Linoleic acid enhances the secretion of plasminogen activator inhibitor type 1 by HepG2 cells

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Abstract This study was undertaken in order to assess whether triglycerides and/or their fatty acids directly influence the secretion of plasminogen activator inhibitor type 1 (PAI-1) in HepG2 cells. To this end, subconfluent HepG2 cells were incubated with triglyceride-rich particles (TGRP) isolated from Intralipid® for 16 h, and PAI-1 levels were determined in conditioned medium using a specific ELISA. TGRP (1 to 6 mg triglycerides/ml) concentration-dependently increased PAI-1 secretion by cells, concomitantly with significant increases in intracellular triglyceride (TG) levels. Fatty acid analysis indicated that the incubation of cells with 3 mg of TG per ml of TGRP induced significant accumulation of 18:2 n–6 (linoleic acid, LA) and 18:3 n–3 (linolenic acid), reflecting the fatty acid composition of the added triglycerides. We then tested the comparative effects on PAI-1 secretion by HepG2 cells of LA and 18:1 n–9 (oleic acid, OA). LA, as a bovine serum albumin (BSA) complex, concentration-dependently (1 to 35 μmol/L) increased the secretion of PAI-1 by cells, whereas OA-BSA only minimally affected it at the highest concentration used (35 μmol/L). Incorporation of LA into cell pools, in the presence of increasing concentration of the FA in the medium, was studied by the use of a preparation containing [^{14}C]LA. LA accumulated in all lipid classes including diacylglycerol, the incorporated LA being converted into arachidonic acid (AA) as assessed by HPLC radiochromatography of the fatty acid methyl esters. It is concluded that PAI-1 secretion in HepG2 cells is modulated by triacylglycerols and by linoleic acid and/or its metabolic products.—**Banfi, C., P. Risé, L. Mussoni, C. Galli, and E. Tremoli.** Linoleic acid enhances the secretion of plasminogen activator inhibitor type 1 by HepG2 cells. *J. Lipid. Res.* 1997. **38:** 860–869.

Supplementary key words PAI-1 secretion • urokinase-type plasminogen activator • fibrinolysis • triglycerides • free fatty acid • cultured cells • cellular lipid composition

The fibrinolytic system is responsible for dissolving the fibrin network on the surface of intravascular thrombi. Fibrinolysis is initiated by the release of plasminogen activators, namely tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA), which convert plasminogen into plasmin (1). The activity of the fibrinolytic system is controlled by two homologous proteins, namely α_2-antiplasmin, which inactivates plasmin, and plasminogen activator inhibitor type 1 (PAI-1) (2, 3). PAI-1 is a 50 kD glycoprotein that forms equimolar complexes with both t-PA and u-PA that are inactive. PAI-1 is an essential regulatory protein of the fibrinolytic system, as indicated by the association of PAI-1 deficiency with hemorrhagic diathesis (4). In addition, transgenic mice overexpressing the human PAI-1 protein exhibit fibrin- and platelet-rich venous occlusions (5).

Plasma PAI-1 levels are elevated in patients with venous thrombosis and in young survivors of myocardial infarction (6–8), in whom they are predictive of reinfarction (9). Moreover, a nested case-control analysis of 60 atherosclerotic patients at higher risk of thrombosis who suffered from coronary cerebral and/or peripheral ischemic events during the first year of follow-up showed impaired fibrinolytic activity, secondary to PAI-1 elevation, in the PLAT study (10). Indeed, several established risk factors for coronary heart disease are associated with altered fibrinolytic function and, specifically, with elevated serum levels of PAI-1 (11). A positive correlation between plasma levels of triglyceride-rich lipoproteins and PAI-1 has been described by several authors including our group (11–14). In vitro studies in cultured endothelial cells have shown that very low density lipoproteins (VLDL) enhance PAI-1 protein formation (12, 15). Moreover, studies by our group (16) demonstrated that incubation of HepG2 cells with 100 μg protein/ml VLDL isolated from normotriglyceridemic subjects induced intracellular accumulation of lipids, mostly of triacylglycerol, and this was associated with en-

Abbreviations: PAI-1, plasminogen activator inhibitor type 1; u-PA, urokinase-type plasminogen activator; VLDL, very low density lipoprotein; TG, triglyceride; PL, phospholipid; FA, fatty acid; AA, arachidonic acid; TL, total lipid; TGRP, triglyceride-rich particles; LA, linoleic acid; OA, oleic acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; UI, unsaturation index; BSA, bovine serum albumin; TLC, thin-layer chromatography; HPLC, high pressure liquid chromatography.

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hanced PAI-1 secretion. These in vitro findings provide a potential explanation for the elevated plasma PAI-1 levels in hypertriglyceridemia and suggest an additional pathogenic role for VLDL in atherothrombosis.

The activation of PAI-1 biosynthesis by VLDL involves the interaction of this lipoprotein with the apoB/E receptor, cellular uptake of lipoprotein, and intracellular accumulation of lipids (16). However, the relationships between intracellular triacylglycerol and PAI-1 biosynthesis have not been yet investigated.

In this study we have evaluated the effects of apolipoprotein-free triglyceride-rich particles (TGRP) on the secretion of PAI-1 by HepG2 cells. These particles were extracted from Intralipid® and have been shown to substitute for VLDL in neutral lipid-exchange processes (17). Because incubation of cells with TGRP induced accumulation of linoleic acid (18:2 n–6) in all cellular lipid classes, including free fatty acids, we then evaluated the effects of this polyunsaturated fatty acid on PAI-1 secretion, in contrast to those exerted by the monounsaturated fatty acid oleic acid (18:1 n–9).

