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REDUCTION OF NITRIC OXIDE SYNTHASE ACTIVITY IN HUMAN NEUTROPHILS BY OXIDIZED LOW-DENSITY LIPOPROTEINS

REVERSAL OF THE EFFECT OF OXIDIZED LOW-DENSITY LIPOPROTEINS BY HIGH-DENSITY LIPOPROTEINS AND L-ARGININE

JAWAHAR L. MEHTA,* JERRY L. BRYANT, JR. and PAULETTE MEHTA

Departments of Medicine and Pediatrics, University of Florida College of Medicine and the VA Medical Center, Gainesville, FL, U.S.A.

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Abstract—Oxidized low-density lipoproteins (ox-LDL) inhibit vascular relaxation by decreasing the synthesis or rapid degradation of endothelium-derived relaxing factor (EDRF), now identified to be nitric oxide (NO). We examined the regulation of NO synthase activity in human neutrophils, which also generate NO, by lipoproteins. Isolated human neutrophils were incubated with native-LDL, ox-LDL (10–50 μg protein/mL), high-density lipoproteins (HDL, 100 μg protein/mL) or HDL + ox-LDL, and NO synthase activity was measured as conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline. Ox-LDL, but not native-LDL or HDL, significantly decreased NO synthase activity in human neutrophils. This effect of ox-LDL was incubation time and concentration dependent. The incubation of cells with HDL or L-arginine diminished the effects of ox-LDL on NO synthase activity. Thus, ox-LDL decreases the activity of NO synthase enzyme, and this effect of ox-LDL can be modified by HDL and L-arginine.

Key words: L-arginine; lipoproteins; neutrophils; nitric oxide

The link between high levels of LDL† in plasma and atherosclerosis is now well established [1]. In contrast, low levels of HDL are a potent risk factor in atherogenesis [2]. Recent studies show that oxidation of LDL is necessary for deposition of cholesterol in vascular tissues destined to develop atherosclerosis [3]. ox-LDL decreases endothelium-dependent relaxation [4–6], and this reduced vasorelaxation is believed to be due to diminished synthesis or release of EDRF, although other studies suggest no dimunition in its synthesis but excessive breakdown by superoxide radicals [7].

Synthesis of EDRF, now identified to be NO, derived from the amino acid L-arginine, is believed to be ubiquitous. Although NO synthesized in different tissues may have different characteristics relative to calcium (Ca^{2+}) and calmodulin requirements and inducibility, NO generated from all sources is a potent vasodilator [8].

There is a paucity of data on the contribution of neutrophils in atherosclerosis except that several pieces of information suggest involvement of these cells in atherogenesis. For example, neutrophil counts and activity are independent predictors of outcome and extent of atherosclerosis [9]; neutrophils are often seen in blood vessels subjected to angioplasty [10], and these sites frequently develop atherosclerosis-type lesions; neutrophils rapidly accumulate in tissues and blood vessels after temporary arterial occlusion and contribute to the phenomenon of "reperfusion injury" [11]; neutrophils oxidize LDL [12]; and neutrophils are an important source of superoxide radicals and NO, and these species cause endothelial dysfunction [13] and exert cytotoxic effects [14, 15], respectively.

The present studies were conducted to determine the regulation of NO synthase activity by native LDL and ox-LDL, as well as HDL. Human neutrophils were used as an easily available source of human tissues capable of generating NO. Recent data suggest that NO synthase activity in neutrophils parallels the changes in vascular tissues [16, 17]. It is possible, therefore, that the effects of lipoprotein fractions on NO synthase activity in neutrophils mimic the effects of lipoproteins on NO synthase activity in the endothelial cells.

MATERIALS AND METHODS

Materials

[^{3}H]L-Arginine (77 Ci/nmol; 1 Ci = 37 GBq) was obtained from Amersham (Arlington Heights, IL). All chemicals were obtained from the Sigma Chemical Co. (St. Louis, MO), or as stated.

