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EXPERIMENTAL STUDIES

An Endogenous Inhibitor of Nitric Oxide Synthase Regulates Endothelial Adhesiveness for Monocytes

Rainer H. Böger, MD, Stefanie M. Bode-Böger, MD, Philip S. Tsao, PHD, Patrick S. Lin, BS, Jason R. Chan, BS, John P. Cooke, MD, PHD

Stanford, California

OBJECTIVES We sought to determine whether asymmetric dimethylarginine (ADMA) inhibits nitric oxide (NO) elaboration in cultured human endothelial cells and whether this is associated with the activation of oxidant-sensitive signaling mediating endothelial adhesiveness for monocytes.

BACKGROUND Endothelial NO elaboration is impaired in hypercholesterolemia and atherosclerosis, which may be due to elevated concentrations of ADMA, an endogenous inhibitor of NO synthase.

METHODS Human umbilical vein endothelial cells (ECV 304) and human monocytoid cells (THP-1) were studied in a functional binding assay. Nitric oxide and superoxide anion (O_2^-) were measured by chemiluminescence; ADMA by high pressure liquid chromatography; monocyte chemotactic protein-1 (MCP-1) by ELISA and NF-κB by electromobility gel shift assay.

RESULTS Incubation of endothelial cells with ADMA (0.1 μM to 100 μM) inhibited NO formation, which was reversed by coincubation with L-arginine (1 mM). The biologically inactive stereoisomer symmetric dimethylarginine did not inhibit NO release. Asymmetric dimethylarginine (10 μM) or native low-density lipoprotein cholesterol (100 mg/dL) increased endothelial O_2^- to the same degree. Asymmetric dimethylarginine also stimulated MCP-1 formation by endothelial cells. This effect was paralleled by activation of the redox-sensitive transcription factor NF-κB. Preincubation of endothelial cells with ADMA increased the adhesiveness of endothelial cells for THP-1 cells in a concentration-dependent manner. Asymmetric dimethylarginine-induced monocyte binding was diminished by L-arginine or by a neutralizing anti-MCP-1 antibody.

CONCLUSIONS We concluded that the endogenous NO synthase inhibitor ADMA is synthesized in human endothelial cells. Asymmetric dimethylarginine increases endothelial oxidative stress and potentiates monocyte binding. Asymmetric dimethylarginine may be an endogenous proatherogenic molecule. (J Am Coll Cardiol 2000;36:2287–95) © 2000 by the American College of Cardiology

Hypercholesterolemia reduces the biological activity of nitric oxide (NO), impairs endothelium-dependent vasodilation (1), increases platelet aggregation (2) and enhances monocyte adhesion (3). The mechanism by which hypercholesterolemia impairs the L-arginine/NO pathway is probably multifactorial and dependent upon the stage of atherosclerosis (4,5). Most studies have reported that the defect is reversed by exogenous L-arginine (6–9). Recently, asymmetric dimethylarginine (ADMA) has been characterized to be an endogenous, competitive inhibitor of NO synthase (10). Asymmetric dimethylarginine has been shown to be synthesized by human endothelial cells (11). The plasma level of ADMA is elevated, and endothelial NO elaboration is impaired in hypercholesterolemic rabbits as well as in humans with atherosclerosis or with risk factors for atherosclerosis (12–17).

Chronic supplementation with the NO precursor L-arginine restores endothelial function and inhibits atherosclerosis (18–22) and even induces regression (22). Conversely, chronic inhibition of NO synthase promotes monocyte adhesion and atherogenesis (23–26). Therefore, it is possible that elevated ADMA levels in hypercholesterolemic humans may promote atherosclerosis. Indeed, we have recently reported that ADMA is a better predictor of carotid artery intimal-medial thickness (as measured by B-mode ultrasound) than the traditional risk factors (17).

An important mechanism by which NO may act as an antiatherogenic molecule is by inhibiting endothelial adhesiveness for monocytes (27). Reduced NO elaboration has been shown to be associated with increased oxidative stress in endothelial cells (28), which, in turn, activates the oxidant-responsive transcription factor NF-κB (29), leading to enhanced endothelial adhesiveness associated with expression of adhesion molecules like vascular cell adhesion molecule and monocyte chemotactic protein-1 (30–33).

In this study we determined that ADMA affects endothelial cell-monocyte interactions in vitro, and we investigated the mechanism leading to this effect.

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Abbreviations and Acronyms

ADMA	=	asymmetric dimethylarginine
BHT	=	butylated hydroxytoluene
DDAH	=	dimethylarginine dimethylaminohydrolase
ECV 304	=	a line of transformed human umbilical vein endothelial cells
ELISA	=	enzyme-linked immunosorbent assay
HBSS	=	Hanks balanced salt solution
HPLC	=	high-pressure liquid chromatography
LDL	=	low-density lipoprotein
LNMMA	=	L, N-monomethylarginine
MCP-1	=	monocyte chemotactic protein
nLDL	=	native low-density lipoprotein
NO	=	nitric oxide
OPA	=	o-phthaldialdehyde
oxLDL	=	oxidized low-density lipoprotein
SDMA	=	symmetric dimethylarginine
THP-1	=	a line of human monocytoid cells

METHODS

Cell culture. The spontaneously transformed human umbilical vein endothelial cell line ECV 304 (ATCC, Rockville, Maryland [34]) was cultured in medium M199 (Irvine Scientific) containing 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco BRL, Grand Island, New York). This cell line retains many of the characteristics of primary endothelial cells including the synthesis of angiotensin-converting enzyme, NO and prostacyclin and the expression of 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 (35).

