Clinical Investigation and Reports

Endothelial Dysfunction Induced by Hyperhomocyst(e)inemia
Role of Asymmetric Dimethylarginine

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Background—Endothelial function is impaired by hyperhomocyst(e)inemia. We have previously shown that homocyst(e)ine (Hcy) inhibits NO production by cultured endothelial cells by causing the accumulation of asymmetric dimethylarginine (ADMA). The present study was designed to determine if the same mechanism is operative in humans.

Methods and Results—We studied 9 patients with documented peripheral arterial disease (6 men; 3 women; age, 64 ± 3 years), 9 age-matched individuals at risk for atherosclerosis (older adults; 9 men; age, 65 ± 1 years), and 5 young control subjects (younger adults; 5 men; age, 31 ± 1 years) without evidence of or risk factors for atherosclerosis. Endothelial function was measured by flow-mediated vasodilatation of the brachial artery before and 4 hours after a methionine-loading test (100 mg/kg body weight, administered orally). In addition, blood was drawn at both time points for measurements of Hcy and ADMA concentrations. Plasma Hcy increased after the methionine-loading test in each group (all, P < 0.001). Plasma ADMA levels rose in all subjects, from 0.9 ± 0.2 to 1.6 ± 0.2 μmol/L in younger adults, from 1.5 ± 0.2 to 3.0 ± 0.4 μmol/L in older adults, and from 1.8 ± 0.1 to 3.9 ± 0.3 μmol/L in peripheral arterial disease patients (all, P < 0.001). Flow-mediated vasodilatation was reduced from 13 ± 2% to 10 ± 1% in younger adults, from 6 ± 1% to 5 ± 1% in older adults, and from 7 ± 1% to 3 ± 1% in peripheral arterial disease patients (all, P < 0.001). Furthermore, we found positive correlations between plasma Hcy and ADMA concentrations (P = 0.03, r = 0.450), as well as ADMA and flow-mediated vasodilatation (P = 0.002, r = 0.623).

Conclusions—Our results suggest that experimental hyperhomocyst(e)inemia leads to accumulation of the endogenous NO synthase inhibitor ADMA, accompanied by varying degrees of endothelial dysfunction according to the preexisting state of cardiovascular health. (Circulation. 2003;108:933-938.)

Key Words: endothelium • dimethylarginine • atherosclerosis • nitric oxide • peripheral arterial disease

Hyperhomocyst(e)inemia has been purported to be a risk factor for cardiovascular disease.1–4 In the Physicians’ Health Study,1 modest increases in plasma homocyst(e)ine (Hcy) level conferred a 3-fold increased relative risk for myocardial infarction and mortality from cardiovascular disease. The investigators calculated that 7% of the myocardial infarctions in that study could be attributed to hyperhomocyst(e)inemia.

The mechanism by which hyperhomocyst(e)inemia increases cardiovascular mortality is still unclear. However, recent studies have suggested that an impairment of endothelial function by hyperhomocyst(e)ine may be responsible. Chronic elevation of plasma Hcy impairs endothelium-dependent, flow-mediated vasodilation (FMVD) of peripheral arteries.5,6 Acute increases of Hcy induced by oral methionine are also associated with a disturbance of endothelial vasodilator function.7 The mechanism for endothelial dysfunction in hyperhomocyst(e)inemia is controversial. However, administration of antioxidant vitamins can prevent the impairment of endothelial vasodilator function induced by experimental hyperhomocyst(e)inemia.8 This finding suggests that oxidative stress is involved in the effect of Hcy.

Endothelium-derived NO is a potent endogenous vasodilator and antiatherogenic agent.9 The synthesis of NO can be inhibited by naturally occurring analogues of the NO precursor L-arginine such as N⁶-monomethyl-L-arginine (LNMMA) or asymmetric dimethylarginine (ADMA).10 A preclinical trial has shown that plasma concentration of the endogenous NO synthase inhibitor ADMA is elevated in cynomolgus monkeys receiving a diet to induce hyperhomo-

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cyst(e)inemia. Accordingly, we hypothesized that the effect of Hcy on endothelial vasodilator function may be mediated by ADMA.

