

Plasminogen Activator Inhibitor Type-1 Synthesis and mRNA Expression in HepG2 Cells Are Regulated by VLDL

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Abstract The effect of VLDL on plasminogen activator inhibitor type 1 biosynthesis in HepG2 cells was investigated. Exposure of HepG2 cells to VLDL (range, 10 to 100 μ g protein per milliliter) for 16 hours resulted in an enhanced release of PAI-1 antigen and PAI activity into conditioned medium, accompanied by the accumulation of intracellular triglycerides. By using a monoclonal antibody (IgG C7) specific to the LDL receptor, we showed that the effect of VLDL is mediated by its interaction with the LDL receptor. Enhanced PAI-1 release was due to increased biosynthesis: PAI-1 mRNA was doubled, mainly because of the effect on the 2.2-kb PAI-1 mRNA rather than the 3.2-kb transcript. Addition of insulin with the VLDL

further enhanced PAI-1 antigen release and PAI-1 mRNA accumulation. The effect of VLDL on steady state levels of PAI-1 mRNA was apparently not due to an increase of gene transcription but to stabilization of both PAI-1 mRNA transcripts. The enhancing effect of VLDL on PAI-1 biosynthesis in HepG2 cells may raise PAI-1 antigen levels not only in hypertriglyceridemic states but also in those conditions in which both insulin and VLDL are elevated. (*Arterioscler Thromb Vasc Biol.* 1996;16:89-96.)

Key Words • VLDL • plasminogen activator inhibitor type-1 • HepG2 • gene expression • insulin

Tissue plasminogen activator initiates fibrinolysis by activation of plasminogen to plasmin in association with fibrin.^{1,2} The activity of TPA is regulated by its major physiological inhibitor, PAI-1.³ Much evidence indicates that elevated levels of PAI-1 are associated with the incidence of atherothrombotic episodes.⁴ Moreover, elevated levels of PAI-1 are associated with obesity,⁵ non-insulin-dependent diabetes,⁶ and/or insulin resistance⁷ and, more generally, with the features of syndrome X.⁸

Epidemiological studies suggest that elevated plasma triglycerides constitute a risk factor for vascular disease.⁹ In the Framingham Study, men and women with triglyceride levels >1.7 mmol/L and HDL levels <1.03 mmol/L had a high rate of coronary heart disease,¹⁰ and the PROCAM study showed hypertriglyceridemia to be a powerful risk factor when combined with a high ratio (>5.0) of plasma LDL to HDL cholesterol.¹¹

In hypertriglyceridemic patients, the plasma fibrinolytic capacity is reduced because of elevated levels of PAI-1 in plasma,¹²⁻¹⁵ and a direct correlation between triglyceride levels and PAI-1 activity or antigen has been reported.^{15,16}

The synthesis of PAI-1 by endothelial, smooth muscle, or liver cells is regulated by several substances.¹⁷ To explain the link between PAI-1 and plasma triglyceride levels, studies in human umbilical vein endothelial cells have been performed.^{15,18} The results indicate that VLDL, the major lipoprotein subfraction responsible for

triglyceride transport, increases PAI-1 biosynthesis in endothelial cells. This effect is more pronounced when cells are incubated with VLDL isolated from plasma of hypertriglyceridemic patients, which suggests a direct effect of the lipoprotein on PAI-1 antigen biosynthesis.^{15,18} The greater effect of VLDL from hypertriglyceridemic patients than that from normolipidemic subjects may be due to the presence in the former of larger VLDL particles containing newly transferred apoE that have high affinity for the apoB/E receptor present on endothelial cells.¹⁸

We have previously demonstrated^{15,19} that VLDL from both normal subjects and hypertriglyceridemic patients increases the release of PAI-1 antigen by HepG2 cells, a hepatoma cell line that possesses most of the characteristics of human hepatocytes. This atherogenic lipoprotein subfraction may thus be directly involved, by an as yet unidentified mechanism, in the regulation of PAI-1 biosynthesis not only in the vascular tree but also in the liver.

In this study we show that VLDL increases PAI-1 biosynthesis by HepG2 cells in vitro through an interaction with the LDL receptor, influencing PAI-1 mRNA accumulation, mainly via (a) posttranscriptional mechanism(s). This effect is further enhanced by insulin, a known inducer of PAI-1 biosynthesis.²⁰

Methods

Materials

All disposable materials were from Costar; Hybond N filters, random primer kit (Multiprime DNA labeling system), and ³²P-labeled dCTP and UTP were from Amersham Corp. All the culture media, antibiotics, and amino acids were from GIBCO Lab. PMSF, cycloheximide, actinomycin D, bovine insulin, and aprotinin were purchased from Sigma Chemical Co.

