Stimulating Effect of Oxidized Low Density Lipoproteins on Plasminogen Activator Inhibitor-1 Synthesis by Endothelial Cells

Y. Latron, M. Chautan, F. Anfosso, M.C. Alessi, G. Nalbone, H. Lafont, and I. Juhan-Vague

Oxidized low density lipoproteins (ox-LDL) are thought to accelerate atherogenesis. It was recently demonstrated that patients with coronary heart disease have defects in plasma fibrinolysis due to increased plasminogen activator inhibitor-1 (PAI-1) levels. Investigation of PAI-1 synthesis by endothelial cells may allow insight into the effect of native LDL (N-LDL) and ox-LDL on endothelial cells. In the present study, secretion of PAI-1 by human umbilical vein endothelial cells (HUVEC) in culture was evaluated after incubation with N-LDL and ox-LDL. Ox-LDL were obtained by peroxidation under ultraviolet radiation, which induced compositional changes in LDL, namely, a decrease in the levels of arachidonic acid, eicosapentaenoic acid, docosahexaenoic acid, and \( \alpha \)-tocopherol and an increase in the malondialdehyde content. Ox-LDL induced a dose-dependent increase in PAI-1 secretion by HUVEC as assayed by an enzyme-linked immunosorbent assay. After a 24-hour incubation, a twofold increase in the PAI-1 content was observed with 50 \( \mu \)g/ml ox-LDL protein. Studies with inhibitors of protein synthesis and metabolic labeling with \(^{35}\)S)methionine confirmed that PAI-1 synthesis was stimulated by ox-LDL. N-LDL had no detectable effect on PAI-1 secretion. Binding studies with radiolabeled lipoproteins showed that the effect of ox-LDL was independent of the B/E receptor. Our experiments indicate that ox-LDL stimulate PAI-1 secretion from HUVEC and that this effect may involve a scavenger receptor.

Arteriosclerosis and Thrombosis 1991;11:1821-1829
synthesis by human umbilical vein endothelial cells (HUVEC) in culture.  

PAI-1 is synthesized by several kinds of cultured cells including endothelial cells, hepatocytes, and smooth muscle cells. In a previous study, we showed that while N-LDL did not modify PAI-1 synthesis by HUVEC, they did increase PAI-1 synthesis by a HepG2 hepatoma cell line. The purpose of the present study was to investigate the effect of ox-LDL on PAI-1 synthesis by endothelial cells.

Methods

Materials

Fetal calf serum and cell culture reagents were purchased from Gibco BRL, Cergy Pontoise, France. Cycloheximide, actinomycin D, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, Mo. [15S]Methionine was purchased from Amersham International, Amersham, UK. [125I]Isosodium iodine was provided by NEN, Paris, France. Monoclonal antibodies 15H12, 12A4, and 7D4, specific for PAI-1, were kindly given by P. Declercq, Center for Vascular and Thrombosis Research, Leuven, Belgium. A commercial kit for tissue plasminogen activator (t-PA) assay was obtained from Biopool, Umeå, Sweden. The monoclonal antibody against the LDL receptor was a generous gift of J. Chapman, INSERM U321, Paris, France. The Kabi-LAL endotoxin test was from Kabi Diagnostica, Stockholm, Sweden.

Methods

Cell culture. Endothelial cells were prepared from fresh human umbilical cord veins according to the method of Jaffe et al. Cells were used after one passage and were plated into tissue culture flasks coated with gelatin. Cells were grown to confluence in a humidified incubator with 5% CO2 in medium 199 supplemented with 15 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 15 mM NaHCO3, 2 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 20% heat-inactivated fetal calf serum. The cells were identified as endothelial cells by their typical cobblestone pattern and immunofluorescence staining for von Willebrand factor. After reaching confluence, HUVEC monolayers were incubated for 16 hours in serum-free medium supplemented with 0.5% BSA followed by a 24-hour incubation with various concentrations of LDL. After incubation, conditioned medium was harvested and centrifuged at 2,000g to exclude cellular debris. Aliquots were sampled and stored at -20°C. Cytotoxicity was checked by trypan blue dye exclusion and lactate dehydrogenase (LDH) release.