MATERIALS AND METHODS

Cell cultures

HepG2 cells were cultured in minimal essential medium (MEM, Gibco, Life Technologies) supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco) containing 2 mmol/L L-glutamine, 100 U/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% CO_2 at 37°C. The cell line was proved free of mycoplasma (Mycoplasma detection kit, Boehringer Mannheim GmgH). The cells received fresh complete medium every 3 days and were passaged 1:5 on a 4-day cycle.

Preparation of triglyceride-rich particles (TGRP)

TGRP were isolated from Intralipid 20% (Kabi-Vitrum, Stockholm, Sweden) after removal of multilamellar phospholipid (PL) structures (18). Briefly, 2 ml of Intralipid 20% was transferred into SW41 polyallomer tubes and overlayered with KBr–NaCl–Na_2 EDTA (d 1.006 g/ml). The tubes were centrifuged for 15 min at 25,000 rpm in a Beckman SW41 Ti rotor and the lipid "cake" at the top was collected and resuspended in KBr–NaCl–Na_2 EDTA (d 1.006). This procedure was repeated twice in order to remove most of the residual PL. The isolated TGRP had a TG/PL ratio of 175:1 compared with a ratio of 47:1 in the starting preparation, indicating that the bulk of the PL not associated with TG had been removed. Freshly prepared TGRP was incubated at different TG concentrations with cells.

Preparation of fatty acid–BSA complexes (FA-BSA)

FA-BSA complexes were prepared essentially according to the method of Spector and Hoak (19). Briefly, 25 mg of FA (18:2 n–6, linoleic acid or 18:1 n–9, oleic acid, Sigma) was dissolved in 7.5 ml hexane and 800 mg celite was added. After mixing, the solvent was removed under N_2, samples were vacuum-desiccated, and BSA free of fatty acid (25 ml of 0.25 mmol/L, Sigma) was added. The mixture was stirred for 1 h at room temperature with constant N_2 passing over the surface. After centrifugation at 800 *g* for 5 min the supernatants were carefully decanted. The FA concentration in FA-BSA complexes (linoleic acid-BSA, LA-BSA; oleic acid-BSA, OA-BSA) was assessed by gas chromatography analysis of the methyl esters, using an internal standard (nonadecanoic acid, 19:0). Samples containing FA-BSA complexes were then filtered through 0.45-μm filters in sterile test tubes and stored at −20°C until used.

In specific experiments, preparations of LA containing 0.1 μCi [^{14}C]linoleic acid ([^{14}C]LA) (DuPont NEN) in the LA-BSA complexes at final concentrations of 0.5, 10, or 35 μmol/L of LA-BSA were used. The radiochemical purity of the FA was assessed by HPLC.

Preparation of phosphatidylcholine liposomes

To 3 mg of chromatographically pure egg phosphatidylcholine (Sigma), dried in a glass tube under N_2 atmosphere and lyophilized at room temperature for 1 h, 0.25 ml of saline (0.9%) was added. The samples were left 1 h at room temperature and sonicated for a total of 40 min in a bath sonicator in order to complete the swelling process. The liposomes were then sterilized by passage through a 0.45-μm filter and used within 24 h (17) in amounts based on the PL content determined by an enzymatic method (Boehringer Mannheim GmgH Diagnostica IC).

Experimental system

HepG2 cells were plated at a density of 5×10^5 cells in 750-mm^2 tissue culture flasks (Costar, Cambridge, MA) or 3×10^4 in 12-well (40-mm^2) cluster plates (Costar) and used at subconfluence, attained within 4–5 days. Cells were then kept in serum-free medium for 24 h and, after washing three times with PBS, were incubated in serum-free medium with the specific lipid preparations. 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. Cell layers were washed in PBS and utilized for lipid analysis.

Cell microscopy

For light microscopy examination, HepG2 cells were cultured on cover glasses in complete medium for 4 days, re-fed with serum-free medium for 24 h, and then incubated with TGRP for 16 h in serum-free medium. The cover glasses were then washed with PBS, fixed for 1 h with 4% formaldehyde in PBS, and then immersed in 60% isopropanol for less then 1 min. Cells were then stained with 1% Oil Red O (Sigma) in 60% isopropanol for 1 h at 4°C, rinsed in PBS, mounted with glycerol, and processed for light microscopy evaluation (20).

Quantitation of PAI-1 and urokinase-type plasminogen activator (u-PA) antigen

The concentrations of PAI-1 in conditioned medium were assayed with specific ELISA (F1-5 Monozyme, Copenhagen, Denmark). The assay, based on the double-antibody principle, allows for the detection of total PAI-1 antigen. u-PA antigen was measured by an ELISA based on two murine monoclonal antibodies (Imubind, American Diagnostica inc.). The possibility of interference of different stimulatory agents with the assay system was excluded using medium containing different concentrations of stimuli as control.

Assays of fibrinolytic inhibitory activity

Fibrinolytic activity was assayed on ^{125}I-labeled fibrin-coated 24-multiwell tissue culture dishes containing: 0.1% gelatin, 0.1 mol/L Tris-HCl, pH 8.1, 5% Triton X-100 (assay buffer), 4 μg/ml human plasminogen, and u-PA (WHO, London, UK) (21). The fibrinolytic inhibitor activity of SDS-treated samples was determined as previously described (22). Briefly, different volumes of inhibitor-containing samples (0 to 300 μl) were added to 0.5-ml tubes containing 0.1 mol/L Tris-HCl, pH 8.1, 5% Triton X-100 (final volume 400 μl/tube). u-PA (0.03 lU, final concentration) was added to each tube, the samples were mixed, incubated for 15 min at 37°C, and aliquots of 430 μl from each tube were added to ^{125}I-labeled fibrin coated wells containing 470 μl of assay buffer, Plasminogen was added and samples were incubated at 37°C. The rate of fibrinolytic activity was determined by measuring the amount of radioactivity released after different times in an aliquot of reaction mixture removed from the surface of the dish. One arbitrary unit of inhibitory activity (AU) was defined as the amount of fraction required for 50% reduction of urokinase-mediated fibrinolytic activity. The inhibitory assay was terminated when the untreated urokinase controls hydrolyzed 25–30% of the total radioactivity.

