Increased Synthesis of Plasminogen Activator Inhibitor–1 by Cultured Human Endothelial Cells Exposed to Native and Modified LDLs
An LDL Receptor–Independent Phenomenon


The effects of native and acetylated low density lipoproteins (LDLs and acetyl-LDLs, respectively) on the release of plasminogen activator inhibitor type 1 (PAI-1) by cultured human umbilical vein endothelial cells (ECs) were evaluated. LDL and acetyl-LDL incubated with ECs for 16–18 hours increased the PAI-1 antigen levels in conditioned medium. At a concentration of 100 μg/mL, LDL and acetyl-LDL increased PAI-1 by 10.8 and 12.0 ng/mL, respectively (p<0.05 and p<0.01 versus control). The increases in PAI-1 antigen levels exerted by the lipoproteins paralleled the changes in PAI-1 activity. The effect of LDL and acetyl-LDL was concentration dependent and specific for PAI-1 because tissue-type plasminogen activator and expression of procoagulant activity were not affected by either lipoprotein. In addition, total protein synthesis evaluated in [35S] methionine-labeled ECs was not affected, and studies with cycloheximide showed that the effect of LDL and acetyl-LDL on PAI-1 release was due to de novo protein synthesis. Experiments using the C7 monoclonal antibody against the LDL receptor and binding-defective LDL indicated that the effect of LDL on the synthesis of PAI-1 was not dependent on the interaction of the lipoprotein with their specific receptors. Finally, extensive oxidation of LDL prevented and even reversed the effect of LDL on PAI-1 release by ECs. It is concluded that LDL specifically increases the synthesis of PAI-1 by ECs with mechanisms that are not receptor mediated. (Arteriosclerosis and Thrombosis 1993;13:338–346)

KEY WORDS • LDL • modified LDL • endothelial cells • plasminogen activator inhibitor
PAI-1 release by ECs, in parallel with another marker of EC activation, the expression of procoagulant activity. We report here that LDLs and acetyl-LDLs stimulate PAI-1 release by ECs, thus increasing the antifibrinolytic potential of these cells.

**Methods**

**Lipoprotein Preparation**

Lipoproteins were separated from freshly prepared (less than 24 hours) human plasma from normolipidemic donors. Blood, obtained from the antecubital vein, was anticoagulated with Na$_2$EDTA (1 mg/mL) and centrifuged at 800g for 18 minutes to obtain plasma. LDLs (density range 1.020–1.050 g/mL) were prepared by differential ultracentrifugation. Plasma was adjusted to a density of 1.020 g/mL with a potassium bromide solution and ultracentrifuged at 40,000 rpm for 20 hours at 4°C in a Beckman SW50Ti rotor. The tubes were then sliced, and the bottom fraction was saved. The density of this fraction was then increased from 1.020 g/mL to 1.050 g/mL with a potassium bromide solution. After another 20-hour ultracentrifugation at 42,000 rpm at 4°C, the tubes were sliced again, and the top fraction was saved.

High density lipoproteins (HDLs) were isolated by the same technique at a density range of 1.06–1.21 g/mL. LDLs and HDLs were exhaustively dialyzed against phosphate buffer (PB) consisting of 1.5 mM Na$_2$HPO$_4$ and 3.5 mM NaH$_2$PO$_4$ (pH 7.5) at 4°C. In some experiments butylated hydroxytoluene (BHT) was added to plasma and to the different buffers (20 μM BHT plus 1 mM EDTA) to avoid possible lipoprotein oxidation. The dialyzed lipoprotein fractions were sterilized by passage through filters (0.22 μm, Millipore Corp., Bedford, Mass.), stored in sterile tubes at 4°C, and used within 1 week. Total protein content in lipoprotein preparations was determined by the Lowry method with bovine serum albumin (BSA, Sigma Chemical Co., St. Louis, Mo.) as the standard. Lipoprotein-depleted serum (LPDS) was obtained as the human serum fraction at density 1.25 g/mL by ultracentrifugation for 72 hours at 40,000 rpm at 12°C followed by extensive dialysis against 0.15 M NaCl and 0.3 mM EDTA, pH 7.4. After sterilization through a Millipore 0.2-μm filter, LPDS was divided in aliquots and stored at −20°C.