Lipoprotein preparation. LDL (density = 1.019-1.063 g/ml) were isolated from the serum of normolipemic blood donors by sequential preparative ultracentrifugation as described elsewhere. LDL protein content was determined before ultraviolet (UV) radiation by the method of Lowry et al using BSA as the standard. The isolated lipoproteins were dialyzed against saline EDTA (0.15 M NaCl, 0.01% EDTA; pH 7.4) for 72 hours. LDL purity was assessed by polyacrylamide gel electrophoresis (PAGE). The presence of endotoxin was determined with the Kabi-LAL test. Briefly, 100-µl samples of LDL were added to 100 µl of reconstituted reagent. The assay tubes were mixed by low-speed vortexing to avoid mixing artifacts. The reaction tubes were incubated in a 37°C water bath for 60 minutes. A negative test is defined as the formation of a firm gel in the bottom of the tube. A positive test is characterized by the absence of such a gel.

Peroxidation of low density lipoproteins. LDL were peroxidized under UV radiation as described by Dousset et al. Lipid peroxidation was measured by quantification of thiobarbituric acid–reactive substance (TBARS) according to a modification of the procedure of Dousset et al.; 100 µl LDL was mixed with 750 µl of a mixture composed of thiobarbituric acid/perchloric acid (2:1,vol/vol) and 100 µl of 0.5% butylated hydroxytoluene in solution. After stirring, the samples were heated for 60 minutes at 95°C and then cooled on ice. The fluorescent component was extracted after adding 2 ml butanol and shaking the tubes for 2 minutes. The mixture was then centrifuged at 700g for 10 minutes, and fluorescence of the butanol phase was measured using a spectrophotofluorometer (λex 532 nm, λem 553 nm). The same procedure was applied for blanks, except they were not heated at 95°C.

Lipid analysis. Total LDL lipids were extracted according to the procedure of Folch et al. and fatty acid methyl esters were prepared as described elsewhere and separated by gas–liquid chromatography (Girdel 3000, Paris, France) using a capillary column (Spirax 1S F493, Spiral, Dijon, France) as previously published. Fatty acid methyl standards (99% pure) were from Interchim, Paris, France. α-Tocopherol was assayed after lipid extraction by high-performance liquid chromatography on an RP8 LiChrosorb (7 µm, 250x4-mm) column (Merck, Darmstadt, Germany). Tocopherol standards were from Sigma.

Radiolabeling of endothelial cell proteins. Confluent HUVEC monolayers were washed and incubated in serum-free medium for 16 hours before the assay. The medium was changed, and the cultures were labeled with 50 µCi/ml [35S]Methionine (Amersham; 370 MBq/ml) in the presence or absence of 50 µg/ml ox-LDL protein at 37°C for various periods. The cells were washed with cold phosphate-buffered saline (PBS) and lysed with 100 µl of a lysis buffer (0.05 M trishydroxymethylaminomethane hydrochloride, pH 8.0, 1% Nonidet P40, 0.15 M NaCl, 100 µg/ml phenylmethylsulfonyl fluoride, and 10 µg/ml aprotinin).

Immunoprecipitation of plasminogen activator inhibitor-1. Radiolabeled PAI-1 was isolated by the sequential addition of 50% (by volume) irrelevant monoclonal antibody coupled to protein A-Sepharose. After removal of the immunoglobulin G (IgG) protein...
Effect of Peroxidation on Low Density Lipoproteins

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The basal lipoperoxide content of 2.38 ±0.27 nmol malondialdehyde (MDA)/mg protein. The production of lipoperoxides under conditions of UV radiation exposure was linear from 30 minutes to 4 hours. Production was 8.65 nmol MDA/mg protein after 2 hours.

