Oxidized Low Density Lipoproteins Induce mRNA Expression and Release of Endothelin From Human and Porcine Endothelium

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Experiments were designed to examine the effect of oxidized low density lipoproteins (Ox-LDLs) on the expression and the release of endothelin from cultured endothelial cells and intact blood vessels. Ox-LDLs (30–300 μg/ml), but not native low density lipoproteins (200 μg/ml), stimulated the expression of preproendothelin mRNA in porcine and human endothelial cells, leading to a time- and concentration-dependent release of the peptide into the culture medium. The Ox-LDL-stimulated release of endothelin was mimicked by acetylated low density lipoprotein and abolished by downregulation of protein kinase C by phorbol ester. In the intact porcine aorta, Ox-LDLs, but not native low density lipoproteins, also increased the release of peptide in an endothelium- and concentration-dependent manner. The maximal effect was observed at a concentration of 100 μg/ml. Incubation of the intact porcine aorta with the scavenger receptor antagonist dextran sulfate decreased the formation of endothelin evoked by Ox-LDLs. The Ox-LDL-stimulated production of the peptide was further augmented in the presence of thrombin (4 units/ml) and was unaffected by nitric oxide–generating compound 3-morpholinosydnonimine (10−4 M). These results suggest that Ox-LDL may be an endogenous mediator of the augmented release of endothelin observed in hyperlipidemia and atherosclerosis. The increased production of the peptide could contribute to vasospastic events and may promote vascular smooth muscle proliferation and progression of atherosclerotic vascular disease. (Circulation Research 1992;70:1191–1197)

KEY WORDS • thrombin • native low density lipoprotein • cultured endothelial cells • intact porcine aorta • dextran sulfate • phorbol esters • acetylated low density lipoprotein • cGMP

The endothelium participates in the regulation of vascular tone by releasing relaxing factors, such as nitric oxide, or contracting substances, such as the peptide endothelin.1,2 Endothelial cells are primary targets for cardiovascular risk factors such as low density lipoproteins (LDLs).3,4 Plasma LDLs can be oxidatively modified by the cells of the blood vessel wall,5–6 and oxidized LDLs (Ox-LDLs) have been found to accumulate in early plaques and atherosclerotic vascular lesions, where they may profoundly affect the endothelial function.7–10

Atherosclerotic arteries are more prone to vasospastic events than are normal blood vessels.11–13 One feature of this disease is the impairment of the relaxations mediated by the endothelium.12,14–16 This endothelial dysfunction16–20 appears to be partially due to the inhibition of the formation of nitric oxide by Ox-LDL, which accumulates within human atherosclerotic plaques.4,5 However, an enhanced production of the potent vasoconstrictor peptide endothelin2 could also participate, particularly since nitric oxide inhibits its release from intact blood vessels.21 Indeed, the circulating levels of endothelin are increased in patients with hyperlipidemia or atherosclerosis.22,23 Hence, this study was designed to investigate the effect of Ox-LDL, as compared with that of native LDL (Nat-LDL) on the expression and release of the peptide by both cultured endothelial cells and intact blood vessels.

Materials and Methods

Blood Vessels

Aortas were obtained from farm pigs killed at the nearby slaughterhouse and placed in modified Krebs-Ringer solution (control solution) of the following composition (mM): NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25.0, calcium EDTA 0.026, and glucose 11.1 (pH 7.4). The blood vessels were cleaned of connective tissue and opened longitudinally. Great care was taken to preserve the intimal surface. In some experiments, the endothelial layer was rubbed.