LDLs of patients belonging to a family affected by a defective apo B100 and characterized by hypercholesterolemia and defective LDL binding (defined as a point mutation at position 3,500 Glu→Arg) were labeled with 125I according to Bilheimer et al. Briefly, 50 μg LDL protein was precipitated with 1 mL of 20% trichloroacetic acid (TCA) plus 0.1% (vol/vol) BHT. After centrifugation, supernatants were collected and 1 mL of a freshly dissolved solution (0.67%, vol/vol) of trichloroacetic acid was added. The mixture was boiled for 45 minutes, cooled, and cleared by centrifugation. Spectrophotometric measurements were made at 532 nm. Blanks were made of distilled water plus 50 μg BSA and processed identically as LDL samples. The amount of malondialdehyde (MDA) equivalent was determined against a standard curve obtained using freshly diluted 1,1,3,3-tetramethoxypropane.

The degree of LDL oxidation was expressed in nanomoles of MDA equivalent per milligram of LDL protein. Under these conditions, the TBARS values in the LDL preparations were below 0.1 nmol MDA per mg protein and, for the ox-LDL preparations, were in the range from 7.0 to 16.0 nmol MDA per mg protein. Agarose gel electrophoresis was used to check ox-LDLs for increased anodic mobility.

Acetyl-LDLs were obtained after extensive dialysis of LDLs in 0.15 M NaCl diluted with an equal volume of saturated sodium acetate and treated with acetic anhydride as previously described. Charge modification was checked by agarose gel electrophoresis. Ox-LDLs and acetyl-LDLs, prepared as described, were shown to bind and to be internalized by macrophages.

**Cell Preparation**

Human ECs were obtained from single, fresh umbilical cords by collagenase (Sigma) digestion using the method of Jaffe et al and grown to confluence in 25-cm$^2$ tissue culture flasks (Costar, Cambridge, Mass.). Cells were cultured in medium 199 (GIBCO, Mascia Brunelli, Milano, Italy) containing 20% newborn calf serum (GIBCO), 2 mM l-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin in the presence of 50 μg/mL porcine intestinal heparin (Sigma), and 50 μg/mL of a crude extract of endothelial cell growth factor (ECGF) obtained from bovine hypothalamus, prepared as previously described. Cells were subpassaged into 75-cm$^2$ culture flasks one to four times before use and allowed to grow to confluence in a humidified incubator at 37°C under a 5% CO$_2$–95% air atmosphere. ECs were characterized by their typical cobblestone morphology and by virtually homogeneous immunofluorescence staining with von Willebrand factor antisera (Nordic Immunobiological Labs.).

Studies were carried out using cells harvested from single cords, used between the second and fourth passages, and plated in 24-well cluster plates (2 cm$^2$) coated with 0.2% calf skin gelatin for 3–4 days in the presence of heparin and ECGF. Confluent cells, after removal of complete medium, were rinsed three times with Hank’s balanced salt solution (GIBCO) and incubated for the indicated period in 1 mL serum-free medium containing antibiotics, l-glutamine, and the indicated stimulus or vehicle. After incubation, conditioned medium (CM) was removed and cells harvested as indicated.
Cell viability was tested by trypan blue staining, and representative cell counts or protein concentrations in disrupted cells were determined by the Bradford method (Bio-Rad, Richmond, Va.). Protein concentrations of adherent cells were performed to exclude a possible toxic effect of lipoprotein incubation in serum-free medium.

Overall protein synthesis was determined by measuring the incorporation of \(^{35}\text{S}\)methionine (Amersham International, Amersham, UK) into the 10% (vol/vol) TCA-precipitable fraction of radiolabeled CM and cell extract. To that end, the cultures were placed in methionine-free medium with 20 \(\mu\text{Ci}^{[35]}\text{S}\)methionine (370 MBq/mL) for the last 4 hours of incubation.