Results

Effect of Peroxidation on Low Density Lipoproteins

Under our experimental conditions, N-LDL have a basal lipoperoxide content of 2.38 ±0.27 nmol malondialdehyde (MDA)/mg protein. The production of lipoperoxides under conditions of UV radiation exposure was linear from 30 minutes to 4 hours. Production was 8.65 nmol MDA/mg protein after 2 hours of radiation (Figure 1). During peroxidation the LDL α-tocopherol content dropped drastically after 30 minutes of UV radiation and disappeared with longer exposures (Figure 1). UV radiation-induced peroxidation caused a change in the fatty acid composition of LDL total lipids (Table 1). Notably, the levels of arachidonic acid (20:4 n-6), eicosapentaenoic acid (20:5 n-3), and docosahexaenoic acid (22:6 n-3) were decreased in ox-LDL as a function of time, particularly after 2 and 4 hours of UV radiation treatment.

Effect of Native and Oxidized Low Density Lipoproteins on Plasminogen Activator Inhibitor–1 Synthesis

Modulation of PAI-1 synthesis by N-LDL or ox-LDL was investigated in HUVEC cultured for 24 hours in serum-free medium. Exposure of HUVEC to ox-LDL caused a significant (p<0.001) dose-dependent increase of PAI-1 secretion. The maximum effect was observed with a 50 μg/ml sample of 2-hour UV-treated LDL protein (Figure 2A). At this dose of ox-LDL, no cytotoxicity was observed either by microscopic examination with trypan blue dye exclusion or by LDH release (LDH content <50 IU/l). Higher doses of ox-LDL were cytotoxic for endothelial cell monolayers (data not shown). Incubation of HUVEC with N-LDL did not significantly modify PAI-1 secretion, even at high doses (200 μg/ml N-LDL protein) (Figure 2B). No cytotoxicity was observed.

Experiments were performed to determine if the increased secretion of PAI-1 by HUVEC after incubation with ox-LDL was due to stimulation of PAI-1 synthesis. HUVEC were labeled with [35S]methionine for 6 and 24 hours, and PAI-1 in cellular extracts was immunoprecipitated with the specific 7D4 monoclonal antibody. Analysis of the resulting samples by SDS-PAGE and autoradiography (Figure 3) revealed a major band with an apparent M, of 50 kd and lighter proteins that had migrated to the dye front. A higher-molecular-weight band of 110 kd, which probably corresponded to PAI-1–t-PA complexes, was also found. A band of very high molecular weight suggests the presence of PAI-1–vitronectin complexes. An increase in radiolabeling of the 50-kd and 110-kd proteins in the treated (lane B) compared with the control (lane A) cell extracts was observed after a 6-hour incubation. After 24 hours, no significant increase was observed (lanes C and D).

It has been suggested that ox-LDL is a carrier for endotoxin, which is known to be a potent stimulator of PAI-1 synthesis. While the effect of endotoxin is temperature-resistant, heating 50 μg/ml ox-LDL protein for 15 minutes at 100°C completely suppressed the ability to stimulate PAI-1 synthesis, suggesting that endotoxin is not involved (Table 2). This assumption is supported by the fact that no endotoxin was found with the Kabi-LAL endotoxin test.

HUVEC secreted another fibrinolytic component, t-PA. To investigate if LDL acted on release of this
component, we assayed t-PA in the medium after a 24-hour incubation. As shown in Table 2, ox-LDL did not modify t-PA release.

Ox-LDL-induced PAI-1 stimulation was blocked by 10 μg/ml actinomycin D and 10 μg/ml cycloheximide, suggesting that this stimulation required messenger RNA and protein synthesis (Table 2).

**Effect of Peroxidation Time on Plasminogen Activator Inhibitor-1 Stimulation**

To study the relation between PAI-1 stimulation and the extent of peroxidation, LDL were treated with UV radiation for various times and then incubated with confluent HUVEC monolayers for 24 hours. As shown above, MDA content increased with the duration of UV treatment (Figure 1). When PAI-1 was assayed, a striking dependency was observed for short peroxidation times. Between 30 minutes and 2 hours of peroxidation, PAI-1 levels increased; the levels then returned to baseline (Figure 4), although the MDA content continued to increase.

