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Asymmetric Dimethylarginine Increases Mononuclear Cell Adhesiveness in Hypercholesterolemic Humans

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Abstract—Asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase, is elevated in hypercholesterolemia. This study was designed to determine the role of ADMA in the increased mononuclear cell adhesiveness observed in human hypercholesterolemia. In patient studies, plasma ADMA levels were determined by high-performance liquid chromatography. Functional mononuclear leukocyte adhesion assays were performed in parallel, and flow cytometry was used to characterize bound monocytes and T lymphocytes. Hypercholesterolemic patients were then placed on an oral L-arginine regimen of 14 or 21 g/d and studied over 12 weeks. In cell culture studies, bovine aortic endothelial cells were incubated with varied concentrations of ADMA. Monocytoid cells were cocultured with these bovine aortic endothelial cells, and their adhesiveness was assessed by use of a binding assay. Flow cytometry was used to quantify adhesion molecule expression. Plasma ADMA levels and adhesiveness of mononuclear cells (specifically, monocytes and T lymphocytes) were elevated in hypercholesterolemic patients. Adhesiveness was inversely correlated with the plasma L-arginine/ADMA ratio. Oral administration of L-arginine normalized plasma L-arginine/ADMA ratios and attenuated monocyte and T-lymphocyte adhesiveness. ADMA had no direct effect on the adhesiveness of mononuclear cells. However, monocytes became hyperadhesive when cocultured with ADMA-exposed endothelial cells. In human hypercholesterolemia, the plasma L-arginine/ADMA ratio is inversely correlated with mononuclear cell adhesiveness. Restoration of the L-arginine/ADMA ratio to control levels normalizes mononuclear cell adhesiveness. Our studies suggest that the elaboration of endothelium-derived nitric oxide affects the behavior of circulating T lymphocytes and monocytes. (Arterioscler Thromb Vasc Biol. 2000;20:1040-1046.)

Key Words: L-arginine ■ atherosclerosis ■ monocytes ■ endothelium ■ T lymphocytes

Monocytes and T lymphocytes are the predominant inflammatory cells found in atherosclerotic plaques. Indeed, the adhesion of mononuclear cells to the endothelium is a key initial event in atherogenesis that precedes the formation of fatty streaks.1-3 In hypercholesterolemic (HC) humans, peripheral blood mononuclear cells exhibit increased adhesiveness for endothelial cells in ex vivo adhesion assays.4,5 Differences in monocyte surface markers have been detected by flow cytometry between HC and normocholesterolemic (NC) humans, although the functional significance is unclear.6 Molecular signaling mechanisms for the altered behavior of mononuclear cells are likely multifactorial and may involve inflammatory cytokines,7 LDL,8,9 platelet-activating factor,10 circulating soluble adhesion molecules,11 and reduced bioactivity of endothelium-derived nitric oxide (NO).

NO has been shown to modulate the behavior of circulating blood elements. In vivo, NO inhibits leukocyte adherence in the early stages of hypercholesterolemia in the rat.12 Furthermore, endothelium-derived NO is able to increase cGMP and reduce the ability of platelets to aggregate as they pass through the microvasculature of the rabbit heart.13 These observations suggest that endothelium-derived NO plays an important role as a modulator of leukocyte and platelet function. Changes in the production or bioactivity of endothelium-derived NO can thus be expected to modulate the adhesiveness of circulating blood cells.

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of NO synthase (NOS) formed by posttranslational methylation of arginine residues,14-16 followed by hydrolysis of methylated protein.17 We have recently reported that plasma ADMA levels are doubled in HC humans. Increased ADMA levels were inversely related to the biosynthesis of NO, as measured by flow-mediated vasodilation of the brachial artery.18 Because NO can modulate the behavior of circulating blood elements and suppress the adhesive behavior of monocytes in vitro,19 inhibition of endothelial NOS by ADMA may contribute to the enhanced adhesiveness of circulating mononuclear cells that is observed in hypercholesterolemia. Accordingly, the present study was designed to determine the contribution of plasma ADMA levels to the increased adhesiveness of mononuclear
cells in human hypercholesterolemia. We hypothesized that by inhibiting endothelial NOS, ADMA can increase mononuclear cell adhesiveness. We further hypothesized that chronic L-arginine administration could reverse the effect of elevated ADMA levels.