Cell Culture

Porcine aortic endothelial cells (PAECs) and human aortic endothelial cells (HAECs) were obtained as
previously described.24 Aortas were placed and washed several times in sterile, cold Earle’s balanced salt solution containing 300 units/ml penicillin and streptomycin. PAECs, obtained by mechanical scraping with a razor blade of the intimal layer, were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine, 20 μg/ml heparin, 100 units/ml penicillin, and 100 units/ml streptomycin. HAECs, obtained intraoperatively from the aorta of a 41-year-old male organ donor patient in the same manner as described for PAECs, were cultured in the same medium as PAECs and supplemented with 20% serum and 150 μg/ml endothelial cell growth factor. Identification of HAECs was confirmed by the presence of factor VIII–related antigen. PAECs and HAECs were used from passages 6 to 12 and from passages 11 to 15 respectively. Confluent cultures were rendered quiescent by incubation in endothelial cell growth factor–free and serum-free medium supplemented with 0.5% bovine serum albumin and were exposed either to vehicles, Ox-LDLs, Nat-LDLs, or acetylated LDLs (Ac-LDLs).

Preparatory LLD

LDLs were isolated from human plasma collected in EDTA (10⁻³ M) and butylated hydroxytoluene (BHT, 10⁻⁵ M) using sequential ultracentrifugation with density adjustments by potassium bromide.26 The isolated LDLs were dialyzed against phosphate-buffered saline (PBS) in the presence of EDTA and BHT and then sterilized by filtration (filter pore size, 0.45 μm; Gelman Sciences Inc., Ann Arbor, Mich.). Protein concentration was determined as described using bovine serum albumin as a standard.27 LDL samples were stored at 4°C in the dark and used within 2 weeks. LDLs prepared under these conditions are referred to as Nat-LDLs.

Before oxidation, LDLs were dialyzed against PBS to remove EDTA and BHT. Then they were oxidized at a concentration of 200 μg/ml by exposure to 5 μM CuCl₂ for 24 hours at 37°C.28 The entire procedure was performed with sterile agents under sterile conditions to prevent any bacterial contamination. The extent of lipid peroxidation was estimated as for thiobarbituric acid-reactive substances.28,29 Tetramethoxypropane was used as a standard, and results are expressed as nanomoles of malondialdehyde equivalents per milliliter of the diluted solution. The average degree of oxidation subsequently increased from values below 0.2 to 4.3±0.4 nmol malondialdehyde equivalents per 100 μg proteins for Nat-LDL and Ox-LDL, respectively (n=6 preparations).

Northern Blot Analysis

Quiescent HAECs and PAECs were exposed to sterile Ox-LDLs (100 μg/ml) or Nat-LDLs (200 μg/ml with 10⁻⁵ M BHT) in RPMI 1640 with 0.2% bovine serum albumin for 1–10 hours. The medium was removed, and cell layers were washed twice with PBS before lysis in guanidinium-

isothiocyanate buffer, which was added directly onto the culture dishes. Total RNA was collected on CsCl gradients as described before.30,31 For Northern analysis, 9 μg (HAECs) to 20 μg (PAECs) of total RNA was electrophoresed through a 1.2% agarose gel containing 2.2 M formaldehyde at a constant voltage of 50 V for 6–8 hours in MOPS buffer. The gel was vacuoblotted onto HYbond N membranes (Amersham Corp., Arlington Heights, Ill.) with 20× standard saline citrate as transfer buffer. Blotted RNA was fixed to the membranes by ultraviolet irradiation at 302 nm for 3 minutes. Blots were hybridized to a random-primed cDNA probe specific for endothelin (1.3 kb, gift of Dr. J. Powell, Hoffmann-La-Roche, Basel, Switzerland)30,31,32 according to the method of Church and Gilbert,33 washed at high stringency (0.1× standard saline citrate/0.1% sodium dodecyl sulfate at 65°C), and then exposed to Kodak X-Omat films at −70°C overnight using one intensifying screen. To assess for variabilities in sample RNA, blots were rehybridized to a 1.5-kb cDNA probe specific for major histocompatibility complex (MHC) class I antigens (clone pMF 48)34 and exposed for 3–6 hours to X-Omat film. Densitometric analysis of hybridization signals was performed by scanning (525 nm) autoradiographs; the given arbitrary optical density (OD) units were obtained by normalization of signals with respect to the OD values obtained for the MHC internal control (MHC OD value of unstimulated cells was arbitrarily taken as 100%).

