Effect of Native and Oxidized Low-Density Lipoprotein on Endothelial Nitric Oxide and Superoxide Production
Key Role of L-Arginine Availability

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Background—Native and oxidized LDLs (n-LDL and ox-LDL) are involved in the atherogenic process and affect endothelium-dependent vascular tone through their interaction with nitric oxide (NO).

Methods and Results—In this study we evaluated directly, by using a porphyrinic microsensor, the effect of increasing lipoprotein concentrations on endothelial NO and superoxide (O$_2^-$) production. We investigated where lipoproteins may affect the L-arginine–NO pathway by pretreating cells with L-arginine, L-N-arginine methyl ester (L-NAME), and superoxide dismutase. Bovine aortic endothelial cells were exposed for 1 hour to increasing concentrations of n-LDL (from 0 to 240 mg cholesterol/dL) and ox-LDL (from 0 to 140 mg cholesterol/dL). A stimulated (calcium ionophore) NO concentration decreased to 29% of the control at n-LDL concentration of 80 mg cholesterol/dL and to 15% of the control at 20 mg cholesterol/dL of ox-LDL. L-Arginine partially neutralized the inhibitory effect of n-LDL and ox-LDL on the NO generation. Superoxide dismutase pretreatment did not modify NO production, whereas L-NAME blunted NO generation at all LDL concentrations. O$_2^-$ production was increased at low n-LDL and very low ox-LDL concentrations; this was reversed by L-arginine.

Conclusions—These findings confirm the inhibitory role of n-LDL and ox-LDL on NO generation and suggest that lipoproteins may induce a decreased uptake of L-arginine. The local depletion of the L-arginine substrate may derange the NO synthase, leading to overproduction of O$_2^-$ from oxygen, the other substrate of NO synthase. (Circulation. 2000;101:1261-1266.)

Key Words: nitric oxide ▪ endothelium ▪ lipoproteins

Various pathological conditions such as hyperlipidemia, atherosclerosis, diabetes, and hypertension are associated with short-term or long-term alterations in the structural and functional properties of the vascular endothelium.1,2 Thus in hypercholesterolemia and atherosclerosis, the regulatory function of endothelium-dependent relaxation is impaired, and this defect in vascular tone homeostasis occurs in the early stage of the atherogenic process. It has been suggested that the biological activity of nitric oxide (NO) is impaired, which leads to reduced vascular relaxation, increased platelet aggregation, increased leukocyte adhesion, and migration and proliferation of smooth muscle cells.3-8 Evidence supports a crucial role of native LDL (n-LDL) and oxidized-LDL (ox-LDL) in the hypercholesterolemia-related endothelial dysfunction and strongly links the decreased NO production to the lipoprotein interaction with the arterial wall and to circulating L-arginine.9-11

Recent results also suggest an impaired endothelial NO synthase (NOS III) gene expression or a decreased activity of the normally expressed enzyme. Endothelial cells exposed for prolonged periods to n-LDL or ox-LDL begin to produce superoxide.11-13 The superoxide production can be inhibited by pretreatment with NOS antagonists or by supplementation with l-arginine.14 Huk et al14 suggested that the conformational change in the synthase, caused by a low l-arginine availability, may promote NOS III generation of superoxide. Under oxidative stress, a great number of oxidant-responsive genes may be activated; this situation leads to an increased production of growth factors, chemokines, and adhesion molecules that enhance the inflammatory response involved in the vascular damage observed in atherogenesis.7 NO and superoxide react and lead to an overproduction of cytotoxic peroxynitrite. The presence of peroxynitrite in advanced plaques has been documented by the use of nitrotyrosine antibodies.15

Recent evidence has shown high accumulation of asymmetrical N$^0$ dimethyl-l-arginine (ADMA), which is an endogenous inhibitor of NOS, in plasma from hypercholester-
olic animals. L-Arginine might compete with ADMA for the enzyme, and when the intracellular availability of the amino acid in the vicinity of the synthase is insufficient, L-arginine supplementation restores NOS activity and normalizes NO production. With the porphyrinic sensor placed near the cell surface, the aim of this study was to evaluate locally the effect of increasing n-LDL and ox-LDL concentrations on endothelial NO production and O$_2$- release by bovine aortic endothelial cells. Our results show that both n-LDL and ox-LDL cause a decrease in stimulated NO production and a parallel increase in stimulated O$_2$- production in cultured endothelial cells and that these processes can be reversed by L-arginine supplementation.