Human monocytoid cells (THP-1) (ATCC) were cultured in RPMI medium 1640 (Irvine Scientific) supplemented with 10% fetal calf serum, 100 U/mL penicillin and 100 μg/mL streptomycin. The viability of THP-1 cells was assessed before each experiment using trypan blue exclusion and was always >95%.

Isolation of low-density lipoproteins (LDL). Human plasma from two to three donors was obtained from the Stanford blood bank, and butylated hydroxytoluene (BHT) and EDTA were immediately added to the plasma to achieve final concentrations of 20 μM and 0.1%, respectively. Plasma was then mixed for 15 min at 4°C before sequential gradient ultracentrifugation (1.019 to 1.063 g/mL adjusted with KBr) as described by Pritchard et al. (36). The native LDL (nLDL) solution was then dialyzed against 0.9% NaCl containing 20 μM BHT and 0.01% EDTA overnight at 4°C. The cholesterol content of the solution was measured by using a cholesterol oxidase colorimetric kit (Sigma). The nLDL solution was stored at 4°C and used for experiments within 2 weeks. For the preparation of oxidized LDL (oxLDL), nLDL solution was dialyzed against 0.9% saline with 0.01% EDTA overnight to eliminate the antioxidant BHT, and oxidation was induced by adding 5 μM $CuSO_4$ for 24 h at 4°C. Oxidation was stopped by adding BHT (20 μM) before using the oxLDL for the experiments.

Experimental conditions. Transformed human umbilical vein endothelial cells were seeded onto 75 cm^2 culture flasks and maintained in M199 medium as described above. After 24 h the cells were washed twice with PBS, and fresh medium was added. Cells were then maintained in culture for 1, 2, 3, 5 or 7 days in the presence or absence of nLDL (30, 100 and 300 mg/dL) or oxLDL (3 and 10 mg/dL) before the cells and media were harvested for the measurement of ADMA and symmetric dimethylarginine (SDMA) levels. Endothelial integrity during incubation with nLDL and oxLDL was assessed by measuring the activity of lactate dehydrogenase in conditioned media using a commercially available spectrophotometric assay kit (Sigma).

To assess the effects of dimethylarginines and lipoproteins on NO and monocyte chemotactic protein (MCP-1) production and nuclear factor-κB activation, endothelial cells were grown to confluence on six-well culture plates. Cells were then washed twice with PBS, and fresh PBS containing 0.7 mmol/L Ca^{2+}, 1.1 mmol/L Mg^{2+}, 2.7 mmol/L K^+ and 6.1 mmol/L glucose was added. The cells were then incubated for 4 h or 24 h in the presence or absence of ADMA, SDMA or L, N-monomethylarginine (L-NMMA) (each 0.1 to 100 μmol/L) or with ADMA in the presence of 1mmol/L L-arginine. In other experiments LDL (30, 100 or 300 mg/dL) or oxLDL (1 or 3 mg/dL) or lipoproteins plus 1 mmol/L L-arginine were added to the incubation medium. For the determination of endothelial cell NO formation, the cells were stimulated with calcium ionophore A23187 (1 μmol/L). After the end of the incubation time, media were collected, and the cells were gently detached with 5 mmol/L EDTA. Cells were counted in a hemocytometer and subsequently lysed, and incubation media and cell lysates were stored at −20°C until analysis.

Monocyte-endothelial cell adhesion assay. Transformed human umbilical vein endothelial cells were grown to confluence on six-well culture plates (Nunc, Naperville, Illinois). Endothelial cells were incubated with the appropriate drugs or lipoproteins for 4 or 24 h before the adhesion assays. Thirty minutes before the adhesion assay, endothelial cells were washed with Hanks balanced salt solution (HBSS) containing 2 mmol/L Ca^{2+}, 2 mmol/L Mg^{2+} and 20 mmol/L HEPES (Irvine Scientific, San Francisco, California). Human monocytoid cells were washed with HBSS and diluted to a final concentration of 2×10^6 cells/mL. Human monocytoid cells (2×10^6) were added to each well of endothelial cells. The THP-1 cells were allowed to incubate with the endothelial monolayer at room temperature for 30 min on a rocking platform (Research Products International, Mount Prospect, Illinois). Each well was turned 90 degrees at 15 min to allow uniform distribution of the THP-1 cells across the endothelial monolayer. Nonadherent mononuclear cells were carefully removed by two

washes with HBSS and the adherent cells fixed with 2% glutaraldehyde in HBSS. Adherent cells were counted by microscopy using a computer-aided image analysis system (ImageAnalyst, Automatix Corp., Billerica, Massachusetts). Thirty fields were counted for each 35mm well, and the mean number of adhering cells per field was calculated.

