Enhanced Endothelial Adhesiveness in Hypercholesterolemia Is Attenuated by L-Arginine

Philip S. Tsao, PhD; Leslie M. McEvoy, PhD; Helmut Drexler, MD; Eugene C. Butcher, MD; John P. Cooke, MD, PhD

**Background** We have shown that chronic administration of the nitric oxide (NO) precursor L-arginine normalizes NO-dependent vasodilation and markedly inhibits atherogenesis in a hypercholesterolemic rabbit model. We hypothesized that this antiatherogenic effect is due to modulation of endothelial adhesiveness by endothelium-derived NO.

**Methods and Results** New Zealand White rabbits were fed normal chow (Cont), a high-cholesterol diet (Chol), a high-cholesterol diet supplemented with L-arginine (Arg), or a normal diet supplemented with the NO synthase antagonist L-nitroarginine (L-NA) for 2 weeks. In additional studies, some animals receiving L-NA were also treated with hydralazine to normalize blood pressure. After 2 weeks, thoracic aortas were harvested, opened longitudinally, and placed in a culture dish with the endothelial surface exposed to medium containing WEHI 78/24 cells, a monocytoid cell line. After incubation with the monocytoid cells for 30 minutes on a rocking platform, the aortic segments were washed repeatedly to remove nonadherent cells and adherent cells counted by epifluorescent microscopy. Monocytoid cell binding to aortic endothelium was significantly increased in Chol (P<.001 versus Cont); binding was markedly reduced in arginine-fed hypercholesterolemic animals (P<.05, Arg versus Chol). Monocytoid cell binding to aortic endothelium was also significantly increased in L-NA (P<.05); hydralazine normalized blood pressure but did not reduce monocytoid cell binding. To confirm that alterations in NO activity modulate endothelial cell–monocyte interaction, the release of nitrogen oxides (NOx) by thoracic aortas was assessed by a chemiluminescent technique. The concentration of NOx in the conditioned medium from segments of Arg thoracic aortas was significantly greater than that from Cont aortas, whereas that from L-NA aortas was significantly less.

**Conclusions** Hypercholesterolemia enhances the adhesiveness of aortic endothelium for monocytes; this effect is attenuated by dietary L-arginine. Conversely, inhibition of NO synthesis enhances monocyte binding. The results suggest that endothelium-derived NO plays an important role in regulating the endothelial adhesiveness for monocytes. Alterations in NO activity may play a critical role in atherogenesis. *(Circulation. 1994;89:2176-2182.)*

**Key Words** • atherosclerosis • arginine • endothelium-derived factors

The earliest observable abnormality of the vessel wall in hypercholesterolemic animals is enhanced monocyte adherence to the endothelium,1 which occurs within 1 week of a high-cholesterol diet. This event is thought to be mediated by the surface expression of endothelial adhesion molecules and chemotactic proteins induced by hypercholesterolemia.

Another endothelial alteration that occurs in parallel is a reduced activity of endothelium-derived relaxing factor (EDRF)2-7. This abnormality is observed very early after the initiation of a high-cholesterol diet, well before any structural changes in the vessel wall.2 Indeed, the adverse effects of low-density lipoprotein (LDL) cholesterol on EDRF activity occur within minutes. When isolated vascular rings from normal animals are exposed to LDL cholesterol in concentrations approximating those in hypercholesterolemic individuals, endothelium-dependent relaxation is markedly inhibited after 30 minutes.3 This property of LDL cholesterol is probably dependent on its oxidation or modification and is not shared by high-density lipoprotein (HDL) cholesterol.4,5

EDRF is now known to be nitric oxide (NO) or a related compound derived from metabolism of L-arginine.8-9 NO not only is a potent vasodilator but also inhibits leukocyte–endothelial cell interaction,10-14 platelet adherence and aggregation,15,16 and vascular smooth muscle cell proliferation.17 Because these are critical processes in atherogenesis, the reduction in NO activity induced by hypercholesterolemia may promote atherogenesis. Conversely, an enhancement of NO synthesis may inhibit atherogenesis. We and others have shown that acute administration of the NO precursor L-arginine to hypercholesterolemic animals or humans normalizes NO-dependent vasodilation.18-21 More recently, we showed that chronic dietary supplementation with L-arginine restores NO-dependent vasodilation in hypercholesterolemic rabbits and that this improvement in NO activity is associated with a striking antiatherogenic effect.22 In the present study, we test the hypothesis that the antiatherogenic effect of dietary arginine is mediated by endothelium-derived NO, which inhibits monocyte–endothelial cell interaction.

