Very Low Density Lipoprotein–Mediated Signal Transduction and Plasminogen Activator Inhibitor Type 1 in Cultured HepG2 Cells

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Abstract—In normal subjects and in patients with cardiovascular disease, plasma triglycerides are positively correlated with plasminogen activator inhibitor type 1 (PAI-1) levels. Moreover, in vitro studies indicate that VLDLs induce PAI-1 synthesis in cultured cells, ie, endothelial and HepG2 cells. However, the signaling pathways involved in the effect of VLDL on PAI-1 synthesis have not yet been investigated. We report that VLDLs induce a signaling cascade that leads to an enhanced secretion of PAI-1 by HepG2 cells. In myo-[3 H]inositol–labeled HepG2 cells, VLDL (100 µg/mL) caused a time-dependent increase in [3 H]inositol phosphates, the temporal sequence being tris>bis>monophosphate. VLDL brought about a time-dependent stimulation of membrane-associated protein kinase C (PKC) activity and arachidonate release. Finally, VLDL stimulated mitogen-activated protein (MAP) kinase phosphorylation. VLDL-induced PAI-1 secretion was completely prevented by U73122, a specific inhibitor of phosphatidylinositol-specific phospholipase C, by H7 or by PKC downregulation, and by mepacrine (all P<0.01 versus VLDL-treated cells). 3,4,5-Trimethoxybenzoic acid 8-(diethylamino)-octyl ester, which prevents Ca2+ release from intracellular stores, inhibited VLDL-induced PAI-1 secretion by 60% (P<0.05), and the MAP kinase/extracellular signal–regulated kinase kinase (MEK) inhibitor PD98059 completely suppressed both basal and VLDL-induced PAI-1 secretion. These data demonstrate that VLDL-induced PAI-1 biosynthesis results from a principal signaling pathway involving PKC-mediated MAP kinase activation. (Circ Res. 1999;85:208-217.)

Key Words: plasminogen activator inhibitor type 1 ■ VLDL ■ fibrinolysis ■ signaling ■ hepatoma cell line

A role for plasma triglycerides as a risk factor for cardiovascular disease has been recently proposed. Case-control studies have shown a positive correlation between triglyceride levels and incidence of cardiovascular disease, and most prospective studies have confirmed this relationship.

Hypertriglyceridemia is associated with reduction in HDL levels, glucose intolerance, and insulin resistance. This pathological condition, reduction of the plasma fibrinolytic capacity has been also documented, and several studies report a direct relationship between the levels of plasminogen activator inhibitor type 1 (PAI-1) and plasma triglycerides, the latter being an independent variable determining the levels of PAI-1 in plasma. Biological plausibility for this observation made in clinical settings has been obtained by in vitro studies in cultured cells.

VLDLs have been shown to increase PAI-1 biosynthesis in endothelial cells by inducing transcription of the PAI-1 gene promoter. Similarly, VLDLs increase PAI-1 synthesis in HepG2 cells, enhancing steady-state PAI-1 mRNA levels, because of stabilization of the 3.2- and 2.2-kb PAI-1 mRNA transcripts; the induction of PAI-1 by VLDLs is dependent on the interaction of the lipoprotein with the apolipoprotein B/E receptors and correlates with intracellular triglyceride accumulation.

PAI-1 synthesis is regulated by several second-messenger signaling pathways that are cell specific. Protein kinase C (PKC) activation is positively associated with PAI-1 induction, whereas agonist-induced cAMP accumulation has negative effects. A PKC-mediated mechanism is involved in the induction of PAI-1 synthesis by phorbol esters, tumor necrosis factor-α (TNF-α), and transforming growth factor-β in different cell systems. Different signaling pathways have been proposed for the induction of PAI-1 elevation in bovine aortic endothelial cells by transforming growth factor-β and TNF-α or lipopolysaccharide. A genistein-sensitive phosphorylation step is involved in TNF-α–induced increase of PAI-1 gene transcription in human endothelial cells.

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cells. Finally, calcium-mobilizing agents stimulate PAI-1 synthesis in U937 cells.

LDLs and HDLs have been shown to induce phosphoinositide turnover, PKC translocation, and mitogen-activated protein (MAP) kinase activation in human and rat smooth muscle cells, as well as in human skin fibroblasts. Oxidized LDLs stimulate MAP kinase in smooth muscle cells and in macrophages. In endothelial cells, oxidized LDLs induce PAI-1 expression through activation of the phosphatidylinositol-phospholipase pathway. No information is available on the signaling pathways evoked by VLDLs. In this study, we demonstrate that VLDLs induce several signaling pathways in HepG2 cells and, using various inhibitors, that the induction of PAI-1 secretion results from activating these signaling pathways.