Determination of dimethylarginines. L-arginine, ADMA and SDMA were quantified by high performance liquid chromatography (HPLC) and precolumn derivatization with o-phthaldialdehyde (OPA) using a modification of a previously published method (13). Briefly, 10 μmol/L L-homoarginine as internal standard was added to 1 mL of cell supernatant or cell lysate. Samples and standards were extracted on solid phase extraction cartridges (CBA Bond Elut, Varian, Harbor City, California). The recovery rates were 84 ± 4%. The eluents were dried over nitrogen and resuspended in bidistilled water for HPLC analysis. High performance liquid chromatography was carried out on a computer-controlled Varian Star chromatography system (Varian, Walnut Creek, California) consisting of a ternary gradient HPLC pump (Varian 9010), an automatic injector with sample-reagent mixing capabilities (Varian 9050) and a fluorescence detector (Varian Fluorichrom III). Samples and standards were incubated for exactly 1 min with OPA reagent (5.4 mg/mL OPA in borate buffer, pH 8.4, containing 0.4% 2-mercaptoethanol) before automatic injection into the HPLC. The OPA derivatives of L-arginine, ADMA and SDMA were separated on a 250 × 4.5 mm I.D. 7 μm Nucleosil phenyl column (Supelco, Bellefonte, Pennsylvania) with the fluorescence detector set at λ^{ex} = 340 nm and λ^{em} = 450 nm. Samples were eluted from the column with 0.96% citric acid/methanol 70:30, pH 6.8, at a flow rate of 1 mL/min. The variability of the method was below 7%; the detection limit of the assay was 0.15 μmol/L.

Endothelial cell NO elaboration. The concentration of nitrogen oxides (NO_x) in supernatants from incubation experiments of endothelial cells with dimethylarginines was measured with a commercially available chemiluminescence apparatus (model 2108, Dasibi) after reduction of the samples in boiling acidic vanadium (III) at 98°C as previously described (22,23). Boiling acidic vanadium quantitatively reduces NO_2^- and NO_3^- to NO, which is quantified by the chemiluminescence detector after reaction with ozone. Signals from the detector were analyzed by a computerized integrator and recorded as areas under the curve. Standard curves for NO_x were linear over the range of 50 pmol to 5 nmol/L (R^2 = 0.997). The detection limit of this assay was 65 pmol/L; intra- and interassay variability was <6%.

Superoxide radical release by endothelial cells. For the measurement of endothelial cell superoxide radical release, cells were seeded into 12 mm scintillation counter vials (Packard, Downers Grove, Illinois) and grown to confluence. After 24 h exposure to experimental agents, the cells were washed with fresh PBS and incubated in PBS containing 250 μmol/L of lucigenin. Photon emission was recorded in a Packard Tricarb 1,500 liquid scintillation analyzer in the single photon emission mode until equilibration was reached. The cells were then exposed to phorbol-12-myristate-13-acetate (PMA, 2 μmol/L dissolved in dimethylsulfoxide), and photon emission was recorded for 15 min. The specific chemiluminescence response was expressed as counts per minute minus the average background activity. After the end of the measurements, the cells were gently detached with 5 mmol/L EDTA, and cell protein was measured (37) according to the Lowry method (32). Data were expressed as counts per minute per nanograms of cell protein. This technique of assessing superoxide anion elaboration is semiquantitative but does provide an index of relative oxidative stress.

MCP-1 enzyme-linked immunosorbent assay. Monocyte chemotactic protein-1 secretion by endothelial cells was measured by ELISA with recombinant human MCP-1 (R&D Systems) as a standard. Briefly, 200 μL per well of polyclonal goat-antihuman MCP-1 antibody (R&D Systems) in bicarbonate buffer (pH 9.6) at a final concentration of 5 μg/mL was coated onto 96 well immunoplates overnight. After washing with PBS containing EDTA and Tween 20 (wash buffer), the plates were incubated with blocking buffer (PBS containing 0.2% Triton X-100 and 1% bovine serum albumin, pH 7.2) for 1 h at 37°C. Plates were washed with wash buffer, and 200 μL of serially diluted samples or standards was added. After incubation for 2 h with shaking, the wells were rinsed with wash buffer. Monoclonal mouse-antihuman MCP-1 antibody (200 μL per well of 5 μg/mL) was then added and incubated for 90 min at 37°C. After washing the wells were incubated with alkaline phosphatase-conjugated rabbit-antimouse IgG monoclonal antibody (Sigma) for 90 min at 37°C. Phosphatase substrate (Sigma; 1 mg/mL) was then added. After 30 min of incubation at room temperature, the products were measured at 405 nm by a microplate reader (Biotek). The standard curve for MCP-1 measured by this ELISA was linear from 0.3 to 50 ng/mL; the detection limit was 1 ng/mL.

Electrophoretic mobility shift assay. Endothelial cells were harvested, centrifuged to pellet cells and resuspended in ice-cold PBS. Nuclear extracts were prepared according to a modification of the procedure described by Dignam et al. (32) as previously published. Cells were resuspended in buffer A (in mmol/L: PMSF 0.5, HEPES 10 [pH 7.8], $MgCl_2$ 1.5, KCl 10, DTT 0.5) containing 0.1% Nonidet P-40 and disrupted in a tight fitting Dounce homogenizer. Nuclei were then pelleted by centrifugation (25,000 *g*, 20 min, 4°C). Crude nuclei were resuspended in buffer C (in mmol/L: HEPES 20 [pH 7.8], NaCl 0.42, $MgCl_2$ 1.5, EDTA 0.2, DTT 0.5, PMSF 0.5; 25 vol% glycerol) and incubated on ice for 30 min. The mixture was then spun at 25,000 *g*(20 min 4°C); the supernatant was collected, and protein was quantified. Nuclear proteins were stored at −80°C until gel shift assay. Binding reactions were carried out by mixing nuclear proteins with a double-stranded

oligonucleotide corresponding with the published NF-κB-binding domain. Reactions were performed with ^{32}P-labeled DNA oligonucleotide in the presence of (in mmol/L) $MgCl_2$ 1, EDTA 0.5, DTT 0.5, NaCl 50, Tris-HCl 10 (pH 7.5) and 0.05 μg/mL polydeoxyinosinic-deoxycytidylic acid in 20 vol% glycerol. Samples were separated on a 4% nondenaturating polyacrylamide gel and exposed to x-ray film overnight.