**Methods**

**Animals** Male New Zealand White rabbits were pair fed, receiving one of the following dietary interventions for 2 weeks: normal rabbit...
chow (Cont, n=7), rabbit chow enriched with 1% cholesterol (Chol, n=7) (ICN Biomedical, Cleveland, Ohio), or 1% cholesterol chow supplemented with 2.25% l-arginine HCl in the drinking water (Arg, n=7) ad libitum throughout the course of the study. Supplementation with 2.25% l-arginine HCl (Sigma Chemical Co.) in the drinking water results in a sixfold enrichment of dietary arginine (based on the daily average water and food intake of the animals) and results in a twofold increase in plasma free arginine levels. In a second series of studies designed to further explore the role of endogenous NO on monocyte-endothelial cell interaction, another group of animals was pair fed, receiving a normal rabbit diet supplemented with either vehicle control (n=5) or the NON synthase antagonist nitro-l-arginine (L-NA, 10 mg/100 mL; n=5) administered in the drinking water ad libitum throughout the course of the study (for an average daily dose of 13.5 mg·kg⁻¹·d⁻¹). In a third series of experiments, animals received a normal diet and either vehicle (n=4), L-NA (13.5 mg·kg⁻¹·d⁻¹; n=4), or L-NA and hydralazine (n=4) added to the drinking water for 2 weeks. At this dose, hydralazine (5 mg·kg⁻¹·d⁻¹; Schein Pharmaceutical, Inc) reversed the increase in blood pressure induced by L-NA. In a fourth series of studies, after 2 weeks of treatment, thoracic aortas were harvested from Cont (n=3), Arg (n=3), Chol (n=3), and L-NA (n=4) animals for measurement of nitrogen oxides as described below.

One day before they were killed (after 2 weeks of dietary intervention), animals were lightly sedated with 3 mg subcutaneous injection of acepromazine maleate solution (Ayerst Laboratories), and the central ear artery was cannulated for measurement of intra-arterial blood pressure, followed by collection of blood samples in EDTA. Total plasma cholesterol levels as well as HDL were enzymatically measured by a spectrophotometric assay (Sigma). Aliquots of plasma were deproteinized with 2% sulfosalicylic acid and analyzed for free arginine by use of an automated amino acid analyzer (Beckman 6300). These protocols were approved by the Administrative Panel on Laboratory Animal Care of Stanford University and were performed in accordance with the recommendations of the American Association for the Accreditation of Laboratory Animal Care.

Mononuclear Cell Culture and Isolation

Murine monocytoid cells, WEHI 78/24 cells (ATCC), were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum (FCS) and were kept in an atmosphere of 5% CO₂/95% air. Before binding studies, mononuclear cells were fluorescently labeled. Cells were allowed to incubate in RPMI medium (Gibco/BRL) containing TRITC (3 μg/mL; Molecular Gibco/BRL Probes) for 15 minutes at room temperature. The cell suspension was carefully underlaid with a layer of FCS and then centrifuged at 400g to separate labeled cells from the remaining dye. Cells were washed in complete medium and resuspended in Hank's balanced salt solution (HBSS; Irvine Scientific) containing 2 mmol/L Ca²⁺, 2 mmol/L Mg²⁺, and 20 mmol/L HEPES for binding studies.