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Selected Abbreviations and Acronyms

apo	=	apolipoprotein
LRP	=	LDL receptor-related protein
PAI-1	=	plasminogen activator inhibitor type-1
poly(A)	=	polyadenylation
TPA	=	tissue-type plasminogen activator

Lipoprotein Isolation

Blood, obtained from normolipidemic subjects after overnight fasting, was anticoagulated with Na₂ EDTA (1mg/mL) containing 10 kallikrein units per milliliter (KU/mL) aprotinin and kept on ice. Plasma was separated by low speed centrifugation (600g) at 4°C, and VLDL isolation was started within 6 hours after blood collection. Plasma containing 50 KU/mL aprotinin and 10 mmol/L PMSF was layered under 1.006 g/mL KBr density solution containing 0.15 mol/L NaCl, 1 mmol/L Na₂ EDTA, and 3 mmol/L NaN₃ (pH 7.4) and centrifuged in a Beckman Ti 50.2 rotor (40 000 rpm) for 20 hours at 4°C. VLDL particles that floated to the top were then removed, sterilized through a 0.45- μ m filter (Millipore Corp), stored in sterile tubes at 4°C, and used within 2 weeks of isolation. LDL (density range, 1.020 to 1.050 g/mL) and HDL (density range, 1.06 to 1.21 g/mL) were prepared by differential ultracentrifugation.²¹ LDL and HDL were exhaustively dialyzed at 4°C against phosphate buffer consisting of 0.15 mol/L NaCl and 1 mmol/L Na₂ EDTA (pH 7.4). The dialyzed lipoprotein fractions were sterilized by passage through Millipore filters (0.22 μ m; Millipore Corp), stored in sterile tubes at 4°C, and used within 2 weeks. Total protein content in lipoprotein preparations was analyzed by the Lowry method.²²

Cell Culture Experiments

HepG2 cells were cultured in minimal essential medium supplemented with 10% heat-inactivated fetal calf serum containing 2 mmol/L L-glutamine, 100 IU/mL penicillin, 100 mg/mL streptomycin, 2.2 mg/L sodium bicarbonate, and 1 mmol/L sodium pyruvate under a humidified atmosphere of 95% air/5% CO₂ at 37°C. The cell line was tested for and found free of mycoplasma infection (Mycoplasma detection kit, Boehringer Mannheim GmbH). The cells received fresh complete medium every 3 days and were passaged 1:5 on a 7-day cycle. For experimental purposes, HepG2 cells were plated at the density of 1.3 to 1.5 $\times 10^6$ cells in 250-mm² flasks and used at confluence, which was attained within 6 to 7 days. Cells, which were refed with serum-free medium for 40 hours before the experiments, were then washed three times with PBS and incubated in serum-free medium with or without different agents. After appropriate incubation times, conditioned medium was collected and centrifuged to remove cell debris, and samples were stored at -20°C until analyzed for PAI-1. Cells were washed in ice-cold PBS and dissolved with guanidine isothiocyanate sarcosyl solution for total cellular RNA extraction.

Immunoblotting of LDL Receptor

For blotting experiments, cell layers seeded in 750-mm² flasks were kept for 24 hours in serum-free medium. Cells were then washed three times with PBS, scraped off with a rubber spatula, and centrifuged at 200g for 5 minutes, and the pellet was solubilized by addition of a buffer containing 50 mmol/L Tris-HCl, 2 mmol/L CaCl₂, 80 mmol/L NaCl, 5 mmol/L benzamidine, 1 mmol/L PMSF, 0.5 μ mol/L leupeptin, and 1.0% Triton X-100. The solubilized proteins were centrifuged at 300 000g (Beckman TLA 100.1 rotor) for 40 minutes at 4°C, and the supernatants were then subjected to one-dimensional SDS-polyacrylamide gel electrophoresis on 3% to 15% gels, the proteins being transferred from the gels onto nitrocellulose paper.²³ The nitrocellulose paper was incubated for 60 minutes at 37°C in 50 mL of buffer A (10 mmol/L Tris-HCl at pH 7.4,

0.15 mol/L NaCl, 50 mg/mL BSA, and 0.2% [vol/vol] Tween 20) and then for 2 hours at room temperature in 50 mL of buffer A containing 15 μ g/mL mouse IgG-C7, a monoclonal antibody anti-LDL receptor (Amersham). The nitrocellulose was then washed at room temperature with 200 mL of 10 mmol/L Tris-HCl and 0.15 mol/L NaCl at pH 7.4, containing 0.1% SDS, 1% Nonidet P-40, and 0.5% sodium deoxycholate (buffer B). The washed nitrocellulose paper was then incubated for 30 minutes at room temperature with 50 mL of buffer A containing peroxidase-conjugated rabbit immunoglobulins directed against mouse immunoglobulins (Dako Denmark) diluted 1:5000. The membrane was then rinsed and washed with buffer A. LDL receptor protein on nitrocellulose membrane was detected by using a light-emitting nonradioactive method of detection (ECL, Amersham) on Western blotting.

To analyze the effect of the monoclonal antibody against the LDL receptor (C7) on VLDL-induced release of PAI-1, we first incubated HepG2 cells with serum-free medium containing 15 μ g/mL of C7 or equal amounts of nonimmuno monoclonal antibody for 2 hours at 37°C. VLDL was added at a final concentration of 100 μ g/mL, and the amount of PAI-1 released was determined after 16 hours.

Quantitation of PAI-1 Antigen and Activity

The PAI-1 concentrations were determined by ELISA (F1-5 Monozyme) and the activity by a two-stage indirect chromogenic assay (Ortho Diagnostics System).²⁴ One unit of inhibitory activity was defined as the amount of sample required to inhibit 1 IU of TPA. The possible interference of the lipoproteins with the assay systems was excluded on the basis of experiments in which PAI-1 was determined in medium containing different concentrations of lipoproteins.