Drugs. Asymmetric dimethylarginine and symmetric dimethylarginine were purchased from Alexis (San Diego, California). Unless noted, all other drugs were obtained from Sigma (St. Louis, Missouri).

Calculations and statistical analysis. All data are given as mean ± SEM. Cell binding data are expressed as the mean relative number of cells bound per high power field (cells/hpf) from the analysis of 30 fields per culture well. Statistical significance was tested in all experiments using analysis of variance followed by Fisher protected least significant difference test. Statistical significance was assumed for $p < 0.05$.

RESULTS

ADMA elaboration by endothelial cells. The concentrations of ADMA and SDMA in ECV 304 human endothelial cell incubation medium were 1.1 ± 0.1 and 0.6 ± 0.2 μmol/L, respectively, on day 1. They gradually increased to 3.0 ± 0.5 and 1.9 ± 0.3 μmol/L on day 7 (Fig. 1A). From these data it was calculated that ECV 304 cells released 0.34 ± 0.17 μmol ADMA/10^7 cells/24 h and 0.29 ± 0.14 μmol SDMA/10^7 cells/24 h into the medium. L-arginine concentrations in the medium were 120 to 160 μmol/10^7 cells without significant changes throughout the experimental period. The basal intracellular concentrations of L-arginine, ADMA and SDMA were 426.5 ± 51.5, 3.2 ± 0.6 and 3.9 ± 0.6 μmol/μg of cell protein, respectively. Given a mean endothelial cell volume of approximately 1.02 pL (34), the intracellular concentrations of ADMA and SDMA were calculated to be 7.9 ± 1.3 μmol/L and 6.2 ± 3.6 μmol/L at baseline and increased to 39.4 ± 6.3 and 15.1 ± 6.7 μmol/L on day 7. At any time point, the intracellular DMA levels were 8 to 12-fold higher than the concentrations in the conditioned medium.

Incubation with nLDL or oxLDL concentration dependently increased ADMA release by endothelial cells (Fig. 1B). Transformed ECV 304 human umbilical vein endothelial cells released 0.33 ± 0.12, 0.44 ± 0.06 and 0.52 ± 0.10 ṁol ADMA/10^7 cells/24 h into the medium in the presence of nLDL (30, 100 and 300 mg/dL, respectively; $p < 0.05$ vs. control for 100 and 300 mg/dL nLDL). In the presence of oxLDL (3 and 10 mg/dL), ADMA release was 0.39 ± 0.27 and 0.68 ± 0.14 μmol ADMA/10^7 cells/24 h ($p < 0.05$ vs. control for 10 mg/dL oxLDL). Lactate dehydrogenase activity in conditioned media from endothelial cells incubated with nLDL or oxLDL was not significantly higher than from control cells (data not shown).

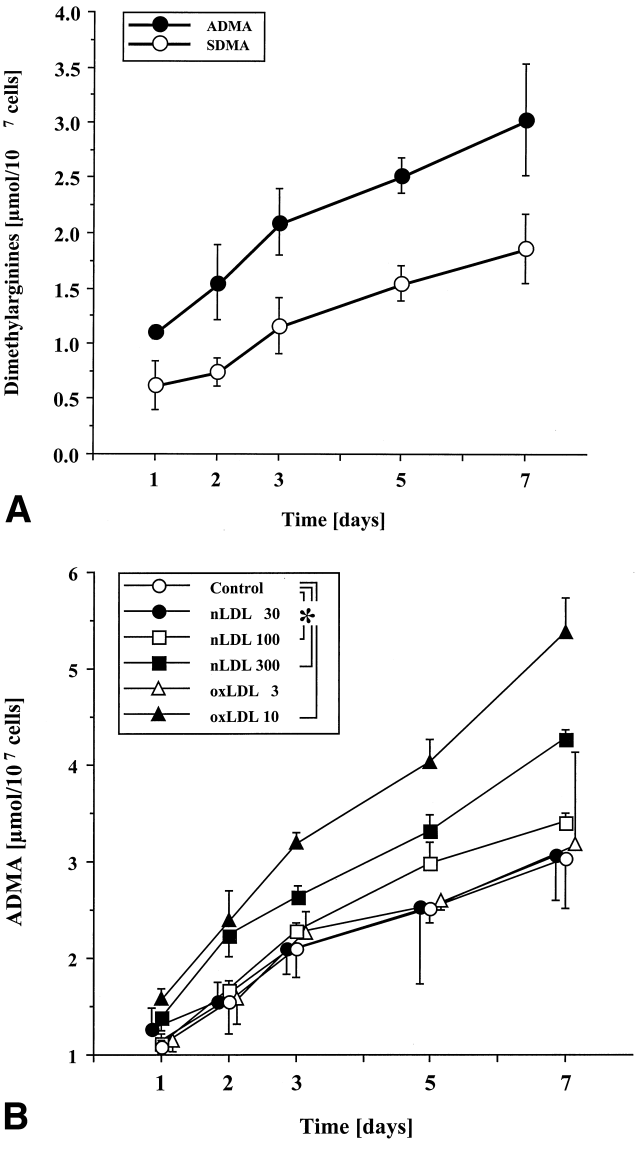

Figure 1. (**A**) The synthesis of ADMA by the cultured human endothelial cell line ECV 304. Data are mean ± SEM for n = 5 to 6 separate experiments. The synthesis rates of ADMA and SDMA were calculated from the mean differences in dimethylarginine concentrations between the experimental days as 0.34 ± 0.17 μmol ADMA/10^7 cells/24 h and 0.29 ± 0.14 μmol SDMA/10^7 cells/24 h. (**B**) The effect of incubating endothelial cells with nLDL or oxLDL on ADMA elaboration. Concentrations of lipoproteins are given as mg/dL in legend. Data are mean of n = 4 to 6 separate experiments. *$p < 0.05$ versus control. ADMA = asymmetric dimethylarginine; nLDL = native low-density lipoprotein; oxLDL = oxidized low-density lipoprotein; SDMA = symmetric dimethylarginine.