Materials and Methods

Materials

MEM and FCS were from Life Technologies. The mycoplasma detection kit and anti-mouse IgG- horsederash peroxidase antibody were from Boehringer Mannheim. Plastic ware for cell culture was from Costar. Sterile filters were from Millipore Corp. The F1-5 ELISA for PAI-1 antigen detection was from Monozyme. [γ-32 P]ATP (specific activity, 3000 Ci/mmol), myo-[3H]inositol (specific activity, 17.7 Ci/mmol), [5,6,8,9,11,12,14,15-3H]arachidonic acid (specific activity, 211 Ci/mmol), [1-14C]palmitic acid (specific activity, 55 mCi/mmol), and an enhanced chemiluminescence (ECL) detection system were from Amersham. Solvents and silica gel plates were from Merck. Liquid scintillation Formula 989 was from NEN. Monoclonal antibody (4G10) was from Upstate Biochemicals, Inc. Rabbit polyclonal antibody against phosphophosphorylated MAP kinase was from New England Biolabs. Other reagents were from Sigma.

Chemicals

1-[(6-[[17β-3-Methoxyestra-1,3,5(10)- trien-17-yl] amino]hexyl]-1H-pyrrrole-2,5-dione (U73343) were from ICN Biomedicals; triclocedecan-9-yl-xanthate (D609) was from Calbiochem; butylated hydroxytoluene (BHT), 3,4,5-trimethoxybenzoic acid 8-(diethylamino)-octyl ester (TMB-8), nordihydroguaiaretic acid (NDGA), butylated hydroxyanisole (BHA), and ethylated hydroxyanisole (BHE) were from Calbiochem; phenylmethanesulfonfluoride (PMSF), and phorbol 12-myristate 13-acetate (PMA) were from Sigma; and (2-[2-amino-3- methoxyphenyl]-4H-1-benzopyran-4-one) (U73343) were from Sigma. Other reagents were from Merck. Liquid scintillation Formula 989 was from NEN.

Lipoprotein Preparation

Blood was obtained from normolipidemic subjects among the medical staff attending the E. Grossi Paulettoni Center at Niguarda Hospital (Milan, Italy). Blood drawn from the antecubital vein after overnight fasting was anticoagulated with Na2-EDTA (1 mg/mL) containing 10 kallikrein units/mL aprotinin and kept on ice. Plasma was separated by centrifugation (600g) at 4°C, and VLDLs were isolated as previously described. VLDL particles were sterilized through a 0.45 μm filter, stored in sterile tubes at 4°C, and used within 2 weeks. Total protein content in VLDLs was measured by the Lowry et al method. The lipoproteins were essentially free from contamination by other lipoproteins as determined by nondenaturing gradient gel electrophoresis. In the study, a total of 20 individual VLDL preparations were used. Average VLDL lipid composition, expressed as percentage of total mass (sum of triglycerides, cholesterol, cholesterol ester, phospholipids, and proteins), was as follows: triglycerides, 59.36±5.9%; free cholesterol, 4.75±0.6%; cholesterol ester, 11.15±1.1%; phospholipids, 16.75±2.4%; and proteins, 8.0±0.8%. The composition is given as the mean value of 6 preparations.

Measurement of Inositol Phospholipid Formation

Subconfluent HepG2 cells were incubated with MEM containing 2% FCS with 10 μCi/well of myo-[3H]inositol. After 24 hours of labeling, the cells were incubated for 20 minutes with MEM containing 20 mmol/L HEPES (pH 7.3), 0.1% BSA, and 20 mmol/L LiCl. Cells were incubated with or without VLDL for different time periods at 37°C. The reaction was terminated by rapid removal of the medium by aspiration, and the cells were rinsed twice with ice-cold PBS and scraped off into 250 μL of trichloroacetic acid 5%. Cells were then pelleted by centrifugation at 15 000g for 5 minutes at 4°C. The supernatants were extracted with diethyl ether saturated with water, neutralized with 1 mol/L NaOH, evaporated under N2, and resuspended in 250 μL of H2O. Inositol phosphates were separated by HPLC connected to a radio detector (Radiomatic Flo-One Beta, Canberra Packard).

