Secretion of Plasminogen Activator Inhibitor-1 from Cultured Human Umbilical Vein Endothelial Cells Is Induced by Very Low Density Lipoprotein

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Clinical studies have demonstrated an impaired fibrinolytic function in patients with angiographically ascertained coronary artery disease or previous myocardial infarction. This decreased fibrinolytic function is to a major extent explained by the presence of high plasma levels of plasminogen activator inhibitor-1 (PAI-1) and is most common in patients with hyperlipoproteinemias type IIB and IV. To further investigate the association between hypertriglyceridemia and elevated plasma levels of PAI-1, cultured human umbilical vein endothelial cells were exposed to purified lipoproteins isolated from normo- and hypertriglyceridemic (NTG and HTG) individuals. We found that very low density lipoprotein (VLDL) from both NTG and HTG subjects stimulated the secretion of PAI-1 from endothelial cells in a dose-dependent manner. HTG-VLDL at a concentration of 100 μg/ml gave rise to a 73% increase in PAI-1 secretion as compared to control cultures, whereas NTG-VLDL only gave rise to a 30% increase (p<0.05), indicating that HTG-VLDL is a more potent stimulus to PAI-1 secretion than is NTG-VLDL. Experiments in which endothelial cells were exposed to VLDL subfractions indicated that large VLDL particles, in particular, induce PAI-1 release. Binding experiments demonstrated a specific cellular binding of both NTG- and HTG-VLDL to the cells, but HTG-VLDL bound about four times more effectively than NTG-VLDL. Exposure of the endothelial cells to an LDL receptor antibody was found to block 75% (p<0.005) of the VLDL-induced secretion of PAI-1 from the cells. Taken together, our experiments indicate that VLDL stimulates the secretion of PAI-1 from human umbilical vein endothelial cells and that the VLDL-induced secretion of PAI-1 is dependent on the binding of VLDL particles to B,E receptors on the cells. (Arteriosclerosis 10:1067–1073, November/December 1990)

The fibrinolytic system is a proteolytic enzyme system with many physiological functions, of which degradation of fibrin deposits in blood vessels is the best known and, possibly, the most important. Several of the well-established risk factors for coronary heart disease, such as smoking, hyperlipoproteinemia, hyperinsulinemia, diabetes, and obesity, are associated with decreased fibrinolytic activity. However, most of the previous epidemiological and clinical studies have been done with methods that simultaneously reflected the function of both fibrinolytic activators and inhibitors. In the past few years, new sensitive and specific methods have been developed to determine the individual components of the fibrinolytic enzyme system. Data from our group have indicated that reduced fibrinolytic capacity, mainly due to elevated plasma levels of plasminogen activator inhibitor-1 (PAI-1), may have pathogenic importance in myocardial infarction, particularly in patients with hypertriglyceridemia, and in deep-vein thrombosis. PAI-1 was discovered independently a few years ago in plasma by several groups. PAI-1 has a molecular weight of about 50 000, and its amino acid sequence has been predicted from cDNA studies. Functionally active PAI-1, in contrast to inactive PAI-1, is bound to a discrete plasma protein, which was recently identified as vitronectin.

The positive and fairly strong relationship between serum triglycerides and PAI-1 levels in plasma is of particular interest, since it raises the possibility that hypertriglyceridemia is connected with a predisposition to thrombosis through a coexisting increase in PAI-1 concentration. It has been proposed that plasma insulin is the major physiological regulator of PAI-1 activity in plasma and is secondary to that of fibrinolytic activity. The mechanism by which insulin influences the release of PAI-1 may be either direct or indirect through an effect of insulin on plasma triglyceride concentration.

It is generally assumed that fibrinolysis in vivo in humans is triggered by tissue-type plasminogen activator (t-PA), which is released from endothelial cells, whereas the origin of the biologically active PAI-1 remains unclear. Like t-PA, PAI-1 is produced in relatively large amounts by...
cultured human endothelial cells. In addition, PAI-1 is synthesized by hepatocytes in culture and is contained in the α-granules of blood platelets. However, the mechanisms regulating PAI-1 secretion from various cells to plasma have been poorly established. It has been shown that endothelial cells are able to increase their PAI-1 production upon stimulation with lipopolysaccharide, interleukin-1, or tumor necrosis factor. Furthermore, Alessi et al. recently showed that insulin induces a dose-dependent increase of the PAI-1 secretion by HepG2 cells in culture, whereas no effect of insulin in this respect was obtained on endothelial cells from human umbilical vein. Thus, insulin stimulation of hepatic PAI-1 synthesis may be one mechanism determining PAI-1 levels in human plasma. However, no published experimental studies on the influence of triglyceride-rich lipoproteins have explained the consistent and fairly strong correlations between triglyceride levels and PAI-1 concentration.

