Mechanisms of Uptake and Secretion of Apolipoprotein B Containing Triacylglycerol-Rich Lipoprotein in a Liver Cell Model

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A Thesis

in

The Department

of

Chemistry and Biochemistry

Presented in Partial Fulfillment of the Requirements
for the Degree of Master of Science at
Concordia University
Montreal, Quebec, Canada

September, 1994

ISBN 0-612-01333-2

ABSTRACT

Mechanisms of Uptake and Secretion of Apolipoprotein B Containing Triacylglycerol-Rich Lipoproteins in a Liver Cell Model

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This thesis examines mechanisms of triacylglycerol-rich lipoprotein (TRL) uptake and the effects of its components, triacylglycerol and cholesterol ester, on the subsequent secretion of apolipoprotein B (apoB) containing lipoproteins. Using a human liver cell model, uptake of TRL by the liver was found to be dependent on the presence of lipolytic activity, as measured by the intracellular accumulation of triacylglycerol and cholesterol ester. This effect was not specific to lipoprotein lipase, as had been previously reported, but was also observed using structurally unrelated triacylglycerol lipases. In addition, inhibition of cholesterol ester mass accumulation was observed using substances which bind or modify cell surface proteoglycans. These data suggest a possible role for cell surface proteoglycans in mediating receptor-ligand recognition events prior to cellular uptake of TRL. Since decreases in cholesterol ester accumulation were not accompanied by decreases in triacylglycerol accumulation, this further suggests that the mechanism for cholesterol ester and triacylglycerol uptake are independent.

The same inhibitors of cholesterol ester uptake were then used to dissociate cholesterol ester and triacylglycerol accumulation within the cell and study the effects of each TRL component, under limiting conditions, on the apoB secretion. A strong correlation was found between cholesterol ester accumulation

and apoB secretion with a value of $r^2=0.76$ from linear regression analysis of the data. Inhibitors of the enzyme ACAT may therefore be useful in limiting the biosynthesis of cholesterol ester and subsequent secretion of the apoB containing lipoproteins which are associated with increased risk of coronary artery disease.

ACKNOWLEDGEMENTS

Plasma for isolation of lipoproteins used in all the experiments presented in this work was kindly provided by Mrs. Diane Liebling, Dr. David Blank and Dr. Mark Sherman.

I would also like to thank Mr. David Kalant for his assistance with the culture of HepG2 cells.

I would also like to express sincere gratitude to Dr. Ann English and Dr. Joanne Turnbull who were always very generous with their time. Their continued support over the last two years has played an significant role in the development of this work.

In particular, I would like to convey my deep gratitude to Dr Katherine Cianflone who is largely responsible for the direction of this work. Over the last several years Dr. Cianflone has not only been my teacher, but has helped shape my very approach to experimentation.

I would also like to thank Dr. Allan D. Sniderman for whom education is an ever ongoing process.

Finally, thanks to Dr. Allain Baldo for nitpicking over the manuscript.

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ABBREVIATIONS

ACAT	acyl CoA:cholesterol acyltransferase
ApoB	apolipoprotein B
ApoE	apolipoprotein E
BSA	bovine serum albumin
DGAT	diacylglycerol acyltransferase
ELISA	enzyme linked immunosorbent assay
FCS	fetal calf serum
GPAT	glycerol phosphate acyltransferase
LDL	low density lipoprotein
LPL	lipoprotein lipase
LRP	low density lipoprotein receptor-related protein
MEM	minimum essential medium
PL	*Pseudomonas* lipase
PPH	phosphatidate phosphohydrolase
RL	*Rhizopus arrhizus* lipase
TLC	thin layer chromatography
TRL	triacylglycerol-rich lipoprotein

1. INTRODUCTION

Dietary lipids are carried through the body in the form of a complex assembly of lipid and protein called lipoproteins. The function of these heterogeneous particles is to transport hydrophobic lipids through the bloodstream to the tissues which require them for storage or fuel. The hydrophobic core of lipoproteins is composed of triacylglycerol and cholesterol esterified with fatty acid (Figure 1) while the outer phospholipid monolayer contains apoproteins required for function. Lipoproteins are classified based on their size as well as lipid and protein composition. Figure 2 shows the lipid and protein content of low density lipoprotein (LDL) and triacylglycerol-rich lipoprotein (TRL). TRL is composed of approximately 60% triacylglycerol and 10% cholesterol ester, while LDL usually contains up to 45% cholesterol ester with no more than 10% triacylglycerol (1) Both these lipoproteins contain characteristic apoproteins at their surface including a single copy of apolipoprotein B (apoB).

Synthesis of lipoproteins commences in the intestine following the absorption of a meal. TRL arises from the necessity to package the lipid absorbed. The resulting intestinal TRLs called chylomicrons are released into the circulation. In peripheral tissue, lipolysis of much of the triacylglycerol contained within the TRL particle occurs through the action of lipoprotein lipase (LPL) which is localized on the endothelial cell surface of the blood vessel wall (2). This lipolysis of triacylglycerol allows cellular uptake of the released free fatty acids for

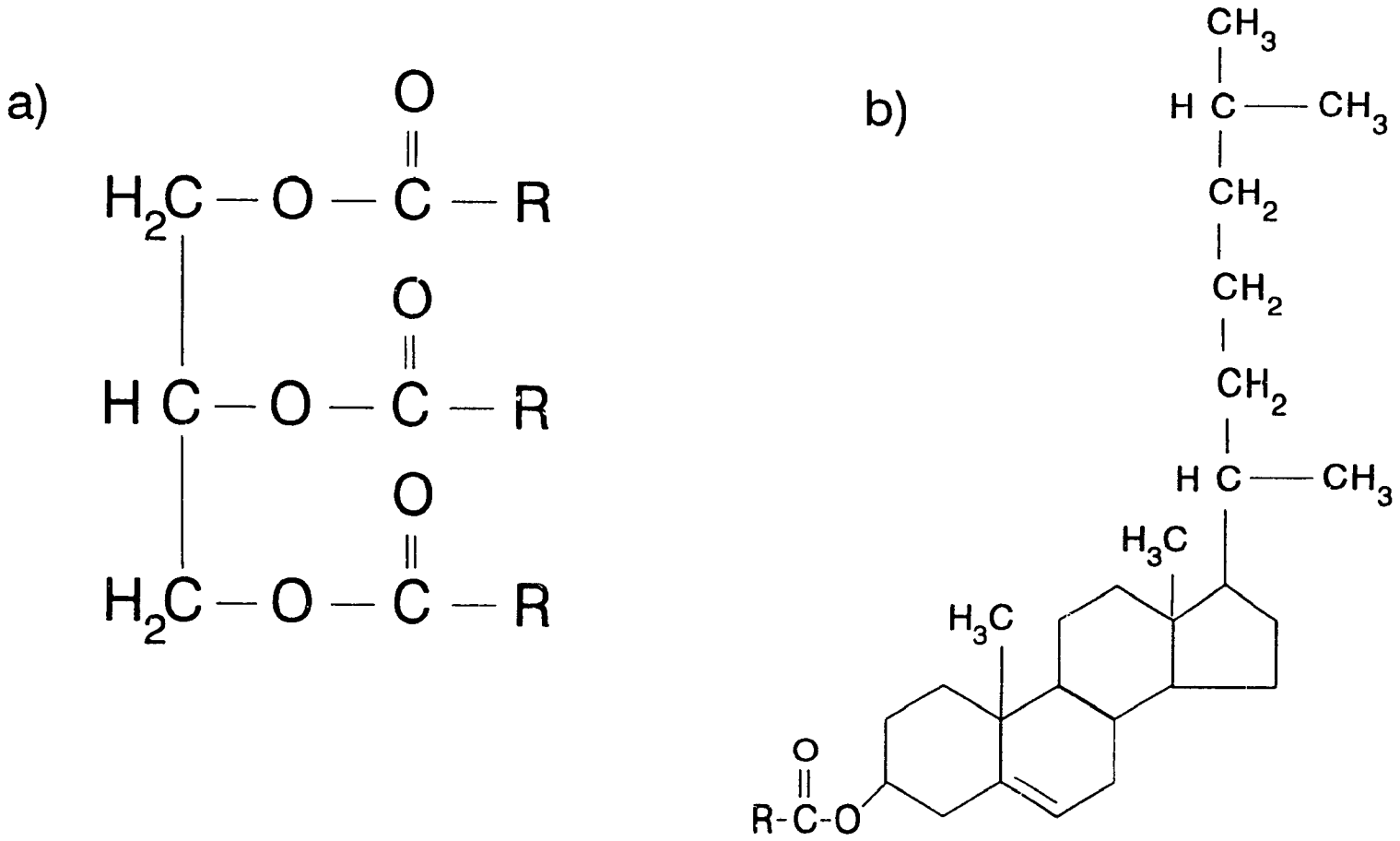

Figure 1 Structure of lipoprotein core lipids (a) Triacylglycerol, (b) Cholesterol Ester
R fatty acid side chain

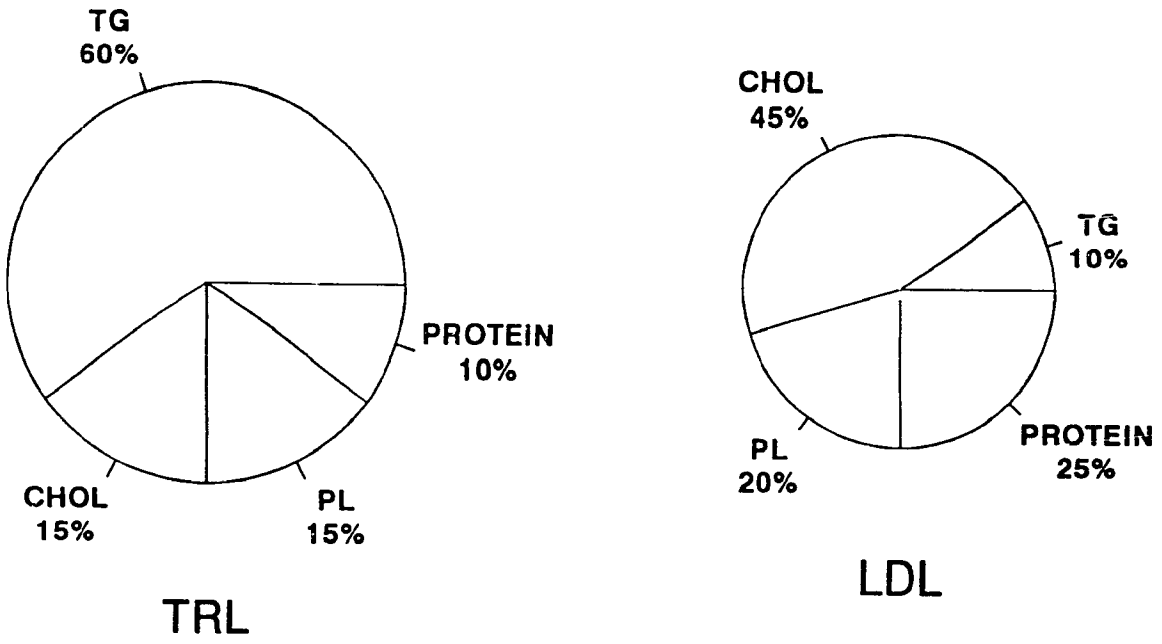

Figure 2 Percent composition of triacylglycerol-rich lipoprotein (TRL) and low density lipoprotein (LDL) TG. triacylglycerol, PL. phospholipid, CHOL. free and esterified cholesterol.

short term storage by the liver. Because they are toxic to cells at elevated concentrations, free fatty acids are re-esterified by enzymatic reactions within the cells, and triacylglycerol is regenerated for subsequent storage mainly in adipose tissue (3). Figure 3 shows the reaction scheme for the biosynthesis of triacylglycerol. Fatty acids are re-esterified onto a glycerol-3-phosphate backbone through the sequential action of glycerol phosphate acyltransferase (GPAT), phosphatidate phosphohydrolase (PPH) and diacylglycerol acyltransferase (DGAT) (4).

The remnant lipoprotein particle generated following LPL action is also taken up by the liver. Endocytosis of the remnant particle, followed by acid lysosomal hydrolysis of the remaining lipids and apoproteins, results in production of free fatty acids, free cholesterol and amino acids (5). The lipids are in turn re-esterified by enzymes localized to the endoplasmic reticulum. Free fatty acids undergo the reactions described above for peripheral tissue, while free cholesterol is acylated through the action of acyl-coA:cholesterol acyltransferase (ACAT) to yield cholesterol ester as shown in Figure 4 (6). Triacylglycerol and cholesterol ester thus formed are stored as cytoplasmic lipid droplets within the liver cell and then reassembled into a complex particle and secreted into the circulation as a TRL (7). Figure 5 summarizes the metabolism of lipoprotein particles by the liver. The liver secretes its own TRL, each containing a single copy of the full length hepatic apoprotein, apoB 100. This apoprotein is approximately twice as long as the intestinally secreted apoB 48. The hepatic

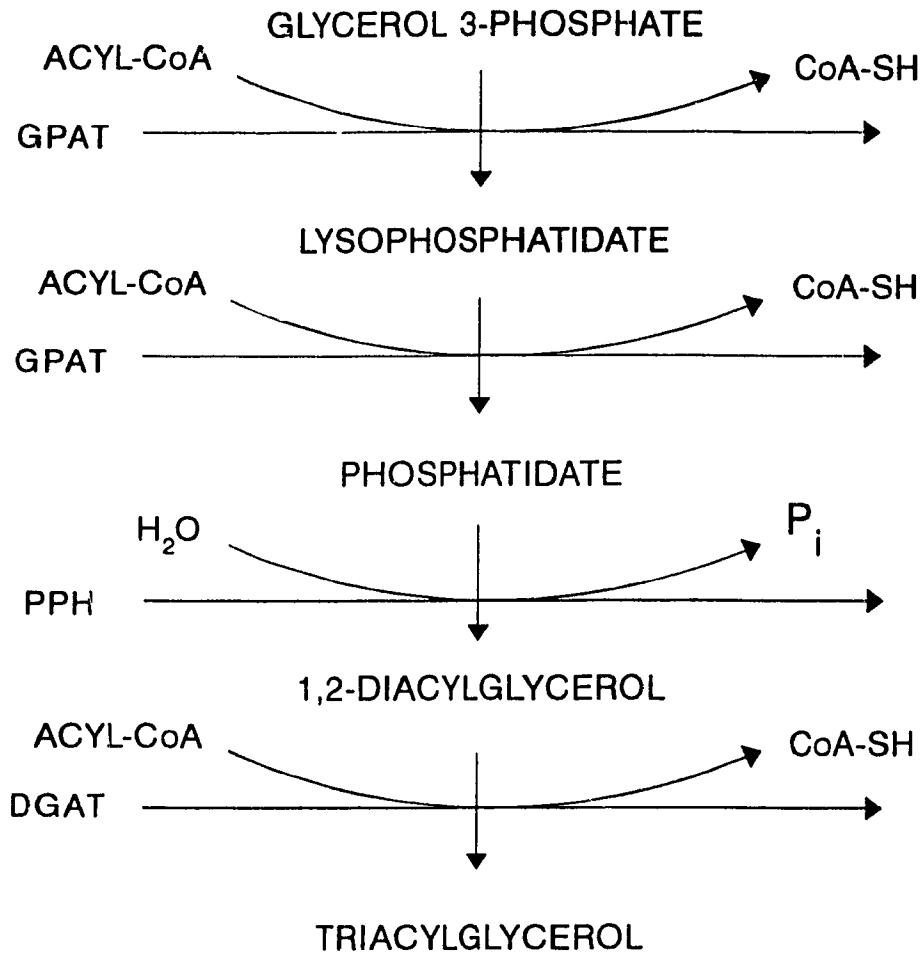

Figure 3 Biosynthesis of triacylglycerol. GPAT: glycerol phosphate acyltransferase, PPH: phosphatidate phosphohydrolase, DGAT: diacylglycarol acyltransferase ACYL-CoA: acyl coenzyme A, CoA-SH coenzyme A

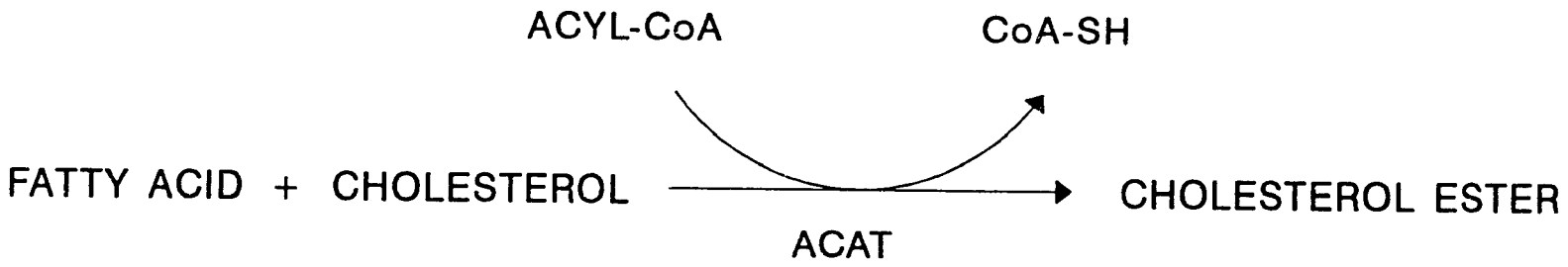

Figure 4 Biosynthesis of cholesterol ester ACAT acyl-CoA cholesterol acyltransferase, ACYL-CoA acyl-coenzyme A, CoA-SH coenzyme A

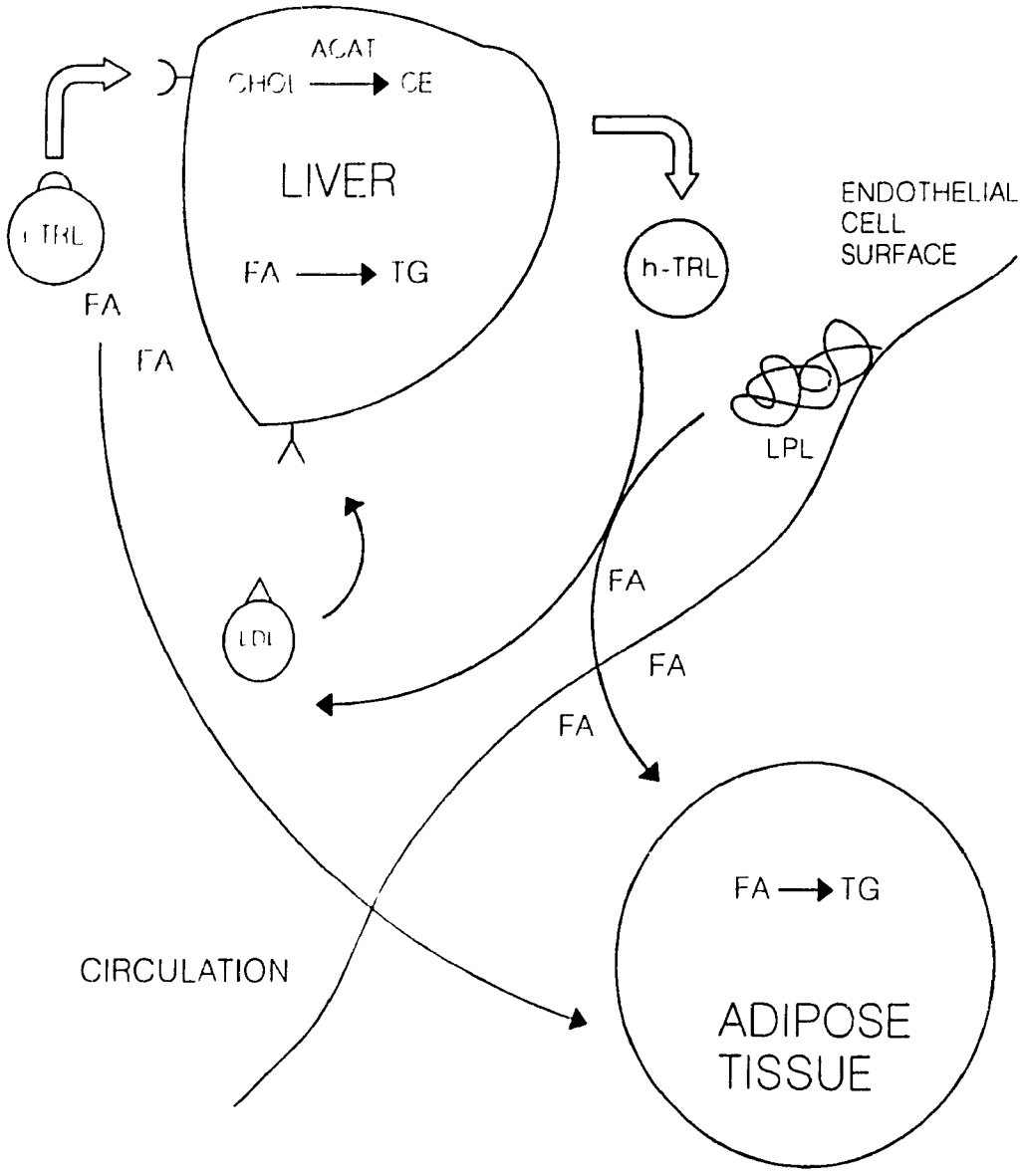

Figure 5 Metabolism of triacylglycerol-rich lipoprotein
i-TRL intestinally derived triacylglycerol-rich lipoprotein,
h-TRL hepatically derived triacylglycerol-rich lipoprotein,
LPL lipoprotein lipase, LDL low density lipoprotein,
FA fatty acid, TG triacylglycerol, CE cholesterol ester, CHOL free cholesterol, ACAT acyl-coA cholesterol acyltransferase

lipoprotein-derived triacylglycerol may be used as fuel or sent for storage in the adipose tissue (3). Like intestinal lipoproteins, hepatic TRL also undergo hydrolysis by LPL once in the circulation and a large portion are eventually converted to LDL (8). It is this form of the lipoprotein, when present in elevated amounts, which is considered atherogenic, since these cholesterol-rich lipoproteins may be oxidized (9) and absorbed into the arterial wall (10). LDL's extended residence time in plasma (2-3 days) as compared to TRL (30 minutes-5 hours) exacerbates this atherogenic effect (11).