Methods

Subjects
HC study subjects (HC group, n = 24; 9 male, 15 female) were recruited from the Stanford University Preventive Medicine and Vascular Medicine clinics. Volunteers with a previous history of cardiovascular disease, diabetes, hypertension, renal disease, and liver disease were excluded. All subjects, including NC controls (NC group, n = 18; 5 male, 13 female), were nonsmokers. Those deemed suitable for our studies had a previous history of hypercholesterolemia and elevated total cholesterol levels >240 mg/dL and/or elevated LDL levels >160 mg/dL. None of the subjects was taking antioxidant or lipid-lowering medication. HC subjects were age-matched to NC subjects (45.0 ± 2.7 and 41.9 ± 2.6 years, respectively; P = NS). A subset of these HC subjects (n = 10) was subsequently placed on a daily regimen of 14 or 21 g/d L-arginine hydrochloride (3 times daily, Tyson, Inc) and studied at baseline, at 2 to 3 weeks, at 4 to 6 weeks, and at 10 to 12 weeks. Compliance with the treatment regimen was monitored by the Medication Event Monitoring System (MEMS cap, APREX Inc), which consists of a bottle cap equipped with a sensor and computer chip to record bottle openings. The study protocol was approved by the Stanford University Review Board for Human Studies, and each subject gave written informed consent.

Biochemical Studies
Venous blood samples (10 mL) were collected in Vacutainer-EDTA tubes (Becton Dickinson Inc) for biochemical analyses. Fasting lipid analyses were performed by indirect β quantification for total cholesterol, HDL cholesterol, and triglycerides by use of an Abbott Spectrum II autoanalyzer. LDL levels were calculated according to the Friedwald formula. Other biochemical parameters were assessed, including measures of hepatic function (serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, alkaline phosphatase, and albumin), renal function (blood urea nitrogen and creatinine), and endocrine function (growth hormone and insulin). These studies were performed in the Stanford University Hospital Laboratory with the use of standard clinical laboratory methods.

Arginine and Dimethylarginine Determination
Plasma concentrations of L-arginine, N°,N°-dimethylarginine (ADMA), and N°,N°-dimethylarginine (symmetric dimethylarginine [SDMA]) were measured by high-performance liquid chromatography (HPLC) after column derivatization with o-phthalaldialdehyde (OPA) with use of a modification of a previously described method. Briefly, 10 μmol/L L-homoarginine (Sigma Chemical Co) 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). The recovery rates were 83 ± 4%. The eluates were dried over nitrogen and resuspended in double-distilled water for HPLC analysis. HPLC was carried out on a computer-controlled Varian Star chromatography system consisting of a ternary gradient HPLC pump (Varian 9010), an automatic injector with sample-reagent mixing capabilities (Varian 9050), and a fluorescence detector (Varian Fluorochrome 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 × 4.4-mm-ID 7-μm Nucleosil phenyl column (Supelco) with the fluorescence detector set at 340-nm excitation and 450-nm emission. 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. The variability of the method was <7%, and the detection limit was 0.15 μmol/L.