Against this background, we considered it of interest to investigate the relationships between very low density lipoprotein (VLDL) and low density lipoprotein (LDL) and their subclasses and low density lipoproteins and Their Subclasses and Low Density Lipoproteins

Methods

Materials

Plastic culture dishes were from Costar (Cambridge, MA); sera and media, from Gibco BRL (Grand Island, NY); and 125I, 3H-leucine, and the C7 monoclonal antibody against the LDL receptor from Amersham (Buckinghamshire, UK). Collagenase was purchased from Worthington Biochemical Corporation (Freehold, NJ) and thrombin, from Sigma Chemical (St. Louis, MO). The COATEST Limulus assay purchased from Kabi Vitrum (Stockholm, Sweden) showed no detectable endotoxin levels in the bovine serum albumin as the protein standard. All samples, including the standards, were extracted with chloroform after color development to remove any turbidity.

Oral Fat Loading and Separation of Postprandial Lipoproteins

Postprandial lipoproteins were separated as described above from two healthy 35-year-old normolipidemic men 4 hours after they had ingested 280 ml of cream. The fat load was consumed over a period of 5 minutes, and the two participants were deprived of any source of energy for the next 4 hours.

Cell Culture

Human umbilical cords were obtained at normal deliveries. The umbilical vein was cannulated and perfused with 50 to 100 ml of phosphate-buffered saline (PBS) to remove all traces of blood. The vein was then filled with 10 to 20 ml of 0.1% collagenase dissolved in PBS.
with an equal volume of Medium 199. The cells were spun down to a pellet by centrifugation at 200 g for 4 minutes, were washed twice in Medium 199, and were seeded out in 12-well plates in Medium 199 supplemented with 20% fetal calf serum (FCS) and 50 μg/ml of gentamycin. The medium was changed three times a week. The cells usually reached confluence within 4 to 5 days.

**Determination of Plasminogen Activator Inhibitor Release**

Confluent primary cultures of human umbilical vein endothelial cells were washed twice in Medium 199 and were incubated for 48 hours at 37°C in 1.0 ml of Medium 199/0.1% bovine serum albumin (BSA) with or without additions. At the end of the incubation period, the conditioned medium was removed, was centrifuged at 15 000 g for 5 minutes to remove cellular debris, and was kept at -20°C until analyzed. The amount of PAI-1 in the medium was analyzed with a double antibody radioimmunoassay system as described. Purified "latent" PAI-1 from HT 1080 cells was used as the standard and, after labeling with 125I, was used as a tracer. To analyze the effect of a monoclonal antibody against the LDL receptor on VLDL-induced release of PAI-1, confluent cultures of endothelial cells were incubated with 0.9 ml of Medium 199 containing 30 μg of the C7 LDL receptor antibody or equal amounts of human polyclonal IgG for 2 hours at 37°C. One hundred microliters of HTG-VLDL (1 mg/ml) was then added to the culture medium, and the release of PAI-1 during a 48-hour period was determined as described above.

**Determination of Protein Synthesis**

Confluent endothelial cells were incubated for 48 hours in Medium 199 containing 10 μCi/ml of 3H-leucine with or without the addition of 100 μg/ml of HTG-VLDL. The cells were then dissolved by incubation in 0.05% Triton X-100 and subsequent sonication. One-tenth milliliter of human polyclonal IgG for 2 hours at 37°C. One hundred microliters of HTG-VLDL (1 mg/ml) was then added to the culture medium, and the release of PAI-1 during a 48-hour period was determined as described above.

**Binding of Very Low Density Lipoprotein to Endothelial Cells**

Confluent primary cultures of endothelial cells grown in 12-well plates were incubated in lipoprotein-deficient serum for 48 hours. They were then added to the lysate, and the protein content of the sample was precipitated by the addition of 0.5 ml of 20% trichloroacetic acid (TCA). The precipitated protein was washed twice in TCA, once in ethanol, and was then dissolved in 0.2 ml of 1 M NaOH. The radioactivity was determined in a beta counter.