Determination of Cellular Triglyceride Content

Layers of HepG2 cells were washed three times with buffer containing 0.15 mol/L NaCl, 50 mmol/L Tris (pH 7.4), and 0.2% BSA and three more times with the same buffer without BSA. Lipids were extracted from cell monolayers by the hexane-isopropanol method,²⁵ ie, 2 mL of hexane-isopropanol mixture (3:2 vol/vol) was added to cell monolayers for 30 minutes at room temperature, and the solvent was transferred to a glass tube and dried under N₂. Triglycerides were determined by enzymatic methods after dissolving the lipids in isopropanol using a commercial kit (Boehringer Mannheim GmbH Diagnostica IC).

Preparation of RNA and Northern Blot Analysis

Total cellular RNA was obtained according to Chomczynski and Sacchi.²⁶ Briefly, 10 to 20 μ g of total RNA, determined spectrophotometrically, was subjected to electrophoresis in formaldehyde-agarose gel.²⁷ Samples were then transferred to the nylon membrane Hybond N⁺ (Amersham) by capillary blotting. Membranes were first hybridized for at least 4 hours at 42°C and then hybridized overnight at the same temperature in 50% formamide, SSPE 5 \times (0.75 mol/L NaCl, 0.05 mol/L NaH₂PO₄ · H₂O, 5 mmol/L EDTA), Denhardt's solution 4 \times , 100 mg/mL dextran sulfate, 100 μ g/mL salmon sperm DNA, and 200 μ g/mL baker's yeast tRNA. Hybridization was performed with cDNA probes labeled with [³²P]dCTP to 5 $\times 10^8$ to 1 $\times 10^9$ cpm/ μ g of DNA by the random primer method. Membranes were then washed four times for 30 minutes at 42°C (two washes with SSPE 5 \times , one with SSPE 1 \times containing SDS 0.1%, and one with SSPE 0.1 \times containing SDS 0.1%). Bands were quantified by exposing the membranes to autoradiography film (Hyperfilm MP, Amersham) with intensifying screens at -80°C and densitometry. The data were expressed as amounts of PAI-1 mRNA relative to GAPDH mRNA, which was not influenced by VLDL treatment. The probe used for PAI-1 was a human 2.2-kb EcoRI-Bam HI cDNA fragment²⁸ kindly provided by Dr A. Riccio (International Institute of Genetics and Biophysics, National Council of Research, Na-

ples, Italy) and a 1.4-kb *Bam*H1 cDNA fragment of human GAPDH kindly provided by Dr P. Castelli (Consorzio Mario Negri sud, Santa Maria Imbaro, Chieti, Italy). All probes were isolated by agarose-gel electrophoresis and purified by using the gene clean kit (Bio 101, Inc).

Run-on Assay of RNA Transcripts

Isolation of nuclei, transcription run-on, and isolation of nascent RNA transcripts were performed according to Greenberg and Ziff,²⁹ with minor modifications.³⁰ Nuclei isolated from stimulated and control cells were resuspended in 250 μ L ice-cold buffer (50 mmol/L Tris-HCl, pH 8.3; 40% glycerol; 5 mmol/L MgCl₂; 0.1 mmol/L EDTA, pH 8). For run-on assay, equal numbers of nuclei (5 to 10×10^6) were incubated for 30 minutes at 30°C in 300 μ L reaction buffer containing 25 mmol/L Tris-HCl, pH 8; 12.5 mmol/L MgCl₂; 750 mmol/L KCl; 1.25 mmol/L each of GTP, CTP, and ATP; and 100 μ Ci of [α -³²P]UTP (3000 Ci/mmol, Amersham International). Elongated transcripts were isolated by the guanidine/cesium procedure, with 50 μ g of yeast tRNA added as the carrier.³¹ The RNA pellet was resuspended in 180 μ L of ice-cold TNE (0.5 mmol/L Tris-HCl, pH 8; 1.5 mol/L NaCl), denatured with 20 μ L of 2 N NaOH on ice for 10 minutes, and then neutralized by the addition of HEPES, pH 7.2 (0.48 mmol/L final concentration). RNA was then precipitated by adding 880 μ L ethanol, the pellet was resuspended in 100 μ L hybridization solution (10 mmol/L TES, 2% SDS, 10 mmol/L EDTA, 300 mmol/L NaCl), and radioactivity was measured. Equal quantities of labeled RNA transcripts from stimulated and control nuclei were hybridized to DNA immobilized on nitrocellulose filters at 65°C for 48 hours. Filters were then washed three times (30 minutes each) with $0.2 \times$ SSC at 65°C and incubated at 37°C for 30 minutes in $0.2 \times$ SSC with 1 μ g/mL RNase A (Boehringer Mannheim) before exposure for autoradiography. For DNA immobilization on filters, plasmid DNA (5 μ g) was denatured with 0.3 mmol/L NaOH at 60°C for 30 minutes, neutralized with ammonium acetate (4 mol/L final concentration), and spotted onto nitrocellulose filters (Schleicher & Schuell) using a slot-blot apparatus.

Determination of PAI-1 mRNA Half-life

Actinomycin D (10 μ g/mL) was added (time 0) to HepG2 cells after overnight incubation with or without 100 μ g of protein per milliliter VLDL. Cells were harvested at specific times and total cellular RNA was isolated. Northern blot analysis was performed as described above.