Uptake of LDL by the liver or peripheral tissue is known to occur through a receptor-mediated mechanism (12). This receptor has been characterized at the molecular level and is known to interact only with LDL apoB 100 or apoE. It is important to note that apoB 100 on hepatically derived TRL or apoB 48 found on larger intestinally derived TRL cannot interact with this receptor (13). Defects in the LDL receptor sequence have been identified and are believed to be pathogenic for familial hypercholesterolemia, a condition which predisposes individuals to coronary artery disease at an early age. This condition results from impeded uptake of LDL particles which causes increased amounts of LDL to build up and remain in the circulation for extended periods of time, thus increasing its absorbtion onto arterial walls (14).

In contrast to LDL, the metabolism of TRL is less well understood Although a major portion of TRL which are secreted by the liver are converted to LDL through lipolytic degradation in the circulation, some are cleared directly

from the circulation by the liver (15). Particularly in hypertriglyceridemic individuals, a large portion of TRL do not proceed through the sequential lipolysis cascade outlined above (16). It is also known that individuals who are LDL receptor deficient clear intestinally derived TRL from the circulation in a normal manner (17). These observations have led to the conclusion that there is a discrete mechanism for hepatic TRL uptake (18) and since this mechanism of TRL clearance is saturable (19) the uptake mechanism may involve a receptor (20).

One likely candidate for the TRL receptor is the low density lipoprotein receptor-related protein (LRP). This receptor is comprised of four transmembrane domains which bear significant structural homology to the LDL receptor (21). Binding studies have been performed using ^{125}I-labelled apoE, an apoprotein found on the surface of TRL which is thought to be involved in receptor recognition of TRL by the cells Results indicate that this apoprotein is recognized by the LRP (22). However, it has also been found that this receptor binds other biomolecules such as α-2-macroglobulin (23) and lactoferrin (24). These findings suggest that either the LRP is not the receptor involved in specific TRL clearance or that different domains on the receptor are responsible for recognizing different apoproteins (25). A review of the literature indicates that much of the data on uptake mechanisms of TRL are based on binding studies using iodinated TRL apoproteins (26). These apoproteins are highly labile and easily dissociable from the lipoprotein particle. It is thus unclear whether binding of iodinated apoproteins

to the hepatocyte cell surface is an accurate reflection of the amount of TRL lipid actually internalized from an intact lipoprotein. In addition, it has been reported that LPL itself is necessary not only for lipolysis, but also can act as a ligand to facilitate TRL remnant particle uptake (27).

The TRL receptor hypothesis has recently been extended to suggest a role for cell surface proteoglycans in mediating receptor-ligand recognition events prior to cellular uptake of TRL remnant particles. Heparinase treatment of hepatocytes, which removes heparan sulphate proteoglycans on the cell surface was shown to decrease binding of TRL particles (28). Similarly, experiments performed using Chinese hamster ovary cells, which are deficient in cell surface proteoglycans, showed no significant specific binding of TRL (29).

It is important to better understand mechanisms involved in uptake of TRL since it is the uptake of the substrates, fatty acid and cholesterol ester, which is believed to ultimately regulate the secretion of apoB containing lipoproteins from the liver (30). Premature uptake of excess TRL, prior to the hydrolysis of triacylglycerol for storage in adipose tissue, may lead to an overproduction of the hepatic apoB containing lipoprotein, LDL. Thus far, evidence is conflicting as to which of the two lipoprotein core lipids, triacylglycerol or cholesterol ester, regulates secretion of lipoproteins from the liver (31,32). Although, presumably both lipids are essential, cholesterol ester which is found in smaller amounts than triacylglycerol, may be the limiting factor in lipoprotein synthesis. Since cholesterol ester and apoB are both synthesized at the rough endoplasmic

reticulum, the two may already be associated before acquiring triacylglycerol at the smooth endoplasmic reticulum (33).

The two objectives of this investigation were (1) to elucidate the mechanism of TRL uptake by the liver and (2) to determine if either of the major core lipids of TRL, triacylglycerol and cholesterol ester, plays a regulatory role in the synthesis and secretion of apoB containing hepatic TRL. Because human hepatocytes are not readily available, and because they are difficult to grow beyond a primary culture, HepG2 cells are often used as an experimental model of the human liver. HepG2 is a human hepatocarcinoma cell line which contains all the cellular machinery necessary for lipoprotein metabolism and synthesis (34).

The involvement of proteoglycans in TRL uptake was investigated by using competitive inhibitors of TRL uptake or an inhibitor of proteoglycan biosynthesis in the presence of TRL. The resulting intracellular triacylglycerol and cholesterol ester accumulation was quantified as a measure of total TRL particle uptake. These same inhibitors of TRL uptake were then used to dissociate triacylglycerol and cholesterol ester accumulation in the HepG2 cells. Since each hepatically derived TRL particle contains a single copy of apoB, measurement of this apoprotein was used to determine the effect each of the core lipids had on synthesis and secretion of liver lipoproteins.

2. EXPERIMENTAL METHODS

2.1 Materials

All chemicals used were of the highest purity available and were obtained from Fisher Scientific (Montreal, Quebec) unless otherwise indicated. Bovine serum albumin used for standard curves in protein determinations, as well as the dye reagent used for the Bradford protein assay were obtained from Bio-Rad Laboratories (Mississauga, Ontario). Kits used for determination of triacylglycerol and cholesterol content of lipoproteins were purchased from Boehringer Mannheim (Laval, Quebec). HepG2 cells were obtained from the American Tissue Culture Collection (Rockville, MD). All tissue culture media and supplies were from Gibco (Burlington, Ontario) or Flow Laboratories (Mississauga, Ontario). Lactoferrin, asialofetuin, *Pseudomonas* lipase and LPL were purchased from Sigma Chemicals (St Louis, MO). *Rhizopus arrhizus* lipase was purchased from Boehringer Mannheim. Heparin was purchased from Organon Pharmaceuticals Canada LTD. TLC plates were obtained from Mandel Scientific (Rockwood, Ontario). Solvents used for TLC were of the highest available purity and were obtained from Fisher Scientific. TLC lipid standard was obtained from Sigma Chemicals. Sodium oleate and bovine serum albumin were purchased from Sigma Chemicals and complexed by the method of Van Harken prior to addition to the cells (35). Triolein (9-octadecenoic acid 1,2,3-propanetriyl ester) and cholesteryl oleate standards for determination of intracellular triacylglycerol and

cholesterol ester mass were purchased from Sigma Chemicals. Polystyrene microtiter plates for lipid as well as apoB determination were obtained from Dynatech Laboratories (Chantilly, VA). Anti-rabbit IgG conjugated to horseradish peroxidase and o-phenylenediamine dihydrochloride were purchased from Sigma Chemicals.

2.2 Instrumentation

Lipoprotein isolation was performed using a Ti50 type rotor in a Beckman ultracentrifuge (Beckman Instruments, Mississauga, Ontario). Lipid samples were dried using a Jouan vacuum centrifuge (Canberra-Packard, Montreal, Quebec). Spectrophotometer readings were taken using an LKB Ultrospec model 4050 (Fisher Scientific, Montreal, Quebec). The microtiter plate reader used was obtained from Molecular Devices (Menlo Park, CA).

2.3 Lipoprotein Isolation

Fasting blood was obtained from hypertriglyceridemic individuals visiting the Lipid Clinic at the Royal Victoria Hospital. The blood was collected into tubes containing EDTA to a final concentration of 0.15% (w/v). The plasma fraction was immediately isolated by performing a 20 min centrifugation at 2 000 rpm at 4°C. The plasma obtained was layered under an equal volume of solution of density 1.006 g/mL consisting of 0.195 M NaCl, 1 mM Tris, pH=7.4, 1 mM EDTA and 3 mM sodium azide. TRL were isolated by salt density centrifugation following a 2

h centrifugation at 40 000 rpm and 12°C (36). The collected supernatant was washed and concentrated through an equal volume of the same solution and recentrifuged for 18 h at 40 000 rpm at 12°C and collected in as small a volume as possible. The isolated TRL was filtered through a 0.45 μm pore syringe filter into a sterile tube. LDL was isolated by layering one volume of plasma under two volumes of solution of density 1.019 g/mL and centrifuging for 18 h at 40 000 rpm at 12°C in order to remove TRL (36). The infranate was collected and increased to a density of 1.063 g/mL and recentrifuged for 20 h at 40 000 rpm and 12°C.

A Lowry assay, modified by the addition of sodium dodecyl sulphate for samples containing lipid, was used to measure protein content of the lipoprotein isolated (37). Bovine serum albumin (BSA) was used as a standard. Figure 6 shows a typical standard curve obtained using this method. The total cholesterol content of each lipoprotein preparation was determined spectrophotometrically using a commercial kit. The kit contained cholesterol esterase which converts cholesterol ester to cholesterol, and cholesterol oxidase which catalyses the oxidation of cholesterol by O_2 to produce H_2O_2. In a further reaction catalyzed by peroxidase, the H_2O_2 produced oxidizes a chromagen, 4-aminophenazone, to yield a coloured product, which can be detected spectrophotometrically at 500 nm. Figure 7 shows the reaction sequence for the measurement of cholesterol in lipoproteins, and Figure 8 shows a typical standard curve.

Total triacylglycerol for each lipoprotein preparation was determined using

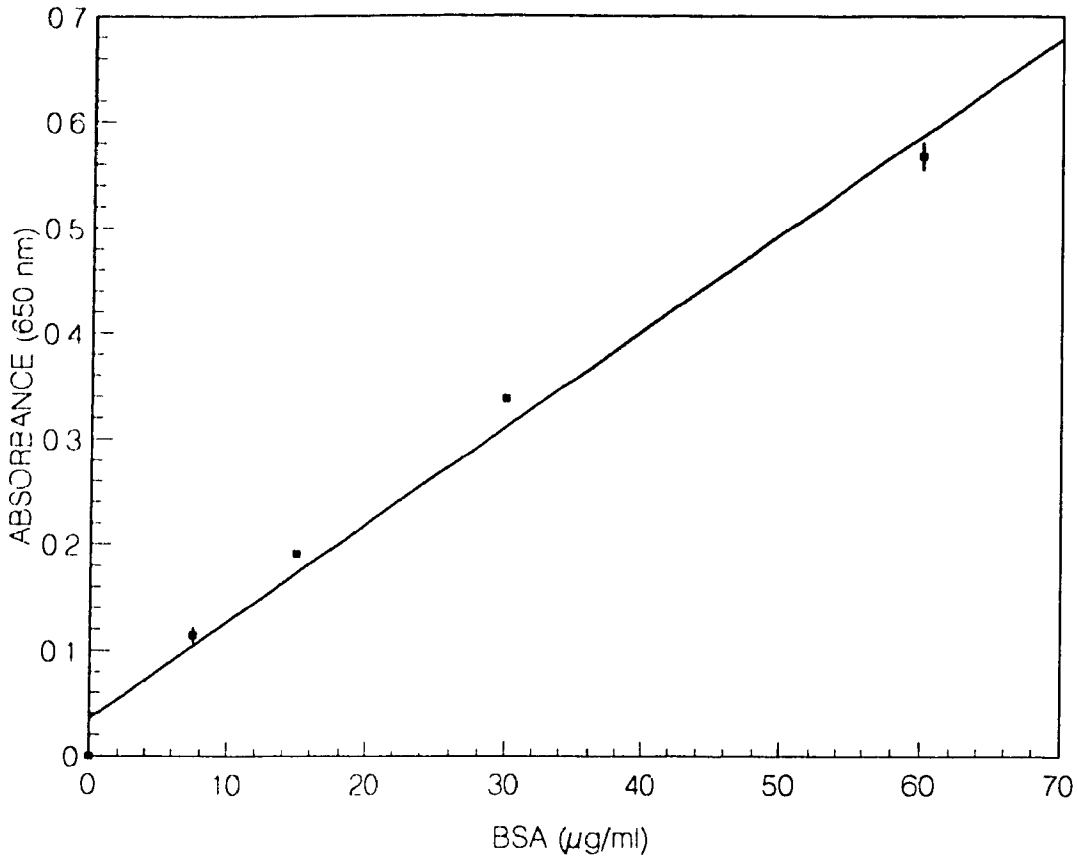

Figure 6. Modified Lowry protein assay. A standard curve for measuring the protein content of lipoprotein was constructed using 0 to 60 μg/ml bovine serum albumin (BSA) and absorbance was read at 650 nm; y-intercept=0 10, slope=0.002, r^2=0.99 for the linear regression The error bars represent deviation between duplicates

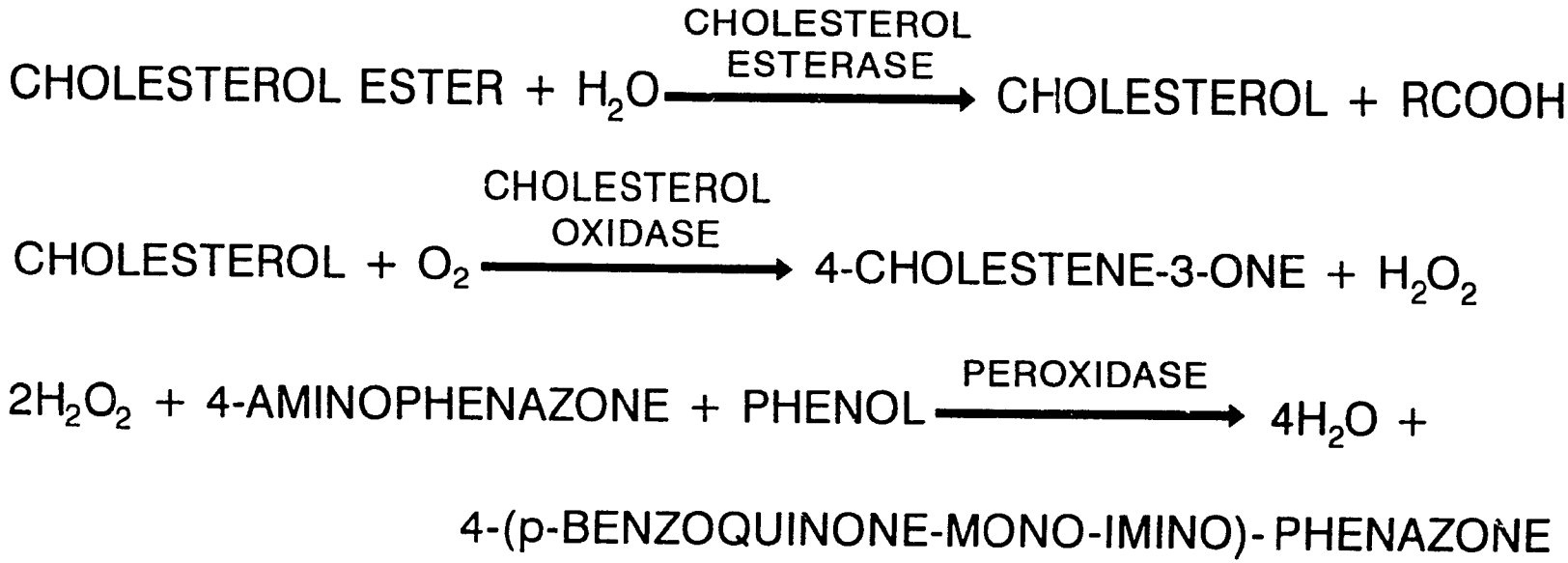

Figure 7 Reaction sequence for the spectrophotometric determination of lipoprotein cholesterol content
R fatty acid side chain

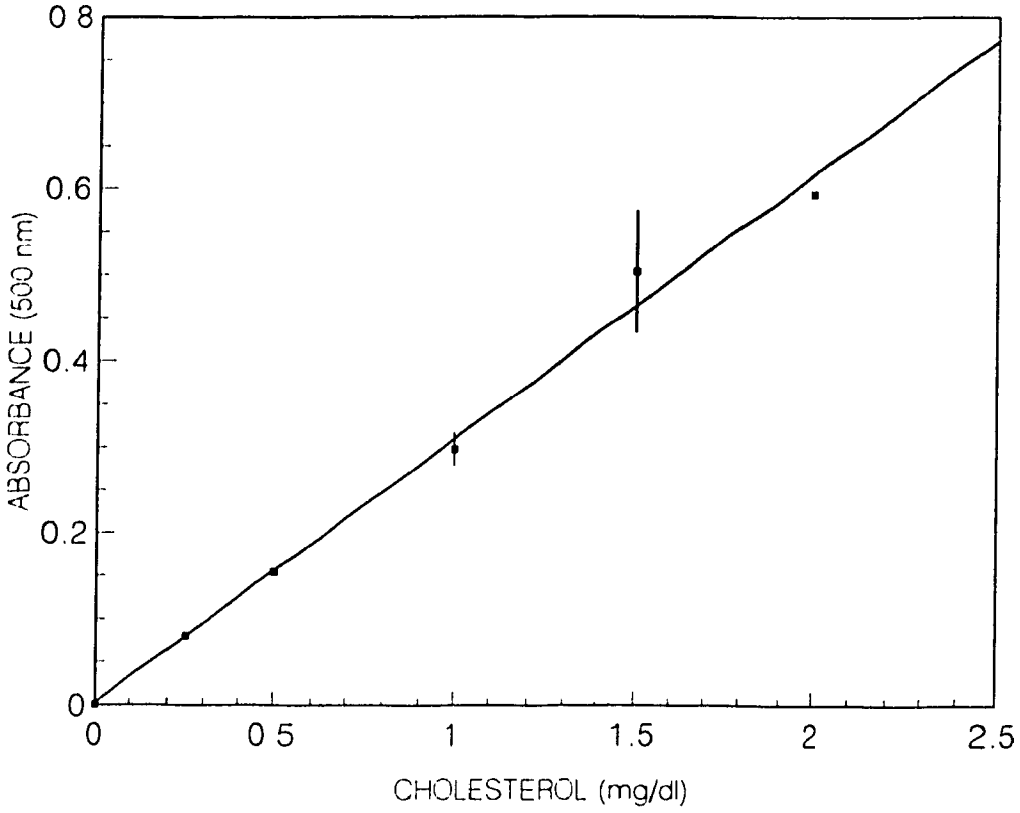

Figure 8. Spectrophotometric cholesterol assay. A standard curve for measuring total lipoprotein cholesterol was constructed using 0 to 2 mg/ml cholesterol and absorbance was read at 500 nm; y-intercept=0.022, slope=0.002, r^2=0 99 for the linear regression. The error bars represent deviation between duplicates.

a similar spectrophotometric analysis. The triacylglycerol kit contained triacylglycerol lipase which converts the triacylglycerol to glycerol and fatty acids. Glycerol kinase then produces glycerol-3-phosphate, which is oxidized by O_2 in a reaction catalyzed by glycerol phosphate oxidase. The H_2O_2 produced will oxidize aminophenazone to yield a coloured product as before, which can be detected spectrophotometrically at 500 nm. Figure 9 shows the detailed reaction sequence for the determination of triacylglycerol, and Figure 10 shows a typical standard curve.