PKC Activity

Cells were incubated for different times in serum-free medium containing 100 μg protein/mL VLDL and 100 mmol/L PMA. At the end of incubation, cells were washed twice with ice-cold PBS, scraped off, and homogenized with a polytetrafluoroethylene glass homogenizer in 0.32 mol/L sucrose buffered with 20 mmol/L Tris-HCl (pH 7.4) containing (in mol/L) EDTA 2, EGTA 10, β-mercaptoethanol 50, and PMSF 0.3, and 20 μg/mL leupeptin (homogenization buffer). The homogenate was centrifuged at 100 000g for 30 minutes at 4°C and the supernatant was collected for PKC determination (cytosolic fraction). The pellet was resuspended in homogenization buffer and centrifuged again. The remaining pellet was sonicated in the same buffer (except containing 0.2% Triton X-100), incubated at 4°C for 45 minutes, and centrifuged at 100 000g for 30 minutes to obtain the particulate fraction. To examine PKC activity, cytosolic and particulate fractions were incubated at 37°C in buffer containing 3 μmol/L Tris-HCl (pH 7.5), 0.8 μmol/L magnesium acetate, and 1 mg Pepsin (Ser2)–PKC<sub>i–j</sub> as kinase-specific substrate. The reaction was started by adding 50 μmol/L [γ-32 P]ATP (0.45 μCi per sample). Basal activity was measured in the presence of 0.1 μmol/L of EGTA, whereas stimulated activity was evaluated in the presence of 10 μg of phosphatidylserine and 1 mg of diolein. The reaction was stopped after 5 minutes by spotting 25 μL of the sample onto P-81 phosphocellulose paper, adding 25 μL of 0.6% H3PO4 to the spot, and washing the paper with tap water.
Radioactivity retained by the phosphocellulose was determined by liquid scintillation counting using Formula 989. Protein content was measured by Bradford method.29

Release of [3H]Arachidonic Acid From Prelabeled HepG2 Cells

Cells were incubated for 16 hours in medium containing 5% FCS and 0.5 µCi/µL of [3H]arachidonic acid.30 After removal of medium, cells were rinsed 3 times with PBS and incubated with or without VLDL for the indicated time. The medium was rapidly removed, and the radioactivity was quantified by scintillation counting. Lipids were extracted from the medium by the method of Folch et al.31 HPLC analysis performed on lipid extracts confirmed that the radioactivity was associated almost exclusively with free arachidonic acid.

Analysis of Phospholipase D (PLD) Activity

HepG2 cells were labeled for 16 hours with 1 µCi of [3H]palmitate in 1 mL of MEM containing 5% FCS.32 After 5 washings with MEM containing 10% serum, cells were incubated for 5 minutes in MEM containing 1% ethanol and then stimulated with VLDL or 1 µmol/L A23187, for various times. The medium was rapidly aspirated, and, after addition of ice-cold methanol, cells were scraped off and lipids by extracted by the method of Folch et al.31 [3H]PEth was separated from the other phospholipids by thin-layer chromatography on silica gel 60 plates. The solvent system was the organic phase of ethyl acetate/isooctane/ acetic acid/water (13:2:3:10, vol/vol/vol/vol). The spots corresponding to phosphatidylethanol (PEth), total phospholipids, and phosphatidic acid were identified by iodine vapor, scraped, and then counted for radioactivity in scintillation liquid in a beta counter.

Immunoblotting With Anti-Phosphotyrosine Monoclonal Antibody

HepG2 cells were incubated with VLDL and 100 nmol/L PMA for the indicated time. The cells were then rinsed with calcium- and magnesium-free PBS and then lysed on ice with 100 µL of 50 mmol/L β-glycerophosphate, pH 7.2, containing (in mmol/L) sodium orthovanadate 100, MgCl2, 2, EGTA 1, and DT 1, and 0.5% Triton X-100, 10 µg/mL leupeptin, and 2 µg/mL aprotinin. Cell lysates, after protein determination by the Lowry et al27 method, were boiled for 5 minutes in Laemmli buffer33 resolved on a 10% SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane. Filters were incubated overnight at 4°C with or without VLDL-Induced PAI-1 Biosynthesis Is Dependent on Inositol Phospholipid Catabolism

We first examined whether VLDL incubated with HepG2 cells induced generation of [3H]inositol phosphates. VLDL induced generation of [3H]inositol trisphosphate (InsP3) and to a lesser extent of [3H]inositol bisphosphate (InsP2), with the maximal increase occurring between 2 and 5 minutes, after which [3H]InsP3 declined, whereas at 10 minutes a 3H content was sustained in InsP2, and it increased further in [3H]inositol monophosphate (InsP) (Figure 1). These data indicate that VLDLs quickly induce phosphatidylinositol breakdown via the activation of phospholipase C (PLC). We therefore investigated the effects of U73122, at concentrations completely inhibiting PLC-dependent processes,35 on PAI-1 secretion. Cells were first incubated for 1 hour with vehicle or with 5 or 10 µmol/L U73122 or its inactive analog U73343 and then exposed to VLDL. Neither U73122 nor U73343 affected basal PAI-1 secretion, whereas 10 µmol/L U73122 completely prevented the effect of VLDL (Figure 2), and U73343 had no effect. D609 (10 µmol/L), a phosphatidylinositol-specific PLC inhibitor,36 did not reduce VLDL-induced PAI-1 secretion, but it augmented it by almost 2-fold (P<0.01 versus VLDL-treated cells, n=4).