Statistical Analysis

Data are expressed as mean \pm SEM. Comparisons between treatment conditions were made by Student's paired *t* test.

Results

HepG2 cells, incubated for 16 hours in serum-free medium, released 13.6 ± 1.8 ng/mL PAI-1 antigen, whereas those incubated with a physiological concentration of VLDL (100 μ g protein per milliliter) released 25.9 ± 3.8 ng/mL ($P < .001$) of PAI-1 antigen, and PAI activity doubled, indicating that PAI-1 released in this condition was in its active form (Table). More marked increases in PAI-1 antigen release were detected in subconfluent than in confluent cells ($+165 \pm 19\%$ and $+61 \pm 4\%$, respectively; $n=3$).

This concentration of added VLDL also increased the intracellular accumulation of triglycerides ($+37.3 \pm 1.7\%$, $n=3$), reflecting both uptake and processing of the VLDL, and this effect was more marked in subconfluent cells ($+112 \pm 23\%$, $n=3$). We hypothesized that the effect of VLDL on PAI-1 release was mediated by an interaction of VLDL with the LDL receptor

Effect of VLDL on PAI-1 Antigen and Activity in Conditioned Medium of HepG2 Cells

	PAI-1 Antigen, ng/mL (n=13)	PAI Activity, IU/mL (n=5)
Untreated cells	13.6 ± 1.8	5.9 ± 1.0
VLDL-treated cells	25.9 ± 3.8	10.8 ± 2.3

Values are mean \pm SEM of separate experiments (n) performed with individual VLDL preparations. HepG2 cells were incubated for 16 hours with serum-free medium (untreated cells) or that containing 100 μ g protein per milliliter VLDL (VLDL-treated cells).

present on HepG2 cells. HepG2 cells were therefore incubated for 2 hours with 15 μ g/mL of a monoclonal anti-LDL receptor antibody (mouse IgG C7) before being incubated for 16 hours in serum-free medium with or without 100 μ g protein per milliliter VLDL. Prior experiments had established that the C7 antibody was specific for the LDL receptor (Fig 1). Exposure of HepG2 cells to the C7 antibody, but not to a nonimmuno antibody, prevented the enhancing effect of VLDL on PAI-1 release (enhancements 92%, 23%, and 100% over control for VLDL, VLDL+C7, and VLDL+nonimmuno antibody, respectively).

The mechanisms involved in the effect of VLDL on PAI-1 biosynthesis in HepG2 cells were investigated. Northern blot analysis showed that VLDL isolated from 13 different individuals doubled the levels of PAI-1 mRNA in HepG2 cells under the above condition, the increase being mainly due to accumulation of the 2.2-kb PAI-1 mRNA transcript (Fig 2). The possibility that the effect of VLDL on PAI-1 biosynthesis was due to contamination of the lipoprotein subfraction with bacterial lipopolysaccharide was excluded, ie, incubation with 10 μ g/mL of lipopolysaccharide did not influence PAI-1 mRNA expression (data not shown) or PAI-1 release into the medium.^{17,32}

We then incubated HepG2 cells with serum-free medium containing increasing concentrations of VLDL

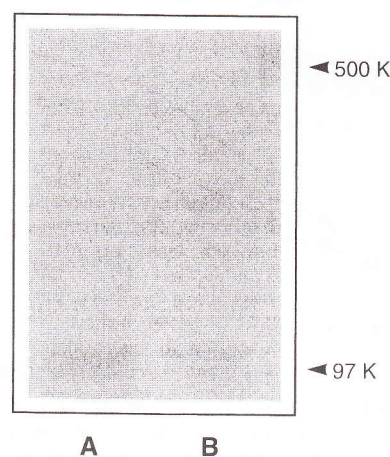


FIG 1. SDS-polyacrylamide gel electrophoresis and immunoblotting of LDL receptor in HepG2 cells. After incubation for 16 hours in serum-free medium, cells were solubilized, and 200 μ g of protein in detergent extracts was subjected to electrophoresis. The proteins were transferred to nitrocellulose paper and incubated with monoclonal anti-LDL receptor antibody (15 μ g/mL of mouse IgG C7). Molecular weight standards are indicated. The LDL receptor preparations were derived from HepG2 cells (A) and normal human fibroblasts (B).