**LDL Uptake and Degradation**

For lipoprotein competition experiments, confluent cells were preincubated for 24 hours at 37°C in medium 199 containing 10% human LPDS to induce LDL receptors. The medium was then removed and replaced by fresh medium containing native \(^{125}\text{I}\)LDL protein (7.5 \(\mu\text{g/mL}\)) along with increasing concentrations of unlabeled LDL. The cells were incubated for 4 hours at 37°C. For determining total uptake (binding plus internalization) of LDL, cell monolayers were directly digested in 0.1% (vol/vol) NaOH solution after standard washing procedures. One aliquot was counted for the cell-associated radioactivity and another aliquot used for cell protein determination. LDL degradation was measured as the accumulation of nondialyzable, TCA-soluble \(^{125}\text{I}\)LDL in the incubation medium in excess of that occurring in the absence of cells. Nonspecific uptake and degradation were determined by adding a 100-fold excess of unlabeled LDLs. Each experimental point represents the average value of triplicate incubations. The amount of unlabeled LDLs required to displace 50% of the \(^{125}\text{I}\)labeled ligand was calculated by linear regression analysis of the logarithm of concentration (micrograms of protein per milliliter) versus probits and read from a probit transformation table.

**Quantification of PAI-1 Antigen**

PAI-1 antigen was measured by enzyme-linked immunosorbent assay (ELISA) (FI-5 Monozyme, Copenhagen, Denmark) in CM, cell extracts, and extracellular matrix stored at \(-20^\circ\text{C}\). Briefly, CM was collected and centrifuged to remove cell debris, and 0.25% (vol/vol) Triton X-100 was added. A cell extract was prepared by lysing the monolayer, rinsed with cold phosphate-buffered saline (PBS), with 500 \(\mu\text{L}\) of 0.25% (vol/vol) Triton X-100 for 20 minutes at 37°C. Extracellular matrix was then washed vigorously with cold bidistilled water and removed with PBS containing 0.1% sodium dodecyl sulfate (SDS). SDS inhibition of PAI-1 binding to antibody was obviated by addition of Triton X-100 to yield a final concentration of 1%. The assay is based on the double-antibody principle, using two different monoclonal antibodies, which allow the detection of total PAI-1 antigen. Appropriate controls were performed to exclude possible interferences of the lipoproteins under study with the assay. None of the lipoprotein preparations contained measurable amounts of PAI-1 antigen.

**Procoagulant Activity (PCA) Assay**

EC PCA was determined by a one-stage clotting assay. Briefly, after medium removal, cells were washed three times with cold PBS and subsequently scraped off at 4°C with a plastic spatula in 300 \(\mu\text{L}\) PBS. Cells were disrupted by repeated (five cycles) freezing and thawing, and clotting times were determined in duplicate at 37°C in prewarmed plastic tubes containing 0.1 mL disrupted cells; 0.1 mL citrated, pooled, normal human plasma; and 0.1 mL CaCl\(_2\) (25 mmol/L). The plasma pool was calibrated using serial dilutions of a preparation of human brain thromboplastin (Thromborel, Behring Corp.) to which arbitrary values (AU) from 2,000 to 3.9 units were assigned. Clotting times for 3.9 and 2,000 AU were 300 and 36 seconds, respectively. Data are expressed as clotting times (in seconds) using triplicate determinations. Assays performed using human plasma deficient in clotting factor VII (Boehringer Mannheim GmbH, Mannheim, FRG) instead of pooled human plasma consistently demonstrated no PCA activity.

**Other Procedures**

C7 monoclonal antibody against the LDL receptor was from Amersham. Immunoglobulin (Ig) \(G_1\) and IgG\(_2\) monoclonal antibody against CD1 and CD2 on T lymphocytes, used as controls in antibody experiments, were kindly provided by P. Allavena (M. Negri Institute, Milan, Italy). Cycloheximide was from Sigma. The L. limulus assay test was from Ortho Diagnostica (Milan, Italy) and Escherichia coli 011:B4 from DIFCO Labs (Detroit, Mich.).

**Statistical Analysis**

Values are expressed as mean±SEM. Statistical significance of the differences was tested by paired Student’s \(t\) test.