Determination of triglyceride (TG) contents

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 cells by a hexane–isopropanol method (23). Briefly, 2 ml of hexane–isopropanol mixture 3:2 (vol/vol) was added to cell layers for 30 min at room temperature, and the solvent was transferred to a glass tube and dried under N_2. Triglycerides were determined enzymatically using a commercial kit (F. Hoffmann-La Roche Ltd, Basel) after dissolving the lipids in isopropanol. Cell proteins in the residue obtained after lipid extraction were dissolved in NaOH (0.1 mol/L) and determined according to Bradford (24). Data were expressed as μg lipids/mg protein.

Lipid extraction

After appropriate stimuli the layers of HepG2 cells were washed with PBS, scraped with a cell scraper, collected into glass tubes, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and cell lipids were extracted with chloroform–methanol 2:1 (Merck D6100 Darmstadt, Germany), containing 5 μg/ml of the antioxidant butylated hydroxytoluene (BHT), according to the method of Folch, Lees, and Sloane Stanley (25), using an Ultra Turrax (T-25 IKA, Staufen, Germany), for homogenization. To the extract was added one-fifth the volume of 0.88% KCl and after thorough mixing the organic and aqueous phases were allowed to separate at −20°C for at least 2 h. The lipid extract was evaporated to dryness under a stream of N_2 and subsequently dissolved in a given volume of chloroform–methanol 2:1 containing BHT. The lipid content in an aliquot of the extract was determined by the use of a Microbalance (C-31, Cahn Instruments, Cerritos, CA) (26) after solvent evaporation.

Lipid analysis

Cholesteryl esters, triglycerides, total phospholipids, free fatty acids, and diacylglycerol in the extracts were separated by thin-layer chromatography (TLC). Silica gel plates were prepared in the laboratory with a spreader using Silica-gel 60 HR to give a final thickness of 300 μm. The solvent system used was hexane–diethyl-ether–acetic acid 70:30:1.5 (vol/vol/vol). Lipids were detected on plates dried under N_2 after brief exposure to iodine vapor and the spots were scraped and collected into vials for radioactivity measurement or fatty acid analysis.

FA analysis

FA methyl esters were prepared from total lipid extracts, from Intralipid 20% or directly from silica-gel spots scraped off plates by transesterification with 3 mol/L methanolic-HCl (Supelco, Bellefonte, PA). Methyl esters were analyzed on a Dani 8510 (Monza, Italy) gas chromatograph, equipped with a flame ioniza-

tion detector, using chemically bonded Supelco omegawax™ 320 (Supelco, Bellefonte, PA) column (30 m, 0.32 mm I.D., 0.25 μm film) and temperature programming (130–220°C). Peaks were identified by using pure reference compounds, and percentage distribution of FA methyl esters was assessed by peak interpolation using a Shimadzu C-R 6A (Kyoto, Japan) recording integrator. FA were quantified by the use of an internal standard (19:0) and calibration curves were obtained with reference compounds.

Analysis of FA-associated radioactivity

The determination of the radioactivity incorporated in individual FAs was carried out by HPLC connected to a radiodetector. The FA methyl esters were separated by a two-solvent system at a flow rate of 1 ml/min: solvent A acetonitrile and solvent B water, according to Moore et al. (27). The column was a LiChrospher 100, RP-18 (5 μm); the HPLC a Jasco Model 880-PU (Japan Spectroscopic, Tokyo 192, Japan). Radioactivity associated with FA methyl esters was detected by using an online radiodetector (Flo-one beta A 200, Radiomatic Instruments Chemicals, A Camberra Company, Tampa, FL) equipped with a 500-μl flow cell, using Flo-Scint A as scintillation fluid.

Accumulation of radioactivity in lipid classes and in individual fatty acids

The amount of FA incorporated into lipid classes at different concentrations of the substrate (0.5, 10, and 35 μmol/L) was calculated on the basis of the specific activity of the substrate and the amount of radioactivity associated with the lipid classes. The amount of arachidonic acid (AA) produced from [^{14}C]linoleic acid was calculated from the specific activity of the incubated precursor and the proportion of radioactivity associated with the AA, separated by HPLC.

RESULTS

Effects of TGRP on PAI-1 biosynthesis and on triglyceride accumulation by HepG2 cells

HepG2 cells were cultured for 16 h in medium alone or in a medium containing 3 mg of TG per ml of TGRP. Staining of cells with Oil Red O consistently showed a larger number of lipid droplets with TGRP than with medium alone (**Fig. 1**). Intracellular triacylglycerol levels increased in proportion to the concentrations of TGRP added to the medium and this effect was accompanied by enhanced secretion of PAI-1 antigen into the medium (**Fig. 2**). Time-course experiments indicated that TGRP increased PAI-1 secretion only after 9 h incubation (not shown). Mean increases in PAI-1 antigen secretion were directly correlated with the mean increments in intracellular triacylglycerol ($r = 0.94$, $P < 0.001$). The increase of PAI-1 secretion was accompanied by increased inhibition of total fibrinolytic activity (5 and 16.7 AU/ml in samples of cells incubated with medium alone or medium containing 3 mg/ml TGRP, respectively).

