Uptake of hypertriglyceridemic very low density lipoproteins and their remnants by HepG2 cells: the role of lipoprotein lipase, hepatic triglyceride lipase, and cell surface proteoglycans

Murray W. Huff,[1],* David B. Miller,* Bernard M. Wolfe,* Philip W. Connelly,[†] and Cynthia G. Sawyez*

Departments of Medicine and Biochemistry and the Robarts Research Institute,* The University of Western Ontario, London, Ontario, Canada N6A 5K8, and The J. Alick Little Lipid Research Laboratory, St. Michael's Hospital,[†] University of Toronto, Toronto, Ontario, Canada M5S 1A9

Abstract Hypertriglyceridemic very low density lipoproteins (HTG-VLDL, S_f 60–400) are not taken up by HepG2 cells. However, addition of bovine milk lipoprotein lipase (LPL) at physiological concentrations markedly stimulates uptake. In the present study, we determined whether: a) LPL catalytic activity is required for uptake, b) LPL functions as a ligand, and c) cell surface hepatic triglyceride lipase (HL) and/or proteoglycans are involved. Incubation of HepG2 cells with HTG-VLDL plus LPL (8 ng/ml) increased cellular cholesteryl ester (CE) 3.5-fold and triglyceride (TG) 6-fold. Heat-inactivation of LPL abolished the effect. Addition of tetrahydrolipstatin (THL, an LPL active-site inhibitor) to HTG-VLDL + LPL, inhibited the cellular increase in both CE and TG by greater than 90%. Co-incubation of HTG-VLDL + LPL with heparin, heparinase, or heparitinase, blocked CE accumulation by 70%, 48%, and 95%, respectively, but had no effect on the increase in cellular TG. Pre-treatment of cells with 1 mM 4-methylumbelliferyl-β-D-xyloside, (β-xyloside) to reduce cell surface proteoglycans inhibited the increase in CE induced by HTG-VLDL + LPL by 78%. HTG-VLDL remnants, prepared in vitro and isolated free of LPL activity, stimulated HepG2 cell CE 2.8-fold in the absence of added LPL, a process inhibited with THL by 66%. Addition of LPL (8 ng/ml) to remnants did not further enhance CE accumulation. HepG2 cell HL activity, released by heparin, was inhibited 95% by THL. The amount of HL activity and immunoreactive mass, released by heparin, was reduced 50–60% in β-xyloside-treated cells.█ These results indicate that physiological concentrations of LPL promote HepG2 cell uptake of HTG-VLDL primarily due to remnant formation and that LPL does not play a major role as a ligand. HL activity and cell surface proteoglycans significantly enhance the subsequent uptake of VLDL remnants.—**Huff, M .W., D. B. Miller, B. M. Wolfe, P. W. Connelly, and C. G. Sawyez.** Uptake of hypertriglyceridemic very low density lipoproteins and their remnants by HepG2 cells: the role of lipoprotein lipase, hepatic triglyceride lipase, and cell surface proteoglycans. *J. Lipid Res.* 1997. **38:** 1318–1333.

Supplementary key words hypertriglyceridemia • apolipoprotein E • hepatocytes • tetrahydrolipstatin • β-xyloside

Very low density lipoproteins (VLDL) are macromolecular complexes composed of a neutral lipid core of triglyceride and cholesteryl ester and contain apolipoproteins (apo) C, E, and B-100. These lipoproteins are synthesized and secreted by the liver into plasma where they interact with lipoprotein lipase (LPL), an enzyme present on endothelial cell surfaces that hydrolyzes triglyceride. The resulting remnant is smaller, enriched in cholesteryl ester, and has an altered apoprotein composition. Further lipolysis converts VLDL remnants into intermediate density lipoproteins (IDL) and subsequently low density lipoproteins (LDL) (1).

Plasma concentrations of VLDL are elevated in subjects with Type IV hypertriglyceridemia. In contrast to normolipidemic VLDL, Type IV hypertriglyceridemic VLDL (HTG-VLDL) is characterized by substantial metabolic heterogeneity, with abnormal aspects being primarily confined to the larger S_f 60–400 lipoprotein particles (2–7). In these subjects, S_f 60–400 VLDL concentrations are elevated due to an overproduction together with a reduced fractional catabolic rate (4–7). Some S_f 60–400 HTG-VLDL is cleared directly, however, the majority is converted to IDL (S_f 12–60) (6, 7). These remnant lipoproteins are almost quantitatively cleared from the circulation (6) by the liver. The mech-

anisms of how HTG-VLDL and their remnants may be taken up by hepatocytes are not completely understood (1). ApoE, a major apolipoprotein constituent of these particles, acts as a ligand for clearance of VLDL remnants by hepatic receptors (8). There is evidence that the LDL receptor, the LDL receptor-related protein (LRP), and/or uncharacterized receptor(s) are involved in hepatic uptake of VLDL remnants (9–16).

In previous studies, we found that VLDL from hypertriglyceridemic subjects (HTG-VLDL) was not taken up by cultured HepG2 cells unless co-incubated with bovine milk lipoprotein lipase (LPL) (17). Uptake resulted in cellular accumulation of cholesteryl ester and triglycerides and, in addition, stimulated intracellular acyl-CoA:cholesterol acyltransferase. These studies indicated that lipolytic remodelling of HTG-VLDL by LPL is a prerequisite for their recognition by hepatic uptake processes. However, it is possible that LPL itself could mediate remnant uptake. It has been proposed that LPL may provide a high-affinity recognition site for the hepatic clearance of lipoproteins (10, 18). LPL can act as a ligand for the LRP (10) and enhance binding of chylomicrons, normal VLDL, LDL, Lp[a], and rabbit β-VLDL to fibroblasts, macrophages, and HepG2 cells (10, 19–29).

It has been reported that LPL-mediated binding of lipoproteins to the LRP is dependent on an intact C-terminal domain (residues 313–348) and the dimeric structure of LPL (20, 21, 27, 30), indicating that the C-terminal domain functions both in binding to lipoproteins and the LRP (20, 27). Choi et al. (31) demonstrated a specific interaction of LPL with the amino-terminal region of apoB of VLDL and LDL. The concept that LPL may act as a ligand facilitating remnant clearance has gained support from the finding that LPL in pre-heparin plasma is associated with lipoproteins (32) and a significant amount of this LPL is associated with triglyceride-rich lipoproteins and their remnants in hypertriglyceridemic subjects (33, 34). Consistent with these in vitro observations, we have demonstrated that in Type IV subjects, an infusion of heparin to release LPL into plasma resulted in a marked reduction in the concentration of S_f 60–400 lipoproteins. The remnants were rapidly cleared from plasma and were not recovered in the IDL or LDL fraction (35). It is not known whether the released LPL enhanced particle clearance by increasing the rate of remnant formation or whether it also acted as a ligand, thereby enhancing hepatic clearance. Importantly, it is not known whether the small amount of LPL associated with triglyceride-rich lipoproteins and their remnants in pre-heparin plasma is capable of functioning as a ligand or bridge to the cell surface.

The interpretation of cell studies, which suggest that LPL functions as a ligand mediating lipoprotein uptake, is not clear. Although the binding of lipoproteins at 4°C was enhanced 30- to 200-fold by LPL (10, 19–28), uptake and degradation were only increased 2- to 6-fold (19, 21–25, 28). In addition, in most studies, high concentrations of LPL were used: 100 to 2000 ng/ml of media (10, 19–28), concentrations significantly higher than the those reported to occur in pre-heparin plasma. Vilella et al. (33) have reported that the concentration of LPL in preheparin plasma is approximately 70 ng/ml and only about 10–15% is associated with triglyceride-rich lipoproteins. Postprandially, plasma LPL mass associated with triglyceride-rich lipoproteins and their remnants increased approximately 2-fold (33, 34). In addition, the major form of LPL in pre-heparin plasma is monomeric whereas the dimeric form is required to enhance binding to the LRP and mediate cell uptake (20, 30).

It has not been firmly established whether the enhanced uptake by LPL requires catalytically active enzyme. It has been shown that chylomicron binding to HepG2 cells is enhanced 30- to 40-fold by LPL and that lipolysis is not required to observe the effect (10). LPL either in its native form, heat inactivated, or treated with partial catalytic inhibitors enhanced both the binding and internalization of normal human ^{125}I-labeled VLDL in fibroblasts (22, 25) and rabbit ^{125}I-labeled β-VLDL in Hep3B cells (30). Whether LPL-enhanced uptake affects intracellular cholesterol or triglyceride metabolism has not been determined. In recently reported perfused rat liver studies, active LPL produced a marked stimulation of hepatic chylomicron clearance, however, a small component of uptake does not appear to require catalytic activity (36).