**Native and Oxidized Low Density Lipoprotein Binding**

To better understand the difference between N-LDL and ox-LDL with regard to stimulation of PAI-1 synthesis, we assessed their binding and internalization by HUVEC at 37°C. Results (Figure 5A) indicated that N-LDL and ox-LDL bind to confluent HUVEC. As previously reported, the shape of the N-LDL binding curve indicates that they bind to a saturable site (Figure 5A). This was confirmed by an internalization curve, which showed a plateau for 20 μg/ml of [125I]N-LDL protein (Figure 5B). In contrast, binding and internalization curves for ox-LDL did not reach a plateau but increased up to the maximum tested (50 μg/ml) (Figure 5). These results suggested that N-LDL and ox-LDL bind to different sites. These findings were confirmed by competitive binding experiments performed at 4°C.
FIGURE 2. Plots of effect of oxidized low density lipoprotein (ox-LDL) and native low density lipoprotein (N-LDL) on secretion of plasminogen activator inhibitor-1 (PAI-1) from human umbilical vein endothelial cells (HUVEC). Confluent monolayers of HUVEC were incubated with increasing doses of ox-LDL (panel A) or N-LDL (panel B). PAI-1 antigen was assayed in conditioned medium after 24 hours' incubation. Results are expressed as mean ± SEM of six experiments, each made in triplicate. *p<0.05, **p<0.001.

Competitive binding curves were obtained by competition between the two LDL preparations and their iodinated counterparts. Incubation with 300 μg/ml N-LDL protein completely displaced the binding of 20 μg/ml [125I]N-LDL protein but had no effect on the binding of [125I]ox-LDL (Figure 6A). Similarly, 300 μg/ml ox-LDL protein had no effect on the binding of [125I]N-LDL, whereas it totally displaced [125I]ox-LDL (Figure 6B). The above results indicate that ox-LDL and N-LDL bind to different receptors on the cell membrane.

To confirm these data, ox-LDL-induced PAI-1 stimulation was studied in the presence of a monoclonal antibody against the N-LDL receptor.44 Cells were incubated for 24 hours together with 50 μg/ml ox-LDL protein and 1/1,000 diluted monoclonal antibody IgG C7 ascitic fluid. Ox-LDL-induced PAI-1 stimulation was not inhibited by this monoclonal antibody.

Discussion

Our results demonstrate that ox-LDL induced an increase in PAI-1 synthesis by HUVEC. This increase is a consequence of in vitro peroxidation since N-LDL do not trigger cells for increased PAI-1 secretion. Ox-LDL-induced PAI-1 secretion required de novo synthesis, as indicated by the inhibition by cycloheximide and actinomycin D. Metabolic

TABLE 2. Effect of Protein Synthesis Inhibitors and Heating on Oxidized Low Density Lipoprotein–Induced Plasminogen Activator–Inhibitor–1 Synthesis

<table>
<thead>
<tr>
<th></th>
<th>PAI-Ag (pg/cell/24 hr)</th>
<th>t-PA Ag (pg/10^3 cells/24 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>1.06±0.08</td>
<td>0.9±0.03</td>
</tr>
<tr>
<td>Ox-LDL (50 μg/ml)</td>
<td>1.98±0.10</td>
<td>0.9±0.02</td>
</tr>
<tr>
<td>Ox-LDL 100°C</td>
<td>1.03±0.07</td>
<td>0.9±0.02</td>
</tr>
<tr>
<td>Ox-LDL+actinomycin D</td>
<td>0.34±0.04</td>
<td>...</td>
</tr>
<tr>
<td>Ox-LDL+cycloheximide</td>
<td>0.60±0.10</td>
<td>...</td>
</tr>
</tbody>
</table>

Ox-LDL, oxidized low density lipoprotein; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue plasminogen activator. Values represent mean of three experiments, each performed in duplicate.
labeling indicated that PAI-1 in cellular extracts was recovered in PAI-1-t-PA complexes, with an apparent molecular weight of 110 kd and in free PAI-1 with a normal weight of 49 kd. The action of ox-LDL is relatively specific for PAI-1 synthesis since ox-LDL did not stimulate t-PA production by HUVEC in conditioned medium.