PAI-1 in cultured cells is present not only as secreted protein, but also as protein associated with the cells and the extracellular matrix (28). No differences in the PAI-1 antigen level of cell extracts or in the extracellular matrix were observed after treatment with TGRP or medium.

Under basal conditions, u-PA antigen in the supernatants of HepG2 cells was below the detection limit of the assay, and did not become detectable after TGRP treatment.

The commercial preparation of Intralipid® contains multilamellar PL in addition to triacylglycerols. Although PL were removed from TGRP by ultracentrifugation (TG:PL ratio 175:1), some effect of the small residual amount of PL on PAI-1 secretion could not be ruled out. Appropriate experiments in which HepG2 cells were incubated for 16 h with 50–200 μg of PL per ml of phosphatidylcholine-enriched vesicles allowed us to exclude this possibility (data not shown).

Effect of TGRP on the fatty acid composition of HepG2 cells

The increased triacylglycerol levels in TGRP-treated cells indicated that lipids were taken up from TGRP-enriched medium. The fatty acid composition of TGRP, reflecting that of the original Intralipid®, was characterized by a high content of polyunsaturates (61%), mainly as 18:2, followed by monounsaturates (22%) and saturates (17%) (**Table 1**). The incubation of cells with 3 mg of TG per ml of TGRP for 16 h induced significant increases in the unsaturation index and levels of PUFA, whereas levels of monounsaturated or saturated fatty acids were not modified (**Fig. 3**); the increases in 18:2 n–6 and 18:3 n–3 were statistically significant ($P < 0.001$ versus untreated cells) (**Table 2**). Up to 9 h of incubation, LA increased predominantly in triglycerides (90-fold) and free fatty acids (8-fold) (**Fig. 4**), slackening off between 9 and 16 h. In contrast, the LA content in phospholipids increased progressively up to 16 h, with a final content about 3 times as high (Fig. 4).

Effect of fatty acid complexed with BSA on PAI-1 secretion by HepG2 cells

To assess the potential role of cellular accumulation of fatty acids on PAI-1 secretion, we incubated HepG2 cells for 16 h with different concentrations of either LA

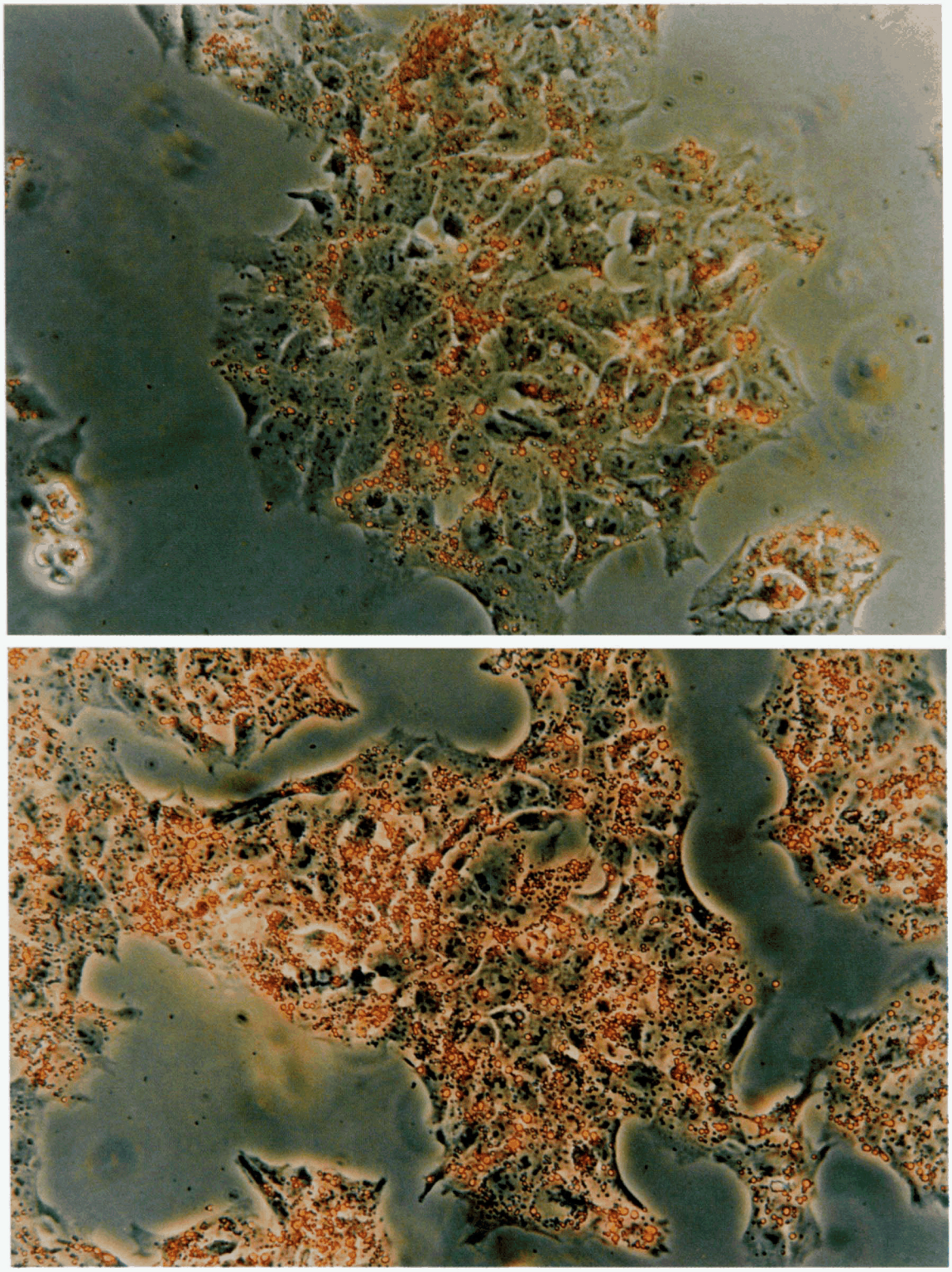

Fig. 1. Light micrographs of HepG2 cells incubated in the absence (upper panel) or in the presence (lower panel) of 3 mg of TG per ml of TGRP. The lipid droplets in the cytoplasm were stained with Oil Red O.