Methods

Cell Culture Techniques

Bovine aorta was filled with 0.1% collagenase (Sigma, type I) in medium 199 (Sigma). After an 8- to 12-minute incubation at 37°C, dissociated bovine aortic endothelial cells were collected, washed by centrifugation at 1000g for 10 minutes at 4°C, and resuspended in DMEM containing 10% FBS and 1% sodium chloride solution, the plasma was centrifuged at 150 000 g for 24 hours, and the chylomicron-rich layers were discarded. The supernatant was used for preparation of LDL. LDL was incubated with thiobarbituric acid (0.5 wt/vol, in H$_2$SO$_4$, 50 mmol/L) for 30 minutes at 100°C. The solution then was centrifuged for 5 minutes, and the difference in absorbency at 532 and 580 nm was calculated. TBARS concentration was determined as MDA equivalents with the use of an MDA standard curve.

LDL Isolation

Venous blood from healthy normolipidemic volunteers was collected into Na-EDTA (1 mg/mL blood) vacuum tubes after a 12-hour fast. Venous blood from healthy normolipidemic volunteers was collected into Na-EDTA (1 mg/mL blood) vacuum tubes after a 12-hour fast. Bovine aorta was filled with 0.1% collagenase (Sigma, type I) in medium 199 (Sigma). After an 8- to 12-minute incubation at 37°C, dissociated bovine aortic endothelial cells were collected, washed by centrifugation at 1000g for 10 minutes at 4°C, and resuspended in DMEM containing 10% FBS and 1% sodium chloride solution, the plasma was centrifuged at 150 000 g for 24 hours, and the chylomicron-rich layers were discarded. The supernatant was used for preparation of LDL. LDL was incubated with thiobarbituric acid (0.5 wt/vol, in H$_2$SO$_4$, 50 mmol/L) for 30 minutes at 100°C. The solution then was centrifuged for 5 minutes, and the difference in absorbency at 532 and 580 nm was calculated. TBARS concentration was determined as MDA equivalents with the use of an MDA standard curve.

Superoxide Assay

The concentration of O$_2^-$ was determined by the method described by Gyllenhammar. O$_2^-$ produced chemiluminescence of lucigenin (bis-N-methylacridinium nitrate), which was detected with a scintillation counter (Beckman 6000 LS, with a single-photon monitor). Endothelial cells grown to near confluence in a 75-cm$^2$ flask were placed into 2 mL of Hanks Balanced Salt Solution (HBSS) adjusted to pH 7.4 and incubated with LPDS and later with LDL. Lucigenin was added to the HBSS to reach a final concentration of 0.25 mmol/L. O$_2^-$ production was measured after a 2-minute incubation in the lucigenin/HBSS solution followed by injection of 10 mL of calcium ionophore A23187 (1.2 mmol/L). Photons were counted for 5 seconds after the addition of A23187. Photon counts were calibrated as O$_2^-$ on the basis of the oxidation of xanthine with xanthine oxidase. In this reaction, O$_2^-$ is produced stoichiometrically from xanthine.

Statistical Analysis

All data are based on at least 3 experiments and are expressed as meanSEM. Statistical analyses were performed by ANOVA followed by Schef?e’s F test. A value of P<0.05 was considered significant.

Experimental Protocol

For each step of this study, bovine aortic endothelial cells were cultured under standardized conditions and placed in LPDS-enriched medium for 14 hours before testing. The release of NO was recorded by placing the active polymerized porphyrin surface in it for 15 seconds and then allowing the completed NO sensor to dry again. Measurements of NO were carried out with an EG&G PAR model 273 voltammetric analyzer with custom software. Amperometric mode of detection was used (constant potential of 670 mV vs silver/silver chloride reference electrode).

Ox-LDL

Ox-LDL was prepared according to the method of Huber et al. A 10-mg sample of n-LDL was dialedyzed against Tris/NaCl Buffer (50 mmol/L Tris in 0.15 mol/L NaCl, pH 8.0) to remove the EDTA. Tris/NaCl buffer was added to the dialedyzed n-LDL to adjust the protein concentration to 30 mg/mL. A 1-mL aliquot of 20 mmol/L CuSO$_4$ was added to 1 mL of dialedyzed n-LDL. Oxidation at 37°C was followed spectrophotometrically (234 nm) over a period of 24 hours until oxidation was complete. The ox-LDL was then dialedyzed at 4°C with 4 L Tris buffer, filtered with a 0.22 μm filter, and stored under nitrogen at 4°C.