NO and superoxide elaboration by endothelial cells. Incubation with ADMA or L-NMMA (0.1 to 100 μmol/L) concentration-dependently inhibited NO release by ECV 304 cells (Fig. 2, Table 1). Coincubation with 1 mmol/L L-arginine reversed the inhibitory effect of ADMA on NO formation.

Concomitantly with the inhibition of NO synthesis, ADMA and L-NMMA increased endothelial superoxide radical elaboration in a concentration-dependent manner (Fig. 3, Table 1). The effect of ADMA on endothelial

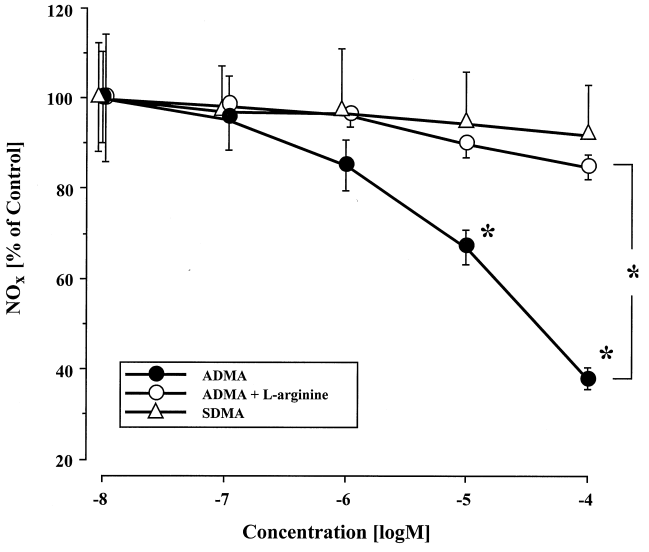

Figure 2. The release of NO_x from ECV 304 human endothelial cells during 4 h of incubation in the presence or absence of ADMA, SDMA or ADMA + L-arginine (1 mmol/L). Cells were stimulated with calcium ionophore A23187 (1 μmol/L). Values are expressed as percent of the NO_x level in conditioned medium from control cells incubated with vehicle alone. Data are mean ± SEM of n = 5 to 6 identical experiments. ADMA = asymmetric dimethylarginine; NO_x = nitrogen oxides; SDMA = symmetric dimethylarginine.

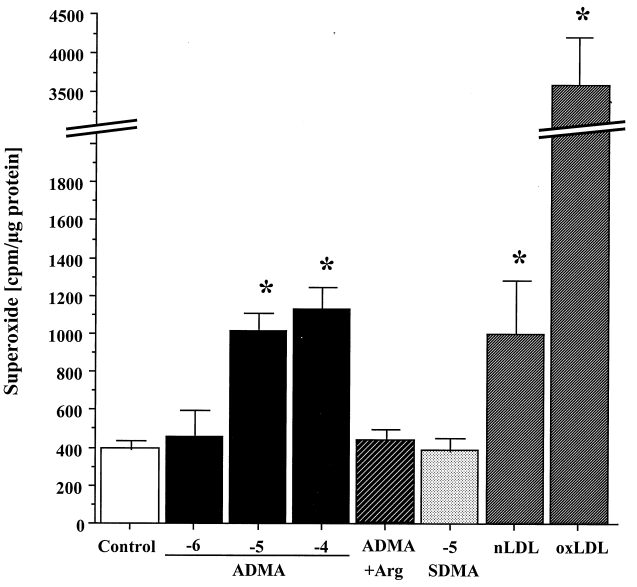

Figure 3. The release of superoxide radicals from ECV 304 human endothelial cells. Cells were incubated for 24 h with the substances indicated before the addition of PMA (2 μmol/L). Data are mean ± SEM of n = 3 to 4 experiments performed in duplicate. ADMA = asymmetric dimethylarginine; nLDL = native low-density lipoprotein; oxLDL = oxidized low-density lipoprotein; SDMA = symmetric dimethylarginine.

superoxide release was reversed by 1 mmol/L L-arginine. Native low-density lipoprotein (100 mg/dL) stimulated superoxide radical release by about two to three-fold, and oxLDL (3 mg/dL) stimulated superoxide formation by about 9 to 10-fold. Symmetric dimethylarginine had no significant effect on endothelial NO or superoxide radical release.

Monocyte adhesion. Asymmetric dimethylarginine and L-NMMA increased the adhesiveness of ECV 304 human endothelial cells for monocytoid cells in a concentration-dependent manner (Fig. 4, Table 1). Symmetric dimethylarginine had no significant effect on monocyte binding.

Incubation with nLDL (100 mg/dL) or oxLDL (3 mg/dL) increased monocyte binding by 60.4 ± 12.4 and 117.2 ± 16.6%, respectively (each $p < 0.05$ vs. control; Fig. 5). The stimulatory effects of ADMA or LDL on monocyte binding were diminished by 1 mmol/L L-arginine or by a neutralizing anti-MCP-1 antibody.