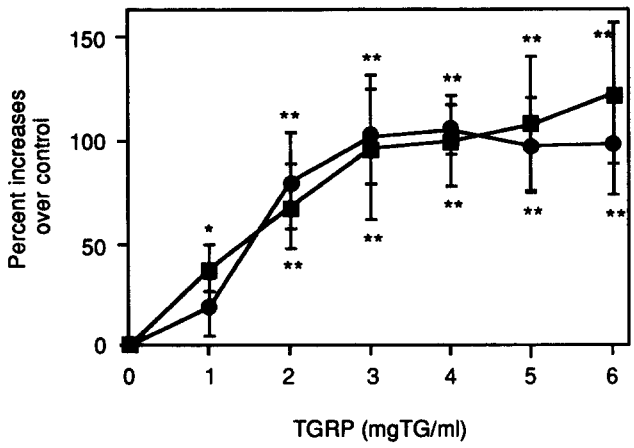

Fig. 2. Effect of TGRP on PAI-1 biosynthesis and on intracellular triacylglycerol accumulation in HepG2 cells. Subconfluent cells were incubated for 16 h with medium containing increasing concentrations of TGRP. The medium was then removed and cells were scraped off. PAI-1 antigen levels were determined in conditioned medium, and intracellular triglyceride levels were determined in lipid extracts of cells. Values are the mean ± SEM of four individual experiments performed in triplicate. Statistical analysis was performed by one-way ANOVA followed by Dunnett's test. $*P < 0.05$, $**P < 0.001$ (●, PAI-1 antigen; ■, triglycerides).

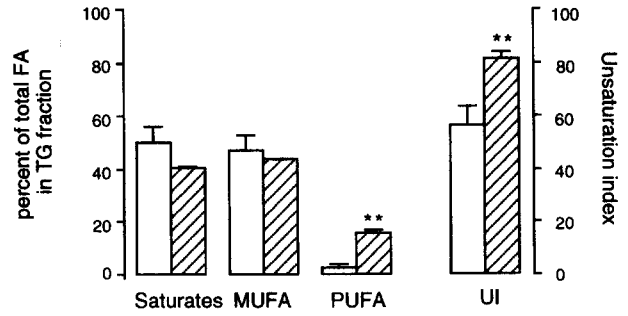

Fig. 3. Fatty acid composition of triglycerides in HepG2 cells incubated with TGRP. Subconfluent cells were incubated with medium containing 3 mg of TG per ml of TGRP. After 16 h the medium was removed, cells were scraped off and analyzed for FA composition. Means ± SEM of three individual experiments. Statistical analysis by unpaired Student's t test. $**P < 0.001$. MUFA: monounsaturates; PUFA: polyunsaturates; UI: unsaturation index. (□, control cells; ▨, TGRP-treated cells).

or OA complexed with BSA (LA-BSA or OA-BSA, respectively). LA-BSA (1–35 μmol/L) concentration-dependently increased PAI-1 secretion into conditioned medium. At the 25 and 35 μmol/L concentrations LA-BSA increased PAI-1 secretion by 31.5 and 44.1%, respectively ($P < 0.001$). In contrast, OA-BSA induced a 10–15% increase in PAI-1 secretion only at the highest concentration used (35 μmol/L) (**Fig. 5**).

Cells were then incubated for different times with [^{14}C]LA-BSA complexes (0.5, 10, and 35 μmol/L final concentration). A concentration-dependent accumulation of LA was detected in several lipid classes between 3 and 9 h in cells incubated with 10 and 35 μmol/L [^{14}C]LA-BSA, whereas at 16 h some reduction was observed in all lipids, except for PL in cells incubated with 35 μmol/L [^{14}C]LA-BSA (**Fig. 6,** panels A to D). At 3 h of incubation, LA increased in a concentration-dependent manner in total lipids (3.2, 108.1, and 311.1 nmol LA/mg total lipids, for cells incubated with 0.5, 10, and 35 μmol/L of [^{14}C]LA-BSA, respectively). At the lowest substrate concentration, the newly incorporated LA accounted for about 10% of the LA initially present with increasing concentrations of LA in the medium; total incorporation was linear but the rate of accumulation in PL declined when the concentration was greater than 10 μmol/L, and LA was preferentially incorporated into TG (**Fig. 7,** panel A). Accumulation in the other lipid classes remained linear up to the maxi-

TABLE 1. Fatty acid composition of TGRP

Fatty Acid	% of Total Fatty Acids
14:0	0.1 ± 0.02
16:0	12.2 ± 0.46
18:0	4.2 ± 0.08
20:0	0.3 ± 0.01
22:0	0.4 ± 0.01
24:0	0.1 ± 0.00
16:1 n–9	0.1 ± 0.00
18:1 n–9	21.5 ± 0.03
20:1 n–9	0.3 ± 0.00
24:1 n–9	0.1 ± 0.00
18:2 n–6	53.8 ± 0.38
20:4 n–6	0.1 ± 0.01
22:5 n–6	0.1 ± 0.00
18:3 n–3	6.34 ± 0.11
20:5 n–3	0.1 ± 0.00
22:5 n–3	0.1 ± 0.00
22:6 n–3	0.2 ± 0.01

Values are the means ± SEM of three individual experiments.