Effect of Calcium on VLDL-Induced PAI-1 Release

The importance of Ca2+ influx from the extracellular environment and Ca2+ mobilization from intracellular stores in the
induction of PAI-1 biosynthesis by VLDL were then evaluated. Measurement of VLDL-induced intracellular Ca\(^{2+}\) changes, using Fluo 3-AM–loaded cells, was not possible because of turbidity produced by the lipoprotein. We therefore analyzed the effects of various compounds interfering with calcium\(^{37}\) on PAI-1 secretion. Neither nifedipine (10 to 50 \(\mu\)mol/L), a calcium channel blocker, nor EGTA (0.1 to 1 mmol/L) incubated with cells for 1 hour affected subsequent basal (5.3 \(\pm\) 0.2 and 4.98 \(\pm\) 0.51 ng/mL for 50 \(\mu\)mol/L nifedipine and 1 mmol/L EGTA versus 5.16 \(\pm\) 0.54 ng/mL for untreated cells \([P<0.01]\) vs unstimulated cells; \(\S\)\(P<0.01\) vs cells stimulated by VLDL without U73122; – indicates absence of U73122.

Figure 3. Effect of TMB-8 and thapsigargin on PAI-1 secretion by HepG2. Top, Cells were kept in serum-free medium for 24 hours and then incubated for 1 hour with the indicated concentrations of TMB-8 or vehicle. Bottom, Cells were preincubated in medium containing vehicle or thapsigargin for 24 hours. Cells were then stimulated for 16 hours in the absence (open bars) or presence (hatched bars) of 100 \(\mu\)g/mL VLDL in the continued presence of the calcium modulators. PAI-1 antigen levels were determined in cell supernatants. Data are mean \(\pm\) SEM of 4 individual experiments performed in duplicate. \(\ast\)\(P<0.01\) vs unstimulated cells; \(\S\)\(P<0.01\) vs VLDL-stimulated cells. – indicates absence of TMB-8 in top panel and absence of thapsigargin in bottom panel.

biosynthesis. To this end, cells were incubated for 24 hours with 1 \(\mu\)mol/L thapsigargin before VLDL addition. A marked increase in PAI-1 secretion in cells incubated with or without VLDL was observed \((P<0.01)\) (Figure 3, bottom).

Figure 2. Effect of U73122 on PAI-1 secretion. HepG2 cells were kept in serum-free medium for 24 hours, incubated for 1 hour with the indicated concentrations of U73122 or vehicle, and then stimulated for 16 hours in the absence (open bars) or presence (hatched bars) of 100 \(\mu\)g/mL VLDL in the continuous presence of U73122. PAI-1 antigen levels were determined in cell supernatants. Each value represents the mean \(\pm\) SEM of 4 individual experiments performed in duplicate. Results are presented as percentage of PAI-1 antigen levels in supernatants of HepG2 cells incubated with medium alone for 16 hours.

\(\ast\)\(P<0.01\) vs unstimulated cells; \(\S\)\(P<0.01\) vs cells stimulated by VLDL without U73122. – indicates absence of U73122.

Figure 1. Time-dependent inositol phosphate generation in response to VLDL. HepG2 cells were kept in serum-free medium for 24 hours and then incubated with 10 \(\mu\)Ci/well of myo-[\(^{3}H\)]inositol. HepG2 cells labeled with myo-[\(^{3}H\)]inositol were treated at 37°C with or without VLDL (100 \(\mu\)g/mL) in the presence of 20 mmol/L LiCl for the indicated times. Inositol phosphates were separated by HPLC with radioactivity detector (see Materials and Methods). Data are given as mean \(\pm\) SEM \((n=3)\) and express percentages of initial \(^{3}H\) content (100%) in each inositol phosphate (control). Actual initial values (time 0, \(n=3\)) were the following: InsP\(_{1}\) (○), 3775 \(\pm\) 541 cpm; InsP\(_{2}\) (□), 560 \(\pm\) 140 cpm; and InsP\(_{3}\) (○), 304 \(\pm\) 139 cpm. \(\ast\)\(P<0.01\) vs corresponding time 0.
VLDL-Induced PAI-1 Release Is Dependent on PKC