Hepatic triglyceride lipase (HL) is synthesized by hepatocytes and is located on the surface of hepatocytes and hepatic endothelial cells (37). It plays a role in the catabolism of remnants of both chylomicrons and VLDL (38–40) by hydrolyzing these remnants, thereby preparing them for hepatocyte uptake. HL deficiency is characterized by an accumulation in plasma of remnant lipoproteins (38, 39). Infusion of anti-HL antibodies into animals delays remnant lipoprotein catabolism (37, 41–43). Hydrolysis of rat chylomicron remnants by HL increases the exposure of apoE (44). Rat hepatoma cells (McA-RH7777) transfected with human HL demonstrated enhanced binding of rabbit β-VLDL compared to non-transfected cells (45). However, a role for hepatocyte cell surface HL, under basal conditions, in the cellular uptake of human HTG-VLDL has not been clearly established.

There is increasing evidence that the uptake of remnants of triglyceride-rich lipoproteins by hepatocytes involve cell surface proteoglycans. Several groups of investigators have reported that treatment of HepG2 cells

TABLE 1. Plasma and lipoprotein lipid concentrations of VLDL donors

	Cholesterol			Triglyceride	
Total	VLDL	LDL	HDL	Total	VLDL
mmol/L				*mmol/L*	
6.92 ± 0.72	2.19 ± 0.49	3.75 ± 0.51	0.99 ± 0.08	4.19 ± 0.79	3.23 ± 0.75

Values are the mean ± SE in mmol/L from twelve subjects with Type IV hypertriglyceridemia. VLDL, S_f > 20, was prepared by ultracentrifugation. HDL cholesterol was determined in the infranatant after ultracentrifugation and precipitation of the apoB-containing lipoproteins with dextran sulfate–$MgCl_2$. LDL cholesterol was calculated by difference.

(25) and normal and FH-fibroblasts (19, 21, 22) with heparinase to deplete cell surface heparan sulfate proteoglycans (HSPGs) inhibited the enhanced binding and internalization of normal human VLDL or LDL mediated by LPL. However, no effect of diminished cell surface HSPGs on VLDL or LDL uptake in the absence of LPL was observed. Ji et al. (46, 47) have demonstrated that addition of excess apoE to β-VLDL from cholesterol-fed rabbits enhanced its uptake by cultured hepatocytes, a process involving cell surface HSPGs. The role of HSPGs in hepatocyte interaction with HTG-VLDL or their remnants is unknown.

The present studies, carried out in HepG2 cells, were designed to determine whether: *1*) LPL, at physiological concentrations, enhances the uptake of human S_f 60–400 HTG-VLDL and HTG-VLDL remnants, beyond its catalytic function, *2*) HL participates in HTG-VLDL remnant uptake, and *3*) cell surface proteoglycans are involved in the uptake of HTG-VLDL remnants without the addition of exogenous LPL or apoE.

EXPERIMENTAL PROCEDURES

Subjects

The lipid and lipoprotein profiles of the patients used in this study are shown in **Table 1.** The Type IV hypertriglyceridemic subjects were classified according to the criterion of Schaefer and Levy (48) after visits to the Outpatient Endocrinology Clinic at University Hospital, London, Ontario, Canada. These subjects all presented with primary hypertriglyceridemia and none displayed fasting chylomicronemia or had a known metabolic disorder such as hypothyroidism, morbid obesity, or renal dysfunction. In addition, none of the subjects used in these experiments were being treated with lipid-lowering agents. The normolipidemic LDL samples were obtained from healthy laboratory personnel. Prior to blood sampling all subjects were required to fast for 12 h. These studies were approved by the University of Western Ontario Health Sciences Standing Committee on Human Research and all subjects gave informed consent.

Lipoprotein isolation

Lipoproteins were isolated from plasma essentially as described previously (17). VLDL (S_f 60–400) was isolated by ultracentrifugation through buffer A (1.006 g/ml density solution containing 0.195 M NaCl, 1 mM Tris, pH 7.4, 1 mM EDTA, 10 μM phenylmethane sulfonyl fluoride, 3 mM NaN_3, 0.10 mM merthiolate) in a Beckman 60 Ti rotor (Beckman Instruments, Mississauga, Ontario) for 2 h at 40,000 rpm at 12°C using a Beckman L8 ultracentrifuge. The S_f 60–400 VLDL fraction was washed through an equal volume of buffer A in a Beckman 70.1 Ti rotor spun at 40,000 rpm, 12°C for 16 h. LDL was isolated from the infranatant, after removal of VLDL and IDL, by ultracentrifugation for 16 h at 50,000 rpm in a 60 Ti rotor in buffer A at d 1.063 g/ml. The LDL was washed in d 1.063 g/ml density solution in a 70.1 Ti rotor spun at 50,000 rpm, 12°C for 16 h. Lipoprotein-deficient serum (LPDS) was isolated as described previously (17).

All lipoprotein samples were analyzed for protein content by a modification of the Lowry method (49) and for total cholesterol and triglyceride using diagnostic kits from Boehringer Mannheim Canada, Laval, Quebec (CHOD-PAP and triglycerides without free glycerol, respectively). All lipoprotein samples were stored at 4°C and used for tissue culture experiments within 1 week of isolation. ApoE phenotypes were determined on all VLDL samples used in these studies by analytical isoelectric focusing gel electrophoresis as described previously (4). The lipid compositions of VLDL and LDL samples used in these studies are listed in **Table 2.**

HepG2 cells

HepG2 cells were cultured in 100-mm culture dishes (Falcon, Fisher Scientific, Ottawa, Ontario) in 10 ml of modified Eagle's media (MEM) with Earle's salts containing; 0.20% sodium bicarbonate, 1 mM sodium pyruvate, 0.3 mg/ml L-glutamine, 10% fetal bovine serum

TABLE 2. Composition of lipoproteins

Lipoprotein	n	C/TG	C/Protein	TG/Protein	FFA/Protein
Type IV VLDL[a]	12	0.14 ± 0.03	0.97 ± 0.16	6.40 ± 0.51	ND
Type IV VLDL					
Control	6	0.16 ± 0.06	1.45 ± 0.28	8.38 ± 0.86	0.008 ± 0.001
Hydrolyzed in vitro[b]	6	0.32 ± 0.06[c]	1.25 ± 0.26[c]	5.14 ± 0.76[c]	0.164 ± 0.037[c]
LDL	5	7.93 ± 2.42	1.63 ± 0.42	0.30 ± 0.10	ND

Values are expressed as means ± SD; C, cholesterol; TG, triglyceride; FFA, free fatty acid; ND, not determined. Ratios are weight ratios.

[a]VLDL S_f 60–400.

[b]VLDL S_f 60–400 from six Type IV hyperlipoproteinemic subjects were incubated with bovine milk lipase (1 mg VLDL cholesterol/unit enzyme) for 3 h and the hydrolyzed lipoproteins were reisolated by ultracentrifugation as described in Experimental Procedures.

[c]$P < 0.05$ compared to control.

(FBS) (ICN Biomedical Inc., Mississauga, Ontario), 10 IU/ml of penicillin, 10 µg/ml streptomycin, and 0.25 µg/ml Fungizone (ICN). Cells were split (1:3) using trypsin-EDTA (ICN) on a 7-day cycle up to a maximum of 10 passages. For all of the experiments HepG2 cells were plated in 24-well (15 mm) culture plates (Falcon) in 1 ml of MEM containing 10% FBS and grown for 5 days. Once the monolayers had become approximately 80% confluent, the media were replaced with MEM containing 5% LPDS for 24 h prior to experiments. The appropriate concentrations of lipoproteins were added to duplicate dishes of cells and were incubated for 5 h (oleate incorporation studies) or 16 h (mass determinations) unless otherwise indicated. The amount of cell protein per dish averaged 0.3 mg.

Heparin, heparinase, heparitinase, and β-xyloside treatment

Heparin, 10 µg/ml of media (Calbiochem, La Jolla CA), heparinase, 4 IU/ml (Sigma, heparinase I, catalogue number H-2519) or heparitinase, 4 IU/ml (Sigma, heparinase III, catalogue number H-8891) were added to cells 4 h prior to incubations. The media were changed and heparin, heparinase, and heparitinase were added at the same concentrations to media containing 5% LPDS at the same time as lipoproteins with or without LPL. Addition of the enzymes prevented regeneration of cell surface HSPGs during the subsequent incubations of cells with lipoproteins and LPL (28). β-Xyloside (4-methylumbelliferyl-β-D-xyloside, Sigma) was incubated with cells at a concentration of 1 mM for 4 days prior to and during the addition of lipoproteins with or without LPL (50).

Determination of cellular lipid content and cholesterol esterification

In the present studies we have used increases in the intracellular mass of cholesteryl ester as well as the incorporation of oleate into cholesteryl esters as measures

of HTG-VLDL lipid uptake. The interpretation of cellular uptake and degradation studies of iodinated triglyceride-rich lipoproteins is complicated by the dissociable nature of the highly labeled C apoproteins, especially under lipolytic conditions. In addition, uptake and degradation studies do not reveal the intracellular consequences on lipid metabolism of particle uptake.