The WEHI 78/24 cells are an excellent model for blood-borne monocytes because they are nonadherent, slightly phagocytic, and, like blood monocytes, express the glycoprotein adhesion molecules MAC-1, LFA-1, L-selectin, and α4. Most importantly, these cells demonstrate selective binding to the endothelium in inflamed but not uninflamed lymph nodes, and their ability to bind to these venules precisely parallels the observed interactions of circulating blood-borne monocytes in vivo with venules at various times after footpad injection of complete Freund's adjuvant (L.M.M., E.C.B., unpublished observations, 1989).

Nevertheless, to confirm the results using WEHI 78/24 cells, in some studies we performed binding studies in parallel using normal rabbit mononuclear cells isolated from fresh whole blood of control rabbits before they were killed. Rabbits were prepared and killed as outlined below. Whole blood (40 mL) was retrieved by cardiac puncture in sodium citrate (10:1 vol/vol) and heparin (10 U/mL). Anticoagulated blood was centrifuged at 400g for 10 minutes at room temperature. Platelet-rich plasma was removed and the volume replaced with HBSS without calcium or magnesium. The samples were then centrifuged at 650g for 10 minutes at room temperature. Theuffy coats were removed and resuspended in 6 mL with HBSS. Containing red cells were lysed by ammonium chloride solution (0.17 mol/L NH₄Cl, 0.01 mol/L KHCO₃, and 92 μmol/L EDTA) for 6 minutes. Total leukocytes were then centrifuged at 1000g for 5 minutes at room temperature. The resulting pellet was resuspended in 3 mL HBSS, carefully layered on to a cushion of 1.081-d Histopaque (Sigma), and centrifuged at 400g for 30 minutes at room temperature. After centrifugation, the mononuclear cells were aspirated from the opaque interface. Cells were then washed and subsequently resuspended in HBSS containing 1 mmol/L Ca²⁺, 1 mmol/L Mg²⁺, and 10 mmol/L HEPES for binding studies.

Preparation of Aortic Endothelium and Binding Assay

Groups of four to six animals (including at least one control animal) were injected intravenously with the thrombin inhibitor D-phenylalanyl-l-prolyl-l-arginy1 chloromethyl ketone (PPACK, 100 ng/kg) to limit platelet interference with the mononuclear cell binding studies (in preliminary studies PPACK had no effect on mononuclear cell binding to endothelium isolated from control or hypercholesterolemic rabbits). Rabbits were killed by intravenous injection of sodium pentobarbital (Abbott Laboratories) (35 mg/kg). Thoracic aortas were removed and placed in cold, oxygenated phosphate-buffered saline. After the adventitia was removed, a 15-mm segment of thoracic aorta was excised from a point immediately distal to the left subclavian artery. The segments were then carefully opened longitudinally and placed into 35-mm culture dishes containing 2 mL HBSS. Aortic strips were then placed on a rocking platform (Research Products International Corp.) at room temperature.

After 10 minutes the HBSS medium was replaced by binding medium (HBSS supplemented with 2 mmol/L Ca²⁺, 2 mmol/L Mg²⁺, and 20 mmol/L HEPES) containing WEHI 78/24 cells (2 mL containing 10⁴ cells/mL). The aortic strips were incubated with the mononuclear cells for 30 minutes, with the dishes rotated 120° every 10 minutes. The medium was then aspirated and replaced by 2 mL fresh binding medium without cells to remove nonadherent cells. After a second washing, the dishes were placed on the rocker platform for an additional 5 minutes. The aortic segments were then removed and placed on a glass slide with the endothelial side up. Adherent cells were counted under epifluorescent microscopy from at least 30 sites on each segment. The data are expressed as a percentage of the number of adherent cells on thoracic aorta from the pair-fed normocholesterolemic animal (Cont) studied in parallel.

