Lipoproteins Inhibit the Secretion of Tissue Plasminogen Activator From Human Endothelial Cells

Eugene G. Levin, Lindsey A. Miles, Gunther M. Fless, Angelo M. Scanu, Patricia Baynham, Linda K. Curtiss, Edward F. Plow

Abstract We studied the effect of lipoprotein(a) [Lp(a)], low-density lipoprotein (LDL), and high-density lipoprotein (HDL) on tissue plasminogen activator (TPA) secretion from human endothelial cells. At 1 μmol/L, Lp(a) inhibited constitutive TPA secretion by 50% and phorbol myristate acetate- and histamine-enhanced TPA secretion by 40%. LDL and HDL also depressed TPA secretion by 45% and 35% (constitutive) and 40% to 60% (stimulated). TPA mRNA levels were also examined and found to change in parallel with antigen secretion. In contrast to TPA, plasminogen activator inhibitor type-1 secretion and mRNA levels were not affected by any of the three lipoproteins. These results suggest that the interaction of lipoproteins with certain cell-surface binding sites may interfere with the proper production and/or secretion of TPA.

Key Words • Lp(a) • tissue plasminogen activator • endothelial cells • plasminogen activator inhibitor type-1

At high levels, lipoprotein(a) [Lp(a)] is a major risk factor for premature development of atherosclerosis, myocardial infarction, and the severity of coronary artery disease.1-4 Low-density lipoprotein (LDL) is also atherogenic, but Lp(a) and LDL are independent risk factors for these diseases, suggesting a difference in their pathogenic mechanisms. Lp(a) is similar to LDL in composition and structural organization but contains an additional apoprotein, apo(a).5 Apo(a) is very similar in structure to plasminogen.6,7 It may contain 36 repeats of the fourth kringle of plasminogen as well as a single kringle 5 and a protease-like region.8 However, Lp(a) is not susceptible to cleavage by plasminogen activators. This similarity in structure between Lp(a) and plasminogen has led to the hypothesis that Lp(a) can interfere with the functions of plasminogen.4 In vitro studies of such effects have shown that Lp(a) inhibits plasminogen activation by streptokinase, suppresses the fibrin and cell surface–dependent enhancement of plasminogen activation by tissue plasminogen activator (TPA), and suppresses plasminogen binding to U937 monocytoid and endothelial cells.9-18 The LDL particle, which lacks apo(a), does not compete for plasminogen binding sites.9,17 Thus, Lp(a) interferes with plasminogen activation by inhibiting several molecular interactions of plasminogen.

In addition to the physical interference of Lp(a) with plasminogen binding to cells and other proteins, a prothrombotic state can be promoted by interference with the normal production and/or secretion of the fibrinolytic components associated with endothelial cells, ie, TPA, the plasminogen activator primarily responsible for blood clot lysis,19 and plasminogen activator inhibitor-1 (PAI-1). For example, inhibition of TPA release or elevation of PAI-1 production would create a prothrombotic state in the endothelial cell environment. It has been shown previously that addition of Lp(a) to human endothelial cells in culture stimulates PAI-1 production up to twofold over a 16-hour period.20 In the present study, we have examined the effects of Lp(a) on endothelial cell expression of the fibrinolytic components PAI-1 and TPA. We report here that the addition of Lp(a) to monolayers of human umbilical endothelial cells suppresses TPA secretion while having a minimal effect on PAI-1 secretion. However, similar results were also observed with LDL and high-density lipoprotein (HDL), suggesting a general mechanism by which lipoproteins might promote a prothrombotic state through the downregulation of TPA secretion without significantly affecting PAI-1 expression.

Methods

Materials

Phorbol myristate acetate (PMA) was from Calbiochem. Monoclonal antibodies to PAI-1 (No. 1105) and TPA were from Monoclonal, respectively.