NF-κB activation. Incubation of ECV 304 endothelial cells with ADMA, L-NMMA or with LDL lipoproteins was associated with a concentration-dependent activation of the transcription factor NF-κB (Fig. 6), while SDMA did not activate NF-κB. The effects of ADMA, L-NMMA and LDL on NF-κB activation were partly reversed by coincubating the cells with excess L-arginine (1 mM) or with the intracellular antioxidant pyrollidine dithiocarbamate (PDTC, 1 μM).

MCP-1 secretion by endothelial cells. Asymmetric dimethylarginine and L-NMMA concentration dependently increased the release of MCP-1 from ECV 304 endothelial cells. These stimulatory effects of the NO synthase inhibitors were significantly diminished by L-arginine (Fig. 7, Table 1); SDMA did not affect MCP-1 production by endothelial cells. Incubation with nLDL or oxLDL also stimulated MCP-1-release; the effect of LDL was diminished by L-arginine.

DISCUSSION

The salient findings of our study are that: 1) intracellular ADMA levels are about 10-fold higher than the reported range for plasma values; 2) inhibition of NO production by ADMA increases endothelial superoxide radical elaboration and NF-κB activation, resulting in enhanced MCP-1 expression and endothelial adhesiveness for monocytes, and 3) these effects of ADMA are reversed by L-arginine.

Table 1. Plasma Lipid Profiles in Human Subjects

	Normocholesterolemic	Hypercholesterolemic
Total cholesterol (mg/dL)	185.3 ± 7.2	262.0 ± 6.1*
Low–density lipoprotein (mg/dL)	109.9 ± 6.3	174.7 ± 4.6*
High–density lipoprotein (mg/dL)	54.6 ± 3.7	47.3 ± 2.6
Triglyceride (mg/dL)	103.3 ± 16.7	178.5 ± 20.7†

*Significantly different from normocholesterolemic individuals ($p < 0.0001$); †significantly different from normocholesterolemic individuals ($p < 0.05$).

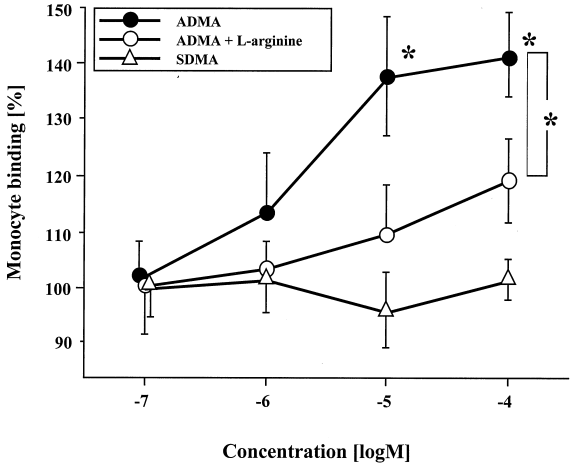

Figure 4. The effect of preincubating ECV 304 human endothelial cells with ADMA, SDMA or ADMA in the presence of 1 mmol/L L-arginine on the adhesion of THP-1 human monocytoid cells. Endothelial cells were coincubated with monocytoid cells for 30 min on a rocking platform. Adhering cells were fixed in glutaraldehyde and counted using a computer-aided image analysis system. Data are mean ± SEM of three separate experiments performed in triplicate. ADMA = asymmetric dimethylarginine; SDMA = symmetric dimethylarginine.

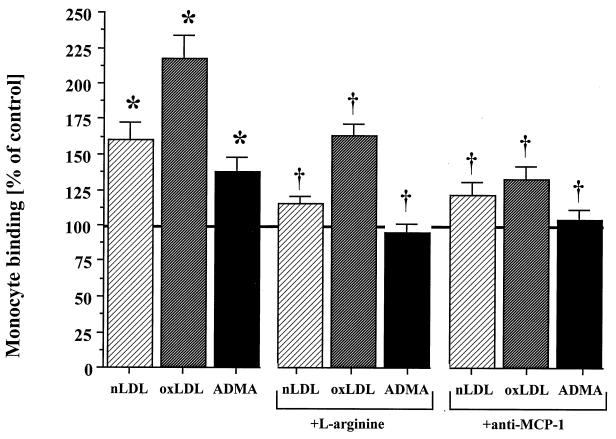

Figure 5. Effect of coincubation with L-arginine (1 mmol/L) or with a neutralizing anti-MCP-1 antibody on the effects of ADMA, nLDL (100 mg/dL) or oxLDL (3 mg/dL) on the adhesiveness of ECV 304 human endothelial cells for THP-1 human monocytoid cells. ADMA = asymmetric dimethylarginine; MCP-1 = monocyte chemotactic protein; nLDL = native low-density lipoprotein; oxLDL = oxidized low-density lipoprotein. * = significantly different from vehicle treated control endothelial cells, $p < 0.05$; † = significantly different from endothelial cells treated with lipoprotein or ADMA.

Vascular NO activity is decreased in hypercholesterolemia and atherosclerosis, leading to impaired endothelium-dependent vasodilation (1), increased platelet aggregability (38,39) and monocyte adhesiveness for the endothelium (3,40). This impaired NO activity may contribute to the development and progression of atherosclerosis. Indeed, in animal models of hypercholesterolemia, pharmacological inhibition of NO synthase promotes atherosclerosis (23–26). On the other hand, enhancement of endogenous NO formation with L-arginine supplementation improves endothelial function (6–9,15), inhibits platelet activation and monocyte adhesiveness for the endothelium (2,3,38–40) and slows progression of lesions or even induces regression (18–22,26). The benefit of L-arginine cannot be attributed to reduced plasma levels of L-arginine in hypercholesterolemia (41,42) but are more likely due to its reversal of the effects of ADMA, an inhibitor of the NOS pathway (43,44).