Measurement of Nitrogen Oxide Release

In some experiments, the aortic segments were prepared as described above and incubated with 2 mL HBSS medium containing calcium ionophore (1 μmol/L; Sigma) and l-arginine (100 μmol/L; Sigma). At selected time points (0, 30, 60, and 120 minutes), samples of the medium (100 μL) were collected for measurement of nitrogen oxides (NO and one-electron oxidation products of NO). Nitrogen oxides (NOx) were measured with a commercially available chemiluminescence apparatus (model 2108, Dasiabi) after reduction of the samples in boiling acidic vanadate (III) at 98°C. Boiling acidic vanadate quantitatively reduces NO₂⁻ and NO₃⁻ 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 NO₂⁻/NO₃⁻ were linear over the range of 100 pmol to 5 nmol.
TABLE 1. Hemodynamic and Plasma Chemistry Measurements

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<th>MAP, mm Hg</th>
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<th>HDL, mg/dL</th>
<th>Plasma Arginine, mmol/L</th>
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<td>76±3.7</td>
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<td>77±4.8</td>
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<td>23.2±4.0</td>
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<td>Nitro-L-arginine (n=5)</td>
<td>101±8.8*</td>
<td>38.8±5.5</td>
<td>22.5±3.6</td>
<td>0.17±0.03</td>
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<tr>
<td>Nitro-L-arginine+hydralazine (n=4)</td>
<td>80±4.0</td>
<td>44.5±7.2</td>
<td>25.3±4.1</td>
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MAP indicates mean arterial pressure; HDL, high-density lipoprotein.

*P<.01.

Superoxide Radical Assay

We hypothesize that the effect of L-arginine to enhance NO activity in the vessel wall is due to its metabolism by NO synthase. Others have suggested the alternative hypothesis that L-arginine enhances NO activity by acting as a scavenger of oxygen-derived free radicals, which are known to degrade NO.23 We therefore examined the activity of L-arginine as a scavenger of superoxide radicals. Xanthine and xanthine oxidase were used to generate superoxide radical synthetically according to the method of McCoD and Fridovich.24 A solution containing potassium phosphate (50 mmol/L, pH 7.8), EDTA (10 mmol/L), ferriytochrome c type III (10 μmol/L), xanthine (50 μmol/L), and potassium cyanide (200 μmol/L) was incubated with L-arginine (0.1 to 10 mmol/L) or superoxide dismutase (SOD) (10 μg/mL) (Boehringer Mannheim). Xanthine oxidase (12.3 U/mL) was added to the reaction mixture at 25°C, and the reduction of cytochrome c was measured spectrophotometrically after 2 minutes at 550 nm.

LDL Isolation and Oxidation

An alternative explanation for the salutary effect of L-arginine is that it may interfere directly with the oxidation of LDL cholesterol. To test this hypothesis, LDL was isolated by a modification of the method outlined by Fong et al.25 Whole peripheral blood was drawn from preprandial normocholesterolemic donors into EDTA (0.2 mL of 10% EDTA wt/vol per 10 mL whole blood) and centrifuged at 100g for 10 minutes at 4°C. The plasma was transferred to a new tube, and the density was adjusted to 1.019 with KBr. Samples were then centrifuged at 38,000 rpm at 6°C for 16 hours. The top lipid layer was carefully removed and its density adjusted to 1.063 with KBr, followed by centrifugation at 38,000 rpm at 6°C for 16 hours. The resulting LDL fraction was removed and dialyzed against phosphate-buffered saline containing 0.01% EDTA wt/vol at 4°C. Protein concentration was measured by the Lowry method.

Oxidation of LDL was achieved by diluting LDL into 1.99 mL F-10 medium containing NaHCO3 and glutamine (Gibco-BRL) to give a final LDL protein concentration of 100 μg/mL. L-Arginine (0.2 to 0.8 mmol/L), urea (10 mg/dL), or butylated hydroxytoluene (BHT) (20 μmol/L) was added immediately before oxidation. Samples were then incubated with copper sulfate (10 μmol/L) at 37°C for 18 to 24 hours. BHT (20 μmol/L) was subsequently added, and LDL was stored at 4°C before use. The extent of oxidation was assessed by measurement of thiobarbituric acid–reactive substances (TBARS) spectrophotometrically at 532 nm using malondialdehyde bis(di-methyl acetal) (Aldrich Chemical Co) as a standard.