The determination of cellular free and esterified cholesterol and triglyceride mass was essentially as described previously (17). Upon completion of the experiment, cells were washed twice in buffer B (0.15 M NaCl, 50 mM Tris, pH 7.4, 0.2% fatty acid-free bovine serum albumin, BSA) and 2 additional washes with buffer B without BSA. Lipids were extracted in situ using two 30-min incubations with 1.0 ml of hexanes–isopropanol 3:2 (v/v) and the residual cell protein was determined (49) after digestion in 0.1 N NaOH. Lipids were separated by thin-layer chromatography and eluted from the silica gel. The cholesteryl esters were determined after saponification as their trimethylsialyl ether derivatives by gas–liquid chromatography. Triglycerides were measured colorimetrically. The cellular lipids measured represent intracellular lipids, as addition of 50 µg/ml of heparin to the wash buffer did not affect the results obtained (17). [^{14}C]cholesterol, [^{3}H]cholesteryl oleate, and tri[^{14}C]oleoylglycerol (Amersham, Oakville, Ontario) were used to assess recovery. The incorporation of [1-^{14}C]oleic acid into cellular cholesteryl esters and triglycerides was determined as described previously (17).

HepG2 cell hepatic triglyceride lipase, apolipoproteins E and A-I

Confluent monolayers were incubated with MEM containing 5% LPDS in the presence or absence of 10 µg/ml of heparin for 16 h. The conditioned media were collected in sterile tubes and stored at −80°C until assayed. The cells were washed 3 times with buffer B without BSA and the cell protein was determined. The

activity of HL in the conditioned media was determined using a stable triolein–gum arabic emulsion as a substrate, prepared as described previously (17). Duplicate 100-µl aliquots of media were added to the incubation buffer (1 M NaCl, 0.4 M Tris, 0.05 g/ml fatty acid-free BSA, pH 8.9) and 100 µl of the substrate. Samples were incubated for 1 h at 37°C and the release of oleic acid was determined as described previously (17). HL activity was expressed in units/mg of cell protein, where one unit of activity was defined as 1 nmole of free fatty acid released per ml of media per h. Apolipoproteins E and A-I in media were assayed using modifications of standard ELISA techniques as described previously (17).

Bovine milk lipoprotein lipase

Bovine skim milk LPL was prepared as described previously (17). The enzyme was in the dimeric form as assessed by elution from heparin-Sepharose (20). In most experiments involving the addition of bovine milk LPL, the enzyme was added at 0.25 units of activity (1 unit is defined as 1 µmole of free fatty acid released per h) per ml of media containing 5% LPDS and lipoproteins as described previously (17). This activity corresponded to 8 ng of LPL/ml of media. Control dishes contained media and enzyme in the absence of lipoproteins. The amount of milk LPL added to the media of HepG2 cells was identical to the amount of LPL activity measured in media conditioned by J774 macrophages (51). Also, this amount approximates the LPL associated with triglyceride-rich lipoproteins and their remnants in pre-heparin plasma (33). In one experiment, a range of LPL concentrations, from 0 to 80 ng/ml, were studied. Heat-inactivated LPL was prepared by incubation of the enzyme for 30 min at 56°C in 2 M NaCl. This preparation was devoid of lipolytic activity as assessed using a $[^{14}C]$triolein–gum arabic emulsion, serum-activated assay described previously (17). In some experiments, the lipase inhibitor tetrahydrolipstatin (THL, Orlistat, Hoffman La Roche) was dissolved in dimethylsulfoxide (DMSO), diluted in media, and added to cells such that the final concentration of DMSO was 2 µl/ml of media. Based on initial experiments in which a range of THL concentrations was used (0.05 to 0.5 µM), subsequent experiments were performed with THL concentrations of 0.5 µM. In the absence of cells, THL inhibited the activity of LPL (8 ng/ml) by 75% at 0.1 µM and 96% at 0.5 µM assessed by measuring free fatty acid released from HTG-VLDL into the media, as described below.

Remnants of HTG-VLDL (hydrolyzed VLDL) were prepared by incubating S_f 60–400 VLDL (1 mg of lipoprotein cholesterol) with 1 unit of LPL in 1 ml of MEM media containing 10% LPDS and 3% albumin (w/w), at 37°C for 3 h. The density was then immediately raised

to d 1.019 g/ml with buffer A at d 1.085 g/ml. These isolation conditions remove LPL from the VLDL surface (52). The extent of VLDL lipolysis was determined by measuring the free fatty acids released, compared to the initial triglyceride content of VLDL. The percent triglyceride hydrolysis was 50 ± 2.5%. Free fatty acid released and the amount of free fatty acid associated with the reisolated lipoprotein were determined using a kit from Wako Pure Chemicals (NEFA C ACS-ACOD, Immunocorp Sciences Inc., Montreal, Quebec). To determine the amount of LPL remaining with the isolated hydrolyzed VLDL, the preparation was diluted with media to 50 µg/ml of VLDL cholesterol and incubated at 37°C for 1 and 2 h in the absence of cells. Free fatty acid release was measured as described above. No further lipolysis was observed. Three hydrolyzed-VLDL preparations were assayed for the presence of LPL mass using a sandwich ELISA described by Cadelis et al. (53). No LPL mass was detected. A volume of 100 µl of hydrolyzed-VLDL was assayed and the limit of detection was 10 ng/well. Typically, 10 µl of remnant/ml media was added to cells (5 µg VLDL cholesterol/µl). Therefore, if present, no more than 0.5 ng of LPL/ml of media would have been added to cells with the hydrolyzed-VLDL preparation.

Detection of media HL by immunoblotting

Media were obtained from HepG2 cells treated with β-xyloside and incubated in the absence of lipoproteins with or without heparin (10 µg/ml). The proteins were separated by electrophoresis on 10% polyacrylamide (2.7% bisacrylamide) gels containing sodium dodecyl sulfate (SDS-PAGE) on a Bio-Rad (Bio-Rad Canada, Mississauga, Ontario) Mini-Protean II apparatus for 1 h at 200 V. Proteins were then transferred to polyvinylidene difluoride (PVDF) membrane (Millipore Immobilon-P, Millipore Canada, Mississauga, Ontario) at 4°C overnight using a buffer of 0.025 M Tris, 0.192 M glycine, 20% methanol, pH 8.3. Hepatic lipase was detected by immunoblot using the Aurora Western Blot chemiluminescence kit (ICN Canada, Mississauga, Ontario). The PVDF membrane was blocked by incubation for 1 h at room temperature with 0.2% Aurora Blocking reagent in PBS, pH 7.4, 0.1% Tween-20. Immunoaffinity-purified primary antibody (10 µl in 10 ml of blocking buffer) was incubated for 1 h with agitation, followed by two 5-min washes with agitation with blocking buffer. The membrane was then incubated with agitation with goat anti-rabbit IgG (1 µl in 10 ml blocking buffer). The membrane was then washed three times (5 min each with blocking buffer) followed by two 5-min washes with Aurora Assay buffer. The membrane was incubated for 5 min at room temperature with 5 ml of chemiluminescent detection solution (Aurora), drained of excess

fluid, and exposed to Kodak XAR X-ray film for up to 5 min. Bands corresponding to HL were quantitated using the JAVA Video Analysis Software, Jandel Scientific. An anti-human hepatic lipase peptide antibody was prepared in New Zealand White rabbits by standard techniques. Briefly, a synthetic peptide corresponding to amino acids 466 to 477 of human hepatic lipase (EIKSK-TSKRKIR) (54) was coupled to ovalbumin using glutaraldehyde. Anti-peptide antibody was isolated by immunoaffinity chromatography using peptide-keyhole limpet hemocyanin conjugated to Bio-Rad Affi-Gel 10.

Statistical analyses

The data were analyzed using an unpaired Student's t-test.

RESULTS

LPL-enhanced HTG-VLDL and HTG-VLDL remnant uptake by HepG2 cells

The effect of HTG-VLDL (S_f 60–400) on the cholesteryl ester content of HepG2 cells is shown in **Fig. 1A.** Incubation of HTG-VLDL (50 μg/ml of VLDL cholesterol) for 16 h did not cause significant accumulation of cellular cholesteryl esters compared to cells not exposed to lipoproteins. Coincubation of HTG-VLDL with 8 ng/ml of bovine milk lipase resulted in a 3.5-fold increase in cellular cholesteryl ester compared to control cells or cells incubated with HTG-VLDL in the absence of LPL ($P < 0.005$). HTG-VLDL did not cause any cellular accumulation of triglyceride unless co-incubated with LPL (Fig. 1B). HTG-VLDL plus LPL increased cellular triglyceride 6-fold over control or incubations in the absence of LPL ($P < 0.005$). HTG-VLDL plus LPL enhanced the incorporation of [^{14}C]oleate into both cholesteryl ester and triglyceride in the same proportions as the increase in cellular mass of these lipids (data not shown).