ADMA metabolism. The pathways by which ADMA is synthesized and degraded and the effect of hypercholesterolemia on these pathways are under investigation. The specific enzyme S-adenosylmethionine: protein arginine N-methyltransferase (protein methylase I) has been shown to methylate internal arginine residues in a variety of polypepetides. Catabolism of these polypeptides generates N^G-monomethyl-L-arginine, N^G, N^G-dimethyl-L-arginine and N^G, $N^{G\prime}$-dimethyl-L-arginine (45–47). The metabolism of ADMA, but not SDMA, occurs via hydrolytic degradation to citrulline by the enzyme dimethylarginine dimethylaminohydrolase (DDAH) (48). Inhibition of DDAH causes a gradual vasoconstriction of vascular segments, which is reversed by L-arginine (46). This latter finding suggests that inhibition of DDAH regulation could reduce NO synthase activity. We have recently found that exposure of vascular cells to oxidized lipoproteins in vitro increases elaboration of ADMA into the conditioned medium, which is temporally related to a decline in DDAH activity (49). Moreover, in the hypercholesterolemic rabbit (where plasma ADMA levels are elevated) DDAH activity is reduced in the aorta, liver and kidney (49). Together, these data and the current investigation suggest that elevated ADMA levels may mediate some of the biological effects of LDL on the endothelium in an autocrine or paracrine manner.

Most importantly, within the concentration range we found in cultured endothelial cells (5 to 40 μM), ADMA induced pathophysiological changes of endothelial biology that also occur in hypercholesterolemia. Inhibition of endothelial NO synthase activity is associated with a concentration-dependent increase of endothelial adhesiveness for human THP-1 monocytoid cells. This effect of ADMA and L-NMMA mimics the increase in adhesiveness observed when endothelial cells are preincubated with nLDL; in both cases, adhesion is diminished by coincubating endothelial cells with L-arginine. Supplementation of cholesterol-fed rabbits with L-arginine has previously been shown to reduce aortic endothelial adhesiveness for monocytes (23).

NO and endothelial adhesiveness. Monocyte adhesion to the endothelium is regulated via a variety of different cell surface receptors on monocytes and endothelial cells (50). In hypercholesterolemia and atherosclerosis, increased expression of vascular adhesion molecule-1 (51) and MCP-1 (52) has been demonstrated. Upregulation of these proteins is induced via increased intracellular oxidative stress, which involves the induction of NF-κB-regulated transcriptional pathways (30). The effects of oxidative stress on NF-κB activation are counteracted by NO, resulting in downregulation of the expression of vascular adhesion molecule-1(32)

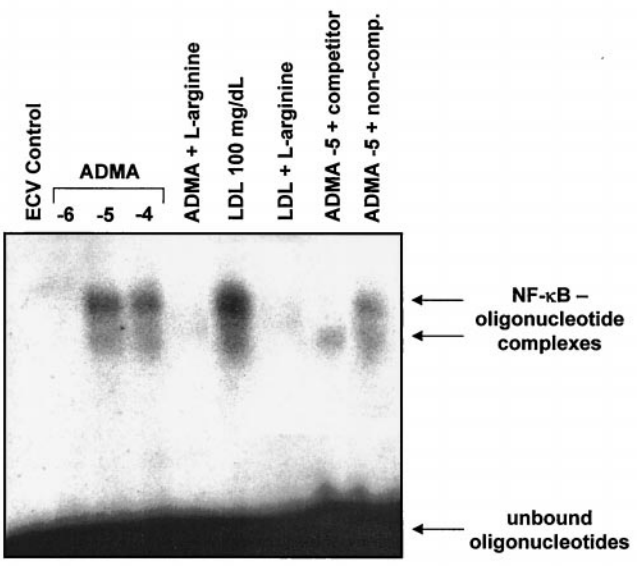

Figure 6. The electrophoretic mobility shift assay showing the effect of ADMA, SDMA and LDL lipoproteins on the activation of NF-κB. Nuclear extracts were isolated from ECV 304 human endothelial cells after incubation with the various substances for 24 h. Binding reactions were carried out by mixing nuclear proteins with a ^{32}P-labeled double-stranded DNA oligonucleotide corresponding to the published NF-κB-binding domain. In some experiments, unlabeled oligonucleotide corresponding to the same NF-κB-binding domain ("competitor") or to the SP-1 binding domain ("noncomp") was utilized. This experiment was replicated 3 times. ADMA = asymmetric dimethylarginine; LDL = low-density lipoprotein; MCP-1 = monocyte chemotactic protein; nLDL = native low-density lipoprotein; oxLDL = oxidized low-density lipoprotein; SDMA = symmetric dimethylarginine.