Data Analysis

All values in the text are mean±SEM of n independent experiments. Differences between specific means were tested by ANOVA with post hoc analysis using the Bonferroni t test. A value of P<.05 was accepted as being statistically significant.

Results

Hemodynamics and Serum Chemistries

Total plasma cholesterol concentration was elevated in the two groups receiving the 1% cholesterol diet (Chol, 803±113 mg/dL and Arg, 846±56.1 mg/dL) compared with those receiving regular chow (Control, 53.8±11.1 mg/dL and L-NA, 38.8±5.5 mg/dL) (P<.01). There were no differences in the HDL fraction among any of the groups (Table 1). Analysis of amino acid levels in blood samples drawn demonstrated that the 1% cholesterol diet had no effect on plasma arginine; supplementation with L-arginine in the drinking water produced a significant twofold increase in free arginine levels (P<.01). In the animals receiving the 1% cholesterol diet, no difference was observed in mean arterial blood pressure compared with control animals (Table 1). However, administration of the NO synthase antagonist L-NA (10 mg/100 mL in drinking water) elevated mean arterial blood pressure after 2 weeks. In those animals simultaneously treated with hydralazine (5 mg·kg-1·d-1), blood pressure was maintained at normal levels.

Monocytoid Cell Adhesion to Rabbit Aortic Endothelium

Exposure of WEHI 78/24 cells to normal rabbit aortic endothelium results in a minimal cell binding in this ex vivo adhesion assay. However, when WEHI 78/24 cells were incubated with aortic endothelium from hypercholesterolemic animals (Chol, n=7), cell binding was enhanced threefold compared with Cont (n=7). As depicted in Fig 1, the aortic endothelium of the hypercholesterolemic animal (B). The fluorescently labeled monocytoid cells adhere in greater numbers to the endothelium of the vessel harvested from the hypercholesterolemic animal. The aggregate data are displayed in Fig 2 and represent the average number of cells binding per high-power field (the value for each vascular segment is derived from quantifying 30 high-power fields). The increased cell binding manifested by aortic endothelium of hypercholesterolemic animals was significantly attenuated by L-arginine supplementation (n=7; Fig 2). Similar results were achieved when adhesion assays were performed in parallel with mononuclear cells that were freshly isolated from Cont animals (n=2) in each of the three groups (data not shown).

Effect of Chronic NO Synthase Inhibition on Endothelial Adhesiveness

To further investigate the role of endothelium-derived NO in modulating endothelial cell–monocyte interaction, an additional series of binding studies was performed using thoracic aorta from animals that received regular chow supplemented with vehicle (n=5) or the NO synthase inhibitor L-NA (n=5). The adhesion of WEHI
78/24 cells was markedly increased when they were incubated with aortic endothelium from L-NA animals compared with control endothelium (Fig 3). This effect could not be attributed to hypertension caused by L-NA, since concomitant administration of hydralazine normalized blood pressure but did not reverse the augmentation of cell binding induced by L-NA (Fig 4).

Biological Studies

**Generation of Nitrogen Oxides**

NO\textsubscript{3} concentration increased in a time-dependent manner in the conditioned medium from culture dishes containing segments of thoracic aorta. At 60 and 120 minutes, the concentration of NO\textsubscript{3} was significantly higher in conditioned medium from the Arg segments (n=3) in comparison with the other groups (Cont, n=3; Chol, n=3; L-NA, n=4; see Fig 5). At 120 minutes, NO\textsubscript{3} levels were significantly lower in conditioned medium from the L-NA group in comparison with the other groups (Fig 5).

**Effect of L-Arginine on the In Vitro Generation of Superoxide Radical**

Superoxide anion was generated by the addition of xanthine oxidase to xanthine, and the reduction of cytochrome c was monitored by absorbance at 550 nm. L-Arginine (0.1 to 10.0 mmol/L) had no effect on the concentration of superoxide anion generated. By contrast, SOD effectively scavenged the superoxide anion (P<.01, Control versus SOD condition; n=5 in each group; Table 2).

