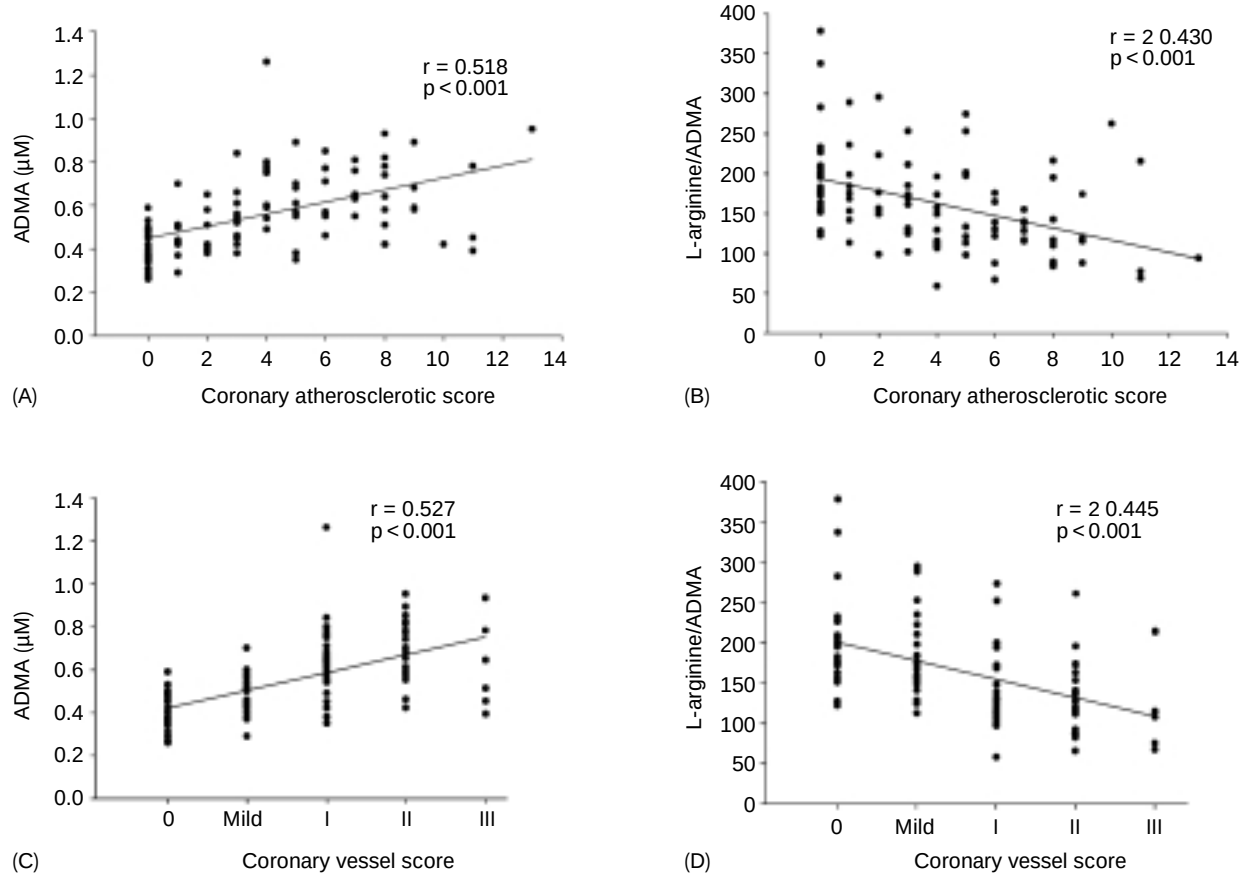

FIG. 3 Correlations between plasma asymmetrical dimethylarginine (ADMA) level, plasma L-arginine/ADMA ratio, and coronary atherosclerotic score (A, B); and between plasma ADMA level, plasma L-arginine/ADMA ratio, and coronary vessel score (C, D).