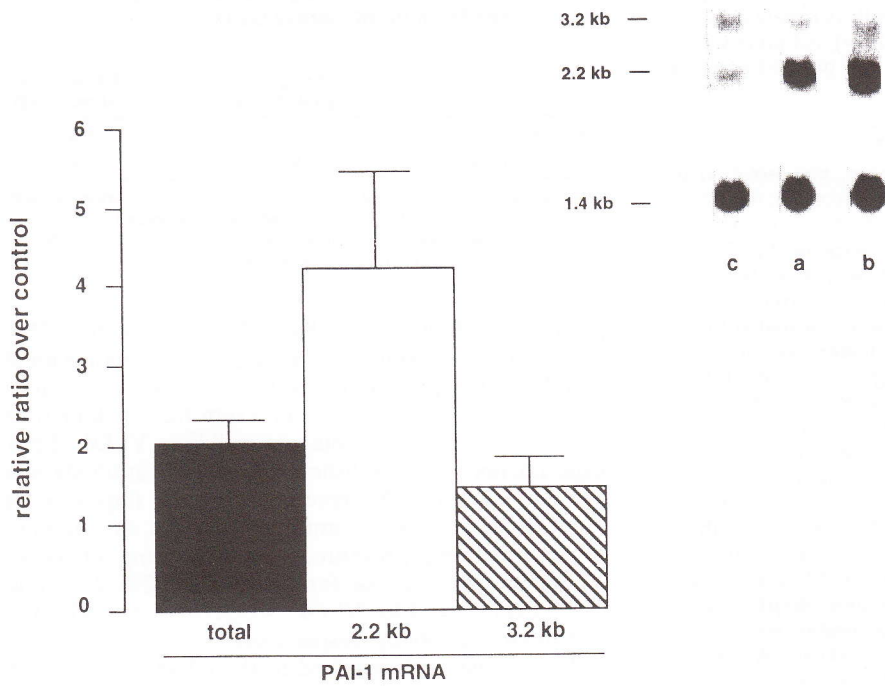


FIG 2. Effect of VLDL on PAI-1 mRNA expression in HepG2 cells. HepG2 cells (3 to 4×10^6 cells) were incubated for 16 hours with serum-free medium in the absence or presence of VLDL. Total RNA was extracted, analyzed by Northern blotting ($10 \mu\text{g}$ of RNA per lane), and hybridized with PAI-1 and GAPDH probes. The intensity of the bands on autoradiographic films was quantified by densitometric scanning, and PAI-1 mRNA amounts were normalized against the corresponding GAPDH signal. The bars represent the mean \pm SEM of PAI-1 mRNA relative value over control, determined in 13 experiments performed with individual VLDL preparations ($100 \mu\text{g}$ protein per milliliter). In the inset is shown a representative Northern blot of untreated cells (c) and those treated with $100 \mu\text{g}$ protein per milliliter of VLDL isolated from two different donors (subjects a and b). The top two bands are 3.2- and 2.2-kb PAI-1 mRNA, and the bottom band is 1.4-kb GAPDH mRNA.

(10 to $100 \mu\text{g}$ protein per milliliter). VLDL induced a concentration-dependent increase in the 2.2-kb PAI-1 mRNA transcript, with a minor effect on the 3.2-kb transcript (Fig 3). Time-course experiments indicated no appreciable change in PAI-1 mRNA expression over 24 hours in control cells. In contrast, in the presence of $100 \mu\text{g}$ protein per milliliter VLDL, enhancement of 2.2-kb PAI-1 mRNA was recorded after 9 hours of incubation, with no effect on the 3.2-kb PAI-1 mRNA over 24 hours (data not shown).

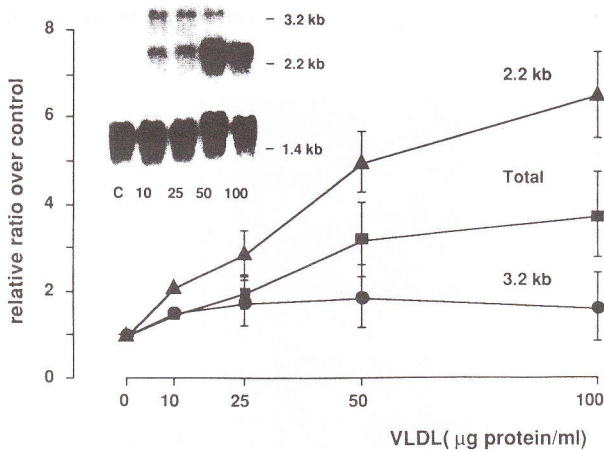


FIG 3. Effect of different concentrations of VLDL on PAI-1 mRNA expression in HepG2 cells, which were incubated for 16 hours in serum-free medium in the absence or presence of different concentrations of VLDL (10 to $100 \mu\text{g}$ protein per milliliter). Total RNA was extracted, analyzed by Northern blotting ($10 \mu\text{g}$ of total RNA per lane), and probed with a PAI-1 cDNA and GAPDH cDNA fragment. The data represent mean \pm SEM of PAI-1 mRNA relative values over control, determined in three experiments performed with individual VLDL preparations. The inset shows a representative Northern blot of control cells (C) and those incubated with different concentrations of VLDL (10 to $100 \mu\text{g}$ protein per milliliter).

The effect of VLDL on PAI-1 biosynthesis was compared with that of HDL and LDL ($100 \mu\text{g}$ protein per milliliter). Neither LDL nor HDL influenced PAI-1 mRNA expression (Fig 4); VLDL alone among lipoprotein subfractions enhanced the accumulation of PAI-1 mRNA.