**Fig 1.** Epifluorescent photomicrographs of thoracic aortas from normal (A) and hypercholesterolemic (B) rabbits after incubation with fluorescent-labeled WEHI 78/24 cells. Note increased monocyte binding to the endothelium of the thoracic aorta harvested from the hypercholesterolemic animal.

**Fig 2.** Bar graph showing quantification of monocytoid cell binding to rabbit aortic endothelium per high-power field in the ex vivo adhesion assay. Segments of thoracic aortas from control (Cont) animals (n=7), those fed a high-cholesterol diet (Chol) (n=7), and those fed a high-cholesterol diet supplemented with L-arginine (Arg) (n=7) were harvested and the functional binding assays performed as depicted in Fig 1. On each of 7 experimental days, binding assays were performed in parallel with thoracic segments from Cont, Chol, and Arg groups. As shown in Fig 1, the number of adherent cells was quantified using epifluorescent microscopy; 30 high-power fields per aortic segment were counted and averaged to give one average value per segment. All values were expressed as a percent of the Cont animal. There was a threefold increase in the number of monocytoid cells bound to the thoracic aortas from Chol animals. Arginine treatment significantly reduced endothelial adhesiveness.

**Fig 3.** Bar graph showing quantification of monocytoid cell binding to thoracic aortas from normocholesterolemic animals receiving vehicle (Cont) or nitro-L-arginine (13.5 mg·kg\textsuperscript{-1}·d\textsuperscript{-1}) for 2 weeks. The functional binding assay was performed as described in "Methods" and as shown in Fig 1. Chronic oral administration of nitro-L-arginine markedly enhanced cell adhesion to the endothelium ex vivo.
incubation with copper sulfate. Neither L-arginine nor urea had any effect on the oxidation of human LDL cholesterol, whereas the antioxidant BHT significantly inhibited the formation of TBARS \((P<.01, \text{Control versus BHT condition; } n=5 \text{ in each group; Table 2})\).

**Discussion**

The salient findings of this investigation are that (1) the endothelium of thoracic aorta from hypercholesterolemic rabbits manifests greater adhesiveness for monocyte cells; (2) this increase in endothelial adhesiveness is attenuated in hypercholesterolemic animals treated chronically with the NO precursor \(L\)-arginine; (3) endothelial adhesiveness is increased in vessels from normocholesterolemic animals treated with the NO synthase antagonist \(L\)-NA; (4) this effect of NO synthase antagonism is not reversed by administration of hydralazine in doses sufficient to normalize blood pressure; and (5) the alterations in monocyte cell binding induced by \(L\)-arginine or \(L\)-NA treatment are paralleled by reciprocal alterations in the elaboration of NO, by the aortic endothelium. The findings are consistent with the hypothesis that NO is an endogenous antiatherogenic molecule by virtue of its inhibition of monocyte–endothelial cell interaction.

Mononuclear cell adherence to the endothelium and subendothelial diapedesis and migration occur within a week after the initiation of a high-cholesterol diet in primates, leading to the development of intimal lesions that contain subendothelial macrophage-derived foam cells interspersed with small numbers of non-lipid-filled macrophages and T lymphocytes. A variety of in vitro models have been developed to investigate the mechanisms that initiate this monocyte–endothelial cell interaction. Monocytes isolated from patients with combined hypercholesterolemia/hypertriglyceridemia adhere to cultured human umbilical vein endothelial cells (HUVECs) to a greater degree than those from healthy subjects. Similarly, when HUVECs are incubated with plasma from cholesterol-fed rabbits or with minimally modified or copper-oxidized LDL, they bind normal monocytes to a greater degree. This increased adhesion induced by treatment with modified LDL cholesterol is selective for monocytes in that neutrophil binding is unaffected.