Peripheral Blood Mononuclear Cell Isolation
In parallel with biochemical studies, additional venous blood samples (40 mL) were collected in Vacutainer-ACD tubes (Becton Dickinson Inc) from HC and NC subjects within 1 hour of each other for adhesion studies. Peripheral blood mononuclear cells were isolated from study subjects by using Ficoll-Hypaque density gradient centrifugation. Peripheral venous blood was centrifuged at 200g for 10 minutes. Plasma was collected and stored at −20°C for HPLC analysis of L-arginine and dimethylarginines as described above. Remaining blood cells were resuspended to their original volume in HBSS (Irvine Scientific, Inc) supplemented with 5 mmol/L EDTA (Sigma), yielding HBSS-EDTA, and washed twice at 200g. Samples were then centrifuged at 1500g, and the buffy coats were removed. Buffy coats were resuspended to 5 mL in HBSS-EDTA, carefully layered over 5 mL Ficoll-Hypaque 1077 (Sigma), and centrifuged at 400g for 30 minutes at room temperature. Peripheral blood mononuclear cells were aspirated from the interface and washed 3 times at 400g with HBSS-EDTA. Cells were then resuspended to a final concentration of 3.5 × 10^6/mL in binding buffer (HBSS, 2 mmol/L CaCl₂, 2 mmol/L MgCl₂, and 20 mmol/L HEPES; Irvine Scientific, Inc) for adhesion studies. Cell viability assessed by trypan blue (Sigma) exclusion was always >98%. The isolation procedure and all subsequent experiments were carried out at room temperature.

Cell Culture
Our adhesion studies used the transformed human umbilical vein endothelial cell line ECV304 (American Type Culture Collection). This cell line retains many endothelial characteristics, including the synthesis of angiotensin-converting enzyme, prostat glandin I₂, and thromboxane A₂. ECV304 cells express endothelial adhesion molecules, including intercellular adhesion molecule-1 and lymphocyte function–associated antigen-3. The expression of these adhesion molecules is upregulated by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide stimulation. Major histocompatibility complex class I antigen is present, but not major histocompatibility complex class II antigen, E-selectin, or P-selectin at the protein level. Cells were cultured in medium M199 (Applied Scientific, Inc) containing 10% FCS (GIBCO-BRL). Two days before adhesion studies, cells were passaged into 35-mm wells on 6-well culture plates (Nunc, Inc). Four hours before use, confluent endothelial monolayers were stimulated with human recombinant tumor necrosis factor-α (Sigma) at a final concentration of 200 U/mL. Thirty minutes before the adhesion assay, endothelial cells were washed with binding buffer.

Human Mononuclear Cell Adhesion Assay
To assess the adhesiveness of mononuclear cells, we modified a nonstatic adhesion assay previously described. Briefly, 7 × 10^6 peripheral blood mononuclear cells (~5% monocytes and 70% T lymphocytes, as determined by flow cytometry) were added to the endothelial monolayer in each well and incubated at room temperature for 30 minutes on a rocking platform (Research Products International Corp). Each well was turned 90° at 15 minutes to allow uniform distribution of the peripheral blood mononuclear cells across the endothelial monolayer. Nonadherent cells were carefully washed off with binding buffer, and adherent cells were counted immediately by microscopy. Thirty-five high-power fields were counted for each 35-mm well using a computer-aided image analysis system (Image Analyst, Automatix Corp). Adhesion assays for each subject were performed in triplicate.

During the course of the adhesion assay, some small aggregates of at least a dozen mononuclear cells were observed. Cells contained within these groups were nonadherent and were removed by washing with binding buffer at the end of the adhesion assay. A similar aggregate formation has also been reported by Lehr and colleagues, who noted that in vivo administration of oxidized LDL induced rolling as well as a tendency of adhesive leukocytes to form aggregates of ≥3 cells.

Flow Cytometry
To characterize the predominant mononuclear cells bound to the endothelial monolayer, we analyzed adherent cells by using a flow
cytometer (FACSscan, Becton Dickinson, Inc). Adherent cells were removed by incubation with HBSS-EDTA for 5 minutes and resuspended in 200 µL binding buffer. Cells were then incubated with monoclonal antibodies conjugated to a fluorescent tag (phycoerythrin and FITC) for 30 minutes at a 1:100 dilution on ice. Anti-human phycoerythrin-CD14 monoclonal antibody (Sigma) was used to detect monocytes. Murine anti-human FITC-CD3 (PharMingen, Inc) monoclonal antibody was used to stain T cells. Some experiments used anti-human FITC-CD19 (Sigma) to characterize B lymphocytes. Dead cells were detected by propidium iodide (Molecular Probes) incorporation and were excluded from our analysis.26 Endothelial cells were eliminated from our analysis by electronic gating.