Activation of PLC leads to PKC activation, and PKC has been shown to enhance PAI-1 biosynthesis in several cell types. We therefore examined whether PKC is involved in VLDL-induced PAI-1 release in HepG2 cells. The capacity of VLDL to stimulate PKC activity was assessed by evaluating the translocation of this enzyme from cytosol to the particulate fraction. In unstimulated cells, up to 95% of cellular PKC activity was recovered in the cytosol fraction. In stimulated cells, up to 95% of PKC activity was recovered in the particulate fraction. In unstimulated cells, up to 95% of cellular PKC activity was recovered in the cytosol fraction. Incubation of cells with VLDL resulted in a maximal increase in membrane-associated PKC at 5 minutes (Figure 4). The relationship between PKC activation and PAI-1 secretion was then investigated. VLDL-induced PAI-1 secretion was completely prevented by prior incubation of cells with H7, a PKC inhibitor (35 μmol/L). In additional experiments, HepG2 cells were treated with vehicle or 100 nmol/L PMA for 24 hours to downregulate PKC. The supernatant was then replaced with serum-free medium or supplemented with 100 μg/mL VLDL or 100 nmol/L PMA. In cells preincubated with vehicle, VLDL and PMA increased PAI-1 secretion by 217±12.2% and 722±92% (n=4), respectively. After downregulation of PKC, VLDL- and PMA-induced PAI-1 secretion was reduced to 76.6±10.1% and 81.4±9.8%, respectively (P<0.01 versus control, n=4), further confirming that PKC activation is involved in VLDL-induced PAI-1 secretion.

Effect of VLDL on Arachidonate Release by HepG2

To determine whether VLDL affected phospholipase-mediated arachidonate release, HepG2 cells were labeled with [3H]arachidonate and then incubated from 0 to 60 minutes with medium alone or medium containing VLDL. A time-dependent [3H]arachidonate release (0 to 60 minutes) was detected in supernatants of VLDL-treated samples; levels of free [3H]arachidonate (confirmed by radio-HPLC) were significantly greater than in controls, with a peak increment at 45 minutes (Figure 5). Mepacrine, a phospholipase inhibitor, and H7 reduced VLDL-induced [3H]arachidonate release by 50% and 40%, respectively. The involvement of phospholipase activation in PAI-1 secretion was then assessed. Prior incubation of cells with 15 μmol/L mepacrine for 1 hour inhibited the VLDL-induced enhancement in PAI-1 by 72.5±9.5% (P<0.01 versus VLDL-treated cells, n=6). Indomethacin, at concentrations completely suppressing cyclooxygenase activity (10 or 20 μmol/L), did not affect PAI-1 secretion by either unstimulated or VLDL-stimulated cells. NDA at 20 μmol/L significantly increased basal PAI-1 secretion by HepG2 cells (5.2±0.4 versus 10.6±1.56 ng/mL [P<0.01 for untreated and NDGA-treated cells, respectively]), whereas this lipoxigenase inhibitor failed to affect VLDL-induced PAI-1 secretion (10.15±1.02 and 11.15±0.75 ng/mL in cells treated with VLDL or VLDL plus NDGA, respectively).

VLDL-Induced PAI-1 Release Involves Tyrosine Phosphorylation

Tyrosine phosphorylation at 5 and 15 minutes after the addition of vehicle or VLDL was visualized by immunoblotting with the monoclonal antibody 4G10, as described in Materials and Methods. Figure 6 shows that VLDL enhanced tyrosine phosphorylation of several proteins, with apparent molecular masses of 40, 44, 70, 80, 110, and 200 kDa. A similar pattern of phosphorylation was observed in cells treated with 100 nmol/L PMA. Preincubation of cells with 35 μmol/L H7 attenuated the effect of VLDL on tyrosine phosphorylation (not shown). Herbinmycin (1 μmol/L), a tyrosine phosphorylation inhibitor, inhibited the secretion of PAI-1 by 81.5±3% in VLDL-treated cells (P<0.01, n=4), which suggests that tyrosine phosphorylation is a prerequisite for VLDL-induced PAI-1 biosynthesis.