### Methods

#### Study Subjects

We examined the effects of experimental hyperhomocysteinemia on endothelial function and plasma ADMA in young healthy adults (YA group), patients with peripheral arterial disease (PAD), and age-matched older healthy adults (OA group). The study population consisted of 23 individuals who were assigned to 3 different groups: Group A consisted of 9 white patients from Stanford University Medical Center (3 women, 6 men; age, 64 ± 3 years) with documented PAD, class II according to Fontaine classification, and an ankle brachial index <0.9. Group B consisted of 9 healthy white patients without PAD, who were age-matched with the PAD patients (9 men; age, 65 ± 1 years) from the University Hospital of Graz, Austria. Group C consisted of 5 young healthy white individuals with no known risk factors for cardiac disease from the Stanford Medical Center staff (5 men; age, 31 ± 1 years). All participants were asked to withhold long-acting vasoactive medications for 24 hours before the study. Individuals with diabetes mellitus, renal insufficiency, hepatic failure, or ongoing infection or inflammation were excluded. The local ethics committees of Stanford University School of Medicine and the Medical Division of the University of Graz, Austria, approved the study.

#### Experimental Protocol

All subjects were asked to give a brief medical history and undergo a physical examination. Duplex ultrasonography to evaluate FMVD of the brachial artery was performed in a well-controlled environment after an overnight fast. Venous blood samples were obtained from the antecubital vein for Hcy and ADMA levels. Immediately after FMVD measurement, an oral methionine-loading test (MLT) was performed by administering L-methionine (100 mg/kg body weight; Ajinomoto Inc) mixed in apple juice (without supplemental B vitamins or antioxidants). Fours hours later, blood sampling and endothelial function studies were repeated. No nutrients or drinks other than water were allowed during the 4 hours between the 2 time points.

#### Measurements of Endothelial Function

Endothelium-dependent FMVD of the brachial artery is induced by the elaboration of endothelium-derived NO. Endothelium-dependent FMVD in response to reactive hyperemia and endothelium-independent, nitroglycerin-induced vasodilatation was evaluated noninvasively by use of high-resolution ultrasound before and 4 hours after the MLT. Ultrasound measurements were performed according to the method described by Celermajer et al. Briefly, with subjects in a supine position, at rest, and in a quiet temperature-controlled room (22°C to 25°C), B-mode scans of the right brachial artery were obtained in longitudinal sections 2 to 8 cm above the elbow by use of a 7.5-MHz linear array transducer and a Hewlett Packard 5500 duplex ultrasound machine. To ensure consistency of the image with serial scans, the transducer position was marked on the skin. The end-diastolic arterial diameter was measured in ECG-gated end-diastolic frames from 1 media-adventitia interface to the other at the clearest section. Reactive hyperemia was induced by inflating a pneumatic cuff to suprasystolic pressures at the level of the forearm, distal to the brachial artery. After 5 minutes, the cuff was deflated, resulting in a brief episode of reactive hyperemia. Measurements of brachial artery diameter were made 1 minute before application of the cuff and every 60 seconds after cuff deflation for 5 minutes. A second baseline measurement was taken after 5 minutes of recovery. Subsequently, sublingual nitroglycerin (0.4 mg) was administered, and brachial artery measurements were obtained after 5 minutes as described above. Images were recorded on super-VHS videotape for quantitative analysis.

#### Biochemical Measurements

Concentrations of ADMA and SDMA and L-arginine in plasma were measured by high-performance liquid chromatography with precol- umn derivatization with o-phthalaldehyde after solid-phase extraction, as described previously. The recovery rate for ADMA was 84%, and the intrasample variation was 2.5%. The detection limit of the assay was 0.05 μmol/L. Plasma samples for measurements of fasting and postmethionine total plasma Hcy concentrations were measured by high-performance liquid chromatography and electrochemical detection according to the method of Smolin and Schneider as described previously. Concentrations of total cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, serum creatinine, and fasting plasma glucose were measured by standard laboratory assays.