2.4 HepG2 Cell Culture

HepG2 cells were plated in 75 mm^2 flasks and grown in 10 ml minimum essential medium (MEM) supplemented with 10% fetal calf serum (FCS) and 100 IU penicillin-streptomycin. The cells were subcultured every 7 days with a split ratio of 1:3 by detaching the cells from the flask by incubating them for 5 min at 37°C in a 0.25% trypsin solution in phosphate buffered saline, pH=7,2 (PBS). For all experiments, cells were plated out in 24 well plates at confluency at a density of $1.3x10^4$ cells per cm^2. Cells were changed to MEM supplemented with 1% BSA for 18 h just prior to confluency. Known amounts of lipoprotein based on cholesterol content (in μg cholesterol/ml culture medium) as well as lipases or inhibitors were then added to the medium and the cells were incubated for an additional 18 h until confluency. One unit of lipolytic activity is defined as 1 μmol of free fatty acid released per ml of enzyme solution per hour. For experiments

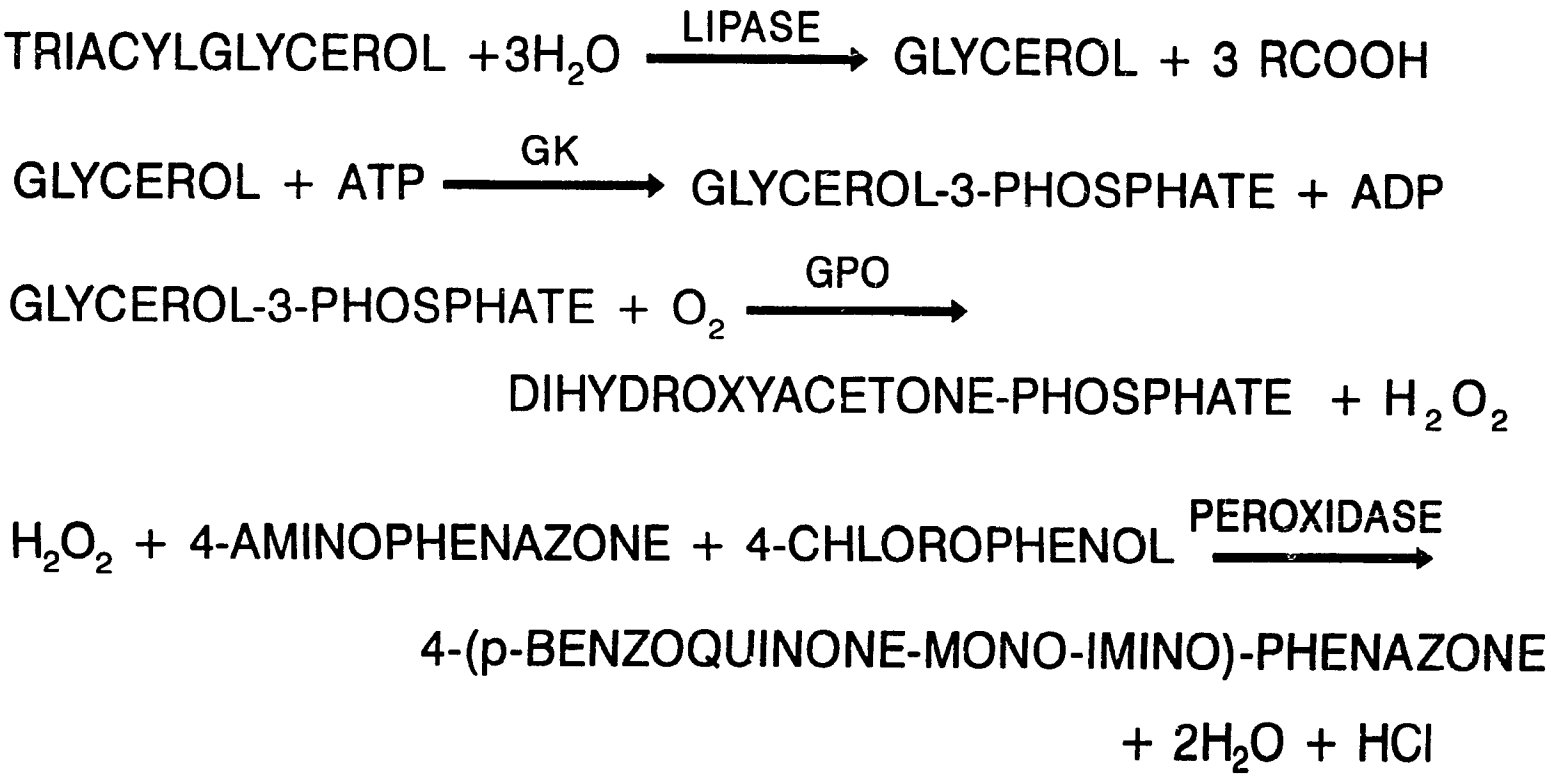

Figure 9. Reaction sequence for the spectrophotometric determination of lipoprotein triacylglycerol content R: fatty acid side chain, ATP: adenosine diphosphate, GPO: glycerol phosphate oxidase, GK: glycerol kinase.

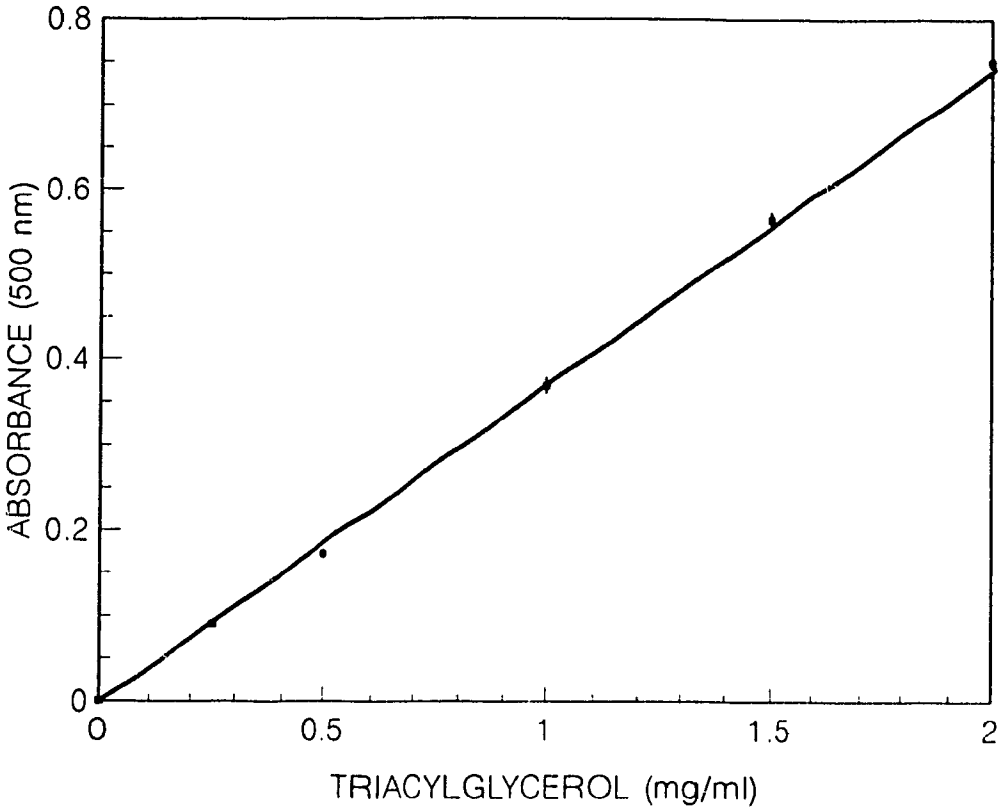

Figure 10. Spectrophotometric triacylglycerol assay. A standard curve for measuring total lipoprotein triacylglycerol was constructed using 0 to 2 mg/ml triolein and absorbance was read at 500 nm; y-intercept = 0.08, slope = 0.003, $r^2 = 0.99$ for the linear regression. The error bars represent deviation between duplicates.

designed to measure apoB secretion into the medium following incubation under various conditions, the medium containing lipoprotein was first removed, the cells were washed with 1 ml of 37°C PBS, and a 0.5-ml aliquot of fresh MEM containing 1% BSA was added for an additional incubation of 8 h. Following this incubation, cells were washed three times with 1 ml ice cold PBS and the intracellular lipids were extracted using 1 ml of 3:2 (v/v) heptane:isopropanol at room temperature for 30 min. The extract was collected into separate test tubes and the cells were washed with an additional 1 ml of heptane:isopropanol which was added to the extract. Cell protein was solubilized using 1 ml of 0.1 M NaOH for 6 h at room temperature and quantified by the method of Bradford (38) using BSA as a standard. This is a rapid sensitive assay which may be used to measure the low protein content of cells. Figure 11 shows a typical standard curve obtained using this method.

2.5 Isolation of Intracellular Lipids

Cell lipid extracts obtained as described in Section 2.4 were evaporated to dryness using a vacuum centrifuge. These were reconstituted in 2x50-μl aliquots of 2:1 (v/v) chloroform.methanol and spotted onto a Silica G thin layer chromatography (TLC) plate which had previously been scored with 1-cm lanes, washed in 2:1 chloroform:methanol and oven dried immediately prior to use. Reference lanes were spotted with a lipid mixture containing cholesterol, cholesteryl oleate, oleic acid and triolein. The plates were developed using

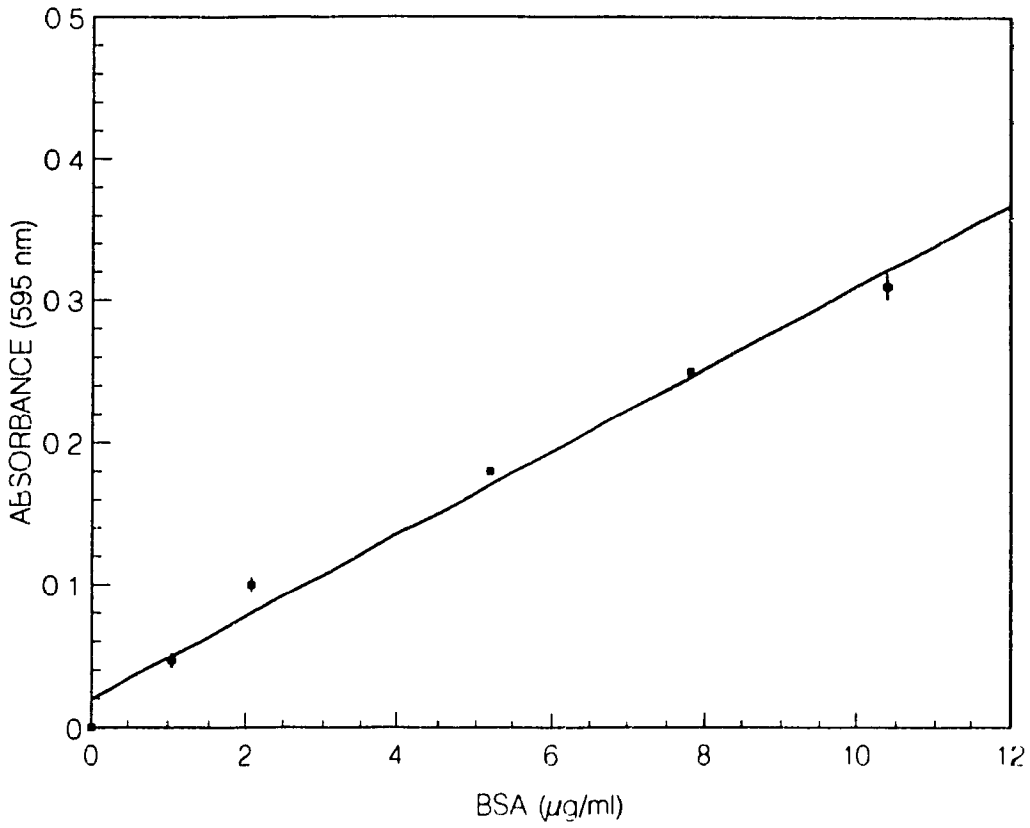

Figure 11. Bradford protein assay A standard curve for measuring total cellular protein was constructed using 0 to 10 5 μg/ml bovine serum albumin (BSA) and absorbance was read at 595 nm, y-intercept = 0.02, slope = 0.003, $r^2 = 0.98$ for the linear regression The error bars represent deviation between duplicates

75:25:1 (v/v/v) hexanes:ethyl ether:acetic acid. Lipid spots were visualized by exposure to iodine vapour and compared to the reference lipids. Table I shows the R_f values for the standards and intracellular lipids resolved. The spots corresponding to triacylglycerol and cholesterol ester were each scraped into 12x75-mm tubes for quantitation.

2.6 Determination of Intracellular Triacylglycerol

Triacylglycerol was extracted by adding 1 ml of isopropanol to the tubes containing the triacylglycerol isolated by TLC. The low levels of triacylglycerol stored intracellularly could not be measured using the colorimetric assay described in Section 2.3 which was designed for measuring human plasma lipid levels. Intracellular triacylglycerol was determined instead by the sensitive method of Neri and Frings (39), a method based on alcoholic KOH saponifying the triacylglycerol to fatty acid and glycerol. Oxidation by periodate converts the glycerol to formaldehyde and glycolaldehyde. Finally, the formaldehyde is reacted with acetylacetone and ammonium acetate to give 3,5-diacetyl-1,4-dihydrolutidine, a coloured product which is measured spectrophotometrically at 405 nm. Figure 12 shows the reaction scheme (40). A 3 mg/ml triolein stock solution was used to prepare standards and the data were analyzed by linear least squares regression. Figure 13 shows a typical standard curve.

LIPID STANDARD	Rf VALUE
PHOSPHOLIPID	0
FATTY ACID	0.30
CHOLESTEROL	0.37
TRIGLYCERIDE	0.61
CHOL ESTER	0.93

Table I. Resolution factors for intracellular lipids Rf values for both lipid standards and intracellular HepG2 lipids applied to thin layer chromatography plates and developed in a 75:25:1 (v/v/v) hexane ethyl ether:acetic acid system.

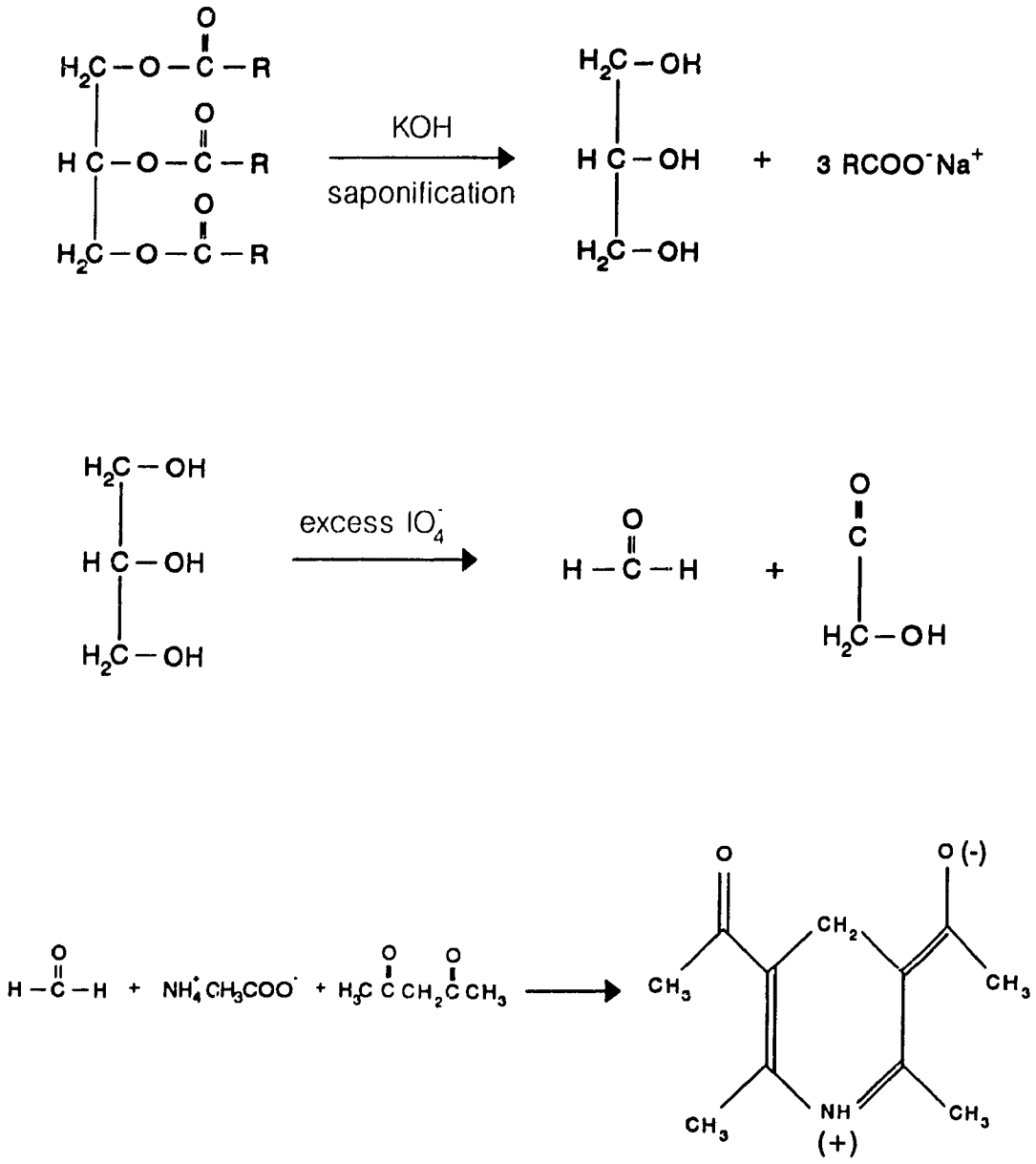

Figure 12. Spectrophotometric determination of intracellular triacylglycerol. R: fatty acid side chain. Adapted from Reference 39.

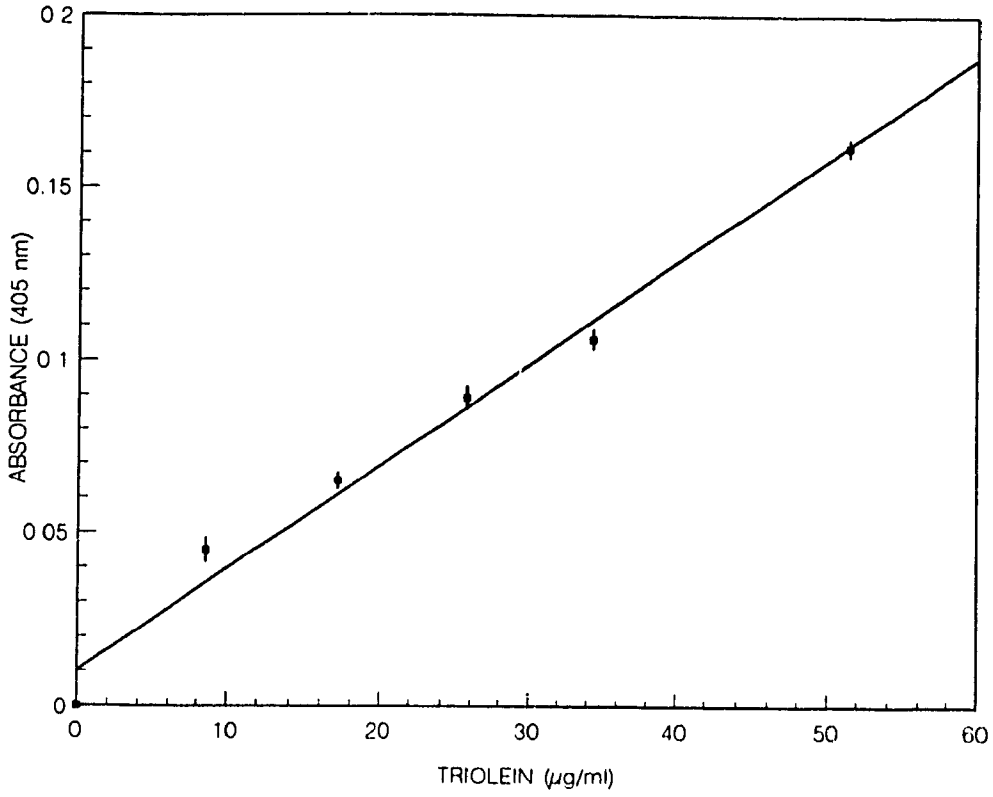

Figure 13. Intracellular triacylglycerol determination. A standard curve was costructed using 0 to 52 μg/ml triolein and absorbance was read at 405 nm; y-intercept=0.01, slope=0.003, r^2=0.98 for the linear regression. The error bars represent deviation between triplicates. See Figure 12 for the reaction scheme.

2.7 Determination of Intracellular Cholesterol Ester

In order to measure cholesterol ester, the method of Rudel and Morris (41) was modified to give a semi-micro assay which was useful for determining low levels of intracellular cholesterol ester. To the tubes containing the cholesterol ester isolated by TLC (Section 2.5), 1.25 ml of 2:1 (v/v) chloroform:methanol was added. Following a 10 min extraction period, 1 ml of extract was evaporated to dryness using a vacuum centrifuge. A 0.25-ml volume of 0.5 mg/ml o-pthalaldehyde in glacial acetic acid was added and the samples were allowed to stand at room temperature for 10 min. To this was added 0.125 ml of concentrated sulphuric acid. Under acidic conditions, the cholesterol ester is hydrolysed and o-pthalaldehyde reacts directly with free cholesterol giving a coloured compound which can be measured spectrophotometrically. The assay samples were transferred to a semi-micro cuvette or a microtiter plate and absorbances were measured at 550 nm. Cholesteryl oleate was used to prepare a standard curve and the data were analyzed using linear least squares regression. Figure 14 shows the reaction for cholesterol derivatization (42) and Figure 15 shows a typical standard curve.