Measurement of Endothelin

Confluent and quiescent endothelial cells were incubated in 1 ml RPMI 1640 supplemented with 0.2% bovine serum albumin and exposed to Ox-LDLs, Ac-LDLs (Biomedical Technologies Inc., Stoughton, Mass.), or Nat-LDLs for up to 24 hours. The incubation medium was centrifuged at 250g to remove nonadherent cells and was used for the determination of endothelin by radioimmunoassay (Peninsula Laboratories, Merseyside, UK). The detection limit of the assay was 1 pg, and cross-reactivity of the antibody was 100% for endothelin-1 (porcine, human), 7% for endothelin-2 and -3, and 35% for big endothelin (porcine, human).

Intact porcine aortas with endothelium (10-cm² intimal surface) were incubated at 37°C in 3 ml control solution containing 0.1% bovine serum albumin and aerated with a mixture of 95% O₂–5% CO₂ as previously reported.21 Endothelin was determined by radioimmunoassay in the incubation medium as described above. Previous work has shown that the release of endothelin from intact porcine aorta was endothelin dependent.21

Measurement of cGMP

Confluent and quiescent endothelial cells (35-mm Petri dishes, second passage) were washed three times and incubated in control solution containing indomethacin (10⁻⁵ M) and isobutylmethylxanthine (10⁻⁴ M) for 45 minutes at 37°C as previously reported.35 Cells were then stimulated either with 3-morpholinosydnonimine (SIN-1, 10⁻³ M) or vehicle. Preliminary experiments have shown that maximal effect of the compound was observed after 2 minutes of stimulation, in agreement with Schini and VanHoutte.26 Incubation medium was removed after 2 minutes of stimulation, and intracellular
cGMP was extracted with 500 μl cold ethanol/1N HCl (1%). Samples were lyophilized and resuspended in 250 μl sodium acetate buffer (0.05 mM, pH 6.2), and cGMP content was determined after acetylation by radioimmunoassay (Amersham-Rahn, Zürich, Switzerland).

**Drugs**

All tissue culture material and chemicals were from GIBCO, Basel, Switzerland, and AMIMED, Basel, Switzerland, with the exception of fetal calf serum (Boehringer, Mannheim, FRG). Lipopolysaccharide (from *Salmonella typhosa*) and thrombin were from Sigma Chemical Co., St. Louis, Mo. SIN-1 was a kind gift from Dr. V. Balteaux, Hoechst Laboratory, Paris. Unless otherwise indicated, all other chemicals were purchased from Fluka, Buchs, Switzerland; Sigma; and Bio-Rad, Glattbrugg, Switzerland.

**Statistical Analysis**

Results are expressed as mean±SEM; in experiments involving cultured endothelial cells (cyclic nucleotide and endothelin measurements), data were obtained from at least three different dishes and were observed with two other cultures. In experiments performed with intact blood vessels, n refers to the number of animals from which the tissues were obtained. In experiments involving densitometric analysis of Northern blots, data are expressed as mean±SEM of percent increase in OD arbitrary value as compared with unstimulated cells (n=3). Statistical comparisons were made using Student's paired t test or Scheffe's test for multiple comparison. Values of p≤0.05 were considered statistically significant.

**Results**

**Expression of Endothelin mRNA**

Ox-LDLs (100 μg/ml) increased endothelin transcription both in PAECs (Figure 1, top panel) and in HAEcs (Figure 1, bottom panel). In PAECs, the increase in mRNA expression could already be observed after 2 hours and was maintained for the following 6 hours of stimulation (Figure 1). In HAEcs, the effect of Ox-LDLs (100 μg/ml) was detected after 3 hours of stimulation, with a maximum after 4 hours (Figure 1). In contrast, Nat-LDLs (200 μg/ml) failed to stimulate preproendothelin mRNA within a comparable time period, both in PAECs and HAEcs (Figure 1).