Separation of leukocytes

Heparinized venous blood from healthy individuals was layered over Mono-Poly Resolving Medium (Flow Laboratories) and was centrifuged at 500 *g* for 30 min at 24° to obtain a neutrophil-rich layer. Red blood cells were lysed by hypotonic lysis. Neutrophils were then removed and washed in Hanks' Balanced Salt Solution (HBSS, pH 7.4) without Ca^{2+} and Mg^{2+}, as described

* Corresponding author: J. L. Mehta, M.D., Ph.D., University of Florida College of Medicine, P.O. Box 100277, Gainesville, FL 32610-0277. Tel. (904) 376-1611, Ext. 6843; FAX (904) 374-6113.

† Abbreviations: LDL, low-density lipoproteins; HDL, high-density lipoproteins; ox-LDL, oxidized low-density lipoproteins; EDRF, endothelium-derived relaxing factor; NO, nitric oxide; TBARS, thiobarbituric acid reactive substances; and L-NNA, N^{ω}-nitro-L-arginine.

previously [9]. Neutrophil suspension (10^7 cells/mL) contained more than 98% neutrophils. Their viability, as determined by trypan blue exclusion, was more than 90%.

Preparation of lipoproteins

Lipoprotein fractions were prepared from fresh human plasma. LDL (density 1.019 to 1.063 g/mL) and HDL (density 1.063 to 1.21 g/mL) were isolated by discontinuous density ultracentrifugation [6]. The isolated lipoproteins were dialyzed for 18 hr against modified Tyrode buffer (composition in M: NaCl, 0.13; $NaHCO_3$, 0.012; $NaHPO_4$, 0.0042; KCl, 0.0027). All buffers contained 0.3 mM EDTA to prevent auto-oxidation of lipoproteins. Native LDL prepared by this method showed no significant oxidation as measured by the TBARS assay (0.22 ± 0.07 nmol/100 μg protein), whereas ox-LDL had a large increase in TBARS (1.39 ± 0.05 nmol/100 μg protein). HDL was further purified from contaminating apolipoprotein B containing lipoproteins by heparin–Sepharose chromatography. Ox-LDL was prepared by exposure of native-LDL to $CuSO_4$ (5 μM) at 37° for 24 hr according to the method of Steinbrecher *et al.* [18].

Determination of NO synthase activity

NO synthase activity was determined in aliquots of neutrophils incubated with native-LDL (10–50 μg protein/mL), ox-LDL (10–50 μg protein/mL), HDL (100 μg protein/mL), or ox-LDL + HDL, or ox-LDL + L-arginine (2 mM). After 1 hr of incubation at 37°, the cells were washed to remove unincorporated lipoproteins. NO synthase activity was measured by monitoring the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, as described previously [19]. Briefly, neutrophils (10^5 cells) and 25 μL of [^{3}H]L-arginine (2 μCi/mL, average count ≈340,000 dpm) were incubated with 200 μL of 25 mM HEPES buffer (140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.4). After 1 hr of incubation, 2 mL of stop buffer (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 24.8 mM $NaHCO_3$, 4 mM EDTA and 5 mM L-NNA, pH 5.5) was added. Each tube was centrifuged twice at 800 *g* for 15 min at 4°. The supernatant was discarded, and the pellet was disrupted by adding 1 mL of 0.3 N $HClO_4$ and neutralized with 65 μL of 3 M K_2CO_3. Aliquots of the cell suspensions were then applied to 2 mL columns of Dowex AG50WX-8 (Na^+ form) (Bio-Rad, Richmond, CA), which were eluted with 6 mL of distilled water. [^{3}H]L-Citrulline in the eluent was quantitated by liquid scintillation spectroscopy.