To determine whether the LPL-enhanced uptake of HTG-VLDL was related to the extent of lipolysis, VLDL (S_f 60–400) that had been incubated with LPL for 3 h in the absence of cells and reisolated by ultracentrifugation was incubated with HepG2 cells. When identical amounts of lipoprotein cholesterol were added, hydrolyzed VLDL caused the same accumulation of cholesteryl esters as HTG-VLDL co-incubated with LPL (Fig. 1). The reisolated hydrolyzed VLDL did not contain any lipolytic activity, however, the preparation contained significantly more free fatty acid than did the pre-hydrolyzed VLDL (Table 2). These fatty acids comprised only 0.85% of the initial triglyceride present and
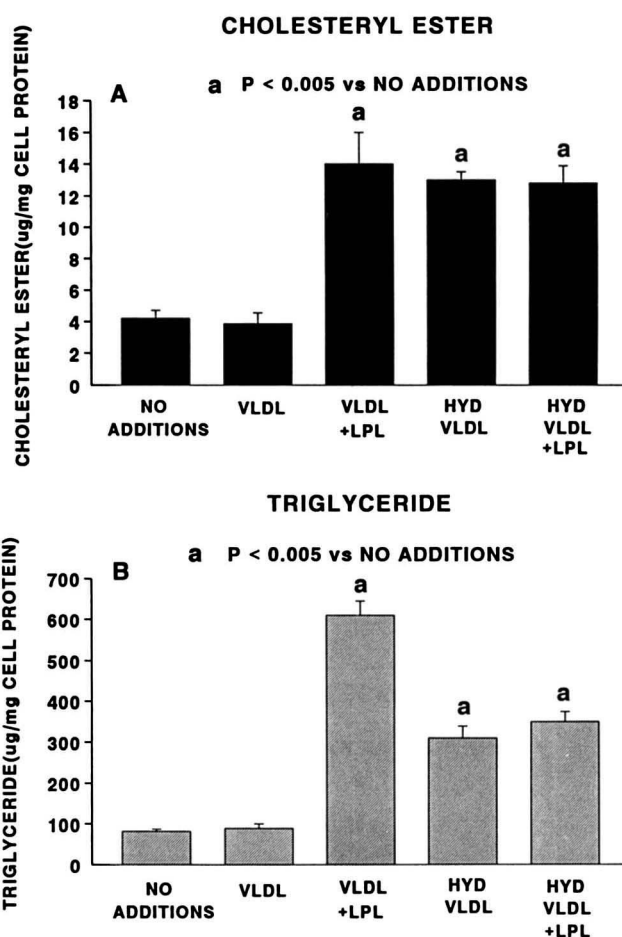

Fig. 1. Effect of lipoprotein lipase on the esterified cholesterol and triglyceride content of HepG2 cells incubated with Type IV VLDL (HTG-VLDL) S_f 60–400 or hydrolyzed HTG-VLDL. Fifty μg of HTG-VLDL (S_f 60–400) cholesterol/ml of media (n = 8) was incubated with HepG2 cells in the absence or presence of 0.25 units (8 ng) of bovine milk LPL/ml of media. Hydrolyzed (HYD) VLDL was prepared by incubating HTG-VLDL with bovine milk LPL (1 mg of VLDL cholesterol with 1 unit of LPL activity/ml of media) for 3 h and then reisolating by ultracentrifugation. Fifty μg of HYD VLDL cholesterol/ml (n = 6) was incubated with HepG2 cells. The cellular cholesteryl ester (A) and triglyceride (B) were determined as described in Experimental Procedures. Results are expressed as mean ± SE; n refers to the number of experiments for each condition.

totalled 8 μg/ml of media when added to cells with the hydrolyzed VLDL. Previously, we had established that addition of 325 μg of oleic acid, complexed to albumin, to HTG-VLDL in the absence of LPL would not stimulate cholesteryl ester accumulation or the incorporation of [^{14}C]oleate into cholesteryl ester (17). Similarly, addition of oleate/albumin to the hydrolyzed VLDL did not enhance cellular cholesteryl ester accumulation above that observed with hydrolyzed VLDL alone (data not shown). LPL (8 ng/ml of media) was added to hydrolyzed HTG-VLDL to determine whether cholesteryl ester accumulation would be enhanced. As shown in
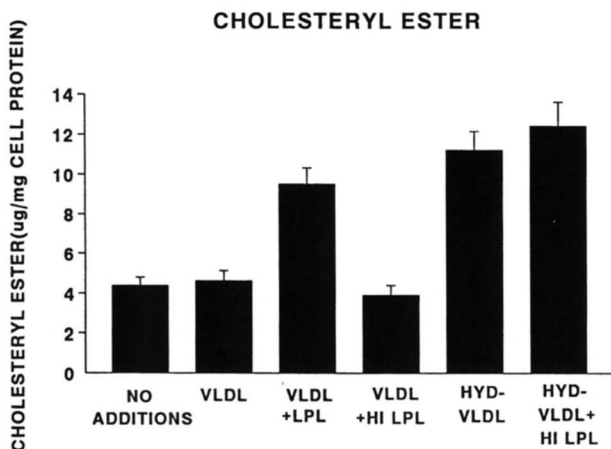
CHOLESTERYL ESTER

Fig. 2. Effect of heat-inactivated lipoprotein lipase on the esterified cholesterol content of HepG2 cells incubated with Type IV VLDL (HTG-VLDL) (S_f 60–400) or hydrolyzed HTG-VLDL. Fifty µg of HTG- VLDL (S_f 60–400) or hydrolyzed HTG-VLDL cholesterol/ml of media (n = 4) was incubated with HepG2 cells in the absence or presence of 8 ng of native or heat inactivated bovine milk LPL/ml of media. Heat-inactivated LPL (HI LPL) was prepared by incubation at 56°C for 30 min in 2 M NaCl as described in Experimental Procedures. Results are expressed as mean ± SE.

Fig. 1, no effect of added LPL was observed. The time-course curves for the accumulation of both cellular cholesteryl ester and triglyceride, from 2 to 16 h, were similar for hydrolyzed VLDL plus LPL, hydrolyzed VLDL alone, and VLDL plus LPL (data not shown).

Addition of 10-fold less LPL (0.8 ng/ml) to HTG-VLDL increased cellular cholesteryl ester content 1.2-fold over no additions. At 40 and 80 ng/ml, LPL produced increases of cellular cholesteryl ester of 1.4- and 1.7-fold, respectively, over the levels observed at 8 ng/ml of LPL. For hydrolyzed HTG-VLDL, no effect of added LPL was observed until 40 and 80 ng/ml of LPL were added. This produced increases of cholesteryl ester of 1.4- and 1.7- fold over 0, 0.8, and 8 ng/ml of LPL (data not shown).

Effect of heat-inactivated LPL and tetrahydrolipstatin on HTG-VLDL and remnant uptake

LPL was heat-inactivated by incubation for 30 min at 56°C, which completely inhibited its catalytic activity. When added to cells with HTG-VLDL, it was unable to stimulate cholesteryl ester accumulation over control cells (**Fig. 2**). Addition of heat-inactivated LPL to hydrolyzed VLDL did not enhance cellular cholesterol ester accumulation beyond that observed for hydrolyzed VLDL. Cellular triglyceride accumulation was also completely inhibited in the presence of the heat- inactivated enzyme (data not shown). As it is not known whether heat-inactivation of LPL preserves the conformation of the enzyme required for binding to cells, we used the active-site inhibitor THL to further investigate the role

of LPL catalytic activity in the uptake of HTG-VLDL by HepG2 cells. As shown in **Fig. 3,** co-incubation of HTG-VLDL with 8 ng/ml of LPL and 0.5 µM THL inhibited cholesteryl ester accumulation by 91%. THL also inhibited LPL-induced HTG-VLDL triglyceride accumulation by 95%. THL had no effect on the lack of HTG-VLDL uptake in the absence of LPL (not shown). THL alone had no effect on cellular cholesteryl esterification, cholesteryl ester or triglyceride concentrations (Fig. 3).