**Release of Endothelin From Cultured Endothelial Cells**

Ox-LDLs stimulated the release of endothelin from PAECs as compared with control conditions (Figure 2A). This effect was concentration dependent in the range of 30–300 μg/ml; concentrations lower than 30 μg/ml (data not shown) did not augment the release of the peptide as compared with that occurring in unstimulated cells. The effect of Ox-LDLs was also time dependent (0–24 hours); after 24 hours, the production rate of the peptide was similar in stimulated and control cells (control cells, 98±4 pg/10⁹ cells per hour; Ox-LDLs [300 μg/ml], 107±6 pg/10⁶ cells per hour) (Figure 2A). Ox-LDLs also caused a 2.3-fold increase of the release of endothelin from HAEcs. Under the same experimental conditions, lipopolysaccharide (100 ng/ml, 4 hours of incubation) did not augment the release of...
the peptide from cultured PAECs (control cells, 1.36±0.10; treated cells, 1.48±0.09 pg/10⁶ cells; n=4).

Release of Endothelin From Intact Blood Vessels

In the intact porcine aorta with endothelium, Ox-LDLs induced a concentration-dependent release of endothelin, with a maximal effect at 100 μg/ml after 5 hours of incubation (=2.7-fold increase, Figure 3A). No detectable amounts of the peptide were observed in the incubating medium of preparations without endothelium (n=4).

Cellular Mechanism of Action

Scavenger receptor. In cultured PAECs, the effect induced by Ox-LDLs was also mimicked by Ac-LDLs (30 μg/ml) but not by Nat-LDLs (200 μg/ml, in the presence of 10⁻⁵ M BHT) (Figure 2B). As in cultured endothelial cells, Nat-LDLs (200 μg/ml, with 10⁻⁵ M BHT) did not increase the release of endothelin from intact porcine aorta (control cells, 30±3 pg/cm²; Nat-LDLs, 26±6 pg/cm²; n=5). Incubation of intact porcine aorta with the scavenger receptor antagonist dextran sulfate (40 μg/ml; molecular weight, 500,000) prevented the release of endothelin induced by Ox-LDLs (100 μg/ml) (Figure 3B). Dextran sulfate by itself did not have any significant effect on the spontaneous production of the peptide (Figure 3B).

Protein kinase C. Chronic treatment of PAECs with phorbol 12-myristate 13-acetate (100 nM, 48 hours) and consequent downregulation of protein kinase C mark-
Discussion

LDLs are a well-known risk factor for atherosclerosis. Plasma LDLs may be oxidized in vivo by either endothelial cells, macrophages, or vascular smooth muscle cells and accumulate in human early plaques as well as fully developed atherosclerotic lesions. Increased circulating levels of endothelin have been observed in hyperlipidemia and in atherosclerosis. The present study demonstrates that Ox-LDLs, but not Nat-LDLs, stimulate the expression and the release of endothelin from cultured and native endothelial cells of intact blood vessels. Therefore, it provides evidence for Ox-LDL as a potential endogenous mediator of the increased release of endothelin in hyperlipidemia and atherosclerotic diseases. The stimulation of the expression of endothelin mRNA and the subsequent release of the peptide were observed both in PAECs and HAECS, emphasizing the importance of these results in humans.

The release of endothelin induced by Ox-LDL most likely involves activation of the endothelial scavenger receptor. Indeed, the production of the peptide induced by Ox-LDL could be reproduced by Ac-LDL, another ligand of the scavenger receptor whereas Nat-LDL, which interacts with its specific receptor, failed to stimulate the release of endothelin. The involvement of the scavenger receptor in the effect of Ox-LDL is further supported by the fact that the scavenger receptor antagonist dextran sulfate totally abolished the release of endothelin induced by Ox-LDL, whereas the substance by itself was without effect. Activation of the endothelial scavenger receptor by Ox-LDL has also been involved in the inhibition of the release of endothelium-derived nitric oxide by Ox-LDL in intact porcine coronary arteries.