**Results**

**Effect of LDL on the Release of PAI-1 by ECs**

ECs were grown until confluence in 16-mm-diameter wells (average number of cells/well =1\(\times10^5\)). PAI-1 activity was detected in CM by a two-stage indirect chromogenic assay with reagents obtained from Ortho Diagnostica Systems (Milan, Italy) as previously described. To activate latent PAI, CM samples (100 \(\mu\text{L}\)) were added to 0.2% SDS and incubated for 30 minutes at 37°C. Triton X-100 was then added to a final concentration of 2%. Samples to be assayed for active inhibitor were not treated with SDS but were made with 2% Triton X-100.

Aliquots of untreated or SDS-treated CM (10–30 \(\mu\text{L}\)) were mixed in 96-well microtiter plates (Flow Labs, Irvine, Scotland) with t-PA standard (0.25 or 0.5 IU/mL) and incubated at room temperature. After 10 minutes, the substrate solution containing human plasminogen, S-2251, and cyanogen bromide fibrinogen fragments in tris(hydroxymethyl)aminomethane HCl (0.05 M, pH 8.3) with 2% Triton X-100 was added and the absorbances read at 405 nm after different times of incubation at 37°C. One unit of inhibitory activity was defined as the amount of sample required to inhibit 1 IU of t-PA.
antigen levels were then determined with a sandwich ELISA in CM and in Triton X-100 extracts of ECs. In the absence of lipoproteins, PAI-1 antigen levels in CM were in the range between 1 and 2 ng/mL and remained constant up to 8 hours, whereas they increased linearly at the rate of 1.5–2 ng/mL per hour from 8 to 24 hours (Figure 1). No cell detachment, as determined by measuring residual proteins after medium removal in each well, occurred with as much as 8 hours of incubation. After 16 and 24 hours of incubation with medium alone, cell detachment (evaluated on the initial cell protein content) ranged between 28% and 34%. Intra-cellular levels of PAI-1 at 2 hours averaged 5 ng/mL and remained constant up to 24 hours (Figure 1).

To assess the effects of native and modified LDLs on PAI-1 synthesis and release, ECs were incubated with or without appropriate concentrations of LDLs or acetyl-LDLs for 16–18 hours. Treatment of ECs with 100 µg/mL LDLs or acetyl-LDLs significantly increased PAI-1 levels in CM by 10.8 ng/mL and 12 ng/mL, respectively (p<0.01 and p<0.05 versus control for LDLs and acetyl-LDLs) (Table 1). Intracellular levels of PAI-1 antigen were not affected by either lipoprotein (data not shown). The effect of lipoproteins on PAI-1 release was concentration dependent (Figure 2). LDLs increased PAI-1 with maximal effect between protein concentrations of 100 and 200 µg/mL (Figure 2A). Similarly, acetyl-LDLs increased PAI-1 release by ECs in a concentration-dependent manner. Interestingly, the effect of acetyl-LDL did not reach a plateau in the range of concentrations tested (Figure 2B). Indeed at 400 µg/mL acetyl-LDL, PAI-1 antigen levels were greater than those detected with a protein concentration of 200 µg/mL. The increases in PAI-1 detected in the CM of LDL or acetyl-LDL–treated cells were not due to PAI-1 antigen detachment from EC matrix because no decrease in matrix-bound PAI-1 was observed in either condition. The effect of the lipoprotein fractions was specific for PAI-1 because they did not influence the levels of t-PA antigen, as determined in

<table>
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<tr>
<th>Conditions</th>
<th>PAI-1 antigen (ng/mL)</th>
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<tr>
<td>Control</td>
<td>20±4.2</td>
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<tr>
<td>Native LDL (100 µg/mL)</td>
<td>31±2.5*</td>
</tr>
<tr>
<td>Control</td>
<td>17±2.5</td>
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<tr>
<td>Acetyl-LDL (100 µg/mL)</td>
<td>29±2.1*</td>
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Endothelial cells (ECs) were incubated in medium 199 alone or in medium containing 100 µg/mL low density lipoproteins (LDLs). After 16–18 hours of incubation, the medium was removed and PAI-1 antigen determined. Values are the mean±SEM from separate experiments performed in quadruplicate on ECs isolated from different cords using individual lipoprotein preparations. Numbers of experiments are in parentheses.