TABLE 2. Fatty acid composition of HepG2 cells before and after incubation with TGRP

Fatty acid	Controls	TGRP-Treated Cells
14:0	4.00 ± 0.63	3.53 ± 0.73
16:0	36.80 ± 2.91	29.74 ± 0.91
18:0	7.10 ± 0.73	5.67 ± 0.18
20:0	2.40 ± 0.25	1.50 ± 0.31
16:1 n–9	13.70 ± 1.61	10.08 ± 0.52
18:1 n–9	31.30 ± 5.01	32.33 ± 0.78
20:1 n–9	1.60 ± 0.14	1.07 ± 0.17
24:1 n–9	0.20 ± 0.02	0.19 ± 0.01
18:2 n–6	1.50 ± 0.20	13.05 ± 0.45[a]
20:4 n–6	0.50 ± 0.03	0.46 ± 0.05
18:3 n–3	0.00 ± 0.00	1.53 ± 0.03[a]
20:5 n–3	0.40 ± 0.04	0.45 ± 0.13
22:5 n–3	0.20 ± 0.03	0.20 ± 0.05
22:6 n–3	0.30 ± 0.11	0.30 ± 0.02

Values are the means ± SEM of three separate experiments.
[a] $P < 0.001$ (unpaired Student's t test).

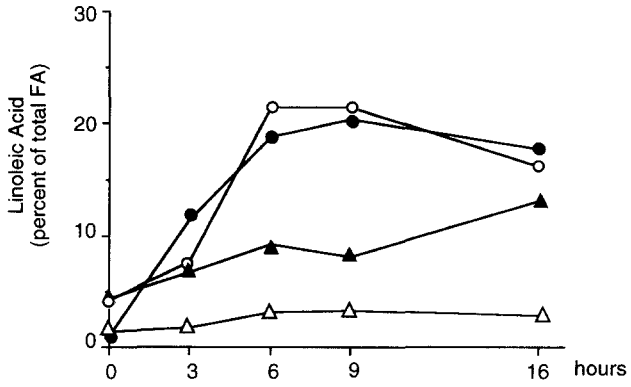

Fig. 4. Percentage of LA in the fatty acids of lipid fractions of HepG2 cells incubated with TGRP for different times. Subconfluent cells were incubated with medium supplemented with 3 mg of TG per ml of TGRP. At different times (0 to 16 h) the medium was removed, cells were scraped off, and lipids were extracted and analyzed for FA content. Data are from a representative experiment performed in duplicate and are expressed as increments in LA content in the different lipid fractions over time. (●, triglycerides; ○, free fatty acids; ▲, phospholipids; △, cholesteryl esters).

mal LA concentration, with some preference for incorporation into diacylglycerol (Fig. 7, panel B).

The incorporation of [^{14}C]LA-BSA was accompanied by its conversion to arachidonic acid (AA). The total lipids in untreated cells (data not shown) contained 30.3 ± 5.3 and 46.4 ± 3.3 nmol/mg of LA and AA, respectively, representing 2.1 ± 0.1% and 3.6 ± 0.2% of total fatty acids. Incubation with increasing concentrations of labeled LA for 16 h resulted in progressive increases in AA, from trace amounts at 0.5 μmol/L concentration to over 4.5% of the FA-associated radioactivity at 35 μmol/L, while the radioactivity associated with LA declined from over 94% to around 86% of the recovered radioactivity (**Fig. 8,** panel A). At the highest substrate concentration, AA was incorporated into cell lipids to the extent of 14.7 nmol/mg (Fig. 8, panel B).

DISCUSSION

This study shows that incorporation of linoleic acid (LA) into HepG2 cells increases the secretion of PAI-1 (antigen and activity) into the surrounding medium, thereby reducing the fibrinolytic potential of the cells. The study involved exposing the cells either to a linoleic acid-rich, apoprotein-free preparation of triglycerides (TGRP) or to LA bound to albumin.

HepG2 cells have been shown (29) to synthesize, in addition to PAI-1, plasminogen activator of the urokinase type (u-PA); however, this finding has not been confirmed (30). Under our experimental conditions only PAI-1 was detectable, both before and after lipid enrichment of cells.

In HepG2 cells treated with TGRP, maximal accumulation of LA occurred in triacylglycerol, the cells' storage lipids, and in free fatty acids, a metabolically labile pool mediating between FA uptake, triglyceride hydrolysis, and resynthesis of various glycerolipids. The fatty acid composition of treated cells began to reflect that of the TGRP, with steadily increasing LA content. Although appreciable amounts of 16:0 and 18:1 were also present in TGRP, the concentration of 16:0 in cells declined and that of 18:1 did not change, probably because glycerol esterifies saturated fatty acids only at position 1, and monounsaturates are displaced from