**Determination of Inhibitor Release**

Confluent primary cultures of endothelial cells were incubated for 48 hours at 37°C in 1 ml of Medium 199/0.1% BSA and were kept at 4°C for 1 hour to arrest endocytosis. Increasing concentrations of 125I-labeled NTG-VLDL or HTG-VLDL, with or without 10 times excess of unlabeled lipoprotein, were then added to the cultures.lodination of the lipoproteins was performed by a modification of the iodine monochloride method of McFarlane. Cultures exposed to lipoproteins were kept on a rocking plate for 2 hours at 4°C. The cells were subsequently rinsed five times with 0.15 M NaCl/50 mM trichloride/0.2% BSA, pH 7.4, lysed by incubation in 0.1 M NaOH for 15 minutes at room temperature. The radioactivity was determined in an LKB gamma counter.

**Data Analysis**

The amount of PAI-1 secreted from the endothelial cells or the amount of VLDL bound to the cells in different experiments was expressed as the mean values and the standard errors of the means (SE). Comparisons between experiments were made by Student’s two-tailed unpaired t test.

**Results**

The amount of PAI-1 secreted from the endothelial cells was determined by measuring the quantity of PAI-1 protein released into the serum-free culture medium during a 48-hour period. The amount of PAI-1 released by control cells varied between 150 and 400 ng/ml. Pretreatment of the cells with 10 μg/ml of the protein synthesis inhibitor cycloheximide for 60 minutes at 37°C before the start of the 48-hour incubation period resulted in a 91.0±0.6% decrease in the amount of PAI-1 secreted. This finding indicates that the PAI-1 present in the conditioned medium represented protein synthesized by the cells during the incubation period or protein released from intracellular pools in response to intracellular signals involving synthesis of other protein/s, rather than PAI-1 dissociated from the tissue matrix.

Exposure of endothelial cells to VLDL caused a dose-dependent increase in the secretion of PAI-1 from the
The effect of very low density lipoprotein (VLDL) subfractions on the release of plasminogen activator inhibitor-1 (PAI-1) from endothelial cells.

Figure 2. The effect of very low density lipoprotein (VLDL) subfractions on the release of plasminogen activator inhibitor-1 (PAI-1) from endothelial cells. VLDL particles of the indicated flotation rates were isolated by cumulative flotation in density gradients. Confluent endothelial cells were then incubated with the isolated VLDL subfractions at a protein concentration of 30 μg/ml for 48 hours, and the amount of PAI-1 in the culture medium was then determined. Each value represents the mean±SE of six determinations with lipoproteins from two different hypertriglyceridemic subjects.

Figure 3. The effect of hirudine on very low density lipoprotein (VLDL) and thrombin-induced secretion of plasminogen activator inhibitor-1 (PAI-1). Confluent endothelial cells were grown in the presence of 100 μg/ml of hypertriglyceridemic (HTG)-VLDL or 1 U/ml thrombin with or without the addition of 2 U/ml of the specific thrombin antagonist hirudine for 48 hours. The amount of PAI-1 in the culture medium was then determined. Each value represents the mean±SE of six determinations with lipoproteins from two different hypertriglyceridemic subjects.

Incubation of the VLDL protein concentration of 100 μg/ml, stimulation with HTG-VLDL resulted in a 100.9%±63.0% greater increase in PAI-1 secretion than NTG-VLDL (p<0.05). Incubation of the cells with lipoproteins at this concentration did not affect cell viability as judged by the morphological appearance and the amount of cell protein remaining in the wells at the end of the incubation period. To further investigate the effect of HTG-VLDL on the total rate of protein synthesis, the incorporation of 3H-leucine was analyzed. HTG-VLDL was found to increase the overall rate of protein synthesis by 31.4%±14.4%.

Incubation of the cells with NTG-LDL gave rise to a small increase in the secretion of PAI-1 (12.7%±6.8%, p<0.05), whereas HTG-LDL was without effect. Oxidation of NTG-LDL by exposure of the lipoprotein to 5 μmol CuSO4 for 24 hours (a treatment known to result in degradation of apolipoprotein B and loss of the ability of the lipoprotein particle to bind to the B,E receptor) markedly diminished the PAI-1 releasing effect.

To investigate if the PAI-1 releasing activity was dependent on the size of the particles, subfractionation of VLDL was performed. VLDL of S, 100 to 400, S, 60 to 100, and S, 20 to 60 isolated from two HTG individuals were added to the cultures at a concentration of 50 μg/ml (Figure 2). This experiment clearly indicated that, in particular, large VLDL stimulate PAI-1 release. Despite the fact that considerably fewer VLDL of S, 100 to 400 were added to the endothelial cells (since the amount of soluble apolipoproteins is higher in large VLDL species), the VLDL of S, 100 to 400 tended to induce secretion of more PAI-1 than the subfractions containing smaller VLDL particles. In contrast, large postprandial lipoproteins, presumably chylomicrons and chylomicron remnants obtained after an oral fat load in two healthy normolipidemic subjects, did not induce a greater increase in the release of PAI-1 from the cells compared with VLDL of S, 60 to 100 or VLDL of S, 20 to 60 obtained in the fasting state (data not shown).