Endotoxin has been shown to increase PAI-1 synthesis by HUVEC in vitro and to generate high PAI-1 plasma levels in vivo. It has also been reported that ox-LDL facilitate the uptake of endotoxin by human endothelial cells. The presence of endotoxin in our preparations is unlikely, since heating ox-LDL totally suppressed the increase of PAI-1 synthesis by HUVEC. Moreover, we also were unable to detect endotoxin using a specific assay.

Our results point out a large discrepancy between the effects of N-LDL and ox-LDL on HUVEC. Previous reports have suggested that LDL may promote atherogenesis by a variety of mechanisms involving prostaglandin I₂ production, derivatives of arachidonic acid, and elevations of intracellular Ca²⁺ content and cytosolic pH. It has also been speculated that LDL facilitate the adhesion of platelets to the endothelial barrier by increasing the synthesis of von Willebrand factor. Nevertheless, in all these reports the degree of peroxidation of LDL was not checked. Our results clearly demonstrate that N-LDL have no effect on PAI-1 synthesis. This finding contrasts with the triggering effect of N-LDL on PAI-1 synthesis by the hepatoma cell line Hep G₂. The discrepancy between ox-LDL and N-LDL does not result from a defect in binding to the cell surface receptor. HUVEC, as well as other cells such as HepG₂, possess high-affinity receptors for LDL. Our binding experiments showed that binding and internalization of LDL by HUVEC are mediated by a high-affinity saturable site. This binding is specific for N-LDL, as shown by competitive binding experiments indicating that N-LDL in excess totally displaced the binding of [¹²⁵I]N-LDL. In contrast, ox-LDL did not compete for this binding site. Endothelial cells, as well as macrophages, possess scavenger receptors for modified LDL such as acetyl-LDL. These receptors, recently characterized, are structurally different from the B/E receptor for LDL. In our work, binding and internalization curves indicated that ox-LDL bound to a nonsaturable cell membrane site. Binding to this site was inhibited by the unlabeled autologous ligand but not by N-LDL, even when used in excess. Furthermore, a monoclonal antibody against the B/E receptor did not block ox-LDL-induced PAI-1 synthesis. Taken together, these findings show that the action of ox-LDL on PAI-1 synthesis is not mediated by the LDL receptor. Steinberg et al showed that ox-LDL competed with acetyl-LDL for binding to the scavenger receptor. It thus seems likely that the effect of ox-LDL on PAI-1 synthesis by HUVEC is mediated by this type of receptor.

In vitro, lipid peroxidation of LDL is often obtained after incubation of the particles with cupric ions. Under these conditions, fragmentation of apolipoprotein (apo) B is observed. Recently, Dousset et al described a new method of peroxidation, using UV radiation, that avoids fragmentation of apo B. Structural changes in the LDL molecule induced by UV radiation peroxidation could alter its metabolism. Although it is not currently possible to determine which UV radiation-induced modification is responsible for the observed effect on PAI-1 synthesis, fatty acid peroxidation products are the prime suspects. It is known that the peroxidation of fatty acids generates cytotoxic aldehydes such as 4-hydroxynonenal and MDA. These compounds efficiently form covalent bonds with apo B through Schiff base formation. This leads to structural changes of the particles, with the appearance of new epitopes. LDL contain a number of natural antioxidants including vitamin E and β-carotene. Experimental studies suggest that vitamin E accelerates lipid peroxidation in vivo and in vitro and that antioxidants, such as butylated hydroxytoluene or vitamin E, prevent LDL oxidation in vitro.
FIGURE 5. Binding (panel A) and internalization (panel B) of iodine-125-labeled oxidized low density lipoprotein (LDL) (●) and iodine-125-labeled native LDL (◆) by confluent monolayers of human umbilical vein endothelial cells at 37°C. Cells were incubated in serum-free medium supplemented with 0.5% albumin in presence of increasing doses of radiolabeled ligand. Binding and internalization were assayed after 3 hours of incubation as described in text. Results are expressed as nanograms of bound radioactivity per milligram of total protein.