2.8 Determination of Apolipoprotein B

An indirect competitive type enzyme linked immunosorbent assay (ELISA) (43) was used to specifically quantify the apoB secreted by the HepG2 cells. Polystyrene microtiter plate with 96 wells were coated with a 3 μg/ml solution of

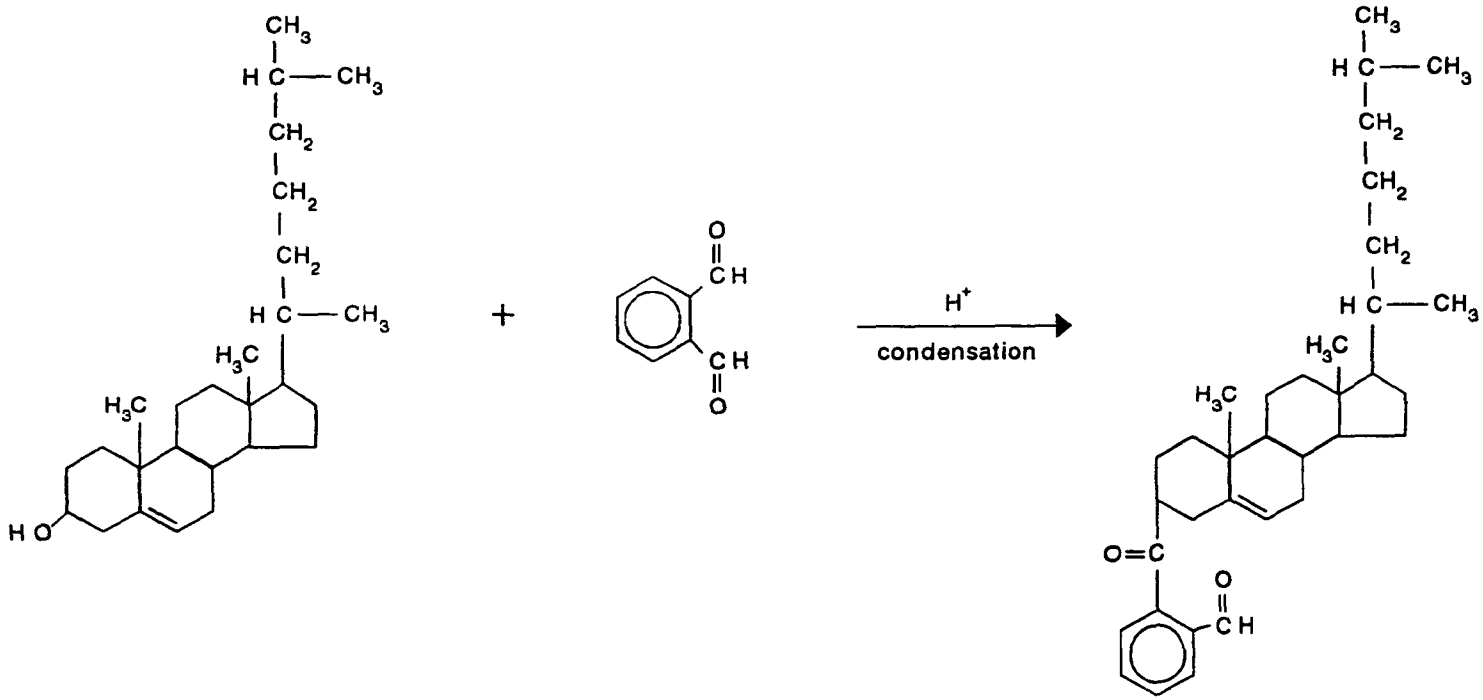

Figure 14 Derivatization of cholesterol for determination of intracellular cholesterol ester Adapted from Reference 41

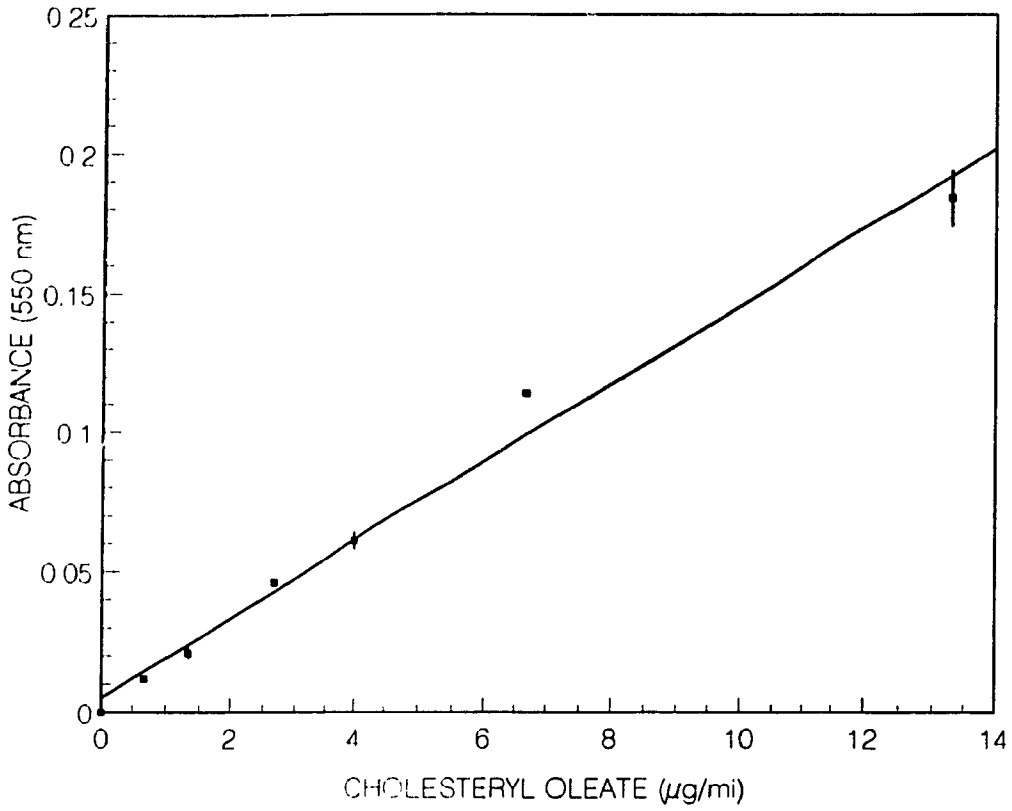

Figure 15. Intracellular cholesterol ester determination. A standard curve was constructed using 0 to 13.5 μg/ml cholesteryl oleate and absorbance was read at 550 nm; y-intercept=0 003, slope=0.014, r^2=0.99 for the linear regression The error bars represent deviation between triplicates See Figure 14 for the reaction scheme.

LDL protein in PBS, (pH=7.2) one day prior to use. Before use, the plate was washed in a 0.9% (w/v) solution of NaCl containing 0.5 ml/l Tween 20 and non specific binding was minimized using a blocking solution consisting of 1.5% BSA (w/v) in PBS for 2 h at room temperature. Standards containing 0.06 to 4 μg/ml LDL-derived apoB were prepared. Unknowns and standards were incubated overnight with a rabbit polyclonal antibody to apoB diluted 1:6000 in PBS containing 0.5 ml/l Tween 20, and 100 μL of each sample was added in triplicate to the microtiter plate for 2 h at 37°C. Following incubation, the plates were washed and a solution of anti-rabbit IgG conjugated to horseradish peroxidase was added to each well, and this was incubated at 37°C for 30 min. Finally, the excess secondary antibody was washed away and a colorimetric reaction was initiated by adding o-phenylenediamine dihydrochloride in the presence of hydrogen peroxide and the plate was read spectrophotometrically at 490 nm. A schematic diagram of the ELISA assay used for apoB determination is shown in Figure 16. A log-log plot of apoB concentration (μg/ml) versus absorbance at 490 nm was constructed and linear least squares analysis of the data was carried out (Figure 17). ApoB controls of known concentration were run with each analysis to ensure reproducibility of the results.

2.9 Statistical Analysis of Data

Figures 18, 20, 21, 22 and 23 show representative experiments performed one or two times where only the intracellular lipids were measured. Deviation is

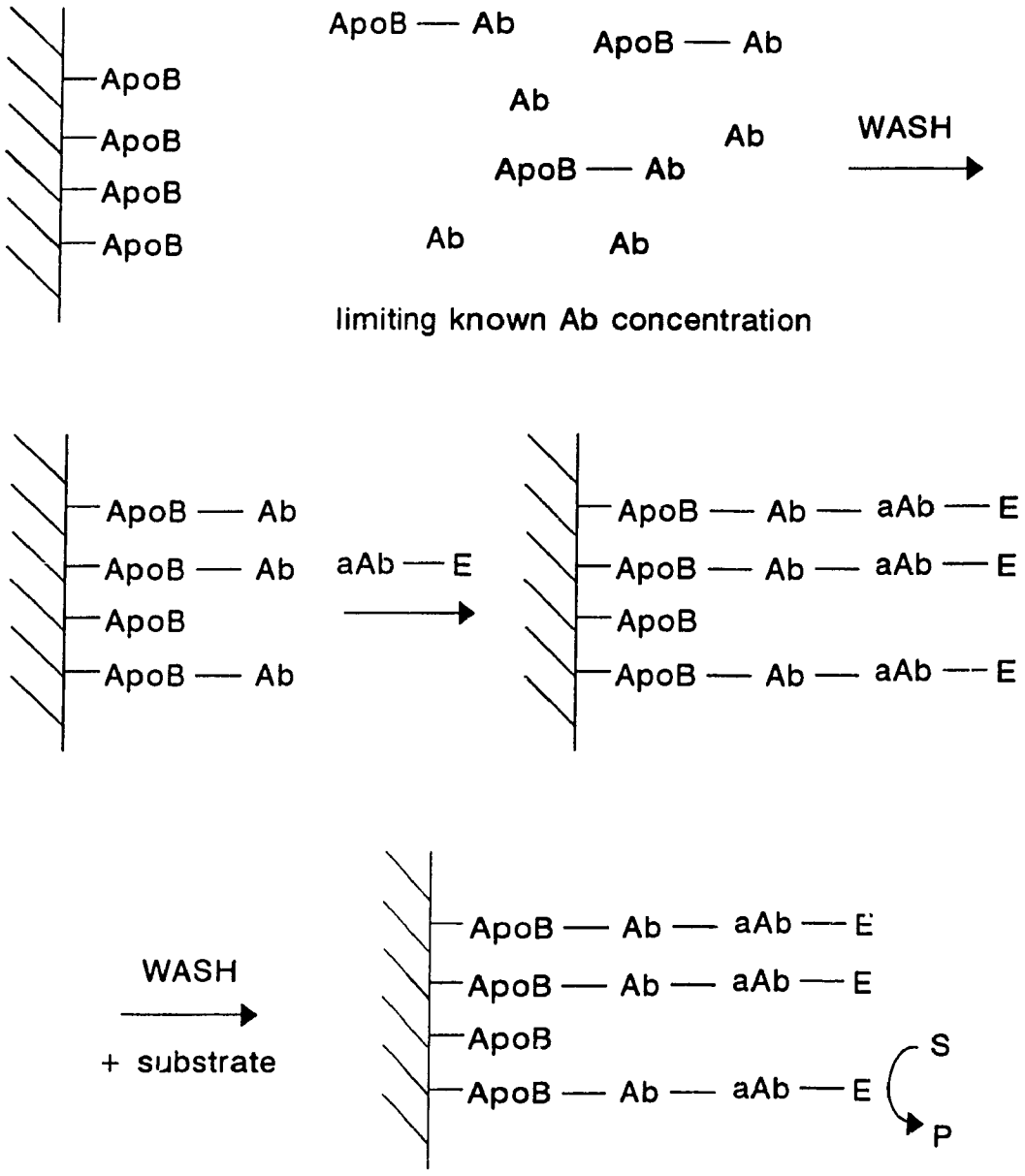

Figure 16 Competitive indirect ELISA for apolipoprotein B determination ApoB: LDL-derived ApoB, Ab: primary antibody to ApoB, ApoB-Ab: LDL-derived ApoB bound to antibody, aAb-E: secondary antibody conjugated to horseradish peroxidase, S: substrate, P: product.

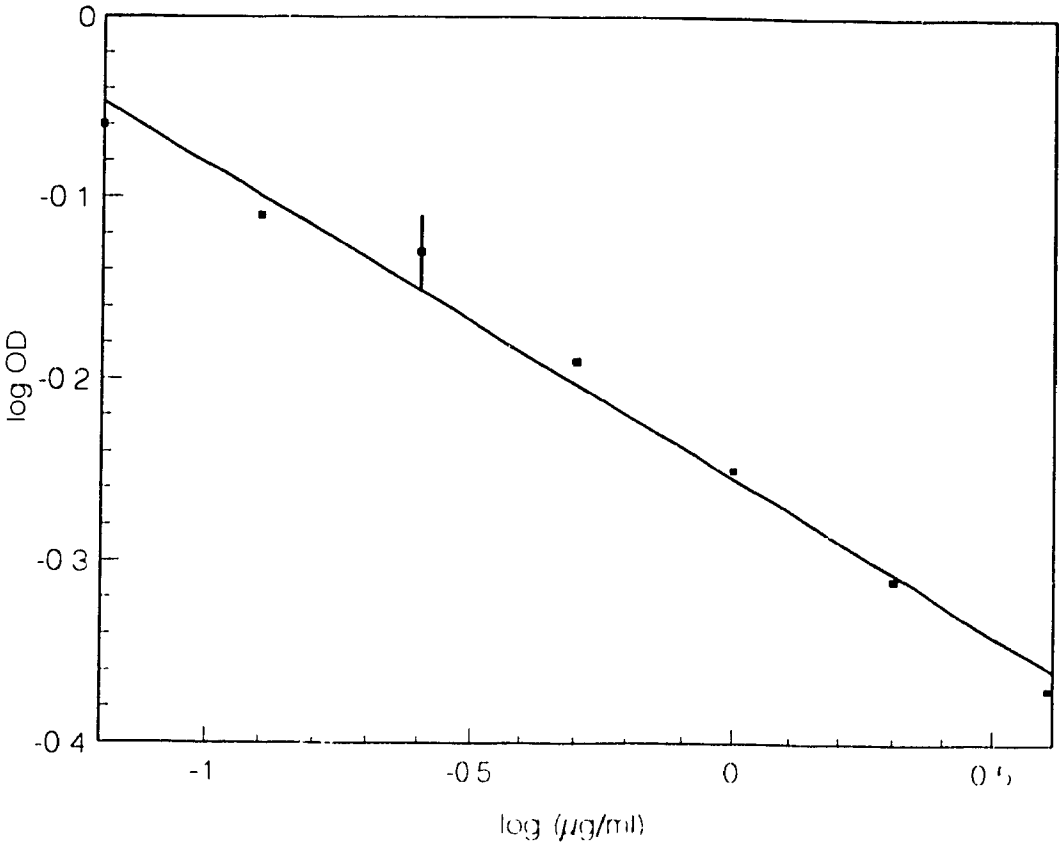

Figure 17. ELISA for the determination of secreted apolipoprotein B (apoB). A standard curve was constructed using 4 to 0 625 μg/ml LDL-derived apoB, and the absorbance was read at 490 nm. Analysis of the log log data yielded the regression line; y-intercept = -0 25, slope = 0 18, r^2 0 99 The error bars represent deviation between triplicates

expressed as standard deviation between triplicate values for each experimental condition. These experiments were then repeated at least three additional times measuring both intracellular lipids as well as apoB secreted. The data are presented in Figures 19, 24, 25 and Tables II-IV which represent the average of a number of experiments (n) as indicated in the figure legends. Deviation is expressed as the standard error of the mean (avg/$\sqrt{n}$). The data for these experiments were averaged and statistical significance was calculated using a paired t test. Statistical significance is defined as the sufficiently small probability of the observed changes occurring by chance alone. A value of $p>0.1$ representing a 10% or greater probability that the experimentally observed changes occurred by chance alone was considered to be not significant ($p=NS$) and was indicated accordingly (43).

3. RESULTS

3.1 Mechanism of Lipoprotein Internalization

It has been previously demonstrated that addition of TRL alone to HepG2 cells is not sufficient to cause an increase in the mass of the intracellular pool of triacylglycerol and cholesterol ester. It has also been reported that breakdown of these lipoprotein particles by LPL is required for intracellular accumulation of both cholesterol ester and triacylglycerol (45).

Figure 18a is a representative experiment showing intracellular triacylglycerol mass accumulation with increasing concentration of TRL added to HepG2 cells in the presence and absence of LPL. The dashed curve shows no significant change in intracellular triacylglycerol accumulation in the absence of LPL despite increasing amounts of TRL added. When increasing amounts of TRL were added in the presence of 0.125 U/ml of LPL, there was a significant increase in triacylglycerol accumulation up to 290% above basal increase at the highest TRL concentration of 100 μg/ml.

Figure 18b shows intracellular cholesterol ester accumulation under the same experimental conditions. There was no significant change in intracellular cholesterol ester accumulation in the absence of LPL. Addition of 0.125 U/ml LPL caused a 138% rise in intracellular cholesterol ester at the highest TRL concentration. These results suggest that LPL is essential for the uptake of both TRL derived fatty acids and cholesterol ester by HepG2 cells. It has been

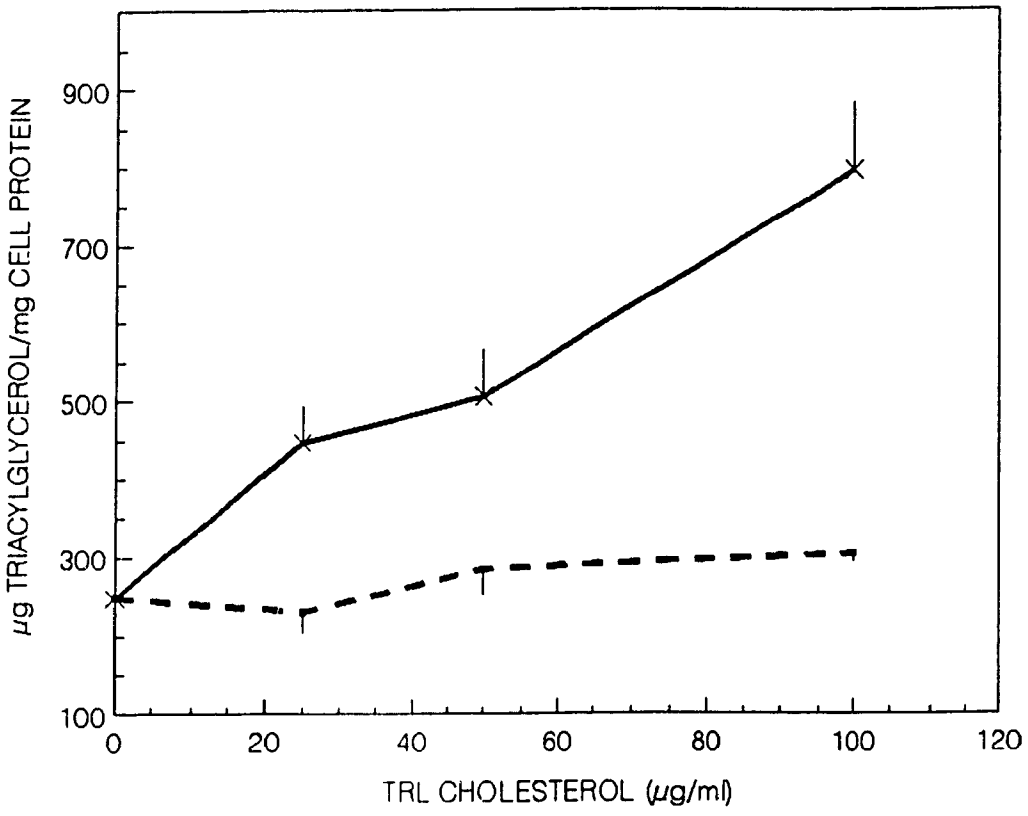

Figure 18a Effect of increasing concentration of triacylglycerol rich-lipoprotein in the absence and presence of lipoprotein lipase on intracellular triacylglycerol accumulation. HepG2 cells were incubated at 37°C for 18 hours with 0, 25, 50 and 100 μg/ml of triacylglycerol-rich lipoprotein (TRL) cholesterol in the absence (---) and presence (—) of 0.125 U/ml of lipoprotein lipase (LPL) Data points represent the average of three values and the error bars indicate the standard deviation. For clarity, only the upper and lower halves of the error bars are shown on solid and dashed curves, respectively