Thrombin at a concentration of one unit per milliliter caused a 101.0%±11.0% (p<0.005) increase in the amount of PAI-1 secreted from the cells (Figure 3). Addition of the specific thrombin inhibitor hirudine (2 U/ml) blocked more than 90% of the thrombin-induced PAI-1 release (p<0.005). Contrarily, there was no effect of hirudine on VLDL-induced secretion of PAI-1, indicating that the PAI-1 releasing activity of VLDL is not due to the presence of thrombin in the lipoprotein preparation.

To determine if VLDL-mediated release of PAI-1 involved binding of the lipoprotein to the B,E receptor, endothelial cells were pre-incubated with the C7 monoclonal antibody (30 μg/ml) against the LDL receptor for 2 hours and then exposed to HTG-VLDL (100 μg/ml) and the LDL receptor antibody (30 μg/ml) for another 48 hours. Exposure of the endothelial cells to the LDL receptor antibody was found to block 75.0%±27.0% (p<0.005) of the VLDL-induced secretion of PAI-1 from the cells (Figure 4). Exposure of the cells to human polyclonal IgG at the same concentration did not influence the VLDL-induced release of PAI-1 from the cells.

Binding experiments demonstrated a specific cellular binding (binding of 125I-labeled NTG- and HTG-VLDL that could be displaced by the addition of 10 times the amount of the respective unlabeled lipoprotein) of both NTG- and HTG-VLDL, but HTG-VLDL bound about four times more than NTG-VLDL (Figure 5). Addition of 10 times the concentration of unlabeled HTG-VLDL removed 64.8%±9.5% (p<0.005) of the binding of 10 μg/ml 125I-HTG-VLDL. Pre-incubation of the endothelial cells with
We then incubated with the indicated concentrations of HTG-VLDL (••) or NTG-VLDL (○○) with or without a 10-fold excess of unlabeled lipoprotein for 2 hours at 4°C, then rinsed and lysed in NaOH. The specific binding of the VLDL particles was calculated by subtracting the binding in the presence of an excess of unlabeled lipoprotein from the binding obtained without the addition of unlabeled VLDL. Each value represents the mean of triplicate cultures.

![Graph showing binding of VLDL](image)

**Figure 4.** The effect of a low density lipoprotein (LDL) receptor antibody on very low density lipoprotein (VLDL)-induced secretion of plasminogen activator inhibitor-1 (PAI-1). Confluent endothelial cells were incubated with 30 μg of the C7 monoclonal antibody against the LDL receptor for 2 hours in 0.9 ml of Medium 199. One hundred microliters of HTG-VLDL (1 mg/ml, giving a final concentration in the culture medium of 100 μg/ml) was subsequently added, and the amount of PAI-1 in the culture medium was determined after an additional 24 hours. Each value represents the mean of triplicate cultures.

Our experiments demonstrate that VLDL stimulates the secretion of PAI-1 from human umbilical vein endothelial cells. At identical protein concentrations, HTG-VLDL was a more potent stimulus to PAI-1 release than was NTG-VLDL. The endothelial cells were also found to bind HTG-VLDL more effectively than NTG-VLDL, suggesting a direct relation between the number of VLDL particles bound and the amount of PAI-1 secreted from the cells. Pre-incubation of the cells with a monoclonal antibody against the B,E receptor decreased both the binding of VLDL and the amount of PAI-1 released in response to stimulation with VLDL. These findings indicate that VLDL-induced secretion of PAI-1 from endothelial cells is dependent on the binding of VLDL particles to B,E receptors on the cells. The persistence of some VLDL binding and PAI-1 release in response to VLDL in the LDL receptor antibody experiments can be explained by recycling of LDL receptors during the 48-hour incubation, which would lead to a depletion of the receptor antibody with time. Furthermore, VLDL at a concentration of 100 μg/ml corresponds to a particle number well above the saturation point of the LDL receptor, which means that a complete shutdown of receptor activity should not be expected in antibody experiments. The extracellular matrix of human umbilical vein endothelial cells has previously been shown to contain active PAI-1. The almost complete disappearance of PAI-1 secretion from the cells obtained by pretreatment with a protein synthesis inhibitor before incubation with VLDL distinctly argues against any significant release from tissue matrix.