conditions of exposure to UV radiation a dramatic decline of α-tocopherol content occurred, associated with a loss of polyunsaturated fatty acids and an increase in TBARS. These observations are consistent with previous studies performed under comparable conditions. Thus, treatment with UV radiation for up to 2 hours results in changes in LDL conformation which, albeit subtle, are sufficient for the particle to be recognized by the scavenger receptor. Curiously, 4 hours of UV radiation abolished the ability of LDL to stimulate PAI-1 secretion. This indicates that after 4 hours the LDL structure is modified in such a way that it cannot trigger PAI-1 synthesis by HUVEC.

Cardiovascular diseases are associated with hypofibrinolysis. Considerable experimental evidence implies that endothelial injury is an important factor in atherogenesis. Several pathways have been implicated in the in vivo generation of ox-LDL. Ox-LDL are highly immunogenic and generate autoantibodies in vivo. Many effects on the endothelium barrier, including endothelial cytotoxicity, impairment of vasorelaxation, and synthesis of tissue factor, have been described. Our work points out a new link between atherogenesis and its well-known counterpart, hypofibrinolysis, induced by an increased synthesis of PAI-1.

The physiological significance of LDL peroxidation can be discussed in terms of which compartment (extravascular and/or intravascular) is involved in the process. It is generally believed that this process occurs mainly in the intima. However, a higher concentration of lipid peroxides and autoantibodies against ox-LDL have been detected in the plasma of atherosclerotic patients and healthy subjects, respectively, suggesting that lipid peroxidation may occur in the circulation. In addition, to our knowledge, the relative distribution of the scavenger receptor between the basal and luminal parts of the plasma membrane of endothelial cells is not known. That means that if the scavenger receptor is uniformly distributed on the plasma membrane, then ox-LDL present in the circulation, as well as in the intima, may exert their pathological effect on the endothelium. Further studies are thus needed to evaluate the relative importance of extravascular and intravascular lipid peroxidation in the atherothrombotic process.
Recently Stiko-Rahn et al. demonstrated that VLDL, especially from hypertriglyceridemic patients, were able to increase PAI-1 synthesis by HUVEC. Those authors also reported that N-LDL slightly stimulated (13%) PAI-1 release. This effect may be due to a slight oxidation of LDL during their 48-hour incubation with endothelial cells, which was twice as long as ours. Also, Mussoni et al. described a strong stimulation of N-LDL on PAI-1 release by endothelial cells. However, in these studies the peroxidation status of the lipoprotein was not available. Also, in these studies PAI-1 release was not affected by cupric ion treatment of LDL. These discrepancies with our 2-hour UV radiation treatment were probably due to the degree and mode of oxidation since we also observed that 4 hours of UV radiation did not induce PAI-1 release.

Inasmuch as the phenomenon observed with HUVEC may be extrapolable to arterial endothelium in vivo, the increase in PAI-1 secretion induced by ox-LDL could facilitate the development of vessel wall damage and thrombotic lesions.

Acknowledgments

The authors express their thanks to B. Bonardo for her skillful technical assistance, to J. Leonardi for fatty acid analysis, to R. Calaf for α-tocopherol analysis, and to N. Billardon (INSERM U321) for preparation of monoclonal antibody IgG C7.

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Arterioscler Thromb Vasc Biol. 1991;11:1821-1829
doi: 10.1161/01.ATV.11.6.1821

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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