The effects of Ox-LDL are unlikely to be influenced by the presence of endotoxin contaminating the preparations of Ox-LDLs, since the lipoproteins were prepared under sterile conditions and because a high concentration of lipopolysaccharide did not stimulate the release of endothelin from cultured cells. Indeed, the stimulating effect of endotoxin on the release of endothelin from cultured endothelial cells requires the presence of serum. Moreover, the release of endothelin and that stimulated by Ox-LDL must involve activation of protein kinase C in endothelial cells, since they were markedly reduced by downregulation of the enzyme by phorbol ester. In this regard, Ox-LDL shares a similar pathway with other agonists releasing endothelin, such as angiotensin II in cultured vascular smooth muscle cells and thrombin in cultured endothelial cells.

The production of endothelin is regulated both by activating and inhibitory mechanisms. Indeed, previous work has shown that the thrombin-induced release of the peptide in the intact porcine aorta is reduced by the concomitant production of endothelin-derived nitric oxide as well as by exogenous nitric oxide–generating compounds such as SIN-1 or nitroglycerin. In contrast to thrombin, however, the release of endothelin evoked by Ox-LDLs was insensitive to SIN-1. One possible explanation could be that Ox-LDLs scavenge and inactivate free nitric oxide or that the modified lipoproteins interfere with the activation of soluble guanylate cyclase by nitric oxide, as it was observed with
purified enzyme. These explanations are unlikely, however, since the accumulation of cGMP induced by SIN-1 by endothelial cells was not impaired in the presence of Ox-LDLs. Thus, in contrast to the response to thrombin, the Ox-LDL–stimulated release of endothelin is not modulated by exogenously added nitric oxide. The absence of a cGMP-dependent regulation of the endothelin release induced by Ox-LDLs cannot be explained by a direct cytotoxic effect of the modified lipoproteins on endothelial cells under the experimental conditions used. Indeed, Ox-LDLs (up to 300 μg/ml) did not modify [3H]adenine release as compared with control cells. Moreover, the rate of endothelin release measured after 24 hours of stimulation with Ox-LDLs was not different from the basal rate, demonstrating that the endothelial cells kept their constitutive release of the peptide even after 24 hours’ exposure to Ox-LDLs.

An increased production of the peptide may have important vascular effects. Indeed, the peptide causes potent and protracted contractions in most blood vessels. In addition, threshold and low concentrations of endothelin potentiate the contraction induced by norepinephrine or serotonin in human blood vessels. This may be of particular importance at sites of platelet activation with release of serotonin and thromboxane A2. Other substances formed or released under these conditions, such as thrombin and transforming growth factor-β, would further augment the production of endothelin already increased by Ox-LDLs. Indeed, the stimulatory effect of thrombin on vascular endothelin production (which may be particularly relevant in myocardial infarction) was fully maintained even in the presence of Ox-LDLs. Hence, locally increased levels of endothelin may upregulate the responsiveness of the blood vessel wall to vasoconstrictor stimuli and may explain that vasospastic events preferentially occur at the site of early atherosclerotic lesions. Inhibition of the release of endothelium-derived relaxing factor by Ox-LDLs may also contribute to vasospastic events, since a locally decreased production of nitric oxide would further augment endothelin production as well as increase the sensitivity of blood vessel to vasoconstrictor stimuli. The Ox-LDL–stimulated release of endothelin may be of particular relevance in aging, which is accompanied by accumulation of oxidized proteins, increase plasma levels of endothelin, and vascular hyperreactivity.

In addition to inducing vasoconstriction, endothelin may facilitate local proliferation of the vascular smooth muscle cells. The peptide evokes intracellular events known to be linked to proliferative responses such as alkalization and expression of c-fos and c-myc proto-oncogenes in cultured vascular smooth muscle and mesangial cells. Endothelin also stimulates the production of autocrine growth factors by increasing the expression of platelet-derived growth factor mRNA transcript in vascular smooth muscle cells. Thus, the release of endothelin from the intact endothelium induced by Ox-LDLs trapped in the vessel wall may contribute to the formation of advanced atherosclerotic lesions containing proliferative smooth muscle cells (i.e., the fibrous cap). An increased release of endothelin induced by Ox-LDLs could also explain why platelet adhesion is not required for smooth muscle proliferation at sites in atherosclerotic lesions where the endothelium remains intact.

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