Oxidation was monitored by the use of measurements of TBARS. Briefly, LDL was incubated with thiobarbituric acid (0.5 wt/vol, in H$_2$SO$_4$, 50 mmol/L) for 30 minutes at 100°C. The solution then was centrifuged for 5 minutes, and the difference in absorbency at 532 and 580 nm was calculated. TBARS concentration was determined as MDA equivalents with the use of an MDA standard curve.

NO Determination

Measurements of NO in cultured endothelial cells were carried out with a porphyrinic microsensor. NO sensors were constructed according to the method first described by Malinski et al. Mono- meric porphyrin, nickel (II) tetrais (3-methoxy-4-hydroxyphenyl) porphyrin (0.25 mmol/L, TMHPP-Ni), in 0.1 mol/L NaOH, was electropolymerized on carbon fibers (Amoco Performance Products, Inc) with the use of cyclic voltammetry (−0.2 to +1.0 V). After the polymerized porphyrin dried (20 minutes), a cation exchanger, Nafion (Alrdich Chemie), in 1% solution in alcohol, was applied by dipping the active polymerized porphyrin surface in it for 15 seconds and then allowing the completed NO sensor to dry again. Measurements of NO were carried out with an EG&G PAR model 273 voltammetric analyzer with custom software. Amperometric mode of detection was used (constant potential of 670 mV vs silver/silver chloride reference electrode).
for 30 minutes before ox-LDL exposure. To evaluate the effect of different concentrations of n-LDL on endothelial cell function, it was necessary to eliminate the possibility of oxidative modification of LDL molecules. An addition of butylated hydroxytoluene to the plasma and to the sterile dialysis solution prevented oxidation of LDL. With this preparative step, n-LDL can be used in tissue culture as a model of hypercholesterolemia.

Results

NO Release

The inhibition of a stimulated NO release by lipoproteins increased exponentially with time of incubation reaching a semiplateau after 20 to 30 minutes for both n-LDL and ox-LDL (Figure 1). Therefore all dose-dependent studies were performed at incubation time of 60 minutes. For the first group, endothelial cells were exposed to increasing concentrations of n-LDL for 1 hour. n-LDL did not significantly modify the stimulated peak NO levels at concentrations from 0 to 60 mg cholesterol/dL (Figure 2). However, NO production was dramatically reduced to 29% of the control levels at n-LDL concentration of 60 mg cholesterol/dL. Beyond 80 mg cholesterol/dL of n-LDL, the NO levels reached a low NO production-rate plateau. A second group of cells was pretreated with L-arginine (0.3 mmol/L, 30 minutes) and exposed for 1 hour to the same range of concentrations of n-LDL. L-Arginine supplementation did not induce any significant increases in the peak NO concentration at low level of n-LDL. However, at n-LDL concentrations from 60 to 130 mg cholesterol/dL, NO concentration was 2 times higher than before L-arginine treatment (Figure 2).

A third group of endothelial LDL–treated cells was supplemented with SOD (100 U/mL) without any significant change in maximally stimulated peak NO concentration (Figure 2). A fourth group of endothelial cells was pretreated with L-NAME (30 minutes) and incubated for 1 hour with increasing concentrations of n-LDL. L-NAME inhibited NO production in controls and at all n-LDL levels (Figure 2).

The same set of experiments was done exposing endothelial cells for 1 hour to increasing concentration of ox-LDL.

Ox-LDL caused a sharper decrease in NO concentration compared with n-LDL; even at low ox-LDL concentration (20 mg cholesterol/dL) NO production was reduced to 15% of the control (Figure 3), which was 5 times lower than the NO production obtained after incubation with the same concentration of n-LDL. L-Arginine pretreatment resulted in a significant increase of NO production in n-LDL–treated cells as well as in ox-LDL–treated cells: At ox-LDL concentration of 20 mg cholesterol/dL, NO concentrations were 3 times higher than before L-arginine supplementation. Also at higher ox-LDL concentration, NO levels were always 2 times higher (Figure 4). The SOD (100 U/mL) treatment did not...
produce significant increase in NO production. The L-NAME pretreatment blunted NO production in control and at every ox-LDL concentration (Figure 4).