Determination of cholesterol content

Cholesterol content of neutrophils was determined enzymatically using a chromogenic substrate (4-aminoantipyrine and *p*-hydroxybenzenesulfonate) that in the presence of peroxidase yields a quinoneimine dye with maximum absorbance at 500 nm [20]. Protein concentrations were determined according to Bradford [21] using bovine serum albumin as the standard.

Statistical significance

The significance of differences between means were based on ANOVA followed by Scheffé F-test and Student's *t*-test for paired samples, as appropriate. All values are based on at least three different experiments, each in triplicate, and are expressed as means ± SD.

Table 1. Effect of duration of incubation of neutrophils with [^{3}H]L-arginine on NO synthase activity in neutrophils

Incubation time (min)	[^{3}H]L-Citrulline formation (%)
15	6.7 ± 0.7
30	7.5 ± 0.8
60	9.1 ± 0.5
120	9.2 ± 0.5

Data are means ± SD from 3 experiments.

RESULTS

Cholesterol content of neutrophils

Total cholesterol content increased markedly in cells incubated with 50 μg/mL of ox-LDL (4.8 ± 0.8 vs 1.0 ± 0.3 μg/10^6 in untreated cells, N = 7, $P < 0.01$). Incubation of neutrophils with 25 μg protein/mL of ox-LDL also increased the cholesterol content modestly (mean 3.8 ± 0.8 μg/10^6 cells, $P < 0.05$). Incubation of cells with native-LDL (50 μg protein/mL) had a small effect on cholesterol content (mean 2.6 ± 0.4 μg/10^6 cells, N = 3). Incubation of neutrophils with HDL attenuated ($P < 0.02$) the ox-LDL (50 μg/mL)-induced increase in cholesterol content to 2.8 ± 0.4 μg/10^6 cells.

NO synthase activity and lipoproteins

Conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline was time dependent, reaching a maximal rate at 1 hr (Table 1). The formation of [^{3}H]L-citrulline was similar in neutrophils suspended in Ca^{2+}-poor (EGTA present) or Ca^{2+}-rich (EGTA absent) buffer. In addition, presence of the NO synthase inhibitor L-NNA markedly decreased [^{3}H]L-citrulline formation. These observations are summarized in Table 2.

Whereas native-LDL had a modest insignificant effect on [^{3}H]L-citrulline formation, ox-LDL caused a marked concentration-dependent inhibition of [^{3}H]L-citrulline formation (Table 3). The suppressive effects of ox-LDL on L-citrulline formation were dependent on the duration of incubation with neutrophils and were maximal at 60 min. $CuSO_4$ used for oxidation of LDL had no effect on NO synthase activity (data not shown).

In contrast to the effects of ox-LDL, HDL had no effect on L-citrulline formation. In parallel experiments,

Table 2. Effects of Ca^{2+}, N^{ω}-nitro-L-arginine (L-NNA) and L-arginine on NO synthase activity

	[^{3}H]L-Citrulline formation (%)
Intact neutrophils incubated with:	
Ca^{2+}-rich buffer (EGTA absent)	8.3 ± 1.0
Ca^{2+}-poor buffer (EGTA present)	8.1 ± 0.9
L-NNA (500 μM)	2.5 ± 1.1*
L-Arginine (2 mM)	8.7 ± 0.2

Neutrophils were incubated for 1 hr in Ca^{2+}-rich or Ca^{2+}-poor buffers followed by measurement of the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline (index of NO synthase activity). Aliquots of neutrophils in Ca^{2+}-rich buffer were treated with L-NNA or L-arginine. Values are means ± SD from 3 to 16 experiments.

* $P < 0.01$ vs NO synthase activity in Ca^{2+}-rich or Ca^{2+}-poor buffer.