The surface expression of L-selectin, which is shed from leukocytes on activation,27 and MAC-1, which is upregulated on activation,28,29 were compared in mononuclear cells isolated by density gradient centrifugation versus mononuclear cells in whole blood collected from the same donor. Surface expression of L-selectin and MAC-1 was assessed by using DREG-200 and IB4 monoclonal antibodies (kindly provided by Dr Eugene C. Butcher, Stanford University School of Medicine, California), respectively, and did not differ between isolated mononuclear cells and those in whole blood, indicating that our isolation procedure was not activating the mononuclear cells (data not shown).

In Vitro Monocyte-Endothelial Coculture Studies

To study the effect of endothelium-derived NO on monocyte adhesiveness, the following studies were performed. Bovine aortic endothelial cells (passages 7 to 9) were grown to confluence in Falcon 3502 tissue culture plates (Fisher Scientific) supplemented with 10% FCS. The following day before use, FCS levels were reduced to 1%. Bovine aortic endothelial cells were grown to confluence in PCMEM (GIBCO-BRL) supplemented with 10% FCS. One day before use, HDC levels were reduced to 1%. Bovine aortic endothelial cells were eliminated from our analysis by electronic gating.

By use of Falcon 3090 (0.4-µm pore size) cell culture inserts (Applied Scientific), 1×105 WEHI 78/24 monocyte cells were cocultured with bovine aortic endothelial cells on a rotating platform (Thermolyne) at 120 rpm for 18 hours. (This coculture system prevents contact between the monocytes and endothelial cells but exposes the monocytes to diffusible substances released by the endothelium into the medium.) Monocyte cells were subsequently removed, and adhesion assays were performed on unstimulated naive ECV304 cells by using 2.5×106 monocyte cells, as described above. Adherent cells were detached by 2 minutes of incubation with HBSS-EDTA, and quantification was made by the addition of 1.5×104 fluorescent beads (DNA Check, Coulter Corp) to each sample before analysis in the flow cytometer. Monocyte and endothelial cells were distinguished by electronic gating. The number of adherent cells in each sample was calculated by determining the ratio of monocyte cell events to fluorescent bead events and multiplying by the number of beads added. Monoclone antibodies (PharMingen) were also used for flow cytometric analysis of surface integrin expression. MAC-1, lymphocyte function-associated antigen-1, very late antigen-4 (VLA-4), and CD18.

Table 2. Plasma Arginine and Dimethylarginine Levels

<table>
<thead>
<tr>
<th></th>
<th>NC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADMA, µmol/L</td>
<td>1.3±0.2</td>
<td>2.1±0.2*</td>
</tr>
<tr>
<td>SDMA, µmol/L</td>
<td>0.7±0.2</td>
<td>0.8±0.2</td>
</tr>
<tr>
<td>L-Arginine, µmol/L</td>
<td>59.8±5.9</td>
<td>59.0±9.2</td>
</tr>
<tr>
<td>L-Arginine/ADMA</td>
<td>54.3±6.6</td>
<td>33.5±3.8†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P<0.05 vs NC group; †P<0.01 vs HC group.

Data Analysis

All data are described as mean±SEM. For adhesion studies, data were expressed as the percentage of added cells (total mononuclear cells, T lymphocytes, and monocytes) that were bound relative to cells from the normal control (total mononuclear cells, T lymphocytes, and monocytes) on each experimental day. HC subjects were age- and sex-matched to NC subjects. In this manner, an index of adhesiveness was obtained for each patient and was analyzed by a 1-sample t test. Biochemical parameters before and after L-arginine treatment were compared by a paired Student t test. Linear regression curves and correlation coefficients were calculated according to the least squares method. All other data were analyzed by ANOVA, followed by the Fisher protected least significant difference post hoc test. Statistical significance was assumed at P<0.05.