**Superoxide Release**

The stimulated O$_2^-$ concentrations were measured under the same experimental conditions as were used for the NO. Endothelial cells incubated for 1 hour with increasing n-LDL concentrations showed a sharp increase of O$_2^-$ production in a dose-dependent manner starting from n-LDL concentration of 40 to 80 mg cholesterol/dL. O$_2^-$ levels rose from 10 nmol/L at 0 to 30 mg cholesterol/dL LDL to 90 nmol/L at 80 mg cholesterol/dL n-LDL. In cells exposed to n-LDL concentrations from 80 to 200 mg/dL, the already high O$_2^-$ concentration did not show any additional increase (Figure 5). L-Arginine pretreatment did not increase O$_2^-$ concentration at low n-LDL concentrations but reduced O$_2^-$ production by 50% when incubated with n-LDL at concentrations >40 mg cholesterol/dL.

Supplementation of the n-LDL treated cells with SOD (100 U/mL) led to a small but statistically significant ($P<0.001$) reduction of O$_2^-$ production at n-LDL concentration >40 mg cholesterol/dL (Figure 5). In L-NAME–pretreated cells, O$_2^-$ production resulting from n-LDL incubation was one third of the controls, decreasing from 67 to 16 nmol/L at 60 mg cholesterol/dL, from 83 to 23 nmol/L at 80 mg cholesterol/dL, and from 92 to 27 nmol/L at 140 mg cholesterol/dL.

Ox-LDL caused a clear increase in O$_2^-$ production starting from ox-LDL concentration of 20 mg cholesterol/dL, and L-arginine pretreatment completely abolished O$_2^-$ production at every ox-LDL dosage (Figure 6). On the other hand, SOD (100 U/mL) supplementation resulted in a decrement in O$_2^-$ production to half of those without SOD. Maximal L-NAME pretreatment of ox-LDL–treated cells reduced O$_2^-$ production to one third at every ox-LDL concentration (Figure 6).

**Discussion**

This study provides direct evidence that n-LDL and ox-LDL cause a decrease of NO production and a parallel increase in O$_2^-$ generation in cultured endothelial cells. With the use of the porphyrinic microsensor, a unique method suitable for measurements of small difference in NO concentration in a time frame of milliseconds, we investigated the effect of n-LDL and ox-LDL on endothelial function measuring directly NO concentration on the cell surface. Other studies on NO production in cells incubated with n-LDL and ox-LDL demonstrated that high levels of n-LDL cause a decrease in
NO production but were carried out with the use of indirect methods based on the determination of NO metabolic products or second-messenger production.\textsuperscript{2,26,27} Furthermore, the precise LDL concentration at which an early loss in NO production can be observed has not yet been established. Our new findings indicate that the endothelial NO production is already altered at low n-LDL concentrations and also demonstrates that physiological concentrations of n-LDL are able to perturb the endothelial cell metabolism and lead to increased $\mathrm{O}_2^-$ production.

The positive effect of $\mathrm{L}$-arginine supplementation on NO production mainly suggests that $\mathrm{L}$-arginine metabolism and/or transport may be impaired in lipoprotein-treated cells and that the decreased NO and increased $\mathrm{O}_2^-$ production may be correlated with an inadequate $\mathrm{L}$-arginine/cNOS coupling.

To maintain smooth muscle relaxation, proliferation, platelet aggregation, and adhesion, endothelial cells must generate a certain concentration of NO on their membrane.\textsuperscript{19,20} cNOS activity is triggered by the calmodulin in response to an increase of intracellular calcium ion concentration.\textsuperscript{28} Any process hindering transport of $\mathrm{L}$-arginine from the kidney to the endothelium or reducing the quantity of $\mathrm{L}$-arginine transported through the cell membrane may cause depletion of this limiting substrate. The depletion of arginine may eventually lead to derangement of the oxidase/reductase domains of cNOS.\textsuperscript{14,29} It has been documented that cNOS, when turned on in an arginine-poor environment, is still able to receive electrons from NADPH and donate them to its other substrate $\mathrm{O}_2$, resulting in one electron reduction to form $\mathrm{O}_2^-$.$^30$ cNOS at the endothelial level appears to be the more active source of $\mathrm{O}_2^-$ in the n-LDL–associated generation of $\mathrm{O}_2^-$. The ability of endothelium to restore a sufficient NO level in the presence of $\mathrm{L}$-arginine, even if this ability is almost lost at concentration of n-LDL $>60$ mg cholesterol/dL, emphasizes the main role of the substrate $\mathrm{L}$-arginine for normal activity of cNOS.