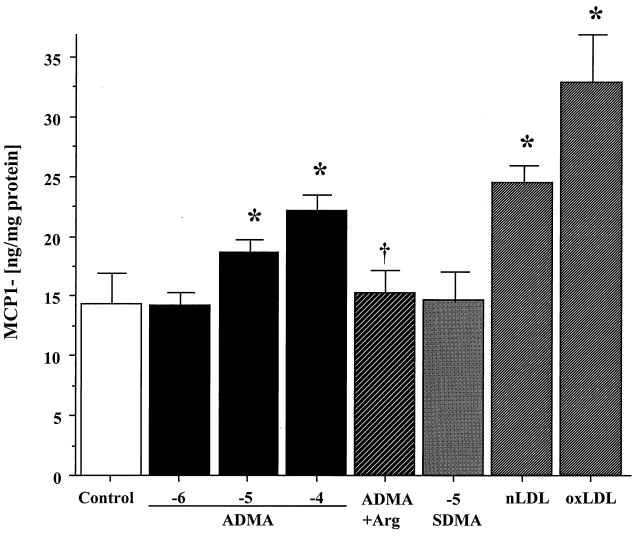

Figure 7. Release of monocyte chemotactic protein from ECV 304 human endothelial cells during a 24 h incubation in the presence or absence of the substances indicated. Data are mean ± SEM of n = 4 to 6 experiments measured in duplicate. * = significantly different from vehicle treated control endothelial cells, $p < 0.05$; † = significantly different from endothelial cells treated with ADMA (10^{-4} m) $p < 0.05$.

and MCP-1 (53). Nitric oxide also stabilizes the inhibitory subunit I-κB, thereby blocking the activation of NF-κB and its binding to specific domains on the DNA (54). Therefore, one can assume that NO activity may regulate mononuclear cell adhesiveness by affecting endothelial cell oxidative stress. Indeed, we found that reduced endothelial NO elaboration by ADMA or L-NMMA was associated with increased superoxide radical-induced oxidative stress, as measured by lucigenin chemiluminescence. L-arginine diminished superoxide anion release. Besides impaired endothelium-dependent vasodilation and increased platelet adhesion and aggregation, this autocrine antioxidant role of NO may play a very important role in its antiatherogenic effects.

Limitations and clinical implications. The current study confirms previous observations of our group and others that oxidized lipoprotein increases the endothelial release of ADMA and that ADMA reduces the synthesis of NO while increasing the generation of superoxide anion. However, in this study it took rather high concentrations (10 μm) of exogenous ADMA to have a measurable effect upon NO synthesis, superoxide anion generation and endothelial adhesiveness. This concentration of ADMA is at the upper range of plasma ADMA levels observed in disease (2 to 10 μm).

However, chronic exposure to the lower levels of ADMA observed in disease states appears to have similar effects on endothelial biology as does short-term exposure to the higher dose used in this study. Specifically, we and others have demonstrated that the two to three-fold increase in plasma ADMA observed in hypercholesterolemic individuals is associated with reductions in endothelium dependent vasodilation and urinary nitrate excretion—effects that are reversed by administration of L-arginine (14,15). Moreover, plasma ADMA levels in the subjects correlate better with endothelial vasodilator function than does LDL cholesterol (15).

The mechanism by which L-arginine exerts its beneficial effects appears to be through enhanced production of endothelium-derived NO (14,23). Originally, this was termed the "arginine paradox," because the effect of L-arginine was surprising, given the low K_m of the purified endothelial NO synthase (2.9 μM) (41) and the relatively high physiological plasma concentrations of L-arginine (60 to 100 μmol/L), which are unchanged in hypercholesterolemia (42).

The "L-arginine paradox" may be explained by the presence of endogenous competitive NO inhibitors such as ADMA (10). The ADMA plasma levels are approximately 1 μmol/L in healthy humans and are elevated two-fold in hypercholesterolemic individuals (15). In elderly patients with peripheral arterial disease and generalized atherosclerosis, plasma ADMA levels range from 2.5 to 3.5 μmol/L, corresponding to the severity of the vascular disease (14). For patients with renal failure, plasma ADMA levels may be elevated nine-fold (10).

Of note, we find that the intracellular ADMA level within endothelial cells is considerably higher than those in the conditioned media. The same may be true for ADMA levels in endothelial cells in vivo with respect to human

plasma. Our observation that endothelial cells are a source of ADMA corroborates the previous finding by Fickling et al. (11). These elevations in plasma ADMA are likely of pathophysiological significance because exogenous ADMA concentrations between 1 and 10 μM affect the activity of the NO synthase in rat mesentery tissue (43), in rat brain (44) and in cultured macrophages (11). Faraci et al. (50) calculated an IC_{50} (concentration inducing half-maximal inhibition of enzyme activity) for ADMA of 1.8 μM in cerebellar tissue (44), and Fickling et al. (11) reported that 2 and 10 μM ADMA inhibited nitrite production in activated macrophages by 17 and 33%, respectively. These data, taken together with the results of this study, suggest that ADMA may be a potential autocrine regulator of endothelial NO synthase.

In conclusion, our study shows that ADMA, an endogenous competitive inhibitor of NO synthesis, increases endothelial superoxide radical formation, increases MCP-1 secretion and enhances adhesiveness of the endothelium for monocytes. Asymmetric dimethylarginine elaboration in endothelial cells is enhanced by LDL. Asymmetric dimethylarginine may act as an autocrine proatherogenic molecule in endothelial cells. Because ADMA is elevated in many disorders associated with atherosclerosis (that is, hypercholesterolemia, hypertension, diabetes mellitus, tobacco use, aging and hyperhomocysteinemia), it may represent one common pathway by which these risk factors induce endothelial dysfunction and atherogenesis.

Reprint requests and correspondence to: Dr. John P. Cooke, Division of Cardiovascular Medicine, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, California 94305-5406. E-mail: john.cooke@ stanford.edu.

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