*p<0.05, †p<0.01 by paired Student’s t test.

PAI-1, plasminogen activator inhibitor–1.

CM (t-PA antigen levels: 1.2–2.7 ng/mL and 1.5–2.8 ng/mL in the presence and absence of either lipoprotein). Similar to that described for PAI-1 antigen, total PAI-1 activity determined in the CM of ECs treated with either lipoprotein was increased. Interestingly, the effect of acetyl-LDLs on PAI-1 release in its active form was greater than that of LDLs (Table 2).

The potency of LDLs and of acetyl-LDLs at the 200 µg/mL concentration on the release of PAI-1 by ECs was similar to that of 10 µg/mL LPS (PAI-1 antigen levels in the CM of ECs incubated for 16–18 hours with 10 µg/mL LPS were 38.5±5.0 ng/mL). The presence of LPS in the lipoproteins used was tested for by the Limulus assay test. LPS levels were consistently below 0.1 ng/mg protein in all preparations, making it unlikely that the increases in PAI-1 antigen levels found in CM were attributable to LPS contamination.

To assess whether the stimulatory activity of LDL on PAI-1 synthesis and/or release in ECs was specific for this lipoprotein fraction, we performed experiments incubating the cells for 16–18 hours with medium containing different concentrations of HDL. These did not influence the release of PAI-1 in concentrations up to 200 µg/mL protein.

**Interactions of LDL with ECs and PAI-1 Release**

Because LDLs and acetyl-LDLs are known to interact with different receptors, both present on ECs, our data excluded the influence of lipoproteins on PAI-1 synthesis and release by ECs via mechanism(s) involving their interaction with specific receptors present on the cells, i.e., the classical apoprotein B/E receptor and the so-called “scavenger receptor.” The possibility, however, existed that LDLs and acetyl-LDLs operated via two different mechanisms. Therefore, a series of experiments were designed to assess the involvement, if any,


of the apoprotein B/E receptor in the stimulation of PAI-1 release induced by LDLs.

To obtain increased expression of apoprotein B/E receptors, ECs were incubated for 24 hours with medium containing 20% LPDS, and then after removal of LPDS, cells were challenged for 16–18 hours with medium containing different concentrations of LDLs. Under this experimental condition, PAI-1 levels were increased at the same extent as in cells conventionally treated (44±6.5% and 49.3±4.3% over control in the presence of 100 μg/mL LDLs in cells treated conventionally or challenged for 24 hours with LPDS, respectively). These data suggest that the stimulatory effect of LDLs on PAI-1 release by ECs is not further enhanced by increased expression of apoprotein B/E receptors on ECs.

Studies using defective LDL binding further proved the hypothesis that the apoprotein B/E receptor was not involved in the observed phenomenon. LDLs isolated from members of a family with a genetic disorder involved in the observed phenomenon. LDLs isolated from the plasma of four family members with familial defective apolipoprotein B-100. Values represent PAI-1 antigen levels of a representative experiment performed in triplicate. PAI-1 levels were determined in supernatants.

PAI-1, plasminogen activator inhibitor–1.

by ECs as that of LDLs isolated from the plasma of normal subjects (Table 3 and Figure 3).

Experiments with the C7 monoclonal antibody against the LDL receptors further ruled out the hypothesis of receptor involvement. In fact, preincubation of ECs for 2 hours with the C7 monoclonal antibody (15 μg/mL) before exposure of the cells to LDLs (100 μg/mL) failed to prevent the stimulatory effect of the lipoprotein on PAI-1 release (Figure 4).