Effect of heat-inactivated LPL and tetrahydrolipstatin on LDL uptake

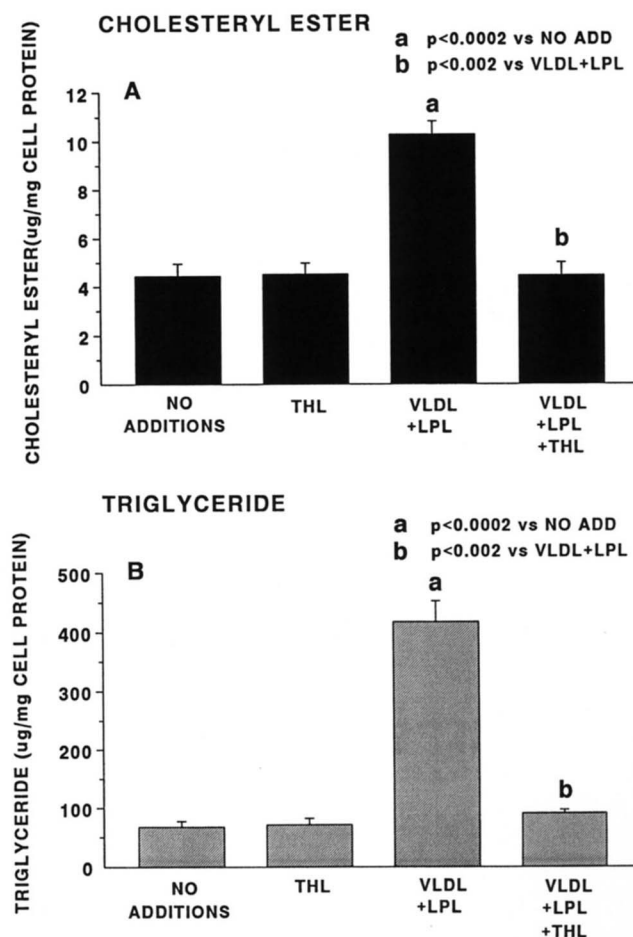In contrast to HTG-VLDL, normal human LDL (150 µg/ml of LDL cholesterol) resulted in a 2.5-fold in-

Fig. 3. The effect of tetrahydrolipstatin on the esterified cholesterol and triglyceride content of HepG2 cells incubated with Type IV VLDL (HTG-VLDL) (S_f 60–400) in the presence or absence of LPL. Fifty µg of HTG-VLDL (S_f 60–400) cholesterol/ml of media (n = 7) was incubated with HepG2 cells in the absence or presence of 0.25 units (8 ng) of bovine milk LPL/ml of media. Tetrahydrolipstatin (THL) dissolved in DMSO was added to cells (0.5 µM) at the same time as LPL as described in Experimental Procedures. A: cholesteryl ester; B: triglyceride. Results are expressed as the mean ± SE. Results for THL plus VLDL without LPL were the same as for No Additions and THL alone.

crease in cellular cholesteryl ester compared to control cells ($P < 0.005$) (**Fig. 4**). This was similar to the extent of cholesteryl ester accumulation observed with 50 µg/ml of HTG-VLDL cholesterol plus LPL. Coincubation of LPL (8 ng/ml) with normal LDL increased cellular cholesteryl ester 1.3-fold over that found with LDL in the absence of LPL ($P < 0.05$). Normal LDL increased cellular triglyceride only 1.4-fold over control reflecting the much lower triglyceride content of LDL (Table 2). LDL plus LPL increased cellular triglyceride 1.7-fold ($P < 0.05$). The modest enhancement of LDL-derived cholesteryl ester accumulation by LPL was partially blocked when the heat-inactivated enzyme was used. The addition of THL to LDL plus LPL completely
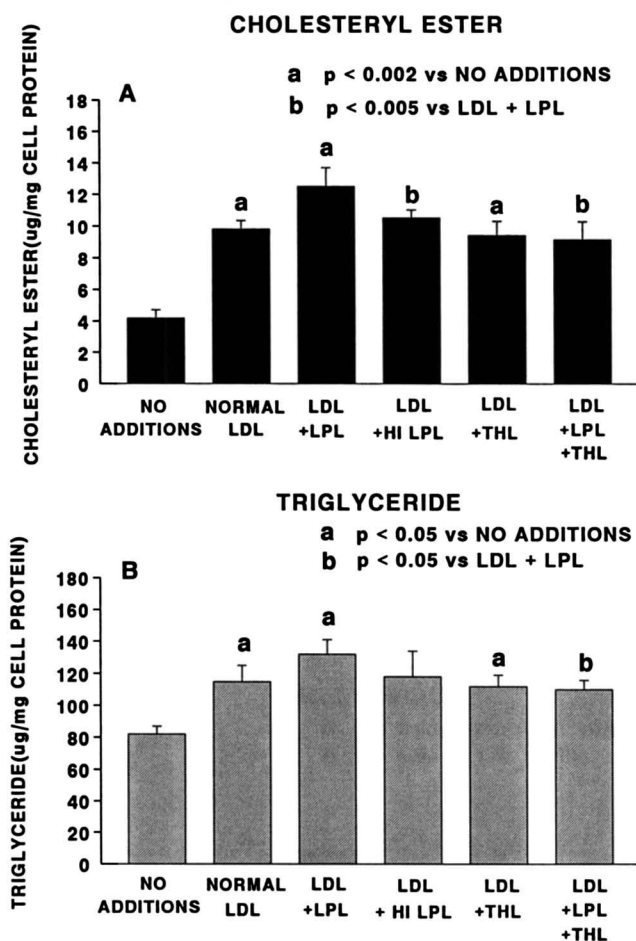

CHOLESTERYL ESTER

a   p < 0.002 vs NO ADDITIONS
b   p < 0.005 vs LDL + LPL

TRIGLYCERIDE

a   p < 0.05 vs NO ADDITIONS
b   p < 0.05 vs LDL + LPL

Fig. 4. Effect of lipoprotein lipase, heat- inactivated lipoprotein lipase, and tetrahydrolipstatin on the esterified cholesterol and triglyceride content of HepG2 cells incubated with normal LDL. One hundred fifty µg of HTG-VLDL (S$_f$ 60–400) cholesterol/ml of media (n = 5) was incubated with HepG2 cells in the absence or presence of 0.25 units (8 ng) of bovine milk LPL/ml of media. Heat- inactivated LPL (HI LPL) was prepared by incubation at 56°C for 30 min in 2 M NaCl. Tetrahydrolipstatin (THL) dissolved in DMSO was added to cells at the same time as LPL. The cellular cholesteryl ester (A) and triglyceride (B) were determined as described in Experimental Procedures. Results are expressed as mean ± SE; n refers to the number of experiments for each condition.

CHOLESTERYL ESTER

a   p<0.005 vs NO ADD'S
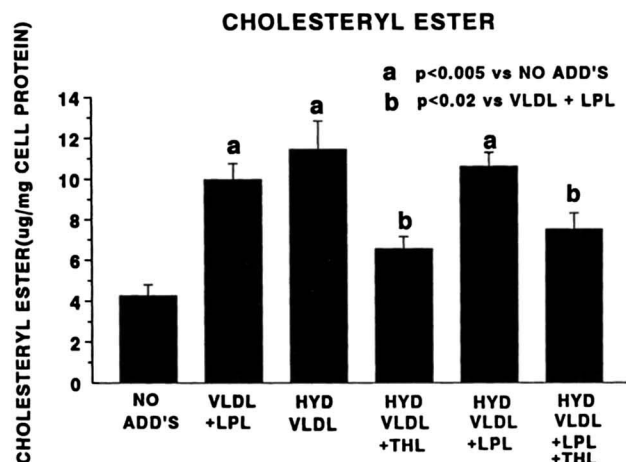b   p<0.02 vs VLDL + LPL

Fig. 5. Effect of lipoprotein lipase or lipoprotein lipase and tetrahydrolipstatin on the esterified cholesterol content of HepG2 cells incubated with Type IV VLDL (HTG-VLDL) (S$_f$ 60–400) or hydrolyzed HTG- VLDL. Fifty µg of HTG-VLDL (S$_f$ 60–400) (n = 6), or 50 µg of hydrolyzed VLDL (HYD VLDL) cholesterol/ml of media (n = 6) was incubated with HepG2 cells in the absence or presence of 0.25 units (8 ng) of bovine milk LPL/ml of media. Tetrahydrolipstatin (THL) dissolved in DMSO was added to cells at a concentration of 0.5 µM. Results are expressed as mean ± SE. NO ADD'S, no additions.

blocked the LPL-stimulated cellular cholesteryl ester accumulation. In the absence of LPL, THL had no effect on LDL-induced cellular cholesteryl ester accumulation (Fig. 4) or cholesterol esterification (data not shown) indicating that THL did not influence LDL-receptor-mediated endocytosis. Consistent with previous studies (55), these results indicate that the LPL-enhanced uptake of LDL was a result of lipolytic modification of LDL.

Inhibition of HepG2 cell HL by tetrahydrolipstatin

THL was added to hydrolyzed HTG-VLDL in the absence of LPL (**Fig. 5**). Unexpectedly, we observed that cholesteryl ester accumulation was inhibited by 66% ($P < 0.02$). Addition of THL to hydrolyzed HTG-VLDL in the presence of LPL inhibited cholesteryl ester accumulation by 50% ($P < 0.02$) (Fig. 5). The lack of effect of THL on LDL-induced CE accumulation (Fig. 4) suggests that the lipolytic activity inhibited by THL is specific for HTG-VLDL remnants.

As hydrolyzed HTG-VLDL did not have any active LPL associated with it, these experiments indicate that THL inhibited a lipase involved in HTG-VLDL remnant uptake other than LPL, most likely hepatic triglyceride lipase. As shown in **Table 3,** HepG2 cells secrete a significant amount of HL activity into the media over 16 h, an amount increased 5-fold by the addition of 10 µg/ml heparin. Addition of 0.5 µM THL to the HL assay inhibited activity by 91 ± 2%. In one experiment, HL was released from HepG2 cells with heparin and incubated with HTG-VLDL and hydrolyzed HTG-VLDL.