Effect of VLDL on MAP Kinase Activity

Enhanced tyrosine phosphorylation of MAP kinase in response to PKC activation has been described in several cell lines. To assess whether VLDL induced MAP kinase

Figure 4. Effect of VLDL on PKC activity in HepG2 cells. Cells were incubated for 24 hours with medium alone and then stimulated with or without 100 μg/mL VLDL for the indicated times. Cytoplasm and particulate fractions were isolated, and PKC activity was determined. Basal PKC-phosphorylating activities in soluble (open bars) and particulate (hatched bars) fractions were 1.54±0.25 and 0.31±0.11 nmol/min·mg of protein, respectively. Data are mean±SEM of 3 individual experiments performed in duplicate. *P<0.01 vs basal.

Figure 5. Arachidonate (AA) release in HepG2 cells incubated with VLDL. Cells were incubated for 16 hours in medium containing 0.5 μCi/mL [3H]AA and then with 100 μg/mL VLDL for the indicated times. [3H] was determined in cell supernatants. Data are mean±SEM of 3 individual experiments performed in duplicate. *P<0.01 vs control. Inset, HPLC chromatogram showing that [3H]AA was the major peak identifiable in the supernatant of HepG2 cells.
activation, experiments were carried out by measuring \(^{32}\)P-labeling of MBP in cytosol extracts of HepG2 cells preincubated for 24 hours with 0.1% FCS and then incubated for 5 and 15 minutes with 100 \(\mu\)g/mL VLDL. After 5 minutes, VLDL doubled MAP kinase activity, with the level remaining elevated at 15 minutes. Similar induction of MAP kinase activity was observed in cells stimulated with 20% FCS (Figure 7A). To confirm that the observed kinase activity results from the stimulation of the ERK family of MAP kinases, phosphorylated ERK1 and ERK2 proteins were identified by Western blotting in cell lysates of HepG2 cells treated with VLDL or 20% FCS. Serum phosphorylated both ERK1 and ERK2, whereas VLDL induced phosphorylation of the ERK2 isoform only (Figure 7B). Preincubation of cells with 35 \(\mu\)mol/L H7 completely prevented ERK2 phosphorylation (Figure 7C). MAP kinase is phosphorylated and activated by MAP kinase/extracellular signal–regulated kinase (MEK), a dual-specificity kinase that phosphorylates serine and tyrosine residues. PD98059 is a highly selective inhibitor of MEK that is commonly used to block MAP kinase activation.\(^40\) PD98059 at a concentration known to inhibit MEK by >50%\(^40\) inhibited both basal and VLDL-stimulated PAI-1 secretion by >75% (\(P<0.01\)), which indicates that MAP kinase was necessary not only for VLDL-induced PAI-1 secretion but also for basal PAI-1 biosynthesis (Figure 8).

**Effect of VLDL on PLD Activation**

The formation of P\(\text{Eth}\), which is generated in the presence of noncytotoxic ethanol concentrations by the PLD-catalyzed transphosphorylation of phosphatidylcholine, is a convenient index of PLD activity.\(^41\) A23187 (1 \(\mu\)mol/L) caused a rapid (5 minutes) ethanol-dependent accumulation of \(\left[\text{\textsuperscript{14}}\text{C}\right]\)P\(\text{Eth}\) in HepG2 cells (from 0.06% of total radioactivity to 0.37%), whereas VLDL did not affect \(\left[\text{\textsuperscript{14}}\text{C}\right]\)P\(\text{Eth}\) over 45 minutes.

**Role of Phosphatidylinositol 3-Kinase (PI 3-K) in VLDL-Induced PAI-1 Secretion**

Wortmannin (0.1 to 1 \(\mu\)mol/L), a PI 3-K inhibitor\(^42\) that was incubated with cells 1 hour before VLDL addition, did not affect basal (5.9±0.44 and 6.12±0.62 ng/mL for untreated and 1 \(\mu\)mol/L wortmannin–treated cells, respectively) or VLDL-stimulated PAI-1 secretion (15.66±1.88 and 16.42±1.6 ng/mL for VLDL and VLDL plus wortmannin, respectively).

**Effect of Antioxidants on VLDL-Induced PAI-1 Secretion**

To evaluate whether oxidation/modification of VLDL was implicated in the effect of the lipoprotein on PAI-1, experiments that included BHT or vitamin E were carried out. BHT (25 \(\mu\)mol/L) did not affect basal PAI-1 secretion (4.5±0.15 and 5.3±0.52 ng/mL for untreated and BHT-treated cells,
respective) or VLDL-enhanced PAI-1 release (9.39 ± 0.77 and 9.8 ± 0.48 ng/mL for VLDL and for VLDL plus BHT, respectively). Similarly, no change in PAI-1 secretion was recorded in the presence of 50 μmol/L vitamin E. These findings rule out the hypothesis that active oxidation of VLDL under our experimental conditions was responsible for the observed effect of the lipoprotein on PAI-1 biosynthesis.