The endothelial alteration or dysfunction that precipitates monocyte binding is not well defined. It seems likely that the induced expression of endothelial adhesion molecules and chemotactic proteins plays a major role. We propose that endothelium-derived NO modulates the expression of these adhesion molecules and/or chemotactic proteins. The first evidence to support this notion came from the work of Bath and colleagues. These investigators found that exogenous NO inhibited mononuclear cell adherence to porcine aortic endothelial cells in culture. In addition, exogenous NO or prostacyclin inhibited in vitro chemotaxis stimulated by \(N\)-formyl-methionyl-leucyl phenylalaine. More recently, Zeiher and colleagues found that inhibition of NO synthase increases mRNA expression and secretion of monocyte chemotactic protein-1 (MCP-1) in primary cultures of HUVECs. Incubation of HUVECs with the NO donor SIN-1 dose-dependently decreased basal and stimulated mRNA expression and secretion of MCP-1 into the medium. To summarize, recent data suggest that endogenous NO may modulate endothelial cell–monocyte interaction. One of the first

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**Fig 4.** Bar graph. The hypertensive effect of nitro-L-arginine (L-NA) cannot explain the increased adhesiveness of aortic endothelium isolated from these rabbits. Reduction of blood pressure with hydralazine (5 mg·kg\(^{-1}\)·d\(^{-1}\)) in rabbits also receiving L-NA had no significant effect on the increased adhesion of WEHI 78/24 cells to aortic endothelium. Each set of bars represents one of four experimental days. On each experimental day, the thoracic aorta was harvested from a control animal (Cont; 2 weeks of treatment with vehicle control; \(n=4\)), and L-NA-treated animal (L-NA; 2 weeks of treatment with L-NA 13.5 mg·kg\(^{-1}\)·d\(^{-1}\); \(n=4\)), or an L-NA–treated animal receiving hydralazine (5 mg·kg\(^{-1}\)·d\(^{-1}\); \(n=4\)). Subsequently, monocyte cell binding studies were performed ex vivo as described in the text.

**Effect of \(L\)-Arginine on Copper-Catalyzed Oxidation of LDL Cholesterol**

Freshly isolated human LDL (100 \(\mu\)g/mL) was incubated with 5 mmol/L copper sulfate in 2 mL of Ham's F-10 medium at 37°C for 18 to 24 hours. At the end of incubation, medium was measured for TBARS. In some experiments \(L\)-arginine (0.2 to 0.8 mmol/L), urea (10 to 40 mg/dL), or BHT (20 \(\mu\)mol/L) was added before the incubation with copper sulfate. Neither \(L\)-arginine nor urea had any effect on the oxidation of human LDL cholesterol, whereas the antioxidant BHT significantly inhibited the formation of TBARS \((P<.01, \text{Control versus BHT condition; } n=5 \text{ in each group; Table 2})\).

**Fig 5.** Graph showing release of nitrogen oxides (NO\(_x\)) by the endothelium of thoracic aortas. Segments of thoracic aorta were incubated in Hanks' balanced salt solution containing 10\(^{-8}\) mol/L calcium ionophore (to stimulate NO synthase activity). The conditioned medium was sampled for chemiluminescence measurement of NO\(_x\) at 0, 30, 60, and 120 minutes. A time-dependent increase in NO\(_x\) levels is observed, reflecting stimulated release of NO from the vessel wall. \(\Delta\), \(\bullet\), and \(\triangle\) indicate conditioned medium from vessel segments in the groups fed a high-cholesterol diet supplemented with \(L\)-arginine (\(n=3\)), normal chow (\(n=3\)), a high-cholesterol diet (\(n=3\)), and a normal diet supplemented with nitro-\(L\)-arginine (\(n=4\)), respectively. *Significantly different value by ANOVA in comparison with the other three groups, \(P<.05\).
endothelial dysfunctions observed in hypercholesterolemic animals and humans is reduced NO-dependent vasodilation; this abnormality occurs before the onset of observable structural changes in the vessel wall.\textsuperscript{37,38} We propose that a reduced activity of NO in the vessel wall facilitates monocyte–endothelial cell interaction in hypercholesterolemia.