tion by L-arginine supplementation.[24, 30, 31] Asymmetrical dimethylarginine, identified in 1970, is generated by methylation of arginine residues in protein and is released as the protein is hydrolyzed.[18] It has been characterized as an endogenous competitive NO synthase inhibitor,[16] and can inhibit endothelial NO elaboration significantly.[17] Vallance *et al.* showed that intra-arterial infusion of ADMA caused a dose-dependent fall in forearm blood-flow.[17] Böger *et al.* demonstrated that, in young patients with hypercholesterolemia, elevated plasma ADMA concentrations were associated with impaired endothelium-dependent vasodilatation.[13] In patients with peripheral arterial occlusive disease, they also reported that there was a significantly progressive increase in plasma ADMA concentrations related to the severity of peripheral arterial occlusive disease.[14] Miyazaki *et al.* reported that, in patients without any symptoms of CAD or peripheral arterial occlusive disease, plasma ADMA concentrations correlated positively with the risk factors for atherosclerosis, including age, glucose intolerance, and mean arterial pressure, and also correlated well with carotid intima-media thickness, an index of early atherosclerosis.[15] Moreover, Böger *et al*. recently demonstrated that, also by inhibition of NO formation, ADMA stimulated the secretion of monocyte chemotactic protein-1, increased endothelial superoxide radical formation, and potentiated monocyte adhesion, which suggested that ADMA might be a proatherogenic molecule.[32] Nevertheless, few data are available on the relation between plasma ADMA level and coronary atherosclerosis. Our study showed for the first time that the plasma ADMA level was an independent predictor of significant CAD and, in addition, a good marker of atherosclerotic burden as assessed by coronary angiography. By using the plasma ADMA concentration of 0.54 μM as the cutpoint, patients are much more likely to have significant CAD (OR = 36.8), with sensitivity 78.4% and specificity 89.1%. Furthermore, the role of ADMA in risk for CAD was recently emphasized by two studies. Valkonen *et al.* reported recently that elevated ADMA level (> 0.62 μM) is associated with an increased risk of acute coronary events among nonsmoking middle-aged men, especially those who have a previous history of CAD.[26] On the other hand, Zoccali *et al.* reported that, in 225 patients with end-stage renal disease, plasma ADMA level is a strong and independent predictor of overall mortality and cardiovascular events.[25] These observations suggested that ADMA may be a novel risk factor of CAD. Finally, the finding of lower plasma L-arginine/ADMA ratio in patients with significant CAD may also provide a possible explanation for the beneficial effects of

L-arginine supplementation in these patients,[13, 24] although this remained controversial.[33] Nevertheless, our study population was rather modest in size, and confirmation by other investigators with more subjects is mandatory.

The mechanism of elevation of plasma ADMA levels in our patients with CAD is not clear. Plasma ADMA is derived from degradation of methylated protein and eliminated from the body by renal excretion and by metabolism by the enzyme dimethylarginine dimethylaminohydrolase (DDAH).[18] Patients with renal insufficiency (serum creatinine > 1.5 mg/dl) were excluded from this study. Furthermore, as expected in this study, plasma levels of SDMA, the inactive stereoisomer of ADMA and neither an inhibitor of NO synthase nor a substrate of DDAH, were not different in patients with and without CAD. Since plasma SDMA is mainly eliminated by the kidneys, elevated plasma ADMA level in our patients with CAD was probably caused by a mechanism other than impaired renal excretion, most likely due to suppression of DDAH activity. MacAllister *et al.* showed that inhibition of DDAH blocked ADMA degradation and caused vasoconstriction in isolated vascular rings.[34] A recent report also demonstrated that reduction of DDAH activity might account for increased production of ADMA by endothelium cell treated with oxidized LDL or cytokines.[35] The DDAH activity in patients with CAD deserves further investigation.

The plasma concentrations of ADMA in our study populations were much lower than those reported before,[13, 14, 22] but were similar to those reported by Miyazaki *et al.*, Valkonen *et al.*, and Teerlink *et al.*[15, 26, 36] The plasma ADMA level in our patients with significant CAD was also close to that reported by Usui *et al.* in Japanese (0.50 μM)[21] and Valkonen *et al.* in Finns (0.56 μM), respectively,[26] suggesting that there may be ethnic differences in plasma ADMA concentrations between peoples. We measured the concentrations of dimethylarginine by HPLC, which is a suitable method for differentiation between ADMA and SDMA. No association of SDMA with significant CAD also supports the accuracy of this assay. Although the plasma levels of ADMA in our patients were much lower than those required for physiologic or pathologic effects,[37] the intracellular ADMA level in endothelium may be much more concentrated, even 10-fold higher than the reported range for plasma values.[29, 35, 38] The intracellular levels of L-arginine and ADMA in patients with CAD remain to be determined.

In contrast to the report from Böger *et al.*, our data showed no relation between the plasma level of ADMA and hypercholesterolemia, which is consistent with the findings of Miyazaki *et al.*[15] This may be related to different ethnic and demographic characteristics (e.g., older age and more cardiovascular risk factors in our patients), as well as the relatively small sample size of our study population.

Conclusion

These results support the notion that plasma ADMA level may be a novel marker of coronary artery atherosclerosis. If further studies with larger populations confirm these findings, determinations of plasma ADMA levels and L-arginine/ADMA ratios may become useful in screening individuals who are at high risk of CAD and undergoing evaluation for suspected CAD.

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