Discussion

In this study, we report that VLDLs activate in HepG2 cells inositol phosphate generation and PKC, which in turn induce MAP kinase phosphorylation. We have shown by pharmacological studies that coordinated activation of this signaling pathway is essential for PAI-1 biosynthesis by these cells. As previously observed for other lipoprotein classes, eg, LDL and HDL, VLDLs induce phosphoinositide catabolism with the formation of inositol phosphates, and the relevance of this pathway to VLDL-induced PAI-1 secretion is supported by the data obtained with a specific inhibitor of this signaling pathway. At variance, inhibition of phosphatidylcholine-specific activity by D609 was associated with VLDL-induced increases in PAI-1 secretion, which suggests that this pathway too is involved in the regulation of PAI-1 by mechanisms not yet understood. In addition, an involvement of PLD or PI 3-K activation in the VLDL-induced PAI-1 secretion was excluded.

Blockage of calcium influx by a calcium channel blocker or by EGTA did not affect the VLDL-induced PAI-1 biosynthesis, whereas TMB-8 significantly reduced it, which suggests that the effect of VLDL requires release of sequestered Ca2+. Thapsigargin, which inhibits the Ca2+-ATPase of intracellular Ca2+ stores and, consequently, activates Ca2+ influx from the extracellular space, strongly induced PAI-1 secretion both in basal and in VLDL-stimulated cells; this finding is in agreement with a previous report on U937 cells exposed to the calcium ionophore A23187.

Cytosolic PKC is normally functionally inactive, whereas after agonist stimulation and generation of inositol phosphates and diacylglycerol, the enzyme translocates to the membrane, where the lipid-rich environment and Ca2+ serve as cofactors for enzymatic kinase activity. The observation that VLDLs induce PKC translocation suggests that activation of this enzyme may be crucial to the signal transduction pathways triggered by this lipoprotein. In HepG2 cells, 2 calcium-dependent (α and βII) and 3 calcium-independent (δ, ε, and ζ) isoforms of PKC have been described. Although we have not explored the role of each PKC isoform, involvement of the calcium-dependent PKCα can be hypothesized. Preliminary experiments have shown that PKCα isoform translocates from the cytosol to the membrane fraction, which supports this hypothesis at least in part (data not shown).

Activation of PKC, brought about by its putative ligand PMA, induces PAI-1 in HepG2 cells. Inhibition of PKC activation by H7 and PKC downregulation prevent VLDL-induced PAI-1 secretion, which indicates that, similarly to other agonists, this lipoprotein fraction increases PAI-1 secretion via this signaling pathway.

We also show that VLDLs induce release of arachidonate from membrane lipids. This increase is time dependent and partially reduced by the PKC inhibitor, H7. Unesterified arachidonate may originate from the activation of several enzymes, among which phosphatidylcholine-specific PLC (PI-PLC) and phospholipase A2 are the most likely candidates. The reduction of arachidonate release by H7 indicates that at least 50% of the release of this fatty acid is related to a PKC-dependent mechanism. PKC has been implicated in phospholipase A2 phosphorylation, and this process is secondary to MAP kinase activation. In addition, the results obtained with inhibitors of arachidonate oxidation rule out the possibility that products of arachidonate metabolism are implicated in VLDL-induced enhancement of PAI-1 secretion.

One downstream event of PKC activation is stimulation of MAP kinases. MAP kinases form a family of serine/threonine kinases uniquely activated by dual phosphorylation of threonine and tyrosine residues. This pathway may originate from several distinct classes of cell-surface receptors such as tyrosine kinases and G protein–coupled receptors. This cascade of events involves activation of Ras, which in turn activates Raf and leads to MAP kinase activation. PKC regulates the MAP kinase cascade in a number of ways. In some cells, PKC regulation of MAP kinase is Ras dependent, and in others it is Ras independent. We show here that both tyrosine phosphorylation and MAP kinase activation are induced by VLDL in HepG2 cells. MAP kinase activation evoked by VLDL, as evaluated by MAPK assay, required shorter time than that of ERK, which indicates that other members of the MAP kinase family might be phosphorylated by VLDL. Interestingly, VLDL induced phosphorylation of ERK2 isoform only, a phenomenon that requires investigation. Inhibition of tyrosine and MAP kinase pathways results in the impairment of the VLDL-induced increase in PAI-1. Indeed, the inhibition of MAP kinase results in almost complete suppression of basal and VLDL-stimulated PAI-1 secretion, which suggests that MAP kinase activation is the final essential step in PAI-1 biosynthesis in HepG2 cells. Interestingly, inhibition of PKC activation by H7 prevented ERK2 phosphorylation induced by VLDL,
which suggests that PKC plays a pivotal role in VLDL-induced MAP kinase phosphorylation. 