TABLE 3. Hepatic lipase activity, hepatic lipase immunoreactivity, apoE and apoA-I released by heparin from HepG2 cells with or without pretreatment with β-xyloside

Variable	Control Cells		β-Xyloside-Treated Cells	
	Medium	Heparin Releasable[a]	Medium	Heparin Releasable
	nmol FFA/h/mg cell protein			
Hepatic lipase activity	58.8 ± 5.8	272.0 ± 20.3[b]	48.8 ± 5.3	116.5 ± 16.5[c,d]
	area/mg cell protein			
Hepatic lipase immunoreactivity	225.5 ± 21.8	475.0 ± 23.4[b]	237.6 ± 15.8	316.4 ± 19.5[c,d]
	μ*g/mg cell protein*			
Apolipoprotein E	15.6 ± 0.98	18.9 ± 0.85[b]	9.4 ± 0.83[b,d]	11.6 ± 0.79[d,e]
	μ*g/mg cell protein*			
Apolipoprotein A-I	2.33 ± 0.12	2.37 ± 0.07	2.35 ± 0.07	2.51 ± 0.08

Values represent hepatic triglyceride lipase activity (n = 8), hepatic lipase immunoreactivity (n = 4), and mass of apoE and apoA-I (n = 8) and are expressed as mean ± SE.

[a]Ten μg of heparin/ml added. Values represent the activity or mass in the media plus the activity or mass released by heparin.

[b]$P < 0.01$ compared to control medium without heparin.

[c]$P < 0.001$ compared to β-xyloside medium without heparin.

[d]$P < 0.003$ compared to control medium plus heparin.

[e]$P < 0.05$ compared to β-xyloside medium without heparin.

The free fatty acid released from HTG-VLDL was 2-fold over background, whereas the net free fatty acid released from the remnant was 5-fold higher than that for HTG-VLDL. Release of free fatty acids from both lipoproteins was inhibited by THL. Collectively, these results indicate that HL plays an important role in enhancing HTG-VLDL remnant uptake in HepG2 cells.

Effect of heparin, heparinase, heparitinase, and β-xyloside on HepG2 cell uptake of HTG-VLDL

Heparin added at 10 μg/ml inhibited the cholesteryl ester accumulation induced by HTG-VLDL in the presence of LPL by 70% ($P < 0.001$) (**Fig. 6**). Heparin has several potential mechanisms to explain this effect other than blocking interaction of lipoproteins with cell surface proteoglycans, such as binding to apoE (4, 5). Therefore, we investigated the effects of pretreating HepG2 cells with heparinase or heparitinase (4 units/ml each) followed by incubation with HTG-VLDL plus LPL in the presence of each enzyme. As shown in Fig. 6A, heparinase or heparitinase significantly reduced cellular cholesteryl ester accumulation by 48% ($P < 0.01$) and 95% ($P < 0.01$), respectively. Heparin, heparinase, and heparitinase did not reduce the increase in cellular triglyceride (Fig. 6B). This indicates that lipolysis by LPL, cellular uptake of free fatty acids, and their subsequent re-esterification are not influenced by these treatments and that their effects are restricted to blocking the uptake of the cholesteryl ester-containing remnant lipoproteins.

To further establish the importance of cell surface proteoglycans, an alternate approach was investigated in which glycosaminoglycan synthesis was blocked. Cells were pretreated for 4 days with β-xyloside, a compound that can substitute for the protein core moiety during proteoglycan synthesis, significantly reducing the appearance of proteoglycans at the cell surface (50, 56). In β-xyloside-treated cells, cholesteryl ester accumulation was inhibited by 78% ($P < 0.001$) after incubation of HTG-VLDL plus LPL (**Fig. 7A**). Cellular accumulation of cholesteryl ester after incubation with hydrolyzed HTG-VLDL in the absence of LPL was inhibited by 80% ($P < 0.01$) in β-xyloside-treated cells. The reduction in cellular cholesteryl ester by β-xyloside was similar for intact HTG-VLDL coincubated with LPL or hydrolyzed HTG-VLDL coincubated with or without LPL (Fig. 7A) indicating that any LPL-enhanced binding of the remnant to cell surface proteoglycans was not rate-limiting for uptake. β-Xyloside had no effect on cellular accumulation of triglycerides after incubation with HTG-VLDL plus LPL, hydrolyzed VLDL alone, or in the presence of LPL (data not shown). β-Xyloside alone had no effect on cellular cholesteryl ester or triglyceride concentrations (data not shown). β-Xyloside had no effect on the small increase in cellular cholesteryl ester after incubation with LDL (Fig. 7B). However, β-xyloside inhibited the additional increase in cholesteryl ester induced by LDL in the presence of LPL.

Effect of β-xyloside on HepG2 cell-secreted HL, apoE, and apoA-I

It is known that HL is bound to hepatocyte cell surface proteoglycans (41) and HepG2 cells secrete he-

patic lipase into the media, an effect enhanced by heparin (Table 3). Hydrolysis of HTG-VLDL by LPL and the remnant by HL may facilitate binding of apoE, secreted by hepatocytes and present on hepatocyte cell surfaces bound to HSPGs (50, 57), to remnant lipoproteins. Uptake of β-VLDL from cholesterol-fed rabbits by rat hepatocytes is enhanced by marked over-expression of HL or apoE, after cellular transfection of either gene (45, 47). Therefore, we investigated whether the inhibition of cellular cholesteryl ester accumulation by HTG-VLDL plus LPL by β-xyloside was due in part to a reduced presence of HL and/or apoE on the cell surface.
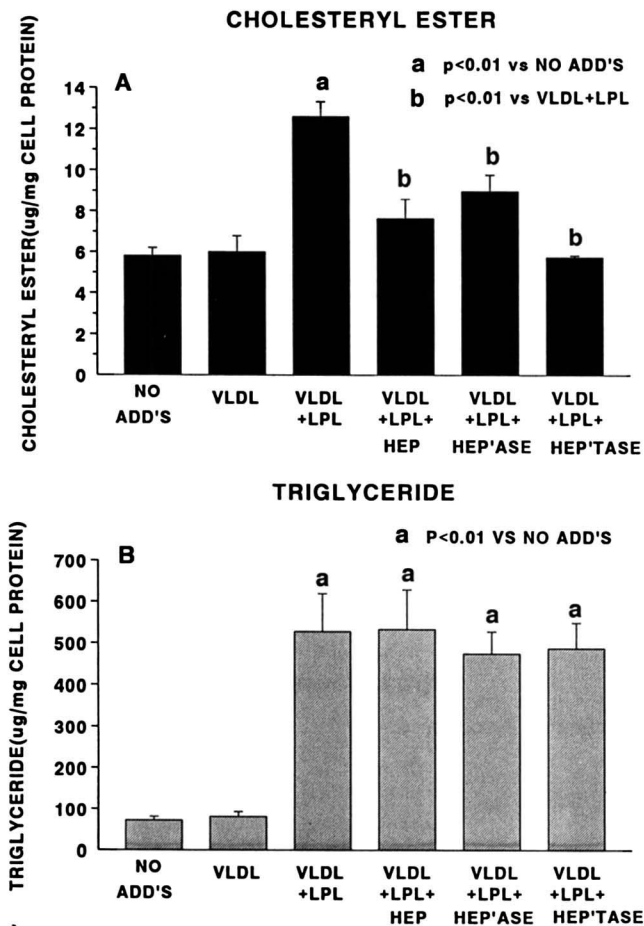
CHOLESTERYL ESTER

TRIGLYCERIDE

Fig. 6. The effect of heparin, heparinase, and heparitinase on the esterified cholesterol and triglyceride content of HepG2 cells incubated with Type IV VLDL (HTG-VLDL) S_f 60–400 in the presence or absence of LPL. Fifty μg of HTG-VLDL (S_f 60–400) cholesterol/ml of media (n = 7) was incubated with HepG2 cells in the absence or presence of 0.25 units (8 ng) of bovine milk LPL/ml of media. HepG2 cells were preincubated with heparin (10 μg) heparinase or heparitinase (4 units/ml of media) for 4 h. The media were changed and the same concentrations of heparin or enzyme were added to the media along with lipoproteins with or without LPL as described in Experimental Procedures. A: esterified cholesterol; B: triglyceride. Results are expressed as the mean ± SE. Results for heparin, heparinase, or heparitinase added without lipoproteins or LPL were the same as No Additions. NO ADD'S, no additions; HEP, heparin; HEP'ASE, heparinase; HEP'TASE, heparitinase.
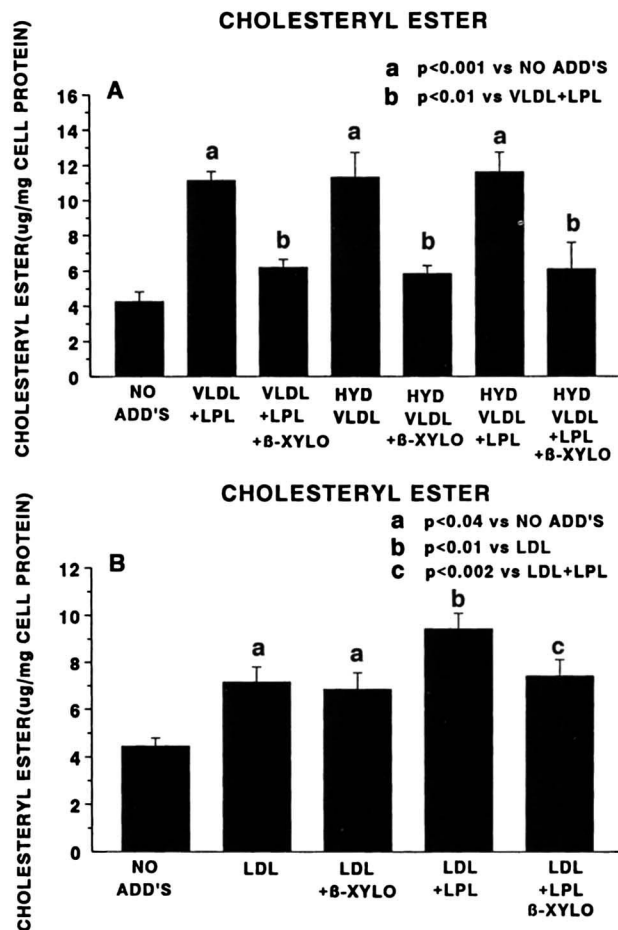
CHOLESTERYL ESTER