#### Calculation and Statistical Analyses

Results are reported as mean ± SEM. Group differences were examined by Statview software (SAS Institute Inc) by using 1-way ANOVA. Post hoc analyses were performed with Fisher’s protected least-significant difference tests. Paired t tests were used to compare values obtained before and after MLT. Pearson’s correlation coefficients were calculated between plasma ADMA concentration, total plasma Hcy, and FMVD. A value of P < 0.05 was considered statistically significant.

### Results

#### Baseline Characteristics

Clinical characteristics of the study participants in the 3 groups are shown in Tables 1 and 2. The age of the patients was significantly higher in the OA group (P < 0.0001 versus YA) and in PAD group (P < 0.0001 versus YA), and body mass index was significantly lower in the YA group (P = 0.02 versus OA; P < 0.05 versus PAD). Plasma creatinine, blood urea nitrogen, and fasting plasma glucose levels were comparable in all groups. However, systolic, diastolic, and mean arterial blood pressure, as well as total and LDL cholesterol, were significantly higher in the OA group compared with the YA and PAD groups. The difference in blood pressure between the PAD and OA groups may be due to hyperlipidemic and antihypertensive medications taken by PAD patients.

#### Plasma Hcy Levels

At baseline, total plasma Hcy was 9.1 ± 1.2 μmol/L in the YA group, 9.3 ± 0.6 μmol/L in the OA group (older individuals without PAD) (P = NS versus YA), and 12.4 ± 1.0 μmol/L in the PAD patients (P = 0.04 versus OA; P = 0.02 versus YA). As shown in Figure 1B, 4 hours after the methionine challenge, Hcy increased to 22.6 ± 3.7 μmol/L in the YA group, to 22.9 ± 2.4 μmol/L in the OA (P = NS versus YA) group, and to 32.5 ± 2.6 μmol/L in the PAD group (P = 0.03 versus YA; P = 0.01 versus OA). The increase of Hcy plasma concentrations was significant in all groups (P = 0.03 in YA; P < 0.0001 in OA; P < 0.0003 in PAD) (Figure 1A). The increase from baseline values to values obtained after methionine loading was 13.4 ± 4.2 μmol/L in the YA group.
13.7 ± 2.2 μmol/L in the OA group, and 20.1 ± 1.9 μmol/L in the PAD group (P = NS).

### Endothelial-Dependent and -Independent FMVD

Endothelium-dependent FMVD was 13.4 ± 2.2% in the YA group, 5.9 ± 0.9% in the OA group (P = 0.0004 versus YA), and 7.0 ± 0.7% in the PAD patients (P = 0.002 versus YA; P = NS versus OA). Four hours after methionine loading, endothelium-dependent FMVD was significantly reduced in each group (P < 0.05 in YA; P = 0.0004 in OA; P < 0.0001 in PAD). Moreover, a significant difference between endothelium-dependent FMVD after MLT was found between groups: FMVD declined to 10.0 ± 0.5% in the YA group, 4.6 ± 0.9% in the OA group (P = 0.0002 versus YA), and 2.8 ± 0.6% in the PAD group (P < 0.0001 versus YA; P < 0.05 versus OA) (Figure 1A).

Endothelium-independent, nitroglycerin-induced vasodilation was 17.3 ± 2.2% in the YA group, 14.9 ± 1.8% in the OA group, and 15.8 ± 1.7% in the PAD group at baseline (P = NS). Four hours after the methionine load, endothelium-independent vasodilation was 15.7 ± 1.0% in the YA group, 14.7 ± 1.6% in the OA group, and 15.7 ± 2.4% in the PAD group (all changes from baseline measurement, P = NS) Essentially, nitroglycerin-induced vasodilation did not change within 4 hours after methionine in any of the groups. No differences were found between measurements of the first and the second baseline diameters of the brachial artery. Furthermore, no differences were detected between baseline artery diameters of each group or between baseline diameters at fasting and 4 hours after the MLT (data not shown).