Effect of Chemical Oxidation on the Capacity of LDL to Induce PAI-1 Release by ECs

To assess the influence of extensive oxidation of LDLs on the interaction of LDLs with ECs, experiments were carried out that involved incubation of EC monolayers for 16–18 hours with serum-free medium supplemented with LDLs extensively oxidized with cupric sulfate. Ox-LDL up to 100 μg/mL did not appreciably influence cell viability, as assessed by trypan blue exclusion by the cells and lactate dehydrogenase determination in supernatants, neither did they increase cell detachment, whereas in the presence of 200 μg/mL, ox-LDL cell mortality was recorded. Therefore, the effects of this lipoprotein fraction were evaluated in the range between 10 and 100 μg/mL. In supernatants of cells incubated with serum-free medium supplemented

![Figure 2. Line plots showing concentration-dependent effect of low density lipoproteins and acetylated low density lipoproteins (acetylated LDLs) on plasminogen activator inhibitor–1 (PAI-1) antigen release by endothelial cells (ECs). Conditioned medium of ECs, refed with fresh medium 199 containing native LDLs (panel A) and acetylated LDLs (panel B), was removed after 16–18-hour incubation. Each point represents the mean of at least six separate experiments performed in triplicate and expressed as percent increase over control incubated in the absence of lipoproteins.](http://atvb.ahajournals.org/)

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with 100 \( \mu \)g/mL ox-LDL, PAI-1 levels were lower than those detected in the absence of this lipoprotein fraction (13.6±4.2 and 6.4±2.6 ng/mL PAI-1 in the absence and presence of ox-LDL, \( n=8, \ p<0.05 \)). The influence of ox-LDL on PAI-1 release by ECs was shown to be concentration dependent up to 100 \( \mu \)g/mL (Figure 5).

**Figure 3.** Representative curves of plasminogen activator inhibitor-1 (PAI-1) antigen (ag) levels in conditioned medium of endothelial cells (ECs) incubated with different concentrations of LDLs isolated from normal subjects (—O—) and from a member of a family with defective LDLs (—*—). Each point represents the average of three determinations.

**Effect of LDL, Acetyl-LDL, and Ox-LDL on PCA Expression**

To assess whether native and chemically modified lipoproteins specifically influenced the synthesis and/or release of PAI-1 antigen or affected other biochemical markers of ECs, the expression of PCA was evaluated in disrupted cells under the same experimental conditions used for evaluating PAI-1. In the absence of lipoproteins, after 16–18 hours' incubation of cells with medium alone, no expression of PCA was detected (clotting times >400 seconds), indicating the absence of cell activation under our experimental condition. Significant shortenings of clotting times were detected in cells stimulated with 10 \( \mu \)g/mL LPS (clotting times of 135±29.9, mean±SEM). LDLs and acetyl-LDLs at the 100 \( \mu \)g/mL concentration failed to influence the clotting times of cell lysates. Concentration–response curves confirmed the lack of effects of LDLs and acetyl-LDLs on this cellular function (data not shown). In contrast to cells previously incubated with ox-LDL, a concentration-dependent shortening of clotting times was detected. In ECs incubated with 25 \( \mu \)g/mL ox-LDL, a significant reduction of clotting times was observed (percent reduction versus basal values: 47.8±7.0, mean±SEM, \( n=7, \ p<0.05 \)). The involvement of tissue factor was confirmed by the observation that clotting times determined using factor VII–deficient plasma instead of pooled normal plasma were consistently >400 seconds.

**Discussion**

Normal endothelium is primarily nonthrombogenic. It has the capacity, however, to synthesize clot-promoting factors such as tissue factor, von Willebrand factor, and PAI-1. Atherogenic lipoproteins present in flowing blood are in close contact with vascular ECs, and under pathologic conditions they influence the nonthrombogenic properties of this lining, leading to EC perturbation and ultimately endothelial injury. 32 In the present study the hypothesis that atherogenic lipoproteins affect the release of fibrinolytic proteins by unstimulated ECs was tested. The overall findings indicate that LDLs increase the release of PAI-1 by human umbilical vein cells. This effect is specific for PAI-1 because t-PA levels and tissue factor expression are not influenced by the presence of LDLs.