Cell Culture

All tissue culture reagents were purchased from sources previously described.21 Endothelial cells were isolated from human umbilical vein cord veins22 and were cultured into 75-cm² tissue culture flasks coated with 20 mg/mL calf skin gelatin. Cells were grown to confluence in RPMI 1640 containing 10% fetal calf serum, 200 U/mL penicillin, 200 μg/mL streptomycin, 10 μg/mL endothelial cell growth factor

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From the Department of Molecular and Experimental Medicine (E.G.L.), Committee on Vascular Biology (E.G.L., L.A.M., P.B.), and Department of Immunology (L.K.C.), The Scripps Research Institute, La Jolla, Calif; the Department of Medicine (G.M.F., A.M.S.) and Department of Biochemistry and Molecular Biology (A.M.S.), University of Chicago (I.I.); and the Center for Thrombosis and Vascular Biology (E.F.P.), The Cleveland Clinic (Ohio).

Correspondence to Dr Eugene G. Levin, Department of Molecular and Experimental Medicine, SBR13, The Scripps Research Institute, 10666 N Torrey Pines Rd, La Jolla, CA 92037.
was stopped by the addition of 100 μL of IN HCl. The plates were read at 450 nm. For PAI-1 measurements, assay samples was added. The substrate mixture was prepared by diluting conjugate, diluted 1:50 into PBS/0.1% BSA, was added and incubated for 2 hours at 22°C. After washing, the wells were incubated with a 1:2500 dilution of horseradish peroxidase–conjugated rabbit anti-
mouse IgG for 2 hours and then washed, and 1 μg/mL of 2.2'-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid in 0.0015% H2O2 was added. Plates were read at 405 nm after 45 minutes of incubation. Standard curves for both TPA and PAI-1 assays were determined by log-logit transformation and linear regression analysis of logit B (absorbance) versus log TPA or PAI-1 concentration. Values in the range of 0.2 to 12.5 ng/mL TPA and 1.6 to 25 ng/mL PAI-1 gave linear dose-response curves.

mRNA Quantitation

RNA was extracted by the method of Chomczynski and Sacchi, and TPA mRNA levels were measured by slot blot analysis as previously described. A full-length cDNA clone encoding the mature TPA protein plus 200 bp of the 3'-untranslated region and a PAI-1 cDNA clone containing the complete coding region, 80 bp of the 5'-untranslated region, and 140 bp of the 3'-untranslated region were used as probes for hybridization. An actin probe was used as a control (furnished by Dr L. Kedes, Stanford University).

Chromium-51 Labeling of Cells

Cells were labeled with 250 μCi/mL 51Cr (Amersham International) for 4 to 6 hours in medium containing 10% fetal calf serum. After the labeling period, cultures were washed twice with RPMI 1640 and then treated with medium containing 5% NuSerum and the indicated agonists in the presence or absence of lipoproteins for 16 hours. Medium was collected and cells were extracted with 0.5% Triton X-100. Both medium and cell extract were counted, and the chromium remaining was calculated as the percentage of cells incubated with agonist in the absence of lipoprotein.

Results

The effect of three different classes of lipoproteins, Lp(a), LDL, and HDL, on constitutive TPA secretion (12.2±4.3 ng/mL in 16 hours; range, 6.5 to 22.0 ng, n=17) was examined by addition of increasing concentrations of each to confluent monolayers of endothelial cells. Lp(a) inhibited TPA secretion in a dose-dependent manner, with 1 μmol/L Lp(a) reducing TPA levels to 49.1±8.1% of control after 16 hours (Fig 1, top). Lp(a) was also effective against PMA-stimulated (100 nmol/L; Fig 1, middle) and histamine-stimulated (10 μmol/L; Fig 1, bottom) TPA secretion. The level of TPA measured in the culture medium after 16 hours, a time when TPA levels reached their maximum levels in both cases, was reduced to 51.1±4.2% and 57±5.6%, respectively.