### l-Arginine and ADMA Plasma Concentrations

Plasma ADMA concentrations were significantly elevated in the fasting state in older individuals without PAD (P < 0.05 versus YA) and in PAD patients (P = 0.009 versus YA). With the methionine challenge, plasma ADMA concentrations increased significantly in all 3 groups: from 1.0 ± 0.2 μmol/L to 1.6 ± 0.2 μmol/L in the YA group (P < 0.0001), from 1.5 ± 0.2 μmol/L to 3.0 ± 0.40 μmol/L in the OA group (P < 0.0001), and from 1.8 ± 0.1 μmol/L to 3.8 ± 0.3 μmol/L in the PAD patients (P < 0.0001). Also, 4 hours after methionine loading, significant differences were found between ADMA levels of each group (OA versus YA, P = 0.02; PAD versus YA, P = 0.0002; and PAD versus OA, P < 0.05). The change in ADMA levels during methionine loading was significantly higher (Figure 1C) in the OA group (1.5 ± 0.2 μmol/L) and in PAD patients (2.0 ± 0.2 μmol/L) than in the YA group (0.7 ± 0.1 μmol/L) (P = 0.002). l-Arginine and ADMA concentrations were comparable in all 3 groups before and 4 hours after the MLT (P = NS).

### Correlations Between Parameters

Univariate and multiple regression analyses were performed to further evaluate the relationship between Hcy, ADMA, and endothelial function. A highly significant correlation was found between plasma ADMA and total Hcy plasma concentrations (P < 0.0001; r = 0.71) (Figure 2). An association between ADMA and Hcy was also observed in the data set obtained before (P = 0.01; r = 0.52) and after MLT (P = 0.3; r = 0.45). There was a strong inverse correlation between ADMA and
endothelium-dependent FMVD before \( (P<0.001; r=0.62) \) and after MLT \( (P<0.0001; r=0.76) \). There was no association between FMVD and Hcy before \( (P=NS; r=0.19) \) or after MLT \( (P=NS; r=0.23) \).

**Discussion**

**Major Findings**

We found that experimentally induced hyperhomocyst(e)ine-mia increases plasma ADMA, an effect that is temporally related to a decline in endothelial vasodilator function. Of note, there is a strong inverse correlation between plasma ADMA and FMVD. There was also a correlation between plasma ADMA and total plasma Hcy levels. By contrast, the correlation between Hcy and FMVD did not reach significance. Finally, endothelium-independent vasodilation to nitroglycerin was not affected by acute elevations in plasma Hcy. These findings are consistent with the hypothesis that the effects of Hcy on endothelial vasodilator function are mediated by ADMA.

**Mechanisms of Hcy-Induced Endothelial Dysfunction**

Previous researchers have suggested that the effects of Hcy are related to its tendency to form disulfide bonds and to generate oxygen-derived free radicals.\(^{16}\) In this paradigm, the oxygen-derived free radicals generated by Hcy react with endothelium-derived NO to reduce its bioactivity. We have suggested an alternate mechanism whereby NO synthesis is inhibited. Both mechanisms may be operative. Furthermore, they may be linked. This linkage has been suggested by observations from our group with regard to oxidative stress and ADMA metabolism.

There is a relationship between ADMA accumulation and oxidative stress. ADMA is derived from the breakdown of proteins that contain methylated arginine residues, a process that occurs in all cell types that have been examined. The methylated arginine residues are excreted in the urine or, in the case of ADMA and L-NMMA, are metabolized by the enzyme dimethylarginine dimethylaminohydrolase (DDAH).\(^{17}\) The activity of DDAH appears to be critical in regulating ADMA levels. In isolated vascular rings, inhibition of DDAH activity results in gradual vasoconstriction.\(^{17}\) This vasoconstriction is reversed by the addition of L-arginine to the medium. These results suggest that ADMA is constantly being produced during
normal protein turnover, and DDAH is essential in preventing accumulation of ADMA.