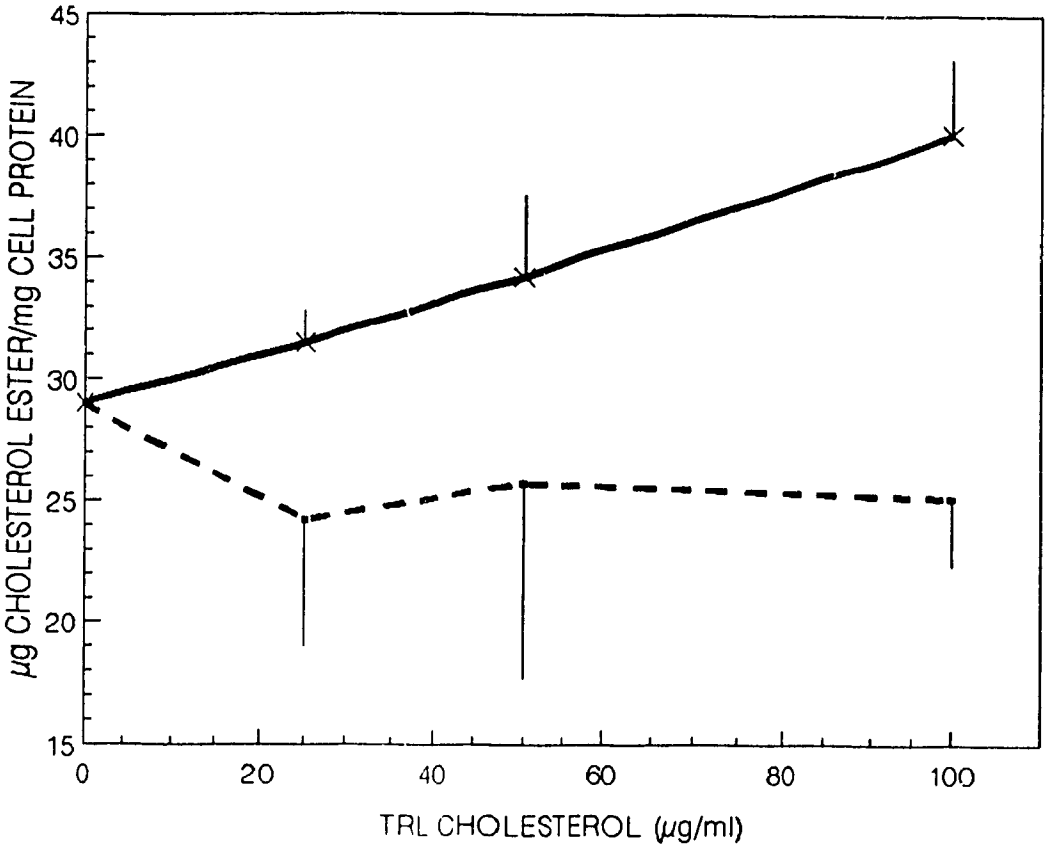

Figure 18b. Effect of increasing concentration of triacylglycerol-rich lipoprotein in the absence and presence of lipoprotein lipase on intracellular cholesterol ester accumulation HepG2 cells were incubated at 37°C for 18 hours with 0, 25, 50 and 100 μg/ml of triacylglycerol-rich lipoprotein (TRL) cholesterol in the absence (---) and presence (—) of lipoprotein lipase (LPL). Data points represent the average of three values and the error bars indicate the standard deviation. For clarity, only the upper and lower halves of the error bars are shown on solid and dashed curves, respectively.

suggested that this uptake may require LPL not only for lipolysis, but also for structural interaction with the cell surface (46). These data confirm earlier reports that LPL is required for internalization of lipoprotein particles (47).

In order to determine whether this effect was specific to LPL, intracellular lipid accumulation was studied using triacylglycerol lipases which are structurally unrelated to LPL. The ability of fungal and bacterial lipases from *Rhizopus arrhizus* (48) and *Pseudomonas*, respectively (48) in allowing accumulation of both triacylglycerol and cholesterol ester were compared to LPL. All three lipases possess similar catalytic subunits and therefore triacylglycerol hydrolase activity, which allows for lipolysis of triacylglycerol and release of free fatty acids in vitro. The binding domains of all three lipases, however, are different so that if some structural property specific to LPL distinct from its catalytic activity is required for binding and uptake of the TRL particles, then both the *Pseudomonas* and *Rhizopus arrhizus* lipases should be ineffective in allowing uptake of TRL particles.

Figure 19a shows accumulation of intracellular triacylglycerol mass in the presence of these three different triacylglycerol hydrolases. Basal intracellular triacylglycerol levels and triacylglycerol levels with the addition of 100 μg/ml of TRL cholesterol alone are comparable. When 0.125 U/ml of LPL was added at the same concentration of TRL, there was a 349% $\pm$ 33%, $p<0.0005$ increase in intracellularly accumulated triacylglycerol mass over basal levels which is consistent with results shown previously (Figure 18a). When 0.125 U/ml of

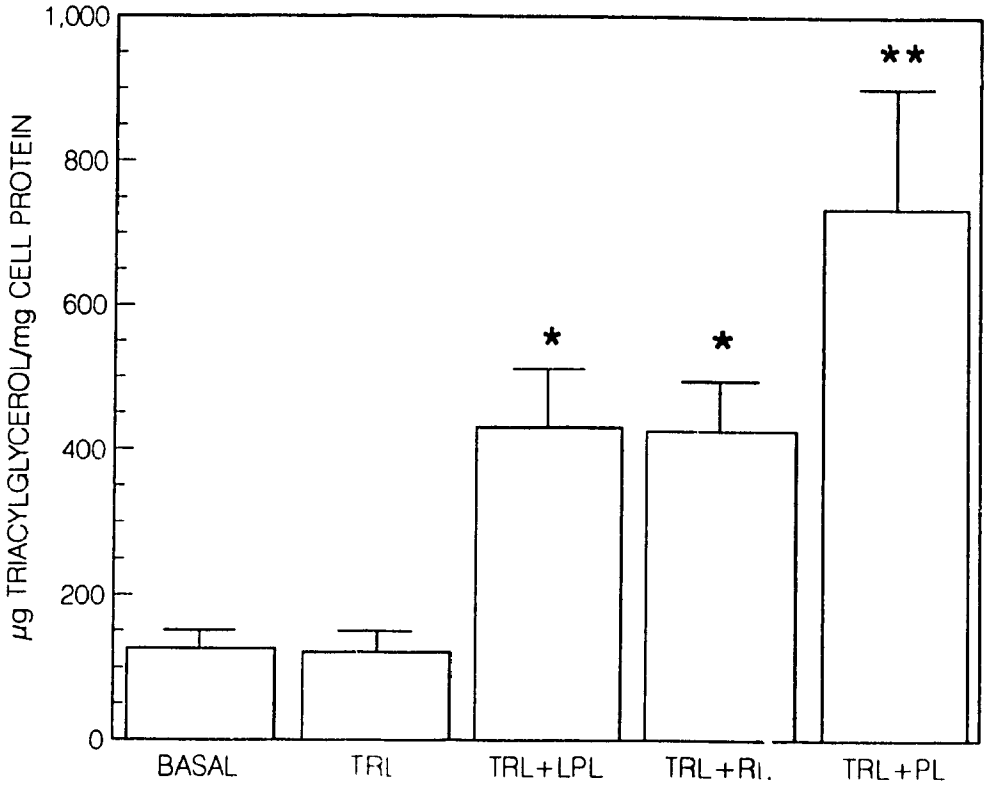

Figure 19a. Effect of structurally unrelated triacylglycerol lipases on intracellular triacylglycerol accumulation. HepG2 cells were incubated at 37°C for 18 hours with 100 μg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol in the absence of lipolytic activity, as well as in the presence of 0.125 U/ml of either lipoprotein lipase (LPL), Rhizopus Arrhizus lipase (RL) or Pseudomonas lipase (PL). This graph represents the average of 5 experiments ± standard error of the mean; *$p<0.005$, **$p<0.025$, significantly different from basal

Rhizopus arrhizus lipase was added to the same amount of TRL, this produced an increase of 472% ± 61%, $p<0.005$ in triacylglycerol accumulation which is comparable to that seen with LPL. Addition of 0.125 U/ml of the *Pseudomonas* lipase produced an equally large 522% ± 99%, $p<0.025$ increase in accumulated triacylglycerol mass.

Figure 19b shows intracellular cholesterol ester mass accumulation in the HepG2 cells for the same experiments. Again there was no significant difference between basal cholesterol ester levels and levels of intracellular cholesterol ester mass accumulated in the presence of 100 μg/ml TRL cholesterol alone, suggesting that LPL is necessary for cholesterol ester uptake. With the addition of 0.125 U/ml of LPL to the 100 μg/ml TRL, there was a 187% ± 14%, $p<0.0005$ increase in intracellular cholesterol ester. A similar increase of 202% ± 25%, $p<0.025$ was seen with the addition of the *Rhizopus arrhizus* lipase to the same amount of TRL. In contrast, there was no significant increase in intracellular cholesterol ester accumulation with the addition of *Pseudomonas* lipase.

The results obtained using structurally unrelated lipases (47,48) suggest that LPL itself is not essential for uptake of TRL particles since increased intracellular lipid accumulation was also observed with *Rhizopus arrhizus* lipase. However, to date the observation of triacylglycerol accumulation had always been coupled with cholesterol ester accumulation inside the cells. Thus the large increase in triacylglycerol, with no corresponding increase in cholesterol ester accumulation, as was the case with the *Pseudomonas* lipase, was unexpected.

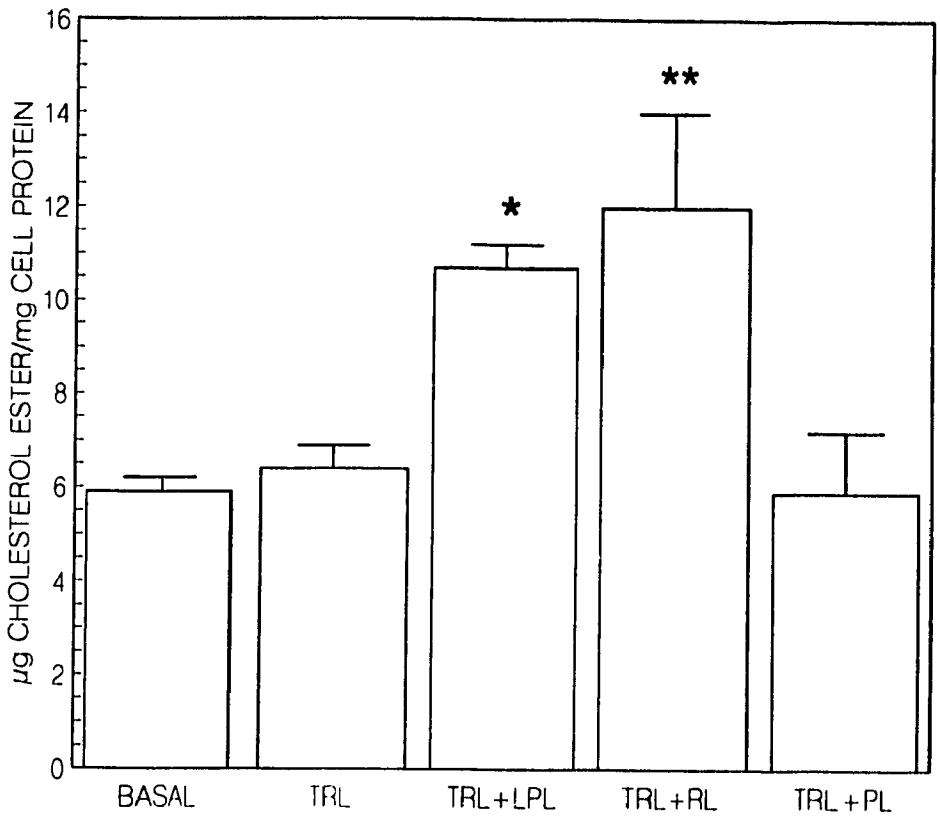

Figure 19b. Effect of structurally unrelated triacylglycerol lipases on intracellular cholesterol ester accumulation. HepG2 cell were incubated at 37°C for 18 hours with 100 µg/ml triacylglycerol rich lipoprotein (TRL) cholesterol in the absence of lipolytic activity, as well as in the presence of 0.125 U/ml of either lipoprotein lipase (LPL), Rhizopus Arrhizus lipase (RL) or Pseudomonas lipase (PL). This graph represents the average of 5 experiments ± standard error of the mean; *$p<0.025$, **$p<0.005$, significantly different from basal.

The failure of this lipase to cause an increase in cholesterol ester suggests that it is somehow selective in allowing fatty acid uptake, but not cholesterol ester uptake. These results would be consistent with a mechanism by which fatty acid and cholesterol ester were taken up through independent mechanisms. Since there is a large quantity of free fatty acid released from the TRL particles in the presence of all three lipases, this would explain the increase in intracellular triacylglycerol mass in all three cases. Furthermore, if fatty acids are released by enzymatic degradation of TRL particle triacylglycerol, then a cholesterol ester enriched remnant particle should remain following this degradation. It is possible that this remnant particle is internalized through a receptor mediated mechanism which recognizes one or more particle surface apoproteins. This mechanism would also explain the results obtained with *Pseudomonas* lipase. This particular lipase is known to possess significant phospholipase activity as well as triacylglycerol hydrolase activity (49). In fact, following an attempt made to hydrolyse and re-isolate TRL, it was found that apparently intact remnant particles were obtained with LPL and *Rhizopus arrhizus* lipase, but not with *Pseudomonas* lipase. If the *Pseudomonas* lipase were hydrolyzing lipoprotein surface phospholipids as well as triacylglycerol to release fatty acids, the resulting intracellular triacylglycerol accumulation would be significantly greater than that observed using LPL, which is, in fact, what was observed. The degradation of surface phospholipids would result in loss of structural integrity of the lipoprotein remnant particle, which would prevent receptor mediated uptake of the particle

based on recognition of surface apoproteins, thus preventing intracellular cholesterol ester accumulation.

LPL is required for uptake of TRL. However, since internalization is also observed in the presence of lipases which contain structurally unrelated binding regions, it is unlikely that the uptake mechanism involves receptor recognition of a specific amino acid sequence on the lipase. One possible alternative mechanism, which would allow TRL targeting to cell surface receptors, may involve affinity between cell surface proteoglycans and those found on the lipases which bind to the lipoprotein particles. Involvement of proteoglycans in cellular uptake of TRL particles was assessed by experiments taking advantage of substances which inhibit proteoglycan interactions at the cell surface.

Heparin is a polysaccharide composed of glycosaminoglycan chains (50). It is known to bind to cell surfaces and may compete with TRL for proteoglycan sites on the putative TRL receptor (51). Figure 20a is a representative experiment showing the effect of adding heparin on intracellularly accumulated triacylglycerol. Addition of 100 μg/ml TRL and 0 125 U/ml of LPL causes a 446% $\pm$ 6% increase in intracellular triacylglycerol above basal level. When 10 U/ml of heparin were added to these same concentrations of TRL and LPL, there is a 569% $\pm$ 8% increase in triacylglycerol accumulation over basal levels. It has been previously reported that heparin stabilizes LPL (52) and this could render the lipase more active and could, therefore, increase levels of triacylglycerol accumulation.

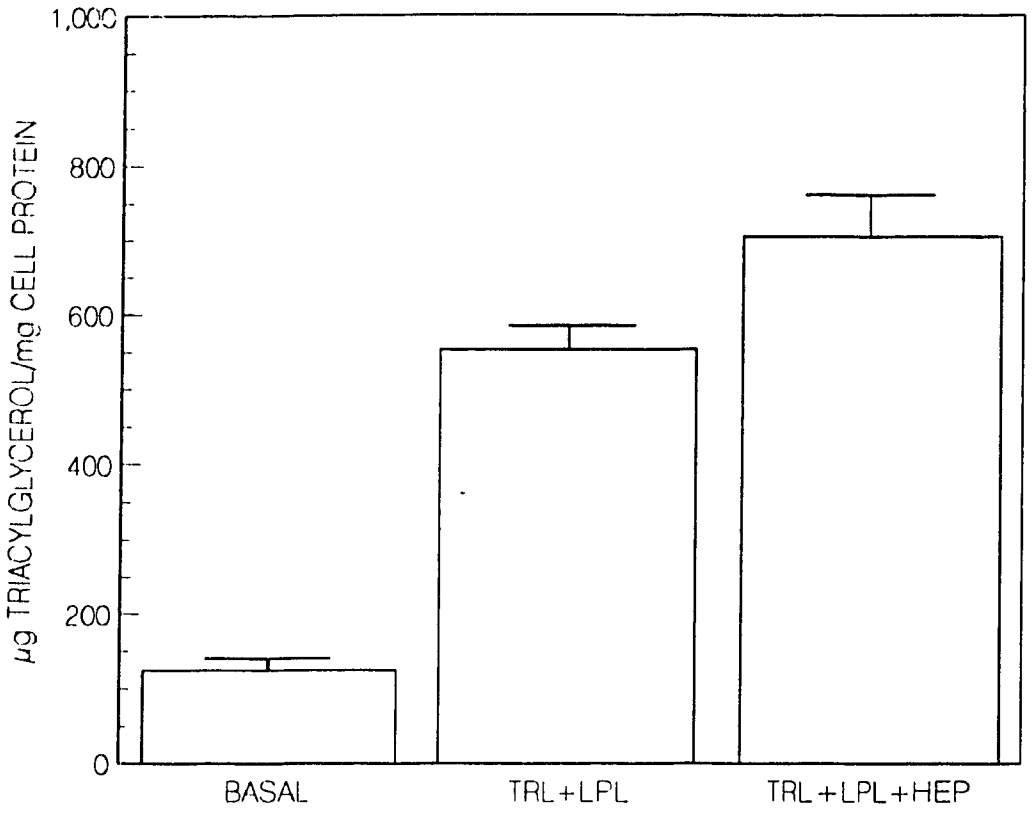

Figure 20a. Effect of heparin on intracellular triacylglycerol accumulation HepG2 cells were incubated for 18 hours with 100 μg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol and 0.125 U/ml lipoprotein lipase (LPL) in the absence and presence of 10 U of heparin (HEP). Data represent the average of three values and the error bars indicate the standard deviation.

Figure 20b shows the effect of heparin on intracellular cholesterol ester accumulation. Addition of 100 μg/ml of TRL and 0.125 U/ml of LPL produced a 280% ± 18% increase above basal levels of cholesterol ester. In contrast to the effects on triacylglycerol, when 10 U/ml of heparin were added under these same conditions, an increase of only 112% ± 30% above basal, a value which was not statistically significant. Therefore, heparin inhibited the TRL induced accumulation of cholesterol ester by 99%. This is in sharp contrast to what was seen with intracellular triacylglycerol where heparin had a positive effect. These observations further strengthen the model proposed that fatty acid uptake is independent of cholesterol ester uptake. These data also suggest that cell surface proteoglycan interactions may be involved in TRL uptake by hepatocytes since heparin is effective in significantly decreasing uptake of cholesterol ester containing particles.

Lactoferrin and asialofetuin are both highly glycosylated proteins which may bind to cell surface proteoglycans. Lactoferrin, in particular, has been shown to bind specifically to the LRP receptor and compete out the binding of TRL (53,54). Addition of lactoferrin in the presence of TRL and LPL resulted in a 292% ± 1% increase in intracellular triacylglycerol accumulation as shown in Figure 21a. This is almost twice the 157% ± 8% increase above basal intracellular triacylglycerol accumulation observed on the addition of only TRL and LPL.