Fig. 7. The effect of β-xyloside on the esterified cholesterol and triglyceride content of HepG2 cells incubated with (A) Type IV VLDL (HTG-VLDL) (S_f 60–400), hydrolyzed HTG-VLDL or (B) LDL in the presence or absence of LPL. Fifty μg of HTG-VLDL (S_f 60–400) cholesterol (n = 5), 50 μg of hydrolyzed HTG-VLDL (HYD-VLDL) cholesterol (n = 5) or 150 μg of normal LDL cholesterol/ml of media (n = 4) were incubated with HepG2 cells in the absence or presence of 0.25 units (8 ng) of bovine milk LPL/ml of media. HepG2 cells were preincubated with or without β-xyloside (1 mM) for 4 days. The media were changed and the same concentration of enzyme was added to the media along with lipoproteins, with or without LPL as described in Experimental Procedures. Results are expressed as the mean ± SE. Values for β-xyloside added without lipoproteins were the same as No Additions. NO ADD'S, no additions; β-XYLO, β-xyloside.

As shown in Table 3, hepatic lipase activity secreted into the media over the 16 h incubation was low and did not differ from that secreted by cells pre-incubated with β-xyloside. However, when 10 μg/ml of heparin was added, β-xyloside-treated cells released 58% less HL activity than untreated cells ($P < 0.003$). As determined by immunoblotting, hepatic lipase mass secreted into the media was unaffected by β-xyloside (**Fig. 8**). Addition of heparin to β-xyloside-treated cells resulted in a 55% ($P < 0.001$) decrease in HL released. The decrease in HL activity and mass in β-xyloside-treated cells is quantitatively similar to the decrease in cellular choles-

HL -

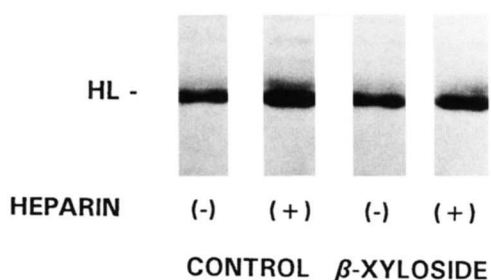HEPARIN   (-)   (+)   (-)   (+)

CONTROL   β-XYLOSIDE

Fig. 8. Immunoblot analysis of media HL. HepG2 cells were incubated for 4 days in the presence or absence of β-xyloside. Cells were then incubated in the absence or presence of heparin (10 μg/ml) for 16 h. The media were separated by SDS-PAGE, blotted, and the HL was detected with a human HL antipeptide antibody.

teryl ester induced by HTG-VLDL plus LPL, hydrolyzed HTG-VLDL, or hydrolyzed HTG-VLDL plus LPL. This suggests a role for HL in enhancing the uptake of HTG-VLDL remnants by HepG2 cells. HepG2 cells secreted apoE into the media over 16 h of incubation, an amount increased by 22% ($P < 0.01$) in the presence of 10 μg/ml of heparin (Table 3). β-Xyloside decreased the amount of apoE secreted into the media by 40% ($P < 0.01$), in the presence or absence of 10 μg/ml of heparin. ApoA-I secretion was not affected by heparin or β-xyloside (Table 3).

DISCUSSION

Lipoprotein kinetic studies in Type IV hypertriglyceridemic subjects have established that a significant proportion of the remnants derived from VLDL (S_f 60–400) are cleared from the circulation prior to conversion to LDL (6, 7). The liver is likely responsible for the majority of this clearance by mechanisms that are now beginning to be understood. A small amount of LPL is present in plasma, some of which is associated with triglyceride-rich lipoproteins and their remnants (33). In the present experiments, HepG2 cells were used as a model to explore the role of physiological concentrations of LPL and HL in mediating the interaction of HTG-VLDL (S_f 60–400) and their remnants with hepatocytes. In particular, we wanted to determine whether, in addition to its catalytic function, LPL would also function as a ligand. In previous studies we established that HTG-VLDL were not taken up by HepG2 cells unless co-incubated with LPL (17). The present studies clearly show that remnant formation by catalytically active LPL is required for HepG2 cell uptake as assessed by cellular cholesteryl ester accumulation. However, LPL does not play a significant role as a ligand in mediating the uptake of HTG-VLDL remnants.

LPL has been shown to enhance the binding, uptake,

and degradation of radiolabeled triglyceride-rich lipoproteins in a variety of cells (19–28). It has been postulated that LPL concentrates lipoproteins at the cell surface by facilitating their binding to cell surface proteoglycans which then enables them to interact with cellular receptors including the LRP and/or the LDL receptor (10, 19, 21–26). However, in most studies high concentrations of LPL have been used (greater than 100 ng/ml), the majority using between 500 and 2000 ng/ml. In human pre-heparin plasma, the concentration of LPL is less than 70 ng/ml (33) which, on a molar basis, is many-fold less than the concentration of triglyceride-rich lipoproteins in plasma.

The results of the present study show that at physiological concentrations, LPL enhances the interaction of HTG-VLDL with HepG2 cells, and that catalytically active enzyme is required. Heat-inactivated LPL could not enhance intracellular cholesteryl ester accumulation. However, as it is likely that heat inactivation results in LPL conformational changes that may influence the lipoprotein binding and cell surface binding functions of LPL, we used the specific lipase inhibitor THL. THL completely inhibited the catalytic activity of LPL and completely blocked the ability of LPL to enhance HTG-VLDL-induced HepG2 cell cholesteryl ester accumulation. It is known that THL binds to Ser152 of pancreatic lipase (58), one of the residues of the catalytic triad. Due to the close structural homology between pancreatic lipase and LPL, it is assumed by analogy that THL binds to Ser134 of bovine LPL (59). Lookene, Skottova, and Olivecrona (59) demonstrated that THL only binds to the dimeric form of bovine LPL and that THL binding to LPL does not appear to change the enzyme conformation or affect its binding to heparin. THL increases, rather than decreases, LPL's interaction with lipoprotein surfaces. THL does not affect the binding of LPL to the LRP or to β-VLDL (20). Collectively this indicates that, in our experiments, the inhibition of LPL-enhanced cellular interaction with HTG-VLDL by THL is related entirely to inhibition of catalytic activity and not to the loss of LPL's ability to bind to proteoglycans, the LRP, or lipoproteins.

The reason that our findings differ from those of Chappell et al. (21, 22) and Mulder et al. (25, 26) is not readily apparent. These investigators showed that either heat-inactivated LPL (26) or LPL that had been inhibited by PNPDC or PMSF (22) did not affect the degradation of iodinated normal VLDL. Their results may be related to the high concentrations of LPL used (0.1 μg/ml and 3.4 μg/ml, respectively), the use of fibroblasts rather than HepG2 cells, the use of iodinated lipoproteins, or incomplete inhibition of LPL. Residual active LPL may have been sufficient to stimulate lipoprotein lipolysis and subsequent cellular degradation (22). The effect of LPL-induced cellular lipoprotein uptake

(other than secreted iodides) on cellular cholesterol metabolism (cellular cholesteryl ester content or cholesterol esterification) was not reported.

In a further attempt to determine whether LPL could function as a ligand and mediate the uptake of HTG-VLDL remnants by HepG2 cells, remnants were prepared in vitro with LPL such that 50% of the VLDL triglycerides were hydrolyzed. These remnants, free of LPL activity, induced the same cellular cholesteryl ester accumulation as native HTG-VLDL incubated in the presence of LPL, indicating that LPL was not required for remnant uptake after initial lipolysis. At 5- and 10-fold higher concentrations of LPL (40 and 80 ng/ml, representing a ratio of LPL to lipoprotein cholesterol of 1:625), modestly stimulated HTG-VLDL remnant uptake (1.4- to 1.7-fold). Although the LPL was active, it is possible that the stimulated uptake was independent of catalytic activity. This is consistent with recent liver perfusion studies in which high concentrations of catalytically inactive LPL (LPL to lipoprotein cholesterol 1:10) stimulated hepatic chylomicron uptake 3-fold (36).