To determine whether the effect of VLDL on PAI-1 mRNA expression required protein synthesis, cells were incubated with $25 \mu\text{g}/\text{mL}$ cycloheximide in the presence or absence of $100 \mu\text{g}$ protein per milliliter VLDL. In the absence of VLDL, cycloheximide caused a net induction of the 3.2-kb without affecting the 2.2-kb transcript (Fig 5), confirming data reported by others,^{33,34} whereas VLDL induced the expected accumulation of the 2.2-kb PAI-1 mRNA. Cycloheximide and VLDL, added to-

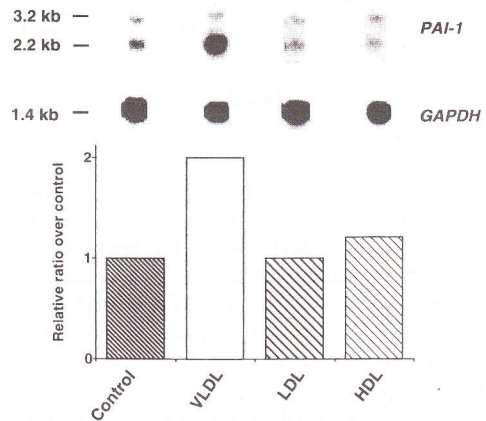


FIG 4. Effect of different classes of lipoproteins on PAI-1 mRNA expression in HepG2 cells. A representative Northern blot of HepG2 cells incubated for 16 hours in serum-free medium in the absence or presence of $100 \mu\text{g}$ protein per milliliter of different lipoprotein fractions (VLDL, LDL, or HDL) is shown. Total PAI-1 mRNA (3.2- plus 2.2-kb transcripts) were normalized against the corresponding 1.4-kb GAPDH signal.

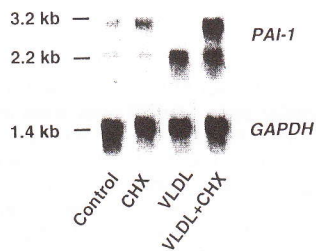


FIG 5. Effect of cycloheximide on PAI-1 mRNA expression in VLDL-treated HepG2 cells, which were incubated in serum-free medium for 16 hours with 100 μ g protein per milliliter VLDL in the absence or presence of cycloheximide (25 μ g/mL). Total RNA was extracted, analyzed by Northern blotting (10 μ g of total RNA per lane), and probed with a PAI-1 cDNA and GAPDH cDNA fragment. Molecular-weight markers in kilobases are shown to the left. A representative blot from a total of three separate experiments is shown. CHX indicates cycloheximide.

gether to HepG2 cells, induced upregulation of both PAI-1 mRNA transcripts.

HepG2 cells were next incubated with VLDL plus insulin. Insulin has been shown to increase PAI-1 synthesis and PAI-1 mRNA accumulation in HepG2 cells by stabilizing the 3.2-kb PAI-1 transcript.³⁴ It was therefore of interest to determine whether VLDL, which specifically increases the 2.2-kb PAI-1 mRNA transcript, could interfere with the effect of insulin on PAI-1 mRNA expression. A representative Northern blotting of HepG2 cells treated with VLDL and/or insulin is shown in the inset of Fig 6. VLDL (100 μ g protein per milliliter) doubled the 2.2-kb PAI-1 mRNA, with no effect on the 3.2-kb PAI-1 mRNA, whereas insulin increased the expression of both transcripts. Interestingly, the combination of the two stimuli induced a further enhancement of the 2.2-kb PAI-1 mRNA species over that of either agent alone. The effect of the combination of the two agents on the 3.2-kb PAI-1 mRNA was more complex, in that VLDL hampered the enhancement of the 3.2-kb PAI-1 mRNA induced by



FIG 7. Effect of VLDL on PAI-1 gene transcription. Slot-blot of transcription run-on studies represents three experiments performed in duplicate with similar results. Nuclei were isolated from HepG2 cells treated with VLDL (100 μ g protein per milliliter) for the indicated times. pBR322 was used as the negative control. Run-on reactions and hybridization of the purified run-on RNA to the indicated plasmid DNA were performed as described under 'Methods.'

insulin (Fig 6), confirming the tendency of VLDL to increase the shorter transcript alone, also in the presence of an agent inducing the stabilization of the longer one. The combination of VLDL and insulin also enhanced PAI-1 protein synthesis more than either agent alone. In fact, PAI-1 levels were increased twofold and fivefold by VLDL and insulin, respectively, but sixfold by the combination (Fig 6).

We then investigated whether the VLDL-induced increase in PAI-1 mRNA was the result of transcriptional or posttranscriptional mechanisms. The transcriptional activity of the PAI-1 gene was evaluated in run-on experiments performed with nuclei isolated from HepG2 cells incubated with VLDL (100 μ g protein per milliliter) for various times. Nascent nuclear transcripts were elongated in the presence of [α -³²P]UTP and hybridized. VLDL apparently did not increase the transcription rate of the PAI-1 gene at any time point (Fig 7); nor did VLDL affect transcription of the GAPDH