Figure 21b shows the effect of lactoferrin on intracellular cholesterol ester accumulation. Incubation with 100 μg/ml TRL cholesterol and 0.125 U/ml of LPL

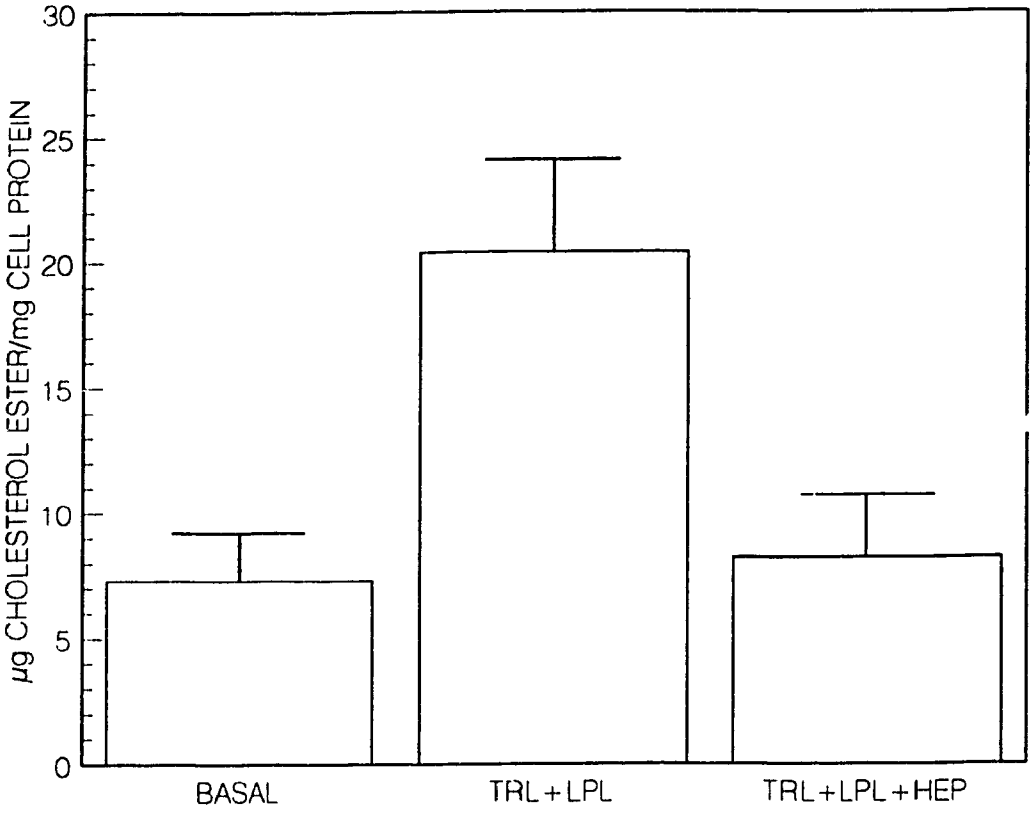

Figure 20b. Effect of heparin on intracellular cholesterol ester accumulation. HepG2 cells were incubated for 18 hours with 100 μg/ml triacylglycerol rich-lipoprotein (TRL) cholesterol and 0.125 U/ml lipoprotein lipase (LPL) in the absence and presence of 10 U of heparin (HEP). Data represent the average of three values and the error bars indicate the standard deviation.

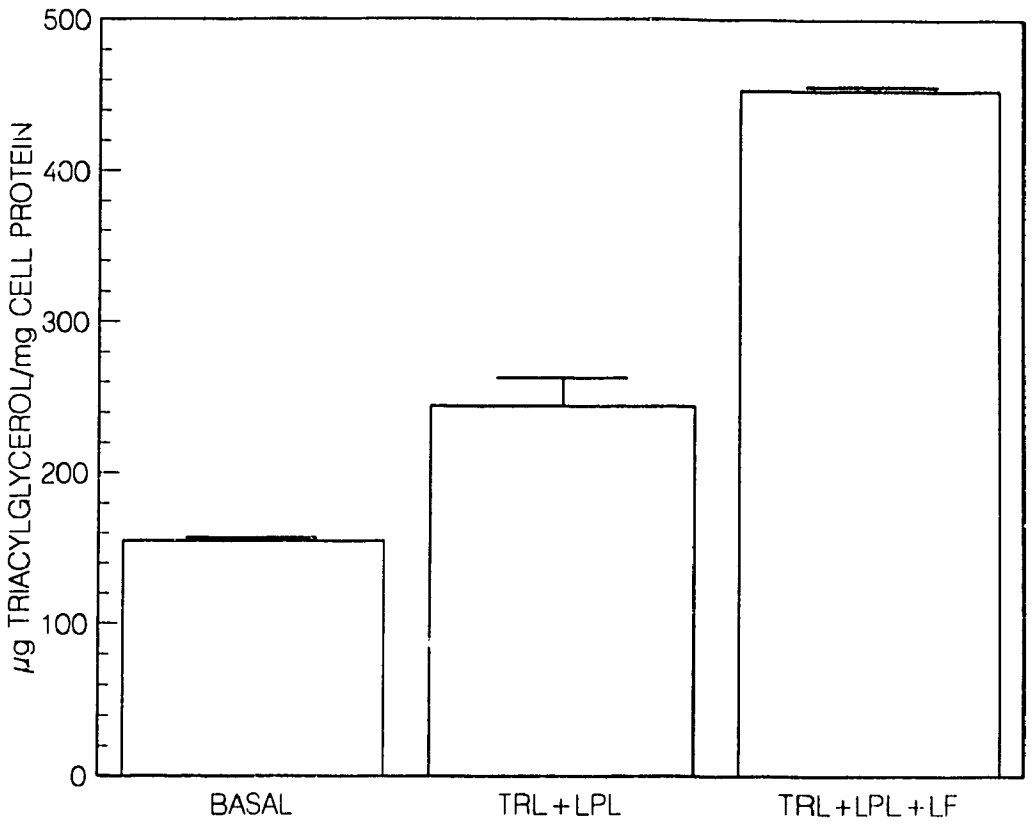

Figure 21a. Effect of lactoferrin on intracellular triacylglycerol accumulation HepG2 cells were incubated for 18 hours with 100 μg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol and 0.125 U/ml of lipoprotein lipase (LPL) in the presence and absence of 0.02 mM lactoferrin (LF). Data represent the average of three values and the error bars indicate the standard deviation.

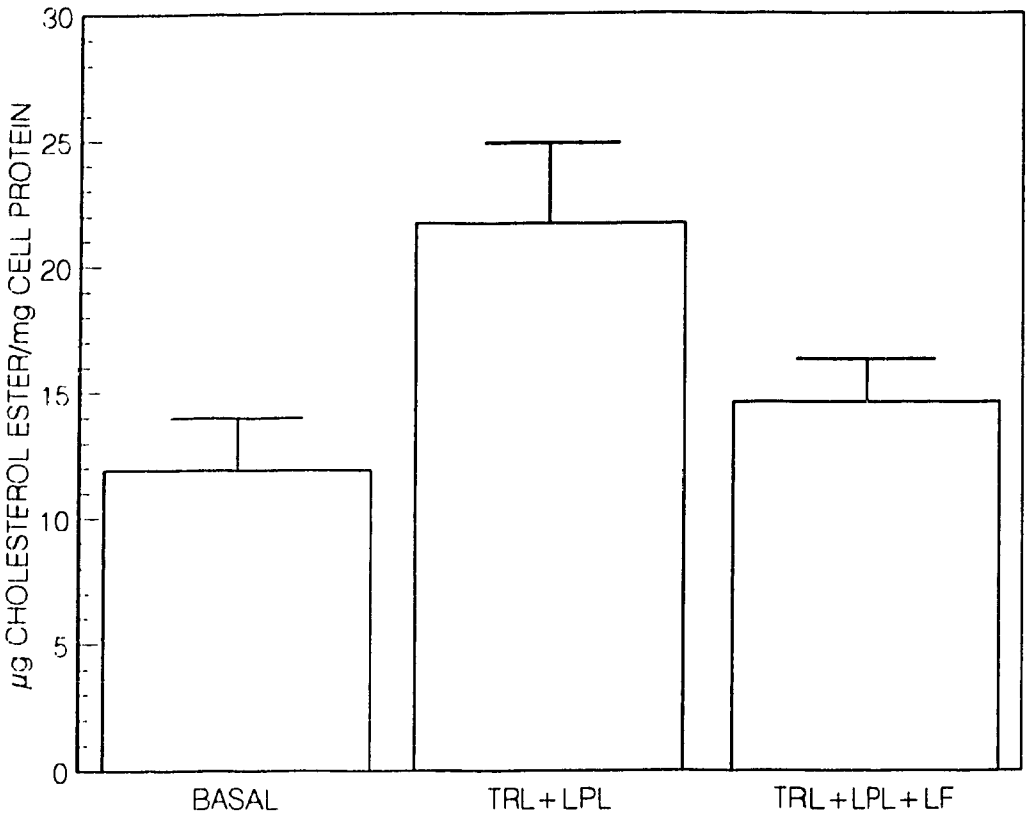

Figure 21b. Effect of lactoferrin on intracellular cholesterol ester accumulation. HepG2 cells were incubated for 18 hours with 100 µg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol and 0.125 U/ml of lipoprotein lipase (LPL) in the presence and absence of 0.02 mM lactoferrin (LF). Data represent the average of three values and the error bars indicate the standard deviation.

caused cholesterol ester to increase by 182% ± 15% for lactoferrin experiments. With the addition of lactoferrin, the accumulated cholesterol ester was found to be 123% ± 12%. This is comparable to basal levels of cholesterol ester produced by the cells in the absence of any TRL addition. Therefore, lactoferrin inhibited the TRL induced increase in cholesterol ester by 72%.

Similar effects are observed with the addition of asialofetuin (55). Figure 22a shows that, with the addition of 100 μg/ml TRL cholesterol and 0.125 U/ml LPL, intracellular triacylglycerol accumulation was 157% ± 8% above basal level. This is less than the triacylglycerol accumulation of 259% ± 5% observed in the presence of asialofetuin. Figure 22b shows cholesterol ester accumulation for the same set of experiments. On the addition of TRL and LPL there was a 279% ± 18% increase in intracellular cholesterol ester. Asialofetuin added to the lipoprotein causes only a 126% ± 11%, which corresponds to a 68% inhibition of cholesterol ester accumulation.

Neither lactoferrin nor asialofetuin were effective in inhibiting uptake of fatty acids leading to marked increases in intracellular triacylglycerol. However, both were effective in almost completely inhibiting the increase in cholesterol ester uptake mediated by cholesterol containing TRL remnant particles. These observations are consistent with the previous data obtained using different lipases and heparin (Figures 19 and 20) which strongly suggest that fatty acid uptake is independent of remnant TRL particle uptake and that the mechanisms for each are dissociable.

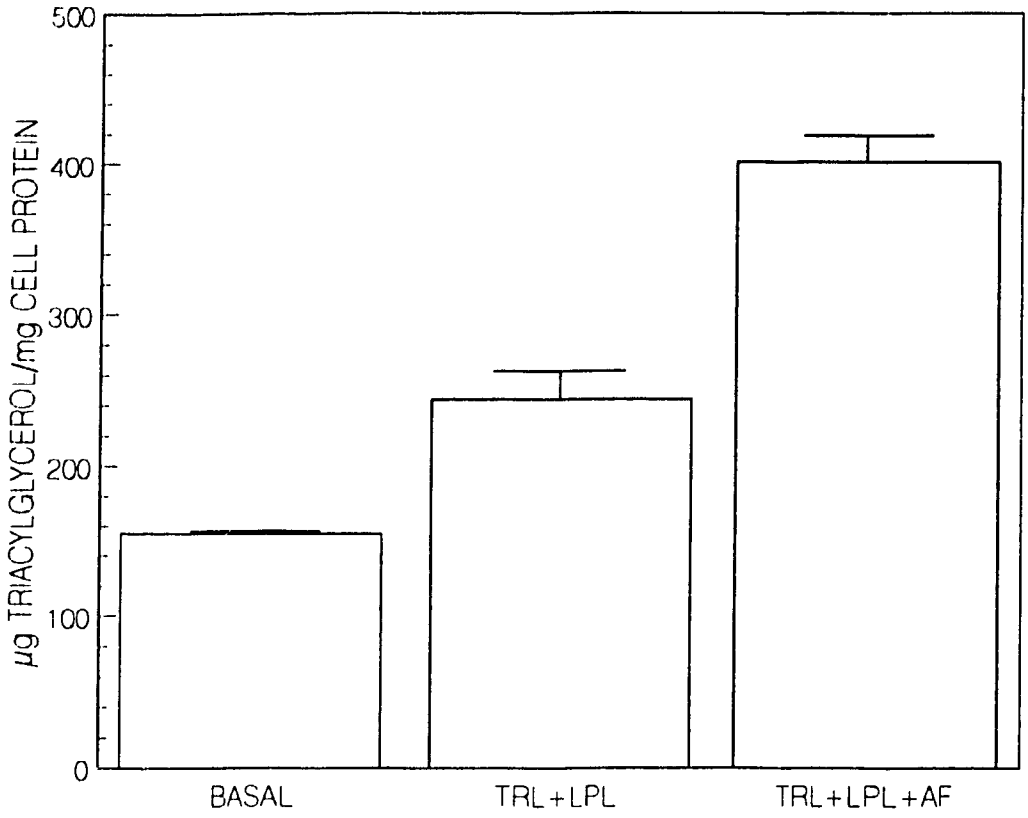

Figure 22a. Effect of asialofetuin on intracellular triacylglycerol accumulation. HepG2 cells were incubated for 18 hours with 100 µg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol and 0.125 U/ml of lipoprotein lipase (LPL) in the presence and absence of 0.2 mM asialofetuin (AF). Data represent the average of three values and the error bars indicate the standard deviation.

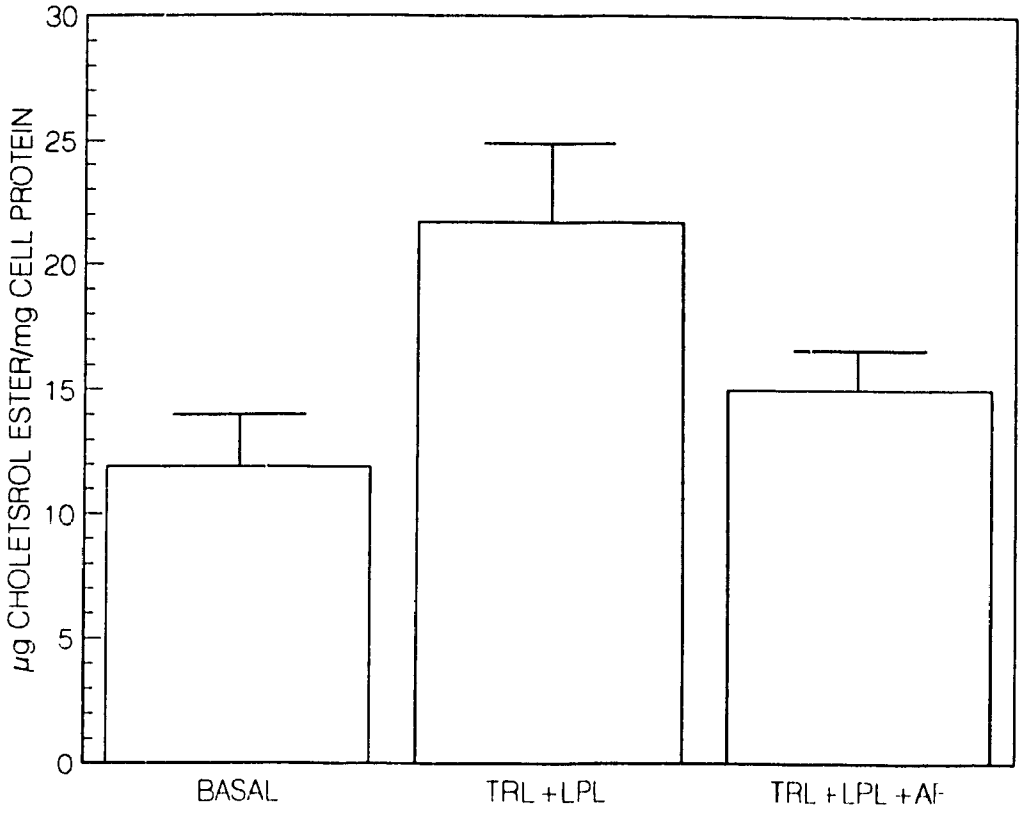

Figure 22b. Effect of asialofetuin on intracellular cholesterol ester accumulation HepG2 cells were incubated for 18 hours with 100 µg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol and 0.125 U/ml of lipoprotein lipase (LPL) in the presence and absence of 0 2 mM asialofetuin (AF) Data represent the average of three values and the error bars indicate the standard deviation

Furthermore, since cholesterol ester is necessary as one of the core components of lipoproteins secreted from the HepG2 cells, the decrease in cholesterol ester accumulation may also prevent triacylglycerol from being secreted by the cell in the form of a TRL. This may be reflected in the large accumulation of triacylglycerol observed in the presence of lactoferrin (Figure 21a) or other inhibitors such as heparin (Figure 20a).

Further experiments related to proteoglycans were performed using the drug, 4-methylumbelliferyl-β-D-xyloside. This substance acts as a specific inhibitor of proteoglycan biosynthesis. The drug competes with xylose units for glycosyltransferases which are the rate-limiting enzymes in lengthening glycosaminoglycan chains of proteoglycans. This results in the synthesis of core protein lacking portions of glycosaminoglycan chain, as well as glycosaminoglycan chains bound to the drug rather than to a protein core moiety (56,57).

The intracellular triacylglycerol and cholesterol ester accumulation for experiments performed using 4-methylumbelliferyl-β-D-xyloside are shown in Figures 23a and 23b, respectively. Cells were grown in the presence or absence of 4-methylumbelliferryl-β-D-xyloside. Optimal concentrations of TRL and LPL were then added and intracellular triacylglycerol and cholesterol ester were measured. There was no significant change in TRL-mediated intracellular triacylglycerol accumulation in the cells incubated both with and without 4-methylumbelliferyl-β-D-xyloside. That triacylglycerol accumulation in the cells was

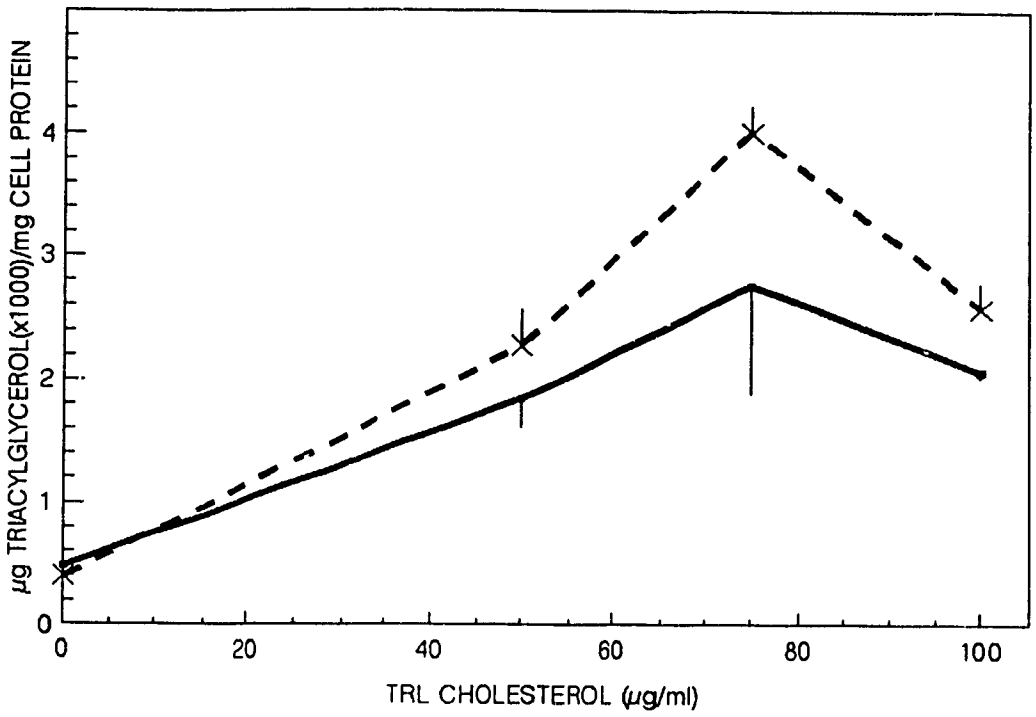

Figure 23a. Effect of 4-methyl-umbelliferryl-ß-D-xyloside on intracellular triacylglycerol accumulation. Cells were grown for 72 hours in the absence (---) and presence (—) of 4-methyl-umbelliferryl-ß-D-xyloside On the fourth day, 25, 50 and 100 µg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol and 0 125 U/ml lipoprotein lipase (LPL) were added and the cells were incubated an additional 18 hours. Data points represent the average of three values and the error bars indicate the standard deviation. For clarity, only the upper and lower halves of the error bars are shown on dashed and solid curves, respectively.