RESULTS

Biochemical Studies

Lipid profiles for the HC and NC groups are summarized in Table 1. HC volunteers had elevated total cholesterol and LDL levels (both P<0.0001). HDL levels were not different, but triglyceride levels were elevated (P<0.05). No differences were detected between HC and NC subjects in any of the other biochemical or endocrine parameters measured (data not shown). Furthermore, in those HC patients receiving L-arginine treatment, no differences were observed in the course of the administration were seen for any of the biochemical or endocrine parameters measured, including total cholesterol, growth hormone, and insulin levels (data not shown).

1-L-Arginine and Dimethylarginine Determination

ADMA levels were approximately doubled in HC patients compared with NC subjects (P<0.05), whereas SDMA levels were not different (Table 2). Plasma L-arginine concentrations were similar, resulting in lower plasma L-arginine/ADMA ratios (P<0.01). Plasma ADMA levels were directly correlated with total cholesterol (R=0.31, P<0.05; not shown). Multiple regression analysis of ADMA versus 3 independent variables (LDL, HDL, and triglyceride levels)
showed that ADMA levels were correlated with LDL (\(R=0.49, P<0.005\); Figure 1). In those patients receiving chronic oral l-arginine, plasma dimethylarginine concentrations were not altered throughout the course of the study (Table 3). Plasma l-arginine concentrations and normalized l-arginine/ADMA ratios remained elevated throughout the study (Figure 2).

### Adhesion Studies

Adhesion assays showed that in HC patients, mononuclear cell adhesiveness is increased by \(47\%\pm 12\%\) (\(P<0.001\)) relative to NC subjects. Fluorescent labeling of adherent mononuclear cells with anti-CD3 and anti-CD14 monoclonal antibodies revealed that T-lymphocyte and monocyte adhesiveness was increased by \(79\%\pm 19\%\) (\(P<0.0005\)) and \(78\%\pm 32\%\) (\(P<0.05\)), respectively (Figure 3). Those mononuclear cells not stained were found to consist mostly of B lymphocytes and did not exhibit enhanced adhesiveness in HC patients (data not shown). Peripheral blood mononuclear cell adhesiveness in the HC patients was inversely correlated with the plasma L-arginine/ADMA ratio (\(R=0.62, P<0.005\); Figure 4). Further analysis showed that T-cell adhesiveness was also inversely correlated to the L-arginine/ADMA ratio (\(R=0.73, P<0.0001\)). Monocyte adhesiveness tended to be inversely correlated with the l-arginine/ADMA ratio (\(R=0.39, P=0.06\)). Daily administration of oral l-arginine attenuated the elevated mononuclear cell adhesiveness (Figure 5A). This normalization was largely due to a reduction in T-lymphocyte and monocyte binding (Figure 5B).

### In Vitro Monocyte-Endothelial Coculture Studies

Monocytoid cells cocultured with ADMA-inhibited endothelial cells were hyperadhesive in functional binding assays in a dose-dependent manner (Figure 6). Monocytoid cells cultured with an l-arginine/ADMA ratio corresponding to that found in HC patients (25:1) were hyperadhesive compared with control cells (no ADMA added) and with cells cocultured with an l-arginine/ADMA ratio corresponding to that found in NC patients (50:1). The biologically inactive stereoisomer SDMA had no effect on monocyte adhesiveness. Flow cytometric analysis did not reveal any significant differences in integrin surface expression of MAC-1, lymphocyte function–associated antigen-1, VLA-4, or CD18 (data not shown). Direct exposure of monocytoid cells with ADMA did not alter their adhesiveness for endothelial cells (data not shown).