Table 3. Incubation time-dependent effects of native and ox-LDL on NO synthase activity

	[^{3}H]L-Citrulline formation (%)		
	Duration of incubation		
	15 min	30 min	60 min
Control neutrophils	7.7 ± 0.1	8.7 ± 0.2	9.7 ± 0.3
+ Native-LDL (25 μg/mL)	7.6 ± 0.1	8.6 ± 0.4	8.8 ± 0.4
+ Native-LDL (50 μg/mL)	7.0 ± 0.1	8.6 ± 0.1	9.2 ± 0.1
+ Ox-LDL (25 μg/mL)	6.3 ± 0.7*	4.7 ± 1.0*	3.7 ± 0.4*
+ Ox-LDL (50 μg/mL)	5.6 ± 0.6*	4.4 ± 1.0*	2.9 ± 0.1*

Neutrophils were incubated with native or ox-LDL for 15, 30 or 60 min in Ca^{2+}-rich buffer before conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline (index of NO synthase activity) was measured. Values are means ± SD from 3 experiments, each in triplicate.
* $P < 0.01$ vs control neutrophils.

the presence of HDL significantly ($P < 0.01$) attenuated the ox-LDL-mediated reduction in L-citrulline formation. Co-incubation of the NO synthase inhibitor L-NNA (500 μM) with ox-LDL further decreased the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline, whereas co-incubation with the NO precursor L-arginine increased ($P < 0.01$) NO synthase activity in neutrophils treated with ox-LDL (Table 4).

DISCUSSION

The presence of the L-arginine–NO pathway in neutrophils has been confirmed by chemiluminescence studies [22], demonstration of cyclic guanosine monophosphate (cGMP)-mediated platelet inhibition by neutrophils [23], and conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline [16, 17, 19]. The synthesis of NO in neutrophils is Ca^{2+} independent and inhibited by ω-substituted L-arginine analogs (Table 2).

In this study we found direct suppressive effects of ox-LDL, but not of native-LDL, on the activity of NO synthase in human neutrophils. Data on the reduction of NO synthase activity in neutrophils parallel the observations of diminished endothelium-dependent relaxation of atherosclerotic vessels of arterial segments exposed to oxidized LDL [4–6]. Our data, however, do not support the observations of Minor *et al.* [7], who noted increased synthesis of nitrosylated compounds, measured by chemiluminescence, in the supernates of atherosclerotic blood vessels. Chin *et al.* [24] have suggested that ox-LDL inactivates NO after its release, but they could not rule out the possibility that ox-LDL may have a direct inhibitory effect on NO synthesis. Most importantly, co-incubation of HDL with ox-LDL in our studies reduced the suppressive effect of ox-LDL on NO synthase activity in neutrophils. Lack of any direct effect of HDL on NO synthase activity and modification of the effects of ox-LDL described herein are in agreement with the observations of modification of ox-LDL-mediated loss of endothelium-dependent vasorelaxation by HDL [25]. HDL is believed to move cholesterol particles from cells to the circulation [26]. A similar extrusion of cholesterol from cytosol of neutrophils occurred when neutrophils were co-incubated with HDL and ox-LDL in our experiments. The inhibitory effect of ox-LDL on constitutive NO synthase activity in neutrophils is similar to that described recently in platelets and may be related to a decrease in NO synthase protein expression [27].

Table 4. NO synthase activity in neutrophils in the presence of ox-LDL and HDL

	[^{3}H]L-Citrulline formation (%)
Control neutrophils	9.9 ± 0.4
+ Ox-LDL (25 μg/mL)	3.6 ± 0.2
+ Ox-LDL + L-NNA (500 μM)	1.1 ± 0.1*
+ Ox-LDL + L-arginine (2 mM)	6.2 ± 0.5*
+ HDL (100 μg/mL)	10.1 ± 0.2
+ HDL + ox-LDL (25 μg/mL)	9.2 ± 0.5†
+ HDL + L-NNA (500 μM)	6.7 ± 0.7‡

Neutrophils were incubated with various agents for 1 hr in Ca^{2+}-rich buffer before measurement of NO synthease activity. Values are means ± SD from 3 separate experiments.
* $P < 0.01$ vs NO synthase activity in neutrophils incubated with ox-LDL.
† $P < 0.01$ vs NO synthase activity in neutrophils incubated with ox-LDL alone.
‡ $P < 0.05$ vs NO synthase activity in neutrophils incubated with HDL.