The percent increases in PAI-1 release exerted by LDLs are comparable to those already described in ECs incubated with very low density lipoproteins (VLDLs)33-34 using the same experimental conditions. It was previously...
proposed that the effects of VLDLs on PAI-1 release were dependent on an involvement of the apoprotein B/E receptor present on ECs. It was thus conceivable to hypothesize similar to that which occurs with VLDLs; the interaction of LDLs with LDL receptors on ECs triggers the synthesis and/or release of PAI-1 by the cells. LDLs, however, stimulate PAI-1 release by ECs by a mechanism(s) that is apparently not dependent on this type of receptor-mediated interaction, as demonstrated by the data we obtained with two different and independent approaches. First, the inhibition of the apoprotein B/E receptor on ECs with the C7 antibody failed to prevent the stimulatory effect of LDLs on PAI-1 release. Second, abnormal LDLs, i.e., those that bind poorly to the apoprotein B/E receptor on ECs, stimulated PAI-1 release as well as did LDLs. On the other hand, chemically modified, negatively charged LDLs, i.e., acetyl-LDLs, which rapidly bind to the scavenger receptor present on ECs, stimulate PAI-1 release in a fashion similar to that of LDLs. It is worth mentioning that other lipoprotein classes, such as HDLs, incubated with cells at up to 200 µg/mL protein concentration, do not modify the release of PAI-1 by ECs.

Extensive oxidation of LDLs leads to the fragmentation of apoprotein B and to the formation of oxidized sterols, which have been previously shown to impair the capacity of ECs to synthesize PAI-1 and von Willebrand factor and ultimately to induce cell toxicity. Under our experimental conditions, extensive oxidation of LDLs prevented and even reversed the effect of LDLs on PAI-1 release by ECs, in accordance with previous data by Stiko-Rahm et al. In addition, the behavior of ox-LDLs differed from that of LDLs and acetyl-LDLs in influencing another marker of EC activation considered in this study, i.e., the expression of procoagulant activity. Ox-LDL concentration dependently (up to 50 µg/mL protein) stimulated tissue factor expression by ECs. Increases in procoagulant activity occurred in the absence of cell toxicity. Indeed cell toxicity, as determined by trypan blue exclusion, cell detachment, and lactate dehydrogenase release, was evident in samples of ECs incubated for 16-18 hours with 200 µg/mL ox-LDLs.

The increases in PAI-1 levels found in the CM of ECs incubated with LDLs or acetyl-LDLs were not due to cell detachment of matrix-bound PAI-1. As shown in experiments in which cycloheximide-treated cells were used, increases were due rather to de novo protein synthesis. In addition, the overall protein synthesis, as assessed in [35S]methionine-labeled cells, was not modified by lipoprotein treatment. LDLs and acetyl-LDLs did not affect EC vitality, nor did they induce cell detachment, as assessed by counting attached cells after incubation with the different lipoproteins and by trypan blue exclusion in cells cultured in the presence of the different lipoproteins. The possibility of contamination of the tested lipoproteins with LPS was excluded on the basis of the lack of effects of both LDLs and acetyl-LDLs on the expression of PCA by ECs. In addition, measurements of LPS levels in the different lipoprotein preparations consistently showed LPS levels below 0.1 ng/mg protein.

The possibility that hardly detectable peroxidation products were responsible for the stimulation of PAI-1 was tested in experiments carried out with additions of BHT and EDTA to plasma with all of the different buffers. LDLs (four different preparations) isolated under these conditions stimulated PAI-1 release by ECs with the same potency as LDLs conventionally isolated (data not shown). It is worth mentioning, however, that there was a significant variability in the effects of LDL preparations from different donors on PAI-1 release by ECs. For experiments described in this study, 20 individual LDL preparations were used. At the concentration of 100 µg/mL protein, two preparations of LDLs failed to influence PAI-1 release, whereas the other 18 preparations increased PAI-1 release by ECs from 17% to 192% (median 49.5%). These data indicate that LDLs isolated from various subjects differ from each other in regard to their capacity to stimulate PAI-1 by ECs and that minor changes in the LDL composition may affect their potency in stimulating the synthesis of this antifibrinolytic protein.