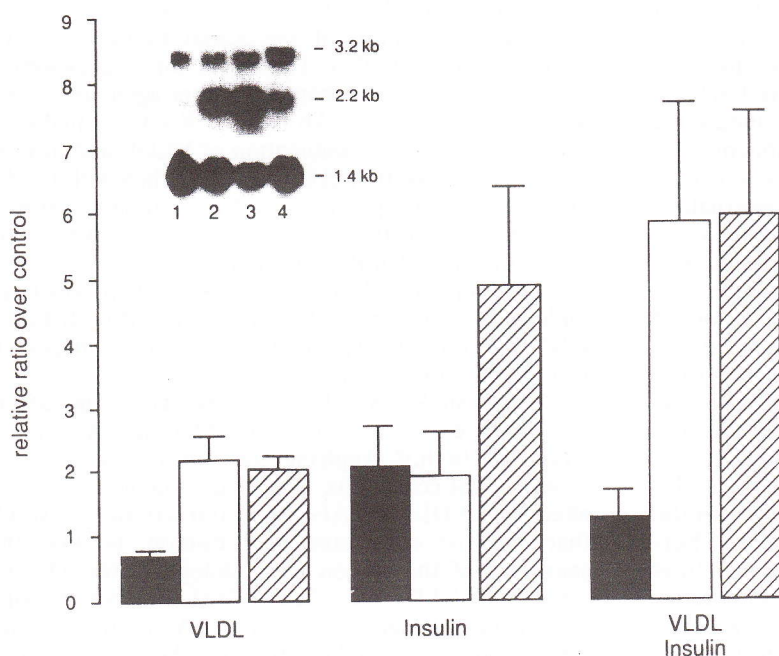


FIG 6. Effect of insulin on PAI-1 in VLDL-treated HepG2 cells, which were incubated in serum-free medium for 16 hours with 100 μ g protein per milliliter VLDL in the absence or presence of insulin (135 nmol/L). Total RNA was extracted and analyzed by Northern blotting (10 μ g of total RNA per lane) followed by hybridization with human PAI-1 cDNA and GAPDH cDNA probes. In the inset is shown a representative Northern blot of control (lane 1), VLDL (lane 2), VLDL plus insulin (lane 3), and insulin (lane 4). The top two bands are 3.2- and 2.2-kb PAI-1 mRNA, and the bottom band is 1.4-kb GAPDH mRNA. PAI-1 mRNA levels (solid bars, 3.2 kb; open bars, 2.2 kb) were quantified by densitometry and normalized against the corresponding GAPDH signal. Levels of PAI-1 antigen (hatched bars) were quantified by ELISA in cell supernatants. The data are expressed as relative values over control and represent mean \pm SEM of four individual experiments.

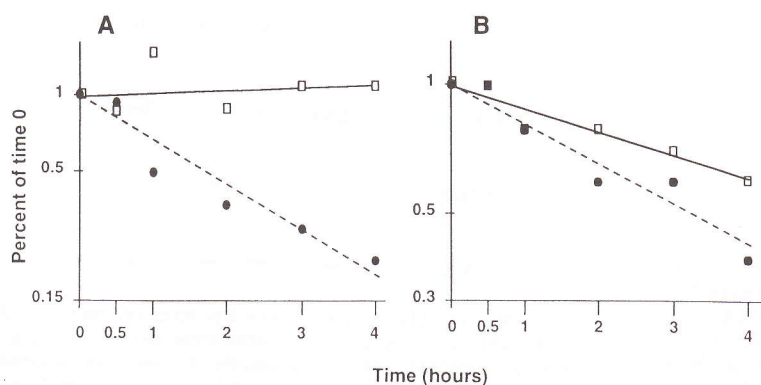


Fig 8. Effect of VLDL on PAI-1 mRNA half-life in HepG2 cells. HepG2 cells were incubated for 16 hours in the absence (●) or presence (□) of 100 μ g protein per milliliter VLDL. Then actinomycin D (10 μ g/mL) was added to the media, and total cellular RNA was harvested at selected time intervals and analyzed as described in 'Methods.' The amounts of PAI-1 mRNA are expressed as relative ratio over time 0. A representative time course of decay of 3.2-kb (A) and 2.2-kb (B) PAI-1 mRNA transcripts is shown.

gene, and no detectable signal was given by the pBR322 used as a negative control (Fig 7).

To determine whether modulation of PAI-1 mRNA levels by VLDL was due to an induced change in stability of PAI-1 mRNA, actinomycin D (10 μ g/mL) was added to cells previously kept for 16 hours in serum-free medium with or without 100 μ g protein per milliliter VLDL. Total cellular RNA was then harvested and analyzed at selected intervals. In control cells, the half-lives of the 3.2- and 2.2-kb PAI-1 mRNA species were, in agreement with previously published data,³⁵ 80 and 190 minutes for the 3.2- and 2.2-kb transcripts, respectively (Fig 8). After exposure of HepG2 cells to VLDL for 16 hours, the half-lives of both species were prolonged, with a marked stabilization of the 3.2-kb and a doubling of the half-life of the 2.2-kb species (Fig 8).

Discussion

In this study we show that VLDL, probably interacting with the LDL receptor on the surface of HepG2 cells, induces triglyceride accumulation, biosynthesis of PAI-1, and enhancement of steady state levels of PAI-1 mRNA.

Incubation of HepG2 cells with VLDL at concentrations like those in the plasma of normolipidemic subjects (10 to 100 μ g/mL) increases PAI-1 release into conditioned medium. This induction might have functional significance, since PAI activity increased in our experiments in parallel with PAI-1 concentration. Interestingly, the enhancing effect of VLDL on PAI-1 release was dependent on cell density, ie, VLDL induced a greater increase in subconfluent than in confluent cells. Since cell density has previously been shown to modulate LDL receptor expression and/or lipoprotein internalization,^{36,37} it is conceivable that the degree of interaction of the lipoprotein with the LDL receptor varies according to cell density, thereby explaining the difference in PAI-1 release; indeed, intracellular accumulation of triglycerides was highest in cells seeded at low density. VLDL interacts with HepG2 cells via both the LDL receptor and LDL receptor-related protein (LRP).³⁸ By using a specific monoclonal antibody (C7) directed against the first cysteine-rich repeat of the LDL receptor,³⁹ we showed that the effect of VLDL on PAI-1 induction depended on interaction specifically with the LDL receptor, since C7 does not recognize LRP. Furthermore, involvement of LRP in the phenomenon is unlikely, because this receptor recognizes chylomicrons or apoE-enriched VLDL (β -VLDL), whereas we used VLDL from normal subjects, which does not normally bind to LRP.⁴⁰ Recently another receptor for VLDL has

been described that binds and internalizes apoE-containing lipoproteins⁴¹; this receptor (VLDL receptor) has been described in skeletal muscle, heart, and kidney but not in hepatic cells.⁴¹