It has been established that the treatment with SOD increases the stability of NO, which suggests that $\mathrm{O}_2^-$ is produced by the endothelial cell along with NO, and inhibits the NO physiological vasodilator function.\textsuperscript{11,31,32} This important finding shed the role of $\mathrm{O}_2^-$ in the impaired NO production and suggests that the amount of $\mathrm{L}$-arginine may play a key role in NO generation and acts complementarily with the increased production of $\mathrm{O}_2^-$ in perturbing the endothelial function. The relatively high levels of $\mathrm{O}_2^-$ produced by endothelial cells incubated with increasing concentrations of n-LDL and ox-LDL were decreased or blunted when we supplemented cells with $\mathrm{L}$-arginine. $\mathrm{L}$-Arginine and L-NAME treatments caused the inhibition of $\mathrm{O}_2^-$ generation in a complementary way. One agent supplies excess substrate for generating NO, which scavenges $\mathrm{O}_2^-$; the other blocks the cNOS activity and decreases the production of both $\mathrm{O}_2^-$ and NO.$^14,33,34$ L-NAME binding to the active site of NO$\mathrm{S}$ decreases electron flux to the heme site of the enzyme.\textsuperscript{35}

Our results indicate that a depletion of $\mathrm{L}$-arginine may cause derangement of cNOS, leading to an overproduction of $\mathrm{O}_2^-$, which can react very rapidly with NO to form ONOO$^-$. The increased generation of ONOO$^-$ by endothelial cells incubated with atherogenic concentration of LDL has been already established and may suggest a mechanism by which n-LDL exposure induces endothelial dysfunction when the deranged cNOS becomes a new source of $\mathrm{O}_2^-$ production.\textsuperscript{12,36} The reaction of $\mathrm{O}_2^-$ with NO is much faster than dismutation of superoxide by SOD. Therefore in the presence of SOD, a recovery of NO concentration was not significant. Our direct measurements proved a decrease of NO and increase of $\mathrm{O}_2^-$ at pathological concentration of n-LDL or ox-LDL. Interestingly, the exposure of endothelial cells to higher physiological concentration of n-LDL (60 mg cholesterol/dL) altered of the $\mathrm{L}$-arginine –NO system. Evidence suggests that ox-LDL exhibit important atherogenic properties that are not present in n-LDL, such as cytotoxicity to vascular cells, modulation of expression of cytokines, and effects on coagulation system.\textsuperscript{37} Moreover, ox-LDLs have been found to be able to alter vascular tone regulation.\textsuperscript{72} The relatively low concentration of ox-LDL led to a rapid loss in NO production. In this way, the n-LDL and ox-LDL uncoupling of cNOS could be one of the mechanisms responsible of the altered endothelial function present in the early stage of the atherogenic process.

In conclusion, our findings demonstrate that n-LDL and even more, ox-LDL, alter NO and $\mathrm{O}_2^-$ generation even at their physiological concentrations. The decrease in NO production suggests that n-LDL and ox-LDL may uncouple cNOS activity, allowing the enzyme to become a source of $\mathrm{O}_2^-$. $\mathrm{L}$-Arginine supplementation limits n-LDL–induced and ox-LDL–induced endothelial dysfunction, increasing the NO generation at any cholesterol concentration. Finally, the perturbation of the cellular membrane that occurs during hypercholesterolemia and the resulting impaired $\mathrm{L}$-arginine transportation through the cell membrane may be one of the main mechanisms of the lipoprotein-induced endothelial dysfunction in atherogenesis.

Acknowledgments

This work was supported in part by US Public Health service grant HL–55397 (T. Malinski). We would like to thank Cristina Bosi for technical assistance and Dr Stephen Patton for valuable discussions.

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_Circulation_. 2000;101:1261-1266
doi: 10.1161/01.CIR.101.11.1261

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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