In the present investigation, the binding of monocytoid WEHI 78/24 cells (as well as freshly isolated rabbit mononuclear cells) to the endothelium of vascular segments from hypercholesterolemic animals was increased threefold compared with vascular segments from control animals. The enhanced adhesiveness of the aortic endothelium from hypercholesterolemic animals was significantly reduced by dietary arginine supplementation (Fig 2). Conversely, when the synthesis of NO was chronically inhibited in normcholesterolemic animals by L-NA, monocytoid–endothelial cell interaction was enhanced (Fig 4). This effect of L-NA cannot be explained by the hypertension that it induced because concomitant administration of hydralazine (which restored normal blood pressure) had no significant effect on the enhancement by L-NA of endothelial adhesiveness. These alterations in endothelial adhesiveness induced by the NO precursor L-arginine as well as the NO synthase inhibitor L-NA are paralleled by changes in the endothelial elaboration of NO.\textsuperscript{39} Taken together, these observations support the hypothesis that chronic endogenous NO modulates the affinity of endothelial cells for monocytes.

Other paracrine factors produced by the endothelium may be involved. The generation of superoxide anion by the endothelium is accelerated in hypercholesterolemia and is a major factor in the initiation and maintenance of a number of atherogenic processes.\textsuperscript{22,39} After 1 month of a 1% cholesterol diet, rabbit thoracic aortas produce threefold more superoxide anion than normal vessels; this increase in the generation of superoxide anion is largely due to the increased activity of endothelial xanthine oxidase.\textsuperscript{40} Antioxidants scavenge superoxide anion, enhance endothelium-dependent relaxation, and reduce lesion formation.\textsuperscript{39,41} Adhesion of leukocytes in vivo, induced by an infusion of oxidized LDL, is significantly inhibited by infusion of SOD or by heparin-induced release of extracellular SOD from the endothelium.\textsuperscript{42} Therefore, it is possible that arginine improves NO activity and decreases monocyte adhesion by acting as an antioxidant. However, our biochemical studies reveal that arginine lacks direct antioxidant properties. Although these studies suggest that the antiadhesive effects of L-arginine are not mediated by a direct antioxidant effect, they do not exclude the possibility that a metabolite of arginine (ie, NO) may interfere with the elaboration of superoxide anion. Removal of the endothelium increases the elaboration of superoxide anion in the thoracic aorta of normolipidemic rabbits.\textsuperscript{40} Moreover, NO may reduce the oxidative modification of LDL by macrophages.\textsuperscript{43} In that recent study, the investigators discovered that the oxidation of LDL cholesterol by macrophages was inversely proportional to their generation of NO.\textsuperscript{44} The inhibition by NO of LDL oxidation may be due to a direct inhibitory effect on enzyme systems that generate oxygen-derived free radicals, such as NADPH oxidase.\textsuperscript{44} It is therefore possible that the ability of NO to modulate endothelial adhesiveness is conferred by its activity against superoxide anion.

To conclude, we have used an ex vivo model of mononuclear cell binding to study the increase in endothelial adhesiveness induced by hypercholesterolemia. We show for the first time that endothelial adhesiveness is attenuated by oral administration of the NO precursor L-arginine. Conversely, inhibition of NO synthase activity by oral administration of L-NA strikingly increases endothelial affinity for monocyte cells in vitro. These alterations in endothelial adhesiveness are paralleled by reciprocal changes in the release of NO, by the vessel wall. The data are consistent with the hypothesis that NO is an endogenous antiatherogenic molecule.

**Acknowledgments**

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**Table 2. L-Arginine Lacks Antioxidant Properties**

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<td>0.4</td>
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*The generation of superoxide radical by xanthine/xanthine oxidase was measured by monitoring the reduction of cytochrome c by spectrophotometry at 550 nm. Values are expressed as absorbance values × 10^5. L-Arginine (0.01 to 10 mmol/L) had no effect, whereas superoxide dismutase (SOD) substantially reduced the levels of superoxide anion.

†The oxidation of low-density lipoprotein (LDL) cholesterol with copper sulfate was assessed by measurement of thiobarbituric acid–reactive substances (TBARS) by spectrophotometry at 532 nm. The generation of TBARS was unaffected by L-arginine and urea but substantially reduced by butylated hydroxytoluene (BHT).
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