### Discussion

The salient findings of the present study are as follows: (1) Elevation of circulating plasma ADMA is associated with increased monocyte and T lymphocyte adhesiveness in HC humans. (2) l-Arginine supplementation restores the l-argi-
nine/ADMA ratio in HC patients and normalizes mononuclear cell adhesiveness without altering cholesterol, ADMA, growth hormone, or insulin levels. (3) In vitro, ADMA has no direct effect on monocyte adhesiveness. However, monocytoid cells become more adhesive when incubated in medium conditioned by endothelial cells exposed to ADMA. With the establishment of endothelium-derived relaxing factor as NO, we postulated that the administration of L-arginine, the precursor for NO, could ameliorate vascular disorders characterized by reduced NO activity. This hypothesis predated the elucidation of the enzyme kinetics of NOS, which predicted that circulating plasma L-arginine levels should not be rate limiting. Nevertheless, in the HC rabbit model, dietary L-arginine normalizes endothelium-dependent vasorelaxation, enhances NO synthesis, reduces vascular generation of superoxide anion, attenuates endothelial adhesiveness for monocytes, attenuates platelet reactivity, prevents atherogenesis in the coronary artery, and even induces the regression of intimal lesions. Human studies have shown that orally and intravenously administered L-arginine improves endothelium-dependent vasodilation in HC patients.

Several years ago the term “arginine paradox” was coined to describe the discordance between in vitro pharmacokinetic studies (which indicated that the $K_m$ of NOS for L-arginine was in a micromolar range and that L-arginine should not be rate limiting) and in vivo studies (which demonstrated that under certain conditions, eg, hypercholesterolemia, L-arginine could enhance endothelium-dependent vasodilation and NO synthesis). This arginine paradox may be explained in part by the existence of endogenous inhibitors of NOS, such as ADMA. We have recently shown that plasma ADMA is elevated in HC patients compared with NC subjects and is associated with reduced generation of endothelium-derived NO, as indicated by attenuated flow-mediated vasodilation of the brachial artery and reduced urinary nitrogen oxide excretion. Intravenous infusion of L-arginine normalized brachial artery vasodilation, indicating that increasing L-arginine levels may overcome the inhibition of NOS by ADMA. Using an independent method, we have confirmed observations made in 1995 by Jongkind et al that peripheral blood monocytes from HC patients are hyperadhesive in ex vivo adhesion assays. In addition, we show that T (but not B) lymphocytes also exhibit increased adhesiveness in hypercholesterolemia. The increased adhesiveness of circulating monocytes and T lymphocytes in HC individuals is consistent with the observation that monocytes and T cells are both present in atherosclerotic plaques and adhere to the endothelium in regions predisposed to atherosclerosis before the formation of fatty streaks.

Chronic oral administration of L-arginine restored plasma L-arginine/ADMA ratios to those seen in NC subjects and was associated with the normalization of mononuclear cell adhesiveness. These in vivo observations were consistent with the in vitro work showing that monocytoid cells become more adhesive when incubated with endothelial cells exposed to ADMA. In these studies, L-arginine/ADMA ratios similar to those found in HC or NC subjects in vivo resulted in qualitatively similar differences in monocytoid cell adhesiveness. This further strengthens the notion that ADMA is a
ADMA, Mononuclear Cells, and Hypercholesterolemia

competitor of L-arginine for endothelial NOS and may provide a possible explanation for the arginine paradox. Leukocyte-endothelial adhesion pathways have been investigated in models of hypercholesterolemia. However, most of this work has been focused on the expression of endothelial adhesion molecules. Indeed, vascular cell adhesion molecule-1 and P-selectin are expressed in regions predisposed to atherosclerosis in the HC rabbit before the accumulation of monocytes and T lymphocytes. The present in vitro coculture study did not show any differences in the expression of integrins CD11b, lymphocyte function–associated antigen-1, MAC-1, and VLA-4 on the surface of monocytioid cells cocultured with ADMA-exposed endothelial cells. However, it is possible that the avidity of some of these integrins may be altered. Indeed, VLA-4 avidity can be regulated by the chemokine monocyte chemotactic factor-1. We have observed that endothelial cells exposed to ADMA produce and release monocyte chemotactic factor-1 (J.R.C. et al, unpublished data, 1999).