Lp(a) was previously shown by Etingin et al to increase PAI-1 antigen synthesis and release by ECs. Therefore the possibility existed that our LDL preparations contained amounts of Lp(a) that sufficed to induce increases in the release of PAI-1. The presence of Lp(a) in our samples was excluded on the basis of the density cuts used for LDL isolation. In addition, specific measurements of Lp(a) in some LDL samples by a specific ELISA consistently showed the absence of this lipoprotein (data not shown).

The data discussed above indicate that LDLs, either unmodified or with a selective modification of the apoprotein B component as acetyl-LDL, stimulate the synthesis of PAI-1 antigen by ECs and that this capacity is lost following extensive oxidation of the lipoprotein. The effect of the two lipoprotein fractions is specific for PAI-1 antigen because t-PA levels remain unchanged and no procoagulant activity was expressed. Extensive oxidation of the lipoproteins suppresses their capacity to influence the antifibrinolytic properties of ECs, resulting in the formation of peroxides and/or small apoprotein B fragments, which in turn stimulate the expression of PCA by these cells. This latter finding is in accordance with the previously published observation that ox-LDLs, both chemically and cell modified, increase the expression of procoagulant activity by ECs with a mechanism partially dependent on binding of the lipoprotein to the scavenger receptor. It is interesting to note that minimally modified LDLs, which are only partially oxidized, are also capable of inducing the synthesis of PCA by ECs.

It has been recently published that ultraviolet light--ox-LDLs stimulate PAI-1 release by ECs. The different behavior of ox-LDLs in the two studies is easily explained by the different degree of oxidation reached. It is worth mentioning that cupric sulfate--ox-LDLs have already been described as failing to increase PAI-1 release by ECs. Concerning LDLs, both a slight stimulatory effect and no effect have been reported. These differences have been ascribed to the different experimental conditions used. These may include differences in cell passage, supplementation with growth factors, time of incubation with lipoproteins, and lipoprotein preparation.

In conclusion, the data described in this study strongly support the concept that cholesterol-rich lipoproteins such as LDLs or acetyl-LDLs affect PAI-1 release by ECs. This effect is comparable to that exerted by native VLDLs previously described by us and by another group.
Stiko-Rahm et al. The studies described in this article have been carried out using amounts of LDL that are far below the quantities present in the plasma of familial hypercholesterolemic subjects, and they suggest that the release of this antifibrinolytic protein by vascular endothelium is under physiological control of LDLs and possibly VLDLs. It may therefore be hypothesized that, in conditions of elevated levels of LDLs, VLDLs, or both, PAI-1 release by ECs is further increased, with concomitant alterations of the fibrinolytic balance. This hypothesis, on the other hand, is supported by clinical findings that suggest a linkage between alterations in the fibrinolytic balance and hyperlipoproteinemias.

In addition, local increases in the release of PAI-1 may account for reduced fibrin removal at the level of specific vascular districts. Indeed, our study demonstrates that both LDLs and acetyl-LDLs increase the release of PAI-1 in its active form, with a more pronounced effect from acetyl-LDLs.

LDLs stimulate the synthesis and/or release of PAI-1 with a mechanism that is not dependent on the interaction with the classical LDL receptor, indicating that other component(s) of the lipoprotein is responsible for the observed phenomenon. The question of which component of the lipoprotein, besides the LDL receptor, mediates this effect was not addressed in this study and deserves further investigation. It has been shown that LDLs induce an increase in the concentrations of inositol trisphosphate, diacylglycerol, and intracellular free Ca²⁺ in rat vascular smooth muscle cells, and another study on human skin fibroblasts showed that LDLs had similar activities in LDL receptor-negative fibroblasts. In addition, LDLs in contact with platelets induce the accumulation of ³H-labeled diacylglycerols and the phosphorylation of a 47-kd protein, a mechanism common to specific platelet agonists. Finally, LDLs and acetyl-LDLs have been shown to have additive effects in stimulating leukotriene B₄ production by human monocyte-derived macrophages. One can therefore hypothesize that LDLs stimulate PAI-1 synthesis and release by ECs by mechanisms that do not involve the specific LDL receptor but probably are dependent on intracellular changes in the levels of either inositol phosphates, Ca²⁺, or both. Studies directed toward the elucidation of such mechanisms are still required.

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