The inhibitory effects of ox-LDL and L-NNA on NO synthase activity were additive. Incubation of neutrophils with L-NNA also resulted in reduction in NO synthase activity even in the presence of HDL. Thus, HDL is not protective against the effects of L-NNA on NO synthase activity in neutrophils. On the other hand, we observed that L-arginine consistently potentiated NO synthase activity in cells treated with ox-LDL. Girerd *et al.* [28] and Creager *et al.* [29] showed that administration of large amounts of L-arginine results in at least a partial restoration of the bioactivity of NO in cholesterol-fed rabbits as well as in hypercholesterolemic humans. Our studies provide direct evidence for reduction in the effect of ox-LDL on NO synthase activity when neutrophils are co-incubated with L-arginine. Other preliminary studies have suggested inhibition of experimental atherosclerosis by administration of L-arginine [30]. Although the effect of systemic L-arginine administration on leukocyte function, especially NO synthase activity, has not been defined, the observations on the effect of L-arginine on NO synthase activity in neutrophils *in vitro* made in this study may be generalizable to blood vessels as well as monocytes and platelets.

Recent studies have shown diminished NO synthase

activity in neutrophils of patients with hypertension or unstable angina pectoris [16, 17]. These changes parallel the reduction in vascular NO synthesis in blood vessels of patients with similar conditions. NO synthase activity is also reduced in patients with hyperlipidemia and atherosclerosis [28, 29]. The direct inhibitory effect of ox-LDL on NO synthase activity in neutrophils documented in this study provides a parallel to the observations in vascular tissues [4–6] and platelets [27].

Observations relative to the modulation of NO synthase activity in ox-LDL-treated neutrophils are provocative. Pretreatment of neutrophils with two unrelated compounds, HDL and L-arginine, almost totally reversed the effects of ox-LDL. Again, these alterations parallel the effects of HDL and L-arginine on vascular tissues [25, 28, 29] and platelets [27]. Modalities, such as raising HDL or administration of L-arginine, may be of therapeutic value in regulating NO synthase activity in a variety of tissues exposed to high concentrations of ox-LDL.

A comment relative to the potent inhibitory effect of ox-LDL, but not native-LDL, on NO synthase activity needs to be made. In previous studies in vascular tissues, only the oxidatively modified form of LDL reduced endothelium-dependent relaxation [4, 6]. Recent studies show that ox-LDL, but not native-LDL, reduces NO synthase activity in macrophages [31]. However, it is not known if neutrophils, like macrophages and endothelial cells, possess receptors for native LDL or ox-LDL. The cholesterol content of neutrophils increased in the present study, particularly when neutrophils were incubated with ox-LDL. An increase in cholesterol may well reflect phagocytic activity of neutrophils, or cholesterol incorporation may be a result of ox-LDL-mediated injury to the neutrophil cell membrane followed by passive diffusion.

Relative to the mechanism of modulation by HDL and L-arginine of the effects of ox-LDL on NO synthase activity, HDL has been shown to reduce the incorporation of cholesterol into tissue [26, 32], which may be the basis of modification of NO synthase activity. The precise mechanism of action of L-arginine is not known, but it is possible that NO synthesis is enhanced as large amounts of substrate for NO synthase become available. In other preliminary studies on the effects of lipoproteins on the L-arginine-NO pathway, both HDL and L-arginine markedly attenuated the effect of ox-LDL on NO synthase activity in the platelet cytosol [27].

In summary, ox-LDL, but not native-LDL, decreased NO synthase activity in human neutrophils. This effect of ox-LDL was reversed rapidly by HDL and L-arginine. These alterations in neutrophil NO synthase activity paralleled the effects of lipoproteins on vascular tissues.

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