We have shown that the accumulation of ADMA in a number of metabolic disorders (including hyperhomocyst(e)einemia) is related to oxidative stress. ADMA accumulates under these conditions because the activity of DDAH is impaired by oxidation. The sensitivity of DDAH to oxidative stress is conferred by a sulfhydryl moiety in the catalytic site that is required for enzyme activity. Modification of this sulfhydryl moiety alters enzyme activity. We have previously shown that Hcy mounts an oxidative attack on DDAH, forming a disulfide bond with the sulfhydryl group in the catalytic site. Endothelial DDAH activity is reduced by Hcy; furthermore, Hcy binds directly to recombinant DDAH to inhibit its activity. In addition to its vulnerability to oxidative stress, this sulfhydryl moiety can be nitrosylated, which provides a mechanism for a reversible form of DDAH inhibition. However, in the present study, we have not determined if this latter mechanism is operative in humans because we did not directly measure oxidative stress or DDAH activity.

ADMA Mediates Endothelial Dysfunction Induced by Risk Factors

Although there was a strong inverse correlation between plasma ADMA and FMVD, no such correlation was observed between Hcy and FMVD. It is possible that this lack of correlation was a type 2 statistical error secondary to the small size of our study population. Alternatively, if the effect of Hcy is mediated by its impairment of DDAH activity, it is quite possible that there is substantial individual heterogeneity in the ability of Hcy to induce oxidative stress and impair DDAH activity. In this case, it would be expected that the elevation in plasma ADMA would be better correlated to endothelial vasodilator dysfunction.

It has previously been shown that cardiovascular risk factors (such as hypercholesterolemia, hypertriglyceridemia, hypertension, hyperglycemia, insulin resistance, renal insufficiency, and aging) are associated with increased plasma levels of ADMA. In each of these disorders, there is an impairment of endothelial vasodilator function that may be secondary to the elevation in plasma ADMA. In the present study, the PAD patients had greater risk factor burden than did the other 2 groups. Indeed, there was a tendency for the plasma ADMA levels to be higher, and the FMVD to be more attenuated, in the PAD patients. Nevertheless, methionine administration to young or old subjects with or without PAD caused qualitatively similar increases in plasma levels of Hcy and ADMA, as well as similar declines in endothelial vasodilator function. This observation indicates that the mechanism of methionine-induced endothelial dysfunction is likely to be similar in all 3 groups.

The inhibition of NO synthase activity by ADMA may represent a common pathway by which cardiovascular risk factors initiate and accelerate atherosclerosis. Antithromogenic properties of NO include its ability to inhibit platelet adhesion and aggregation, to reduce the expression of endothelial adhesion molecules and chemokines, to attenuate monocyte adherence and infiltration, to suppress myointimal hyperplasia, and to reduce oxidative stress. Indeed, pharmacological or genetic inhibition of NO synthase activity accelerates atherosclerosis whereas enhancement of vascular NO synthase activity reduces vascular lesion formation. Extrapolating from these data, it seems reasonable to surmise that by inhibiting endothelial NO synthesis, ADMA may contribute to the initiation and/or acceleration of atherosclerosis. In support of this hypothesis, recent data indicate that plasma levels of ADMA are predictive of cardiovascular mortality. In patients with coronary artery disease or in those with renal insufficiency, plasma ADMA levels are an independent predictor of cardiovascular events. Accordingly, the present investigation supports the notion that the effect of Hcy to increase cardiovascular mortality may be mediated in part by ADMA.

Summary

To conclude, we have found that experimental hyperhomocyst(e)einemia is associated with an endothelial vasodilator dysfunction that is temporally related to, and quantitatively correlated with, elevations in plasma ADMA. ADMA is an endogenous inhibitor of the NO synthase pathway, and it may mediate the adverse effects of Hcy and other risk factors on endothelial function.

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