The source of ADMA in hypercholesterolemia is unclear. ADMA is likely the result of the hydrolysis of methylated proteins. Lipid peroxidation in vivo results in peroxidative damage to tissue proteins and may accelerate the rate of proteolysis. Alternatively, there may be a downregulation or dysfunction of dimethylarginine dimethylaminohydrolase, the enzyme that degrades ADMA to L-citrulline. Hypercholesterolemia may disturb the function or regulation of dimethylarginine dimethylaminohydrolase, thereby leading to intracellular accumulation of ADMA. Indeed, regenerating endothelial cells exhibit endothelial vasodilator dysfunction and produce more ADMA. It is clear from the present study that chronic L-arginine administration did not affect circulating ADMA levels. This observation suggests that exogenous L-arginine is not directly converted to ADMA.

Fickling et al have shown that endothelial cells synthesize ADMA. Furthermore, they have shown that an ADMA concentration of 2 μmol/L in the culture media is able to inhibit the production of nitrite by murine macrophages and therefore would be sufficient to inhibit NO synthesis in neighboring cells. Faraci et al have also shown that 2 μmol/L ADMA can result in 50% inhibition of NOS activity in the rabbit and rat brain. We show that this is approximately the same level of ADMA found in the plasma of HC patients. Because endothelial dysfunction is characteristic of hypercholesterolemia, it is possible that circulating plasma ADMA at 2 μmol/L may inhibit the endothelial production of NO and be partly responsible for the elevated monocyte and T-lymphocyte adhesiveness seen in the present study. Indeed, the reduced production of NO, as measured by urinary metabolites, in patients with peripheral arterial occlusive disease has been attributed to their elevated plasma ADMA levels (between 2 to 4 μmol/L). Furthermore, impaired endothelial vasodilator function in hypercholesterolemia is associated with increased plasma ADMA and is restored by intravenous L-arginine administration.

ADMA is currently assayed by a cumbersome, time-consuming, and expensive procedure involving extraction, chemical derivatization, isolation by reverse-phase HPLC, and detection by fluorescence. Recently, we have developed a new high-throughput enzymatic assay that takes advantage of the absolute specificity for ADMA of the enzyme dimethylarginine dimethylaminohydrolase. Using this assay to detect ADMA in HC individuals, we observe values similar to those seen with the HPLC assay but greater reproducibility. Because the values obtained in HC individuals with either technique are similar, it is not likely that plasma cholesterol is affecting the measurement of ADMA.

Recently, insulin release by high doses of L-arginine has been implicated as a mechanism by which L-arginine might stimulate vasodilation in vivo independent of its serving as a substrate for NOS. Endogenously released insulin may contribute to the vasodilation and inhibition of platelet aggregation that is observed during intravenous L-arginine administration in healthy volunteers. However, this endocrine effect requires large doses of intravenous L-arginine. In the present study, insulin levels were not affected by chronic oral administration of L-arginine (14 g/d over 12 weeks). Growth hormone, which promotes insulin release, was also unaffected. Furthermore, the reduction in monocyte and T-lymphocyte adhesiveness was not associated with a lipid-lowering effect, because L-arginine treatment did not affect cholesterol levels. Taken together, these observations suggest that the decrease in T-lymphocyte and monocyte adhesiveness is most likely due to L-arginine metabolism by the NOS pathway.

In summary, the present study indicates that ADMA alters the adhesive behavior of circulating mononuclear cells in HC humans. Increased adhesiveness of monocytes and T lymphocytes may predispose these individuals to atherosclerosis. Chronic oral administration of L-arginine restored L-arginine/ADMA ratios to normal levels and attenuated mononuclear leukocyte adhesiveness. We propose that supplemental L-arginine may overcome ADMA inhibition in hypercholesterolemia. Such a strategy may be useful in circumventing the development of atherosclerosis by inhibiting the recruitment of monocytes and T lymphocytes to the endothelium during the initial stages of atherogenesis.

Acknowledgments
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