The observation that endothelial cells bind HTG-VLDL more effectively than NTG-VLDL extends the earlier findings of Gianturco et al. who reported that large HTG-VLDL particles, but not large NTG-VLDL particles, bind to the B,E receptor on human fibroblasts and cultured bovine aortic endothelial cells. The concept of involvement of the B,E receptor in the induction of PAI-1 secretion was further supported by the finding that exposure of the cells to normal LDL also resulted in a small increase in the amount of PAI-1 released from the cells. Furthermore, HTG-LDL and oxidized LDL, which are known to bind less effectively to the B,E receptor, failed to stimulate the secretion of PAI-1 from the cells. In fact, HTG-LDL in high concentration appeared to be toxic to the cells. The question of which specific lipoprotein components are responsible for the differing effects on PAI-1 secretion was not directly addressed, since neither the apolipoprotein nor the lipid composition of the lipoprotein fractions exposed to the endothelial cells was determined in the present study. However, the greater PAI-1 release induced by HTG-VLDL compared with NTG-VLDL is most likely explained by a higher proportion of large VLDL particles containing newly transferred apolipoprotein E in hypertriglyceridemia. Apolipoprotein E of an appropriate surface orientation has been shown to be a prerequisite for LDL receptor recognition and uptake of HTG-VLDL of S, 100 to 400 by fibroblasts. In contrast, NTG-VLDL mainly consists of small particles, which utilize apolipoprotein B as a ligand when interacting with the LDL receptor. Thus, because apolipoprotein E has a higher affinity for the B,E receptor than does apolipoprotein B, HTG-VLDL both binds more effectively to this receptor and induces a greater increase in PAI-1 release.
than does NTG-VLDL. Notably, NTG-VLDL added at a VLDL protein concentration identical to that of HTG-VLDL contains a higher number of particles, since: 1) each VLDL particle has one apolipoprotein B molecule, and 2) the proportion of large VLDL particles is higher in HTG-VLDL, and 3) large VLDL particles have a higher content of soluble apolipoproteins per particle. Accordingly, incubation of the endothelial cells with identical particle numbers of various VLDL subfractions from hypertriglyceridemic subjects would most likely further emphasize the potent stimulatory effect on PAI-1 secretion indicated for VLDL of S100 to 400 in our VLDL subclass experiments (Figure 2). The observation that large postprandial lipoproteins, namely, chylomicrons and chylomicron remnants, obtained from two healthy subjects with normal fasting lipoprotein patterns did not produce an increase in PAI-1 secretion from the cells compared with VLDL of S60 to 100 or VLDL of S20 to 60 isolated from fasting plasma is compatible with the notion that intestinally derived apo-

lipoprotein B-48 does not interact with the B,E receptor.

It has been proposed that insulin is the major physiolog-
ical determinant of PAI-1 activity in plasma, either directly by stimulation of PAI-1 secretion from the liver or indirectly through influences of insulin on VLDL triglycer-
ide concentration. Indeed, plasma insulin level corre-
lates with PAI-1 activity and body mass index. Furthermore, it has been argued that insulin stimulates the synthesis and release of free PAI-1 from hepatocytes, while t-PA and PAI-1 are released together from endothelial cells merely due to a nonspecific acute-phase re-
response to chronic vascular disease and subsequently rapidly inactivated by complex binding. However, the present data do not support this hypothesis. Furthermore, hyperinsulinemia is associated with an increased hepatic synthesis of VLDL triglycerides, which means that VLDL is by no means ruled out as the link between high plasma insulin and PAI-1 elevation and as a significant physiological regulator of plasma PAI-1 activity, in spite of a direct effect of insulin on hepatic PAI-1 produc-
tion and release.

The mechanism by which the binding of VLDL to B,E receptors initiates the release of PAI-1 from endothelial cells remains to be clarified. The binding of VLDL to its surface receptor may in itself be sufficient to generate an intracellular signal leading to increased PAI-1 secretion. Alternatively, uptake and degradation of the VLDL parti-
cles may be required. A further mechanism that must be considered is that the lipoprotein-induced stimulation of PAI-1 secretion is due to the presence of endotoxins in the lipoproteins. Morel and coworkers have reported that oxidized LDL may facilitate the uptake of endotoxins in human endothelial cells. However, our finding that oxidized LDL lacked any effect on PAI-1 secretion argues against any major influence of endotoxins in lipoprotein-induced secretion of PAI-1.

In conclusion, the experimental data reported here may provide an explanation to the clinical finding of an associ-

ation between serum triglycerides and plasma levels of PAI-1 and add further support to the notion that hypertri-
glyceridemia is associated with hypercoagulability.

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