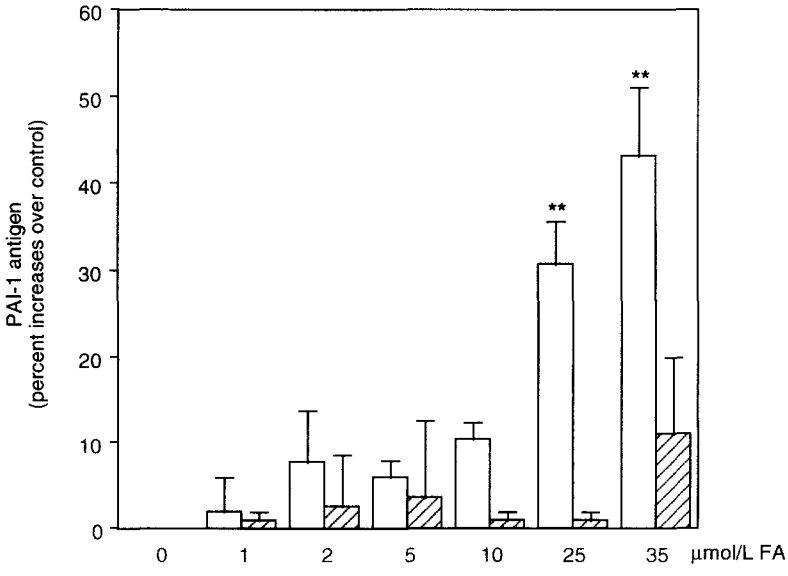

Fig. 5. Effect of 16 h incubation of LA-BSA or OA-BSA with HepG2 cells on PAI-1 secretion. Subconfluent cells were incubated with medium supplemented with different concentrations of each fatty acid complexed to BSA (1–35 μmol/L). After 16 h the conditioned medium was removed and analyzed for PAI-1 antigen determination. Data are the means ± SEM of five individual experiments performed in triplicate. $^{**}P < 0.001$ (□, LA-BSA; ▨, OA-BSA).

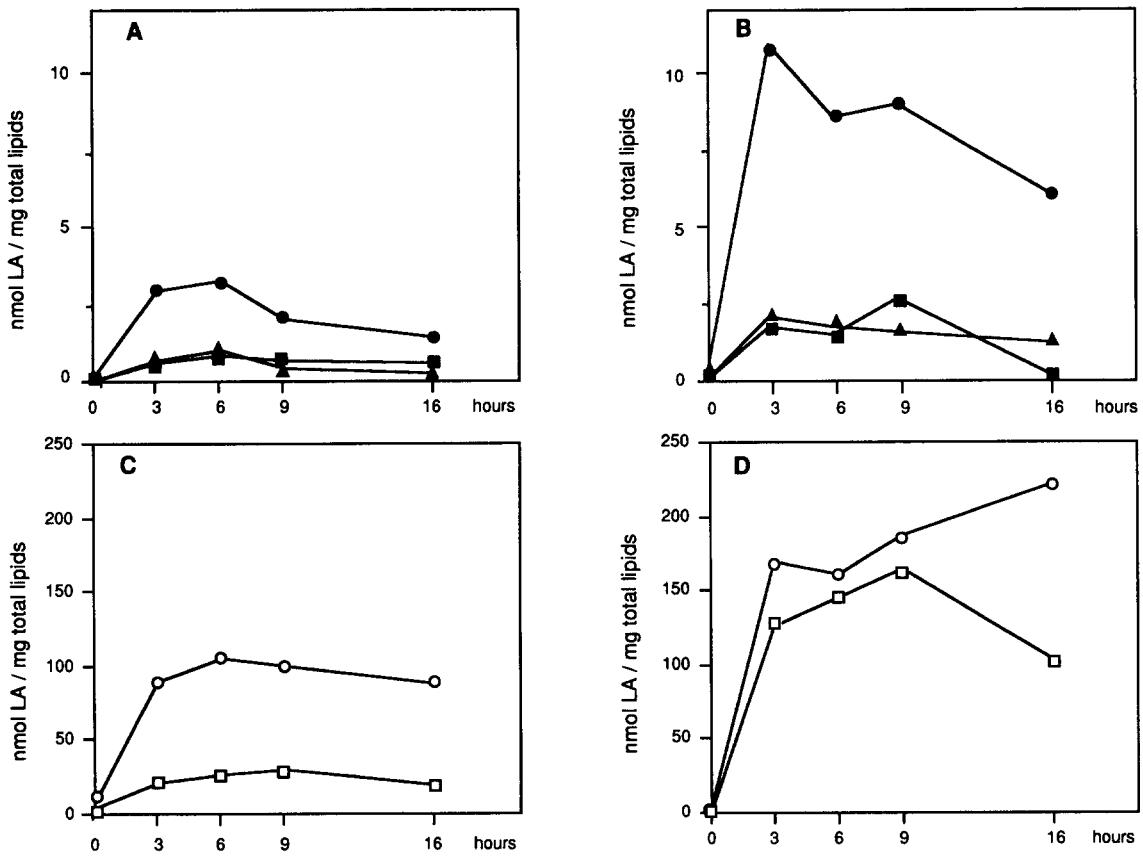

Fig. 6. Levels of [^{14}C]LA in lipid fractions of HepG2 cells incubated with different concentrations of [^{14}C]LA-BSA complexes. Subconfluent HepG2 cells were incubated for different times with medium supplemented with 10 or 35 μmol/L of [^{14}C]LA-BSA. Data are expressed as nanomoles of [^{14}C]LA in the different lipid pools. Panels A and C: [^{14}C]LA content in cells incubated with 10 μmol/L [^{14}C]LA-BSA. Panels B and D: [^{14}C]LA content in cells incubated with 35 μmol/L [^{14}C]LA-BSA. (■, cholesteryl esters; ▲, free fatty acids; ●, diglycerides; ○, phospholipids; □, triglycerides). Data are from a representative experiment performed in duplicate.

position 2 to position 1 by the preferential esterification of polyunsaturates at position 2.