In contrast to the inhibitory effect of Lp(a) on plasminogen binding to cell surfaces, other lipoproteins were also effective at blocking both constitutive and stimulated TPA secretion. LDL and HDL inhibited constitutive release to 55.2±7.3% and 64.9±5% of control, respectively (Fig 1, top), histamine-stimulated TPA secretion to 49.9±7.9% and 67.9±1.5% (Fig 1, bottom), and PMA-stimulated secretion to 30.6±4.8% and 59.8±4.2% (Fig 1, middle), respectively. Addition of plasminogen (1 μmol/L) to the cells under identical conditions had no effect on TPA secretion (data not shown). The effect of LDL on various concentrations of histamine and PMA was also tested. At concentrations ranging from 0.1 to 20 μg/mL histamine, 1 μmol/L LDL inhibited the stimulated rate of TPA secretion by 30%
to 35% at each concentration. At concentrations of $10^{-10}$ to $10^{-7}$ mol/L PMA, 0.5 μmol/L LDL inhibited secretion by 50% at each point.

PAI-1 antigen levels were also examined in replicate samples (Fig 2). Despite previous reports that Lp(a) stimulates PAI-1 secretion from endothelial cells, we detected no significant change in PAI-1 levels after 16 hours of incubation either with this lipoprotein, LDL, or HDL (96.6±16.0%, 98.5±5.9%, and 101.8±8.8%, respectively) (Fig 2).

To ensure that the decline in TPA secretion and the absence of an effect on PAI-1 secretion were not due to loss of cell viability, we performed $^{51}$Cr release assays in cultures treated with each agonist in the presence or absence of lipoproteins (Table). When compared with the level of $^{51}$Cr associated with cells treated with agonist alone, the addition of each lipoprotein for 16 hours did not decrease $^{51}$Cr content by more than 10%. Thus, the effects observed in the presence of lipoproteins were not due to loss of cell viability.

To examine whether TPA mRNA levels are affected in the same manner as antigen levels, we performed slot blot analysis on cultures treated with vehicle, 1 μmol/L LDL, 100 nmol/L PMA/100 μmol/L forskolin, and PMA/forskolin and LDL. Forskolin, which elevates cyclic AMP levels through the activation of adenylate cyclase, potentiates the PMA effect fivefold, making the quantitative measurement of TPA mRNA easier. Cyclic AMP does not, however, change any of the characteristics of the TPA response to PMA. Slot blot analysis of TPA mRNA showed a 67% decline in steady-state levels after 8 hours of LDL treatment (n = 2) (Fig 3). In the presence of PMA/forskolin, TPA mRNA increased 3.6-fold within the same time period but only 2.3 times in the presence of LDL. Whether the same effect on TPA mRNA is observed with HDL or Lp(a) is unknown. PAI-1 mRNA levels were unaffected by 1 μmol/L LDL (data not shown).

**Discussion**

We have demonstrated that the treatment of human umbilical vein endothelial cells with either Lp(a), LDL, or HDL results in a dose-dependent depression in both constitutive and stimulated TPA secretion. This effect on TPA secretion occurs with no significant change in the level of PAI-1 antigen over the same period of time.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Counts per Minute, x10$^{-3}$</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>458±16</td>
<td>100</td>
</tr>
<tr>
<td>Buffer</td>
<td>412±15</td>
<td>90</td>
</tr>
<tr>
<td>LDL</td>
<td>428±3</td>
<td>93</td>
</tr>
<tr>
<td>HDL</td>
<td>427±15</td>
<td>93</td>
</tr>
<tr>
<td>Histamine</td>
<td>439±11</td>
<td>100</td>
</tr>
<tr>
<td>Histamine+LDL</td>
<td>390±8</td>
<td>89</td>
</tr>
<tr>
<td>Histamine+HDL</td>
<td>409±2</td>
<td>93</td>
</tr>
<tr>
<td>PMA</td>
<td>405±7</td>
<td>100</td>
</tr>
<tr>
<td>PMA+LDL</td>
<td>367±8</td>
<td>91</td>
</tr>
<tr>
<td>PMA+HDL</td>
<td>378±13</td>
<td>93</td>
</tr>
</tbody>
</table>

LDL indicates low-density lipoprotein; HDL, high-density lipoprotein; and PMA, phorbol myristate acetate.
Actin tPA