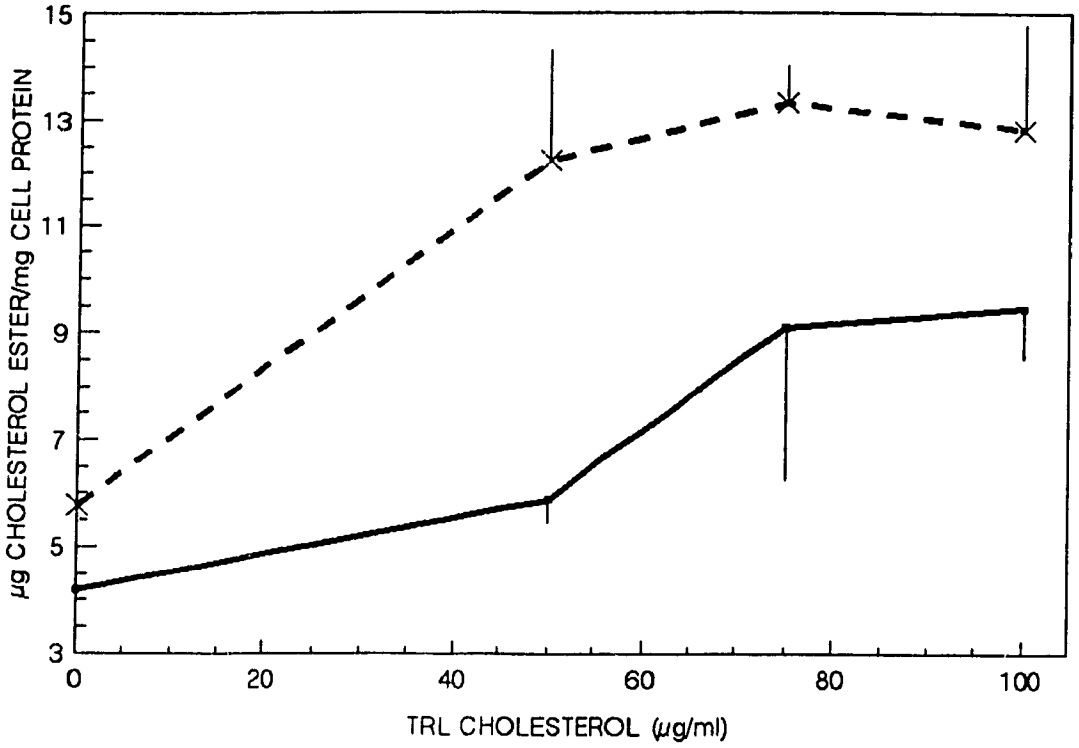

Figure 23b. Effect of 4-methyl-umbelliferryl-ß-D-xyloside on intracellular cholesterol ester accumulation. HepG2 cells were grown for 72 hours in the absence (---) and presence (—) of 4-methyl-umbelliferryl-ß-D-xyloside On the fourth day, 25, 50 and 100 µg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol and 0.125 U/ml lipoprotein lipase (LPL) were added and the cells were incubated an additional 18 hours. Data points represent the average of three values and the error bars indicate the standard deviation. For clarity, only the upper and lower halves of the error bars are shown on dashed and solid curves, respectively.

unaffected by treatment with the drug again supports the idea that fatty acids enter the hepatocytes by an independent mechanism which does not rely on proteoglycan interactions.

In the absence of 4-methylumbelliferyl-β-D-xyloside, cells incubated with increasing concentrations of TRL and 0.125 U/ml LPL showed increased cholesterol ester accumulation up to a maximum of 12.8 ± 2.0 μg at 100 μg/ml of TRL cholesterol added. In contrast, the cells incubated with 4-methylumbelliferyl-β-D-xyloside showed a marked reduction in cholesterol ester accumulation at each concentration of TRL cholesterol with a maximum accumulation of 9.5 ± 0.9 μg at 100 μg/ml of TRL cholesterol. Again these data suggest that proteoglycans may be involved in facilitating uptake of TRL particles by HepG2 cells.

LDL is known to be internalized via a receptor mediated mechanism by hepatocytes (12). The LDL receptor recognizes apoB on the surface of LDL particles and this mechanism of internalization does not involve proteoglycans (58). When LDL was added to the cells grown in the presence of 4-methylumbelliferyl-β-D-xyloside, there was no observed increase in triacylglycerol accumulation consistent with the small quantities of triacylglycerol available from LDL. A 159 % increase in intracellular cholesterol ester is observed, however, comparable to the increase in cholesterol ester obtained when LDL was added to normal cells (data not shown). This suggests that in contrast to TRL uptake, the LDL uptake is unaffected by the disruption of proteoglycans on the cell

surface and is further indirect evidence that the uptake of TRL particles is mediated by proteoglycans.

3.2 Apolipoprotein B Secretion

The effect of accumulation of triacylglycerol and cholesterol ester on apoB secretion in HepG2 cells was investigated to determine the effects of lipid accumulation within these cells on secretion of apoB containing lipoproteins. Figure 24a shows apoB secretion from HepG2 cells over 8 hours following incubation in the presence and absence of LPL with increasing concentrations of TRL. In the absence of LPL (dashed line), there was no significant change in the secretion of apoB for all concentrations of TRL. In the presence of 0.125 U/ml LPL added plus TRL, there was a dose dependent increase in apoB secreted by the cells up to 291% ± 168%, $p<0.025$ at the highest concentration of TRL cholesterol (100 μg/ml). Intracellular lipid accumulation clearly stimulates secretion of apoB containing lipoproteins.

Figures 24b and 24c show the corresponding intracellular triacylglycerol and cholesterol ester accumulation respectively, for these experiments. As previously shown (Figure 18a and b), there was no significant accumulation of either triacylglycerol or cholesterol ester when increasing amounts of TRL were added in the absence of LPL. In the presence of 0.125 U/ml LPL, however, both intracellular lipids show significant dose dependent increases. The amount of intracellular triacylglycerol and cholesterol ester remaining in the HepG2 cells

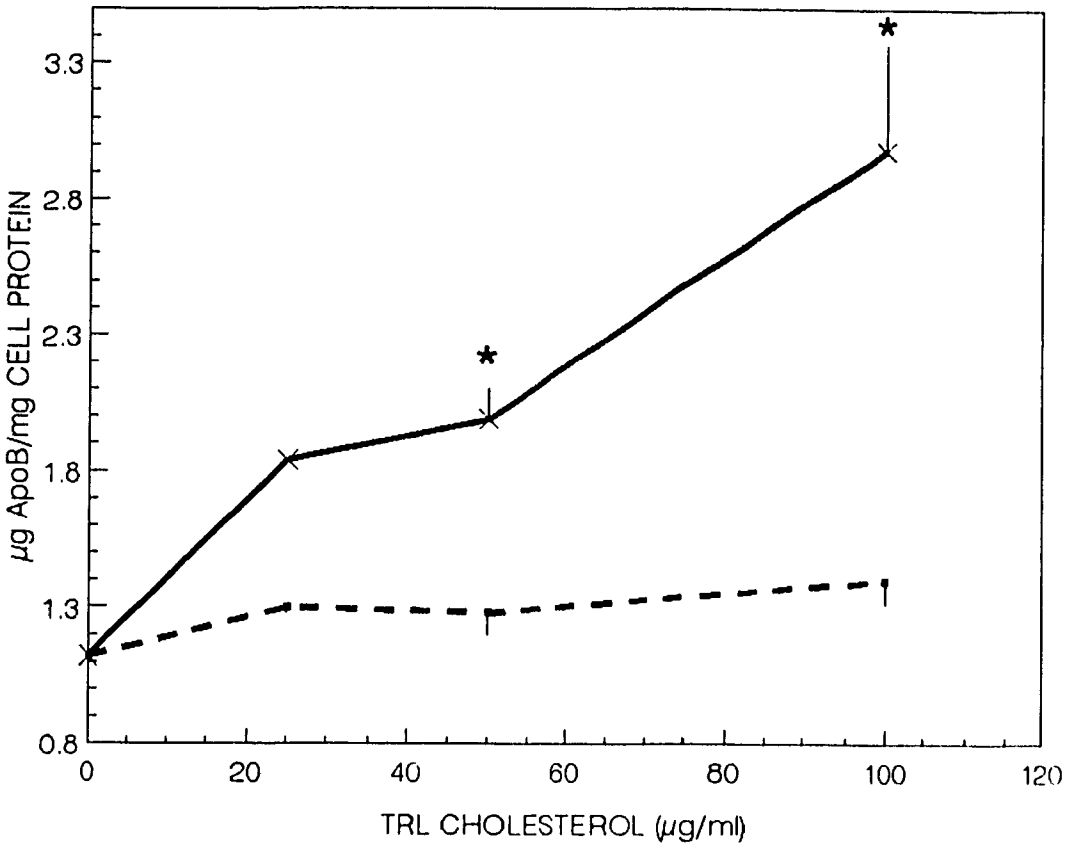

Figure 24a. Effect of increasing triacylglycerol rich lipoprotein concentration on apolipoprotein B secretion HepG2 cells were incubated at 37°C for 18 hours with 0, 25, 50 and 100 µg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol in the absence (---) and presence (—) of 0.125 U/ml lipoprotein lipase (LPL) This graph represents the average of 3 experiments ± standard error of the mean; *p<0 025, significantly different from basal For clarity, only the upper and lower halves of the error bars are shown on the solid and dashed curves, respectively

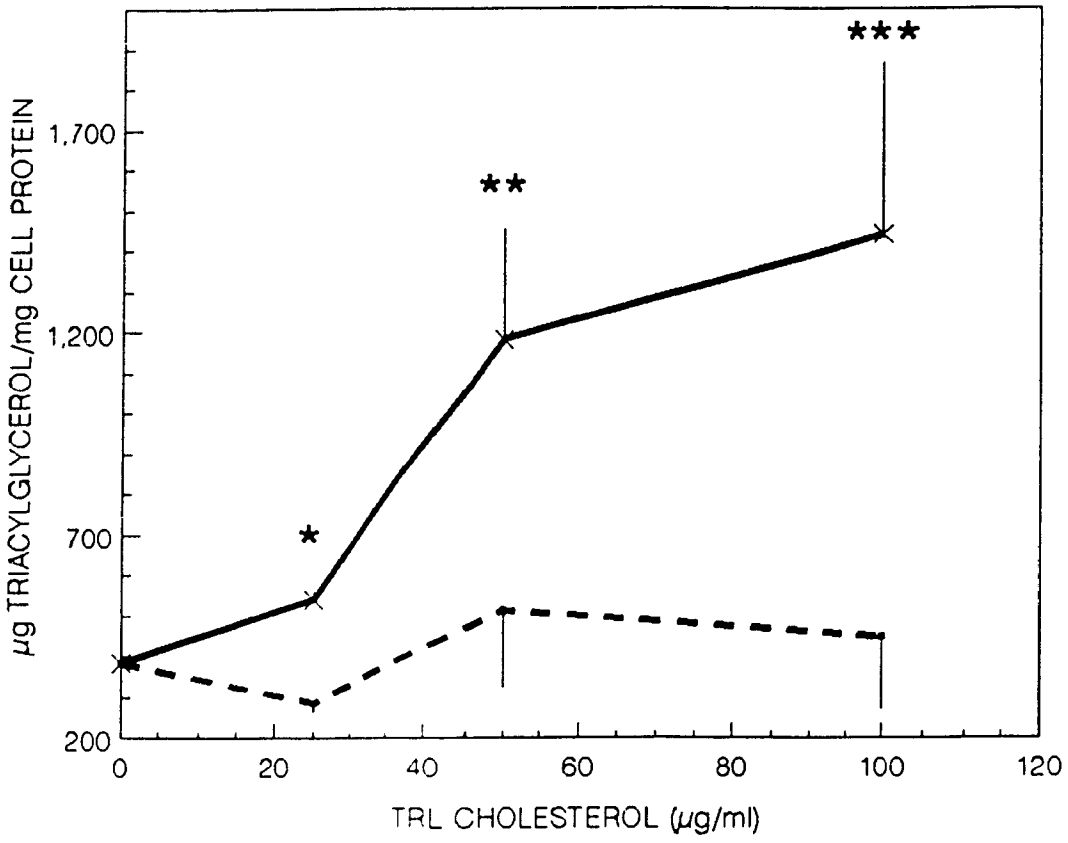

Figure 24b Effect of increasing triacylglycerol rich lipoprotein concentration on intracellular triacylglycerol accumulation HepG2 cells were incubated at 37°C for 18 hours with 0, 25, 50 and 100 μg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol in the absence (---) and presence (—) of 0 125 U/ml lipoprotein lipase (LPL) This graph represents the average of 3 experiments ± standard error of the mean, *p<0.01, **p<0.0125, ***p<0 025, significantly different from basal For clarity, only the upper and lower halves of the error bars are shown on solid and dashed curves, respectively.

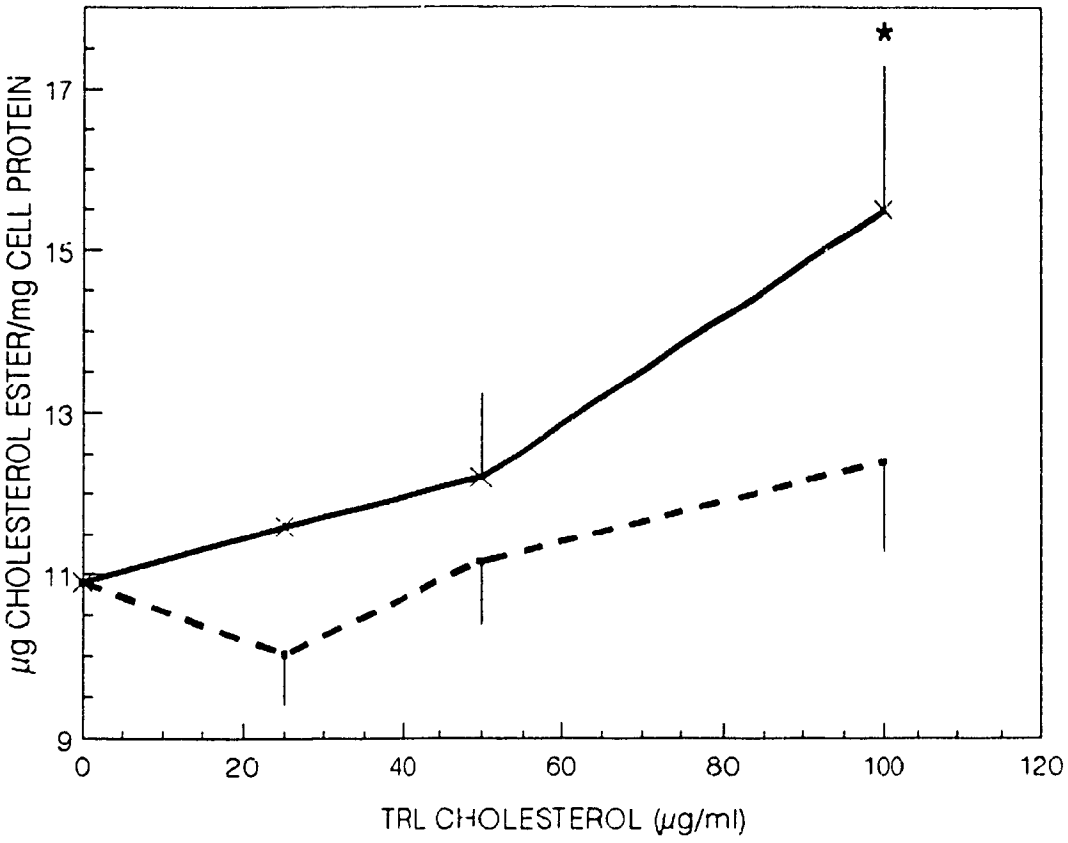

Figure 24c Effect of increasing triacylglycerol rich lipoprotein concentration on intracellular cholesterol ester accumulation HepG2 cells were incubated at 37°C for 18 hours with 0, 25, 50 and 100 μg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol in the absence (---) and presence (—) of 0 125 U/ml lipoprotein lipase (LPL) This graph represents the average of 3 experiments ± standard error of the mean, *p<0 05, significantly different from basal For clarity, only the upper and lower halves of the error bars are shown on solid and dashed curves, respectively

following the 8-hour incubation period used to measure apoB represents 96% and 73% respectively of what was present in the cells immediately following the 18 hour lipid loading with TRL. Therefore, it is felt that the initial intracellular lipid mass is an appropriate representation of this quantity during the 8-hour apoB secretion period.

Addition of LDL and fatty acid to the cells was used to differentiate between the effects of triacylglycerol and cholesterol ester accumulation on apoB secretion. Fatty acid is known to cause significant increases in the intracellular triacylglycerol mass, with much smaller effects on cholesterol ester. Conversely, LDL is almost exclusively a source of cholesterol ester.

Table II shows the effect of adding either fatty acid, LDL or both on intracellular triacylglycerol, cholesterol ester as well as the apoB secretion. There was a 132% ± 21%, p=NS increase from basal apoB secretion with the addition of 225 μg/ml (800 μM) fatty acid. In the presence of 300 μg/ml LDL, an increase of 267% ± 86%, $p<0.0125$ over basal apoB secretion was observed. A similar effect was observed with the addition of both fatty acid and LDL which produces a 199% ± 43%, $p<0.01$ increase in apoB over basal levels. These data suggest that although both lipoprotein substrates are required, apoB secretion more closely follows the intracellular accumulation of cholesterol ester.

Table II also shows corresponding triacylglycerol intracellular accumulation. As expected, there was a significant increase of 328% ± 49%, $p<0.05$ in accumulated triacylglycerol with the addition of 225 μg/ml fatty acid, but no

	ApoB μg/mg	TG μg/mg	CE μg/mg
BASAL	2.98±1.49	256±118	12.1±0.7
LDL	5.24±2.30**	304±171	15.9±0.7*
FA	3.09±1.17	1024±492****	16.3±1.8
LDL+FA	5.22±2.58*	709±330****	19.4±1.8***

Table II. Effect of low density lipoprotein and fatty acid on apolipoprotein B secretion and intracellular lipid accumulation. HepG2 cells were incubated at 37°C for 18 hours in the presence of 300 μg/ml low density lipoprotein (LDL) cholesterol, 225 μg/ml fatty acid (FA) or both. Results are expressed as μg apolipoprotein B or lipid per mg total cell protein and represent and average of 4 experiments ± standard error of the mean; *p<0.01, **p<0.0125, ***p<0.025, ****p<0.05, significantly different from basal.

significant increase with the addition of LDL. Addition of both fatty acid and LDL produced an increase of 265% ± 6%, $p<0.05$. Addition of fatty acid increased the intracellular cholesterol ester mass by 139% ± 20%, p=NS while addition of LDL caused an increase of 133% ± 5%, $p<0.01$ increase in cholesterol ester over basal levels. Addition of both fatty acid and LDL produced and even greater increase of 165% ± 24%, $p<0.025$ above basal. Therefore, under these experimental conditions, an increase in cholesterol ester mass paralleled the increase in apoB secretion, whereas an increase in triacylglycerol mass alone had little effect.

Experiments performed to study mechanisms of TRL particle uptake by HepG2 cells showed internalization of remnant particles could be inhibited following lipolysis by LPL through the use of substances such as heparin (Figure 20b). The resulting selective inhibition of cholesterol ester accumulation was used to further investigate the separate effect of triacylglycerol and cholesterol ester on apoB secretion.

Figure 25a shows the effect of heparin on the apoB secretion. Addition of 100 μg/ml TRL cholesterol and 0.125 U/ml LPL caused an expected 267% ± 34%, $p<0.025$ increase in apoB secretion. However, when 10 U of heparin were also added under the same conditions, there was a 211% ± 46%, $p<0.025$ increase above basal level. Addition of heparin therefore inhibited the TRL induced secretion of apoB by an average of 46% ± 14%, $p<0.05$.