On the basis of the data discussed above, we conclude that VLDL or its components, through activation of phosphatidylinositol-specific PLC, induce InsP₃ and diacylglycerol formation and intracellular Ca²⁺ increases, leading to activation of PKC and its translocation from the cytosol to the membrane fraction. PKC activates MAP kinases, which in turn result in PAI-1 upregulation. This signaling pathway may also induce, in addition to PAI-1, a variety of other cellular functions as well as the synthesis of biologically active proteins.

The mechanisms by which VLDLs induce the signaling cascade discussed above have not been addressed in this study.

The VLDL fraction contains VLDL and VLDL remnants, resulting from lipoprotein lipase-mediated hydrolysis of triglycerides. Both VLDL and VLDL remnants bind and are taken up by a variety of receptors present on liver cells, eg, the LDL receptor and the LDL receptor-related protein. In addition, VLDL may be catabolized by a specific VLDL receptor present on several cell systems, including HepG2 cells. Previous studies by our group have shown that VLDL-induced PAI-1 is fully prevented by the C7 antibody, which specifically recognizes LDL receptor, suggesting a mechanism mediated by the ligand activation of the apolipoprotein B/E receptor. The possibility, however, that in addition to LDL receptor, VLDL receptor is also involved in PAI-1 induction cannot be ruled out.

Interestingly, the C7 antibody reduces S6 kinase activation and phosphoinositide turnover induced by LDL in human vascular smooth muscle cells and Ca²⁺-mediated VCAM-1 and E-selectin expression in human cultured endothelial cells exposed to native LDL. LDL- and HDL-induced surfactant secretion by alveolar type II cells involves activation of phosphoinositide hydrolysis and increases in intracellular calcium concentration and PKC activity, and these effects are inhibited by pertussis toxin, which suggests that the LDL receptor also interacts with a heterotrimeric G protein. The mechanisms, however, by which lipoprotein-receptor interactions initiate signal transduction are unknown.

Thus, ligand activation of the apolipoprotein B/E receptor or lipid components formed after uptake and degradation of VLDL or VLDL remnants may be responsible for the activation of the signaling cascade leading to PAI-1 biosynthesis.

We have previously shown that the exposure of HepG2 cells to VLDL results in intracellular triglyceride accumulation, which in turn correlates with PAI-1 secretion. Incubation of HepG2 cells with linoleic acid, either as triglyceride or complexed to albumin, increases PAI-1 secretion, and a direct effect of unsaturated fatty acids on PAI-1 gene transcription has been recently demonstrated by Nilsson et al in endothelial cells. Interestingly, arachidonic and linoleic acids activate MAP kinase in vascular smooth muscle cells. Finally, experiments with antioxidants indicate that active oxidation of the lipoprotein under in vitro experimental conditions was not likely to mediate the event.

PAI-1 levels have been shown to be elevated in patients with risk factors for cardiovascular disease, eg, hypertension, type 2 diabetes, insulin resistance, and hypertriglyceridemia. Elevated levels of this protein may reduce fibrinolytic activity resulting, in vivo, in a reduced fibrin dissolution and thrombus removal. The interaction of VLDL with HepG2 cells induces increases in PAI-1 antigen in its active form. This effect is obtained at concentrations of the lipoprotein similar to those present in vivo in normolipidemic subjects. In this context, it is worth mentioning that a correlation between PAI-1 levels and triglycerides has been observed not only in patients at high risk of cardiovascular disease but also in healthy subjects with normal lipid profile. PAI-1 levels are not constant during the day but show diurnal variation with remarkable postprandial increases, with particular increments in patients with hypertriglyceridemia. These changes during the day may represent a predisposing condition toward an effect of VLDL in competent cells, leading to increase in PAI-1, which in turn results in reduced fibrinolysis.

On the basis of the present in vitro findings, we propose that VLDLs induce biosynthesis of PAI-1 as the result of a principal signaling pathway involving PKC-mediated MAP kinase activation. However, the role of other potential related signaling pathways in this process cannot be excluded. The finding that selective inhibition of the proposed signaling cascade results in almost complete suppression of the secretion of this antifibrinolytic protein may provide a series of target candidates for pharmacological strategies aimed at reducing PAI-1 biosynthesis.

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