The interaction of VLDL with HepG2 cells doubled the steady state levels of PAI-1 mRNA. This effect is consistently observed with VLDL obtained from plasma of different subjects (n=13) and is dependent on the concentration of VLDL added to the incubation medium. In contrast, neither LDL nor HDL influenced PAI-1 antigen or PAI-1 mRNA levels.

VLDL mostly increased the 2.2-kb PAI-1 mRNA transcript, with a minor effect on the 3.2-kb PAI-1 mRNA. Thus, VLDL profoundly changed the ratio between the two mRNAs in favor of the more stable transcript. It is known that the 2.2-kb PAI-1 mRNA has a longer half-life than the 3.2-kb species because of the lack of the AU-rich sequence in the 3'-untranslated region.⁴²⁻⁴⁵ Phorbol myristate acetate induces the 2.2- and 3.2-kb transcripts at 12 and 3 hours, respectively, which effect decreases in both after 12 hours.⁴⁶ In contrast, VLDL specifically increases the 2.2-kb PAI-1 mRNA transcript in HepG2 cells after 9 to 12 hours of incubation, and this effect lasts for over 24 hours.

Insulin has been shown to induce overexpression of PAI-1 mRNA in HepG2 cells as a result of the increase in steady state levels of the 3.2-kb transcript.³⁴ The combination of VLDL and insulin resulted, as expected, in more PAI-1 release than with either agent alone, but whereas the level of 2.2-kb PAI-1 mRNA was higher in cells exposed to the combination of VLDL and insulin, that of the 3.2-kb transcript was lower than with insulin alone. These data provide a further, although indirect, demonstration of the predominant effect of VLDL on the shorter PAI-1 mRNA transcript.

VLDL added to HepG2 cells exposed to cycloheximide produced a marked accumulation of both PAI-1 mRNA transcripts, with ongoing protein synthesis affecting mostly the 3.2-kb species.

Steady state levels of PAI-1 can be affected by factors that increase gene transcription, PAI-1 mRNA accumulation, or both.¹⁷ Applying the run-on assay in our experimental conditions, we failed to demonstrate any effect of VLDL on PAI-1 gene transcription, a result that must be considered with caution, because the sensitivity of the run-on methodology is low. On the other hand, VLDL did influence PAI-1 mRNA stability, prolonging the half-lives of both PAI-1 mRNA transcripts. Increased stabilization of the 2.2-kb PAI-1 mRNA transcript would favor increased PAI-1 biosyn-

thesis; other mechanisms may be responsible for the unchanged levels of the 3.2-kb PAI-1 mRNA. For instance, VLDL may influence the termination process during PAI-1 mRNA transcription in favor of the shorter transcript. So far, no information is available on the mechanism(s) involved in the selection of one of the two poly(A) sites in the termination of PAI-1 gene transcription. Such selection appears to operate in higher primates, and it has been shown to be dependent on the presence of *cis*-acting sequences in the 3'-untranslated region in the human PAI-1 gene.⁴⁷ Agents that are known to induce PAI-1 mRNA accumulation have been shown to act either through an increase in gene transcription or through stabilization of PAI-1 mRNA, mostly of the 3.2-kb transcript. Thus, in addition to affecting PAI-1 mRNA stabilization, VLDLs are the first agonists so far described that may operate to influence selection of the poly(A) site.

In conclusion, the evidence indicates that PAI-1 mRNA can be regulated by triglyceride-rich lipoproteins at concentrations that occur in plasma of normolipidemic subjects. The capacity of VLDL to induce PAI-1 biosynthesis is specific to this class of lipoproteins and is mediated by the interaction of VLDL with the LDL receptor present on HepG2 cells. The induction of PAI-1 biosynthesis involves at least two major mechanisms, namely PAI-1 mRNA stabilization and a hitherto undescribed change in the selection of the two poly(A) sites in the termination of PAI-1 gene transcription in favor of the shorter 2.2-kb transcript. Even if the physiological role of the two PAI-1 mRNA transcripts has not yet been elucidated, the increase in the level of the more stable mRNA may be expected to have physiological significance in terms of increased PAI-1 biosynthesis. These mechanisms are also operative in the presence of other agents acting on PAI-1 mRNA stabilization, such as insulin.

The effect of VLDL on PAI-1 biosynthesis may be relevant not only in genetic or diet-induced hypertriglyceridemia but also in pathological conditions such as type II diabetes and more generally in the polymetabolic syndrome (syndrome X), conditions characterized by insulin resistance and/or elevated insulin levels as well as by alterations in lipid metabolism leading to elevated triglyceride levels.

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