Control
PMA + fors
PMA + fors + LDL

Fig. 3. Blots show effect of low-density lipoprotein (LDL) on tissue plasminogen activator (TPA) mRNA levels. Cultures were treated with or without 100 μmol/L phorbol myristate acetate (PMA) and 50 μmol/L forskolin (fors) for 8 hours in the presence or absence of 1 μmol/L LDL. Cells were extracted, and RNA was isolated as described in "Methods." TPA mRNA was measured by slot blot analysis; the values presented in the text were derived from comparing the counts per minute in the bands representing the LDL-treated samples with that of the respective control. Slots hybridized with a cDNA probe for actin are shown to demonstrate comparable loading of RNA.

Thus, abnormally high levels of Lp(a) and other lipoproteins may promote a prothrombotic state by manipulating the fibrinolytic capacity within the endothelial cell environment.

Our results contrast with the previous report of Etingin et al.20 who found that Lp(a) enhanced PAI-1 antigen secretion from human endothelial cells in culture by twofold while having no effect on TPA production. It is not clear why this discrepancy exists, although the culture conditions under which these studies were performed were quite different. Etingin et al performed experiments in serum-free medium in contrast to the 1.25% serum used in the present studies. We have determined previously that the level of constitutive and stimulated TPA production and secretion in human umbilical endothelial cells is dependent on the presence of serum and that, in its absence, TPA secretion is unresponsive to modulating compounds.21 Another potential source for the differences in results may be related to the state of lipoprotein oxidation. For example, Latron et al22 and Kugiyama et al23 reported that oxidized but not nonoxidized LDL affects PAI-1 synthesis by endothelial cells. Our findings also are supported to some extent by in vivo observations.29,30 In one study, patients with coronary artery disease and levels of Lp(a) 2.5 to 3.5 times higher than control subjects showed a decreased ability to release TPA in response to venous occlusion (50%), although basal levels of TPA were increased 76%. PAI-1 levels increased an average of only 25%. In the second study, a negative correlation between Lp(a) levels and PAI-1 levels was observed.

The depression of TPA secretion from endothelial cells can also be promoted by the cytokine interleukin-1.31 Prolonged treatment of the cells with interleukin-1 results in a 50% decrease in the constitutive release of TPA, whereas interleukin-1 stimulates PAI-1 expression. Addition of interleukin-1 to cells stimulated with PMA also has a depressive effect (E.G. Levin, unpublished observations). In addition, we have demonstrated recently that the disruption of microtubule organization during treatment of cells with phorbol esters or histamine depresses enhanced TPA secretion by approximately 50%.32 Thus, our observation that TPA secretion can be inhibited by treatment of endothelial cells with lipoproteins is not unusual.

The mechanism by which the three lipoproteins inhibit TPA secretion is not clear, eg, whether it occurs through a protein or lipid moiety or whether it occurs through a general perturbation of the endothelial cell membrane or through a specific receptor or receptors. The lack of specificity of the lipoprotein effect shown in Fig 1 suggests that it is the lipid components of the lipoproteins that are responsible for the biologic activity, because the various lipoproteins do not contain common apoproteins but do contain various common lipid components, although at different relative proportions. A number of the lipid constituents are viable candidates, including the lysolecithins, oxygenated steroids, and free fatty acids. The fact that all three lipoproteins have similar effects also suggests that the site of interaction is similar in each case, a suggestion consistent with recent observations that LDL and HDL can both inhibit Lp(a) binding to endothelial cells and that gangliosides, which inhibit plasminogen binding to endothelial cells, also inhibit both Lp(a) and LDL binding.33 Although HDL and LDL generally exert counterbalancing effects, competition between HDL and LDL for cellular binding sites on endothelial cells34 and other cells35 has been noted. Tabas and Tall36 suggested that the association of HDL with endothelial cells may be dependent on lipid interactions that could be shared by all lipoprotein classes. Lipid transfer mechanisms28 may also be involved and could be shared among the various lipoproteins. The interaction of lipoproteins with certain shared cell surface binding sites such as lipids may interfere with the proper production and/or secretion of TPA.

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