It has been shown that the dimeric form of LPL is required for mediating the interaction of lipoproteins with the cell surface (20, 30) and that the presence of monomers inhibits dimeric LPL-stimulated uptake of rabbit β-VLDL by Hep3B cells (30). The major form of LPL in pre-heparin plasma is monomeric (33). In the present studies, dimeric LPL was used at concentrations that would approximate the amounts associated with triglyceride-rich lipoproteins and their remnants (33). As we were unable to show that active dimeric LPL (8 ng/ml) could enhance cellular cholesteryl ester accumulation by HTG-VLDL remnants, it is unlikely that the inactive monomer would be any different. Whether sufficient LPL exists in human plasma to function as a ligand facilitating remnant hepatic uptake remains an important unanswered question.

On the other hand, these experiments revealed that lipolytic activity other than LPL was important for VLDL remnant uptake (Fig. 4). As shown in Table 3, HepG2 cells secrete active HL that is inactivated by THL. These findings indicate that hepatocyte HL activity has an important function in remnant uptake as measured by cellular cholesterol esterification and cholesteryl ester accumulation. Previous studies have demonstrated that HL is important in the catabolism of remnant lipoproteins. Remnant lipoproteins accumulate in plasma of patients with HL deficiency (38–40), observations consistent with animal studies in which anti-HL antibodies resulted in increased plasma remnants due to decreased hepatic clearance (37, 41–43) or the targeted inactivation of the HL gene in mice resulted in delayed chylomicron clearance (60).

Ji et al. (45) have shown that rat hepatoma cells (McA-RH7777) transfected with the human HL gene secrete high levels of the enzyme which enhances the binding and uptake of rabbit β-VLDL 3-fold over non-transfected cells. In recent studies of Kraap et al. (61), addition of exogenous HL (100 ng/ml) to chylomicrons and rabbit β-VLDL stimulated uptake in HepG2 cells. Our findings clearly show that inhibition of basal activity of HL in HepG2 cells significantly inhibits the ability of human HTG-VLDL remnants to induce cellular cholesteryl ester accumulation. It is important to note that HL activity cannot substitute for LPL activity as HTG-VLDL without prior exposure to LPL fail to induce any cellular increase cholesteryl ester. This is consistent with the concept that large triglyceride-rich lipoproteins are poor substrates for HL unless previously exposed to LPL (41, 62). Our demonstration of a role for HL in HTG-VLDL remnant uptake in HepG2 cells could explain our inability to demonstrate that LPL plays a significant role beyond its catalytic function. In contrast to FH-fibroblasts, HL is present on the cell surface of HepG2 cells and, in addition to its catalytic function, it may also provide a ligand function. Addition of LPL to HTG-VLDL remnants produced no further enhancement of cellular cholesteryl ester accumulation (Figs. 1, 5). It is possible that the HL, already present on the cell surface, was sufficient to mediate uptake. Approximately 30% of VLDL remnant-induced cholesteryl ester accumulation was not inhibitable by THL, suggesting that some uptake of remnants occurs independent of HL catalytic activity. Whether this uptake is independent of HL or reflects a role for HL beyond its catalytic function, as discussed below, remains to be elucidated.

Cell surface proteoglycans are important participants in the cellular catabolism triglyceride-rich lipoproteins in the presence of LPL (19, 21, 22, 25, 26) and catabolism of apoE-enriched rabbit β-VLDL in the absence of LPL (46, 47, 63). The current studies indicate that this is also true for cellular uptake of human HTG-VLDL coincubated with LPL or HTG-VLDL remnants, but not native HTG-VLDL. Treatment of cells with heparinase, heparitinase, or β-xyloside to deplete cell surface proteoglycans blocked cholesteryl ester accumulation induced by HTG-VLDL plus LPL or HTG-VLDL remnants. The increase in cellular triglyceride accumulation was not inhibited, indicating that depletion of cell surface proteoglycans had no effect on the activity of LPL or HL, but only blocked the uptake of the cholesteryl ester contained in the remnant. For HSPGs to facilitate uptake, remnant formation is required; however, after initial lipolysis, the HSPG requirement is observed in the absence of LPL. Depletion of cell surface proteoglycans blocked cellular accumulation of cholesteryl esters induced by hydrolyzed VLDL isolated free of LPL activity. Studies in McA-RH7777 cells have demonstrated that heparinase does not affect the binding of rabbit β-

VLDL unless these cells have been transfected with apoE3 or the β-VLDL was supplemented with exogenous apoE (46, 47, 63). The results of the present study show that the requirement of HSPGs for enhancing HTG-VLDL remnant uptake does not depend on exogenous LPL or exogenous apoE over and above that present on the lipoprotein and/or secreted from HepG2 cells.

Studies presented in this paper indicate that one of the mechanisms whereby cell surface proteoglycans mediate the cellular cholesteryl ester accumulation caused by HTG-VLDL remnants is by anchoring secreted HL. β-Xyloside significantly reduced the amount of HL activity and immunoreactivity released from HepG2 cells with heparin. This result is consistent with the studies of Ji et al. (45) who demonstrated that overexpression of HL by rat McA-RH7777 hepatoma cells significantly enhanced rabbit β-VLDL uptake, a process inhibited by heparinase. However, a role for proteoglycan-bound HL was not apparent in non- transfected cells as heparinase had no effect on β-VLDL uptake under basal conditions. Our experiments with THL and β-xyloside show that HL on HepG2 cell surfaces significantly enhances HTG-VLDL remnant uptake and that the role of cell surface proteoglycans in remnant uptake is, in part, to bind secreted HL.

Hydrolysis of chylomicrons and VLDL by LPL (17, 51, 64, 65) and their remnants by HL (44) greatly increases the exposure of apoE epitopes, facilitating enhanced binding to specific antibodies and lipoprotein receptors. In addition, hydrolysis of remnant surface lipids by HL may facilitate the acquisition of cell surface apoE. ApoE is secreted by hepatocytes and is bound to cell surface HSPGs (50, 57). Addition of exogenous apoE to rabbit β-VLDL (46, 63, 66) or human VLDL (64) or the incubation of rabbit β-VLDL with rat hepatoma cells over-expressing apoE (47) enhances uptake by either the LDL receptor or the LRP. In the present studies, β-xyloside significantly decreased the amount of apoE present on the cell surface and released into the media. This indicates that another mechanism whereby cell surface HSPGs mediate cellular cholesteryl ester accumulation caused by HTG-VLDL remnants is the binding of secreted apoE.

Although our experiments demonstrated that HL lipolytic activity was required for HTG-VLDL remnant uptake, we did not determine whether HL played a role beyond its catalytic function. It is possible that HSPGs bind and internalize remnants after interaction with HL, as suggested previously (45). Alternatively, internalization of remnants as part of a remnant-HL-HSPG complex could occur after hydrolysis, where HL functions as a bridge between the remnant and HSPGs, as suggested recently by Shafi et al. (41). Obunike et al. (24) have suggested that LPL may serve a similar func-

tion in the uptake of VLDL and LDL by macrophages, cells that secrete LPL but not HL. HL could also facilitate HTG-VLDL remnant binding and internalization by mediating binding to the LRP. Others have demonstrated that excess LPL bound to normal VLDL (21) or rabbit β-VLDL (10, 20) mediates binding to the LRP on FH-fibroblasts. Addition of excess, exogenous, catalytically inactive HL to chylomicrons or rabbit β-VLDL has been shown to stimulate uptake in Hep3b cells, an effect greater than that observed for an equivalent amount of LPL (61). Therefore, after lipolysis, HSPG-bound HL could also serve to concentrate the remnant at the cell surface, allowing the transfer of cell surface bound apoE to the remnant prior to uptake by LRP or the LDL receptor.

In vivo, large HTG-VLDL (S_f 60–400) particles are cleared directly from the circulation (6, 7). In addition, most of the remnants formed from these large VLDL are cleared from plasma without conversion to LDL. LPL is able dissociate from endothelial cell surfaces during lipolysis, some of which associates with triglyceride-rich lipoproteins and their remnants (33). The present studies suggest that LPL, at physiological concentrations, does not play a significant role in mediating HTG-VLDL and HTG-VLDL remnant uptake beyond its catalytic function. On the other hand, we provide evidence that HL activity significantly enhances uptake of the HTG-VLDL remnant and that cell surface proteoglycans play an important role in the interaction of HTG-VLDL remnants with HepG2 cells. Whether the importance of heparan sulfate proteoglycans lies in *a* anchoring secreted hepatic lipase and secreted apoE or *b* binding the remnant prior to either internalization directly or transfer to cellular receptors remains to be clarified.∎

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