Similarly, incubation of cells with LA bound to albumin resulted in LA uptake, at low concentrations mostly into phospholipids, but at higher concentration into free fatty acids (8 times the baseline content), diglycerides, and triglycerides (almost 100 times above baseline). The LA incorporated into HepG2 cells at the higher concentrations was in part converted to arachidonic acid (AA), indicative of the amount of time spent in the free fatty acid state, the only form that may undergo chain extension and increased unsaturation.

Incubation of LA with HepG2 cells, either as triglyceride or complexed to albumin, induced PAI-1 secretion from the cells; oleic acid complexed to albumin did not. This is consistent with the observation (31) that other polyunsaturated fatty acids, dihomo-γ-linolenic acid or docosahexaenoic acid, but only to a lesser extent oleic acid, up-regulate PAI-1 mRNA expression in endothelial cells. A fatty acid-responsive regulatory element in the PAI-1 gene has been identified and it has been hypothesized that fatty acids may induce PAI-1 transcription directly (31). It has been shown that oxidized low density lipoproteins induce PAI-1 biosynthesis by endothelial cells concomitantly with activation of phospholipase A2, which hydrolyses arachidonic acid (AA) from phospholipids, thus suggesting a role for AA in PAI-1 biosynthesis (32). As in our study about 4% of the incorporated LA was converted to AA when high concentrations of LA were incubated with the cells, we cannot exclude the possibility that AA or its metabolites contributes to the effect on PAI-1 secretion.

Two major mechanisms may be involved in the enhanced PAI-1 secretion observed in HepG2 cells enriched with LA, namely a direct effect by the accumulated FA on fatty acid-responsive regulatory elements present on the PAI-1 gene or activation of protein kinase C and/or other second messengers. Indeed, long chain polyunsaturated fatty acids have been shown to directly activate this signal pathway (33) and PAI-1 biosynthesis is known to involve activation of protein kinase C (34, 35).

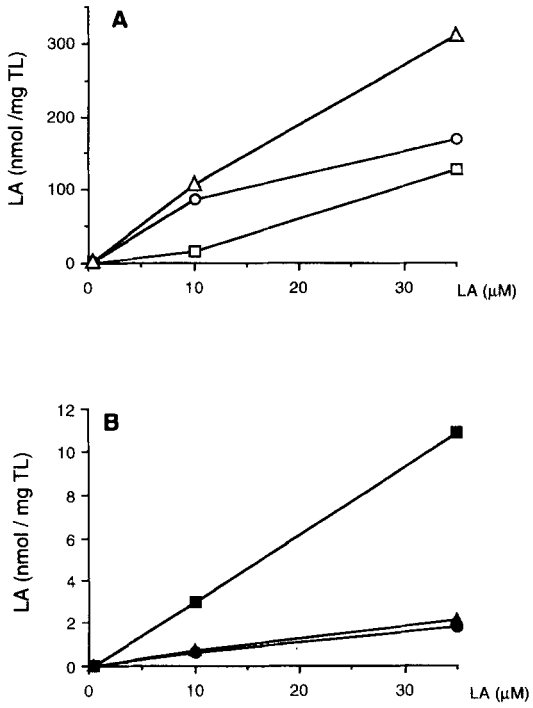

Fig. 7. Levels of [^{14}C]LA in different lipids from HepG2 cells incubated with 10 or 35 μmol/L [^{14}C]LA-BSA for 3 h. Experimental conditions as in Fig. 6. Panel A, [^{14}C]LA content in total lipids (△), phospholipids (○), and triglycerides (□); in panel B are represented the [^{14}C]LA content of diglycerides (■), free fatty acids (▲), and cholesteryl esters (●).

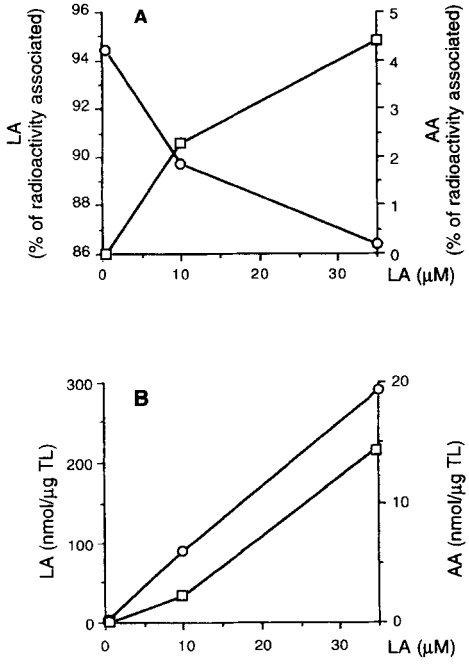

Fig. 8. Levels of [^{14}C]LA and [^{14}C]AA (arachidonic acid) in lipid classes from HepG2 cells incubated with 0.5, 10, and 35 μmol/L [^{14}C]LA-BSA for 3 h (○, LA; □, AA).

In conclusion, our data demonstrating lipid accumulation in HepG2 cells after exposure to triglycerides rich in LA or to LA itself, and the induction of PAI-1 in the cells, may explain, at least in part, the mechanism(s) for the induction of PAI-1 by VLDL and reveal yet another potential effect of circulating VLDL: diminution of the fibrinolytic capacity in HepG2 cells.

The extrapolation of these data to an in vivo situation should, however, take into consideration the fact that there is controversy as to whether PAI-1 biosynthesis and regulation are the same in human hepatocytes as in transformed cell lines such as HepG2 (36, 37). However, our preliminary data clearly show that VLDL also increase PAI-1 biosynthesis in hepatocytes freshly isolated from human hepatic tissue.

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