Figures 25b and 25c show the intracellular accumulation of triacylglycerol

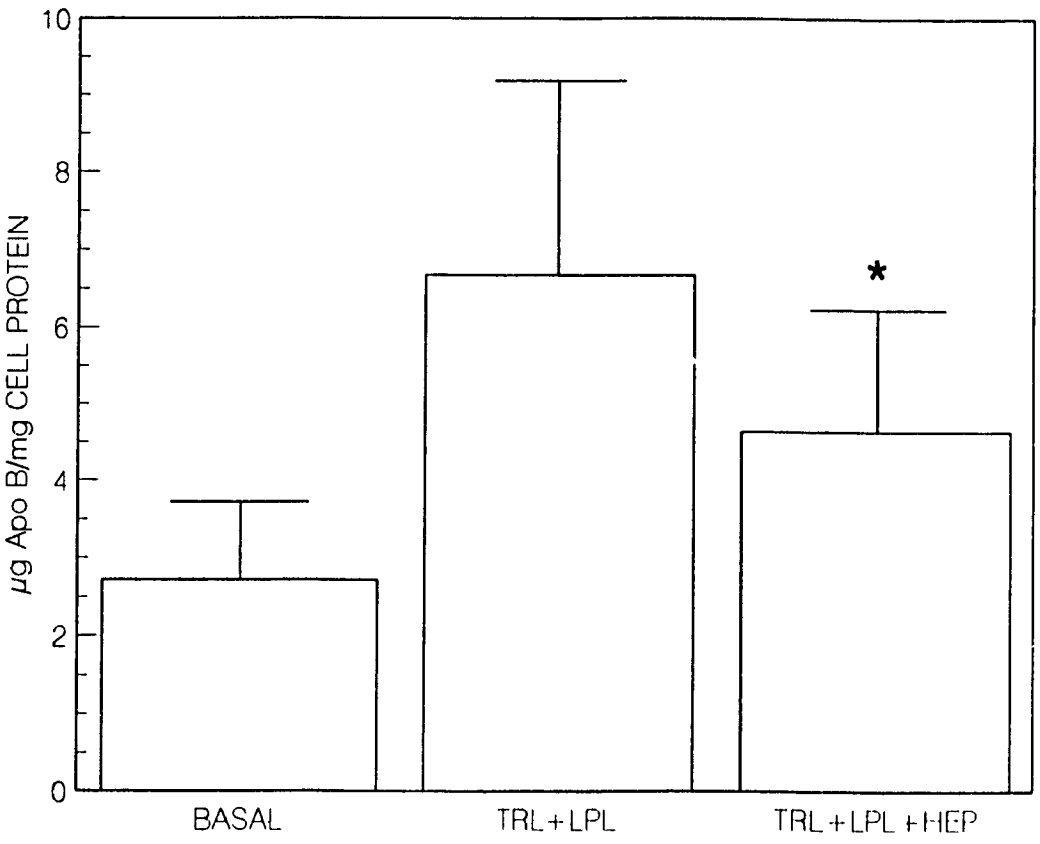

Figure 25a. Effect of heparin on apolipoprotein B secretion. HepG2 cells were incubated at 37°C for 18 hours with 100 µg/ml of triacylglycerol rich lipoprotein (TRL) cholesterol and 0 125 U/ml lipoprotein lipase (LPL) in the presence and absence of 10 U heparin (HEP). This graph represents the average of 5 experiments ± standard error of the mean, *p<0 025,significantly different from incubation without heparin

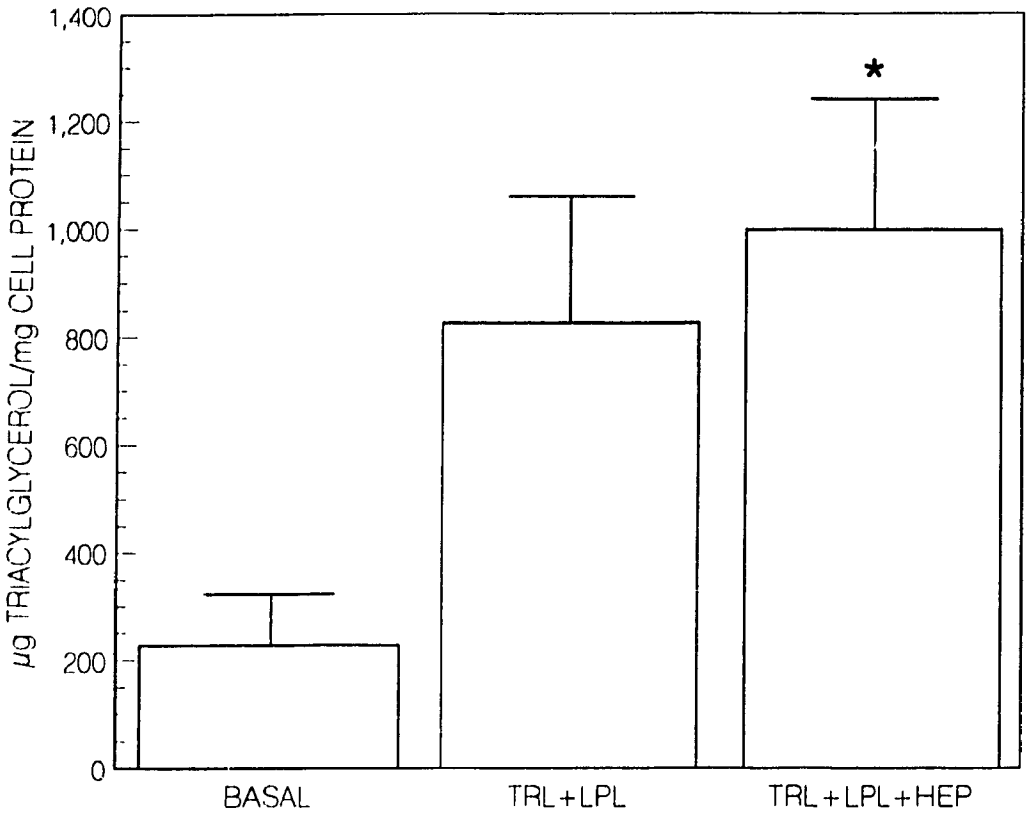

Figure 25b. Effect of heparin on intracellular triacylglycerol accumulation. HepG2 cells were incubated at 37°C for 18 hours with 100 μg/ml of triacylglycerol-rich lipoprotein (TRL) cholesterol and 0.125 U/ml lipoprotein lipase (LPL) in the presence and absence of 10 U heparin (HEP). This graph represents the average of 5 experiments ± standard error of the mean; *$p<0.01$, significantly different from incubation without heparin.

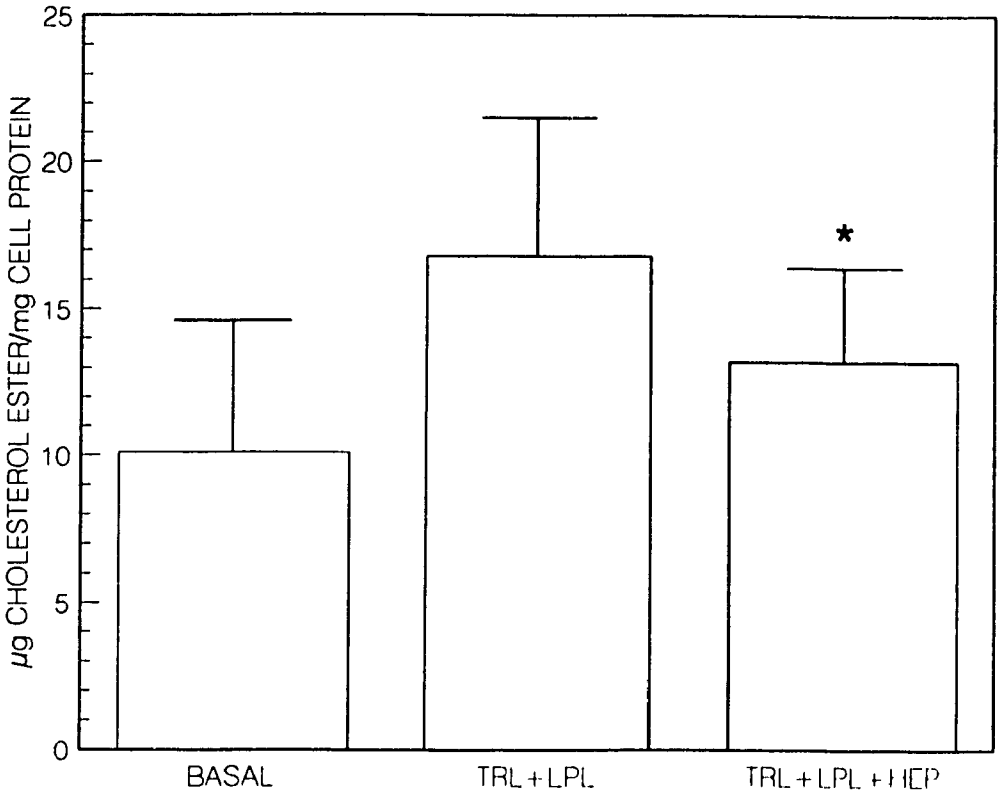

Figure 25c. Effect of heparin on intracellular cholesterol ester accumulation. HepG2 cells were incubated at 37°C for 18 hours with 100 μg/ml triacylglycerol-rich lipoprotein (TRL) cholesterol and 0 125 U/ml lipoprotein lipase (LPL) in the presence and absence of 10 U heparin (HEP) This graph represents the average of 5 experiments ± standard error of the mean; * $p<0.025$, significantly different from incubation without heparin

and cholesterol ester respectively. Addition of TRL with LPL resulted in a 442% ± 74%, $p<0.0125$ increase in accumulated triacylglycerol over basal level. Heparin did not significantly inhibit this TRL stimulation of triacylglycerol mass as shown previously in Figure 20a. Cholesterol ester mass was increased by 183% ± 30%, $p<0.025$ above basal with addition of TRL and LPL. However, when heparin was also added, an increase of only 155% ± 33%, $p<0.025$ over basal in intracellular cholesterol ester mass was observed. Cholesterol ester accumulation was therefore inhibited by 51% ± 15%, $p<0.05$ with the addition of heparin when compared to the effect of TRL and LPL without heparin. These data again suggest that apoB secretion more closely follows cholesterol ester mass in HepG2 cells.

Lactoferrin and asialofetuin were also effective in selectively inhibiting intracellular cholesterol ester mass accumulation in HepG2 cells. Table III shows results obtained for apoB secretion as well as for lipid accumulation in the presence of lactoferrin. As expected based on data shown in Figure 21a, there was a 237% ± 118%, $p<0.05$ increase in apoB secretion above basal, on addition of 100 μg/ml TRL cholesterol and 0.125 U/ml of LPL. Lactoferrin produced a 126% ± 63% $p<0.2$ increase in apoB secretion above basal which corresponds to 118% ± 59%, p=NS inhibition from the amount of apoB secreted on addition of TRL and LPL only.

Triacylglycerol levels were unaffected by addition of lactoferrin. Incubation with TRL and LPL produced a 224% ± 112%, $p<0.05$ increase in triacylglycerol

	ApoB μg/mg	TG μg/mg	CE μg/mg
BASAL	8.20±0.72	355±42	15.0±1.8
TRL+LPL	18.20±4.58	863±41	23.7±2.3
TRL+LPL+LF	11.0±3.88	659±176**	19.7±1.4*

Table III. Effect of lactoferrin on apolipoprotein B secretion and intracellular lipid accumulation. HepG2 cells were incubated at 37°C for 18 hours with 100 μg/ml triacylglycerol rich lipoprotein (TRL) cholesterol and 0 125 U/ml lipoprotein lipase (LPL) in the presence and absence of 0 02 mM lactoferrin. Results are expressed as μg apolipoprotein B or lipid per mg total cell protein and represent and average of 4 experiments ± standard error of the mean, *$p<0.0125$, **$p<0.05$, significantly different from basal.

mass. The addition of lactoferrin with TRL and LPL resulted in an increase of 171% ± 86%, $p<0.05$. In contrast, the cholesterol ester mass accumulation was inhibited. With addition of TRL and LPL, there was a increase in intracellular cholesterol ester mass of 167% ± 83%, $p<0.025$, but this was reduced to 137% ± 68%, $p<0.0125$ with the addition of lactoferrin, corresponding to an inhibition of 39% ± 19%, p=NS. The apparently small inhibition in cholesterol ester accumulation observed in the presence of lactoferrin is due to the averaging of several experiments in which there is a significant variation in cholesterol ester mass between experiments. Basal levels as well as stimulated levels of cholesterol ester mass were measured in each experiment, however, and the small decrease observed is statistically significant.

As shown in Table IV, similar results were obtained with the addition of asialofetuin as an inhibitor of receptor mediated particle uptake. Addition of 100 μg/ml TRL cholesterol and 0.125 U/ml LPL to the HepG2 cells, caused an increase of 253% ± 44%, $p<0.025$ in apoB secretion, while the addition of asialofetuin decreases this to 97% ± 40%, p=NS from basal which represents a 35% ± 14%, $p<0.005$ inhibition from stimulated levels.

Addition of TRL and LPL to the cells caused a 315% ± 25%, $p<0.005$ increase in intracellular triacylglycerol. Addition of asialofetuin caused no significant inhibition of triacylglycerol accumulation, but cholesterol ester mass is slightly reduced to 178% ± 26% from its stimulated level (192% ± 26% above basal). Again as was the case with lactoferrin, averaging of a wide range of

	ApoB μg/mg	TG μg/mg	CE μg/mg
BASAL	5.65±0.90	242±64	9.8±2.3
TRL+LPL	14.67±3.90	706±183	16.9±3.6
TRL+LPL+AF	4.61±1.34	674±155*	15.9±3.7**

Table IV. Effect of asialofetuin on apolipoprotein B secretion and intracellular lipid accumulation. HepG2 cells were incubated at 37°C for 18 hours with 100 μg/ml triacylglycerol rich lipoprotein (TRL) cholesterol and 0.125 U/ml lipoprotein lipase (LPL) in the presence and absence of 0.2 mM asialofetuin. Results are expressed as μg apolipoprotein B or lipid per mg total cell protein and represent and average of 6 experiments ± standard error of the mean, *p<0.0025, **p<0.0125, significantly different from basal

cholesterol ester mass values produced an apparently small decrease in cholesterol ester mass accumulation in the presence of asialofetuin, but since controls were again performed in each experiment, this small decrease is statistically significant.

All of the results obtained using specific inhibitors of cholesterol ester accumulation suggest there is a significant correlation between cholesterol ester mass in HepG2 cells and the quantity of apoB-containing lipoprotein secreted. These results are summarized in Figure 26a and 26b, which show plots of intracellular triacylglycerol and cholesterol ester mass accumulation, respectively, versus apoB secretion. In Figure 26a, the correlation coefficient (r^2) is equal to 0.06 showing no significant correlation between intracellular triacylglycerol mass and apoB secretion. In Figure 26b, $r^2=0.76$ which indicates, in this case, significant correlation between intracellular cholesterol ester accumulation and apoB containing lipoprotein secretion.

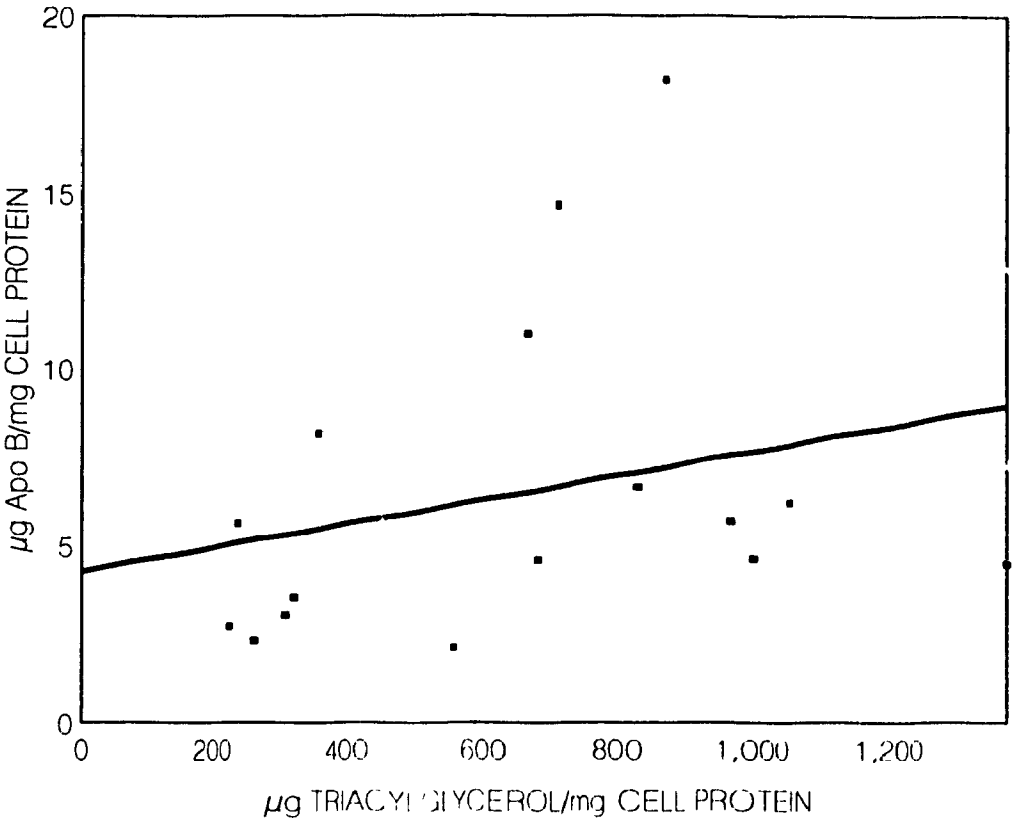

Figure 26a. Correlation between intracellular triacylglycerol accumulation and apolipoprotein B secretion. This graph represents the linear regression analysis of the data shown in Figures 24a-c, 25a-c, Tables III and IV, y-intercept=4.27, slope=0.003 and r^2=0.06

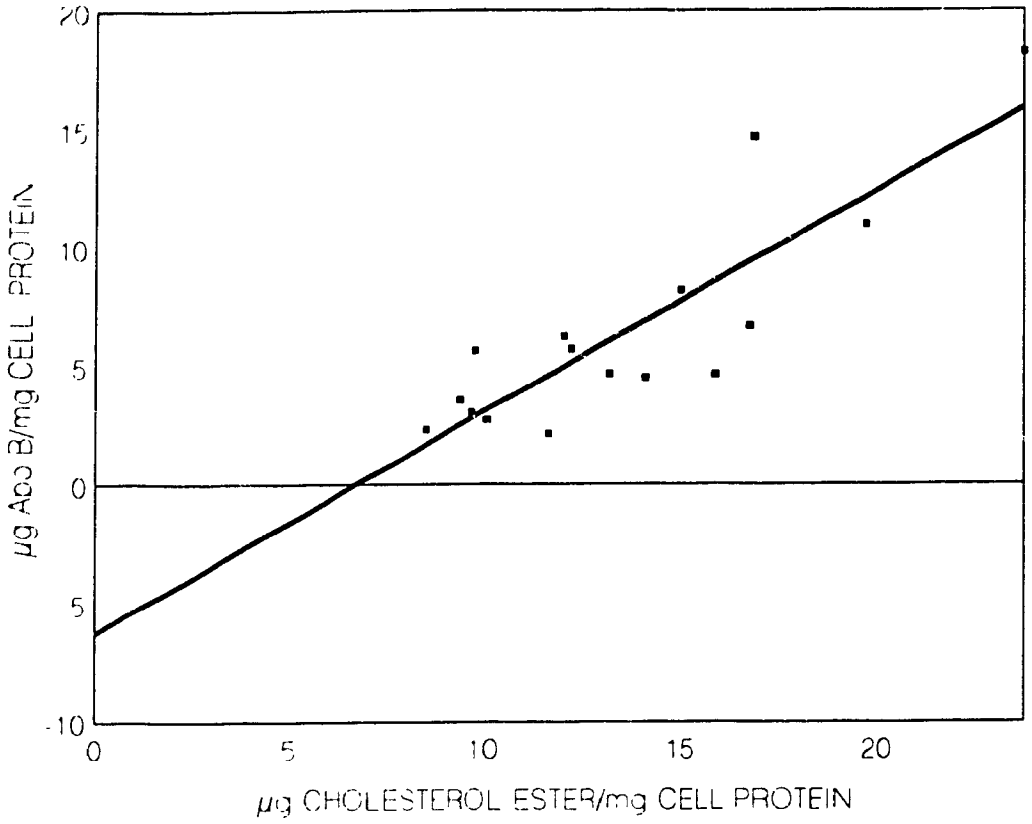

Figure 26b Correlation between intracellular cholesterol ester accumulation and apolipoprotein B secretion. This graph represents the linear regression analysis of the data shown in Figures 24a-c, 25a-c, Tables III and IV, y intercept = -6.28, slope = 0.935 and $r^2 = 0.76$.

4. DISCUSSION

Lipoproteins are produced in the intestine for transport to the liver where they are first metabolized. LDL, which is a product of this metabolism is known to be taken up by a receptor mediated mechanism which recognizes apoB at the lipoprotein surface (12). While the LDL receptor has been characterized for some time now (13), the identity of the receptor which internalizes TRL remains unclear. The rapid clearance of TRL from the circulation, in conditions where LDL clearance is impaired, however, provides some indirect evidence that a discrete uptake mechanism may exist. Because TRL contain apoproteins similar to those required for LDL uptake at their surface, and because TRL particles are relatively large, it is likely that they are taken up by a receptor mediated mechanism similar to that for LDL.

It has been shown by Evans and Huff (45) that lipolysis of TRL particles is a prerequisite for the uptake of this class of lipoprotein by the liver, and that this uptake results in measurable increases in the intracellular mass of both triacylglycerol and cholesterol ester. Effects of LPL, in addition to the lipolysis of the TRL particle, were investigated in this study. Studies performed by Beisiegel (46) using both LPL as well as a structurally unrelated *Pseudomonas* triacylglycerol lipase, showed that only in the presence of LPL was there binding of TRL to the hepatocyte surface. From these data, they concluded that only LPL can act both as a lipolytic enzyme, and as a ligand for binding of TRL to its

membrane receptor. It should be noted, however, that these binding experiments were performed at 4°C using iodinated TRL particles as a measure of lipoprotein uptake. While much work has been done using iodinated lipoproteins, TRL particles are labile and the surface apoproteins dissociate easily. Binding studies using iodinated TRL may, therefore, not be truly indicative of the amount of lipid being internalized.

For this reason, the experiments presented in this work quantify intracellular lipid mass as a measure of TRL uptake by cells. In addition, all experiments were carried out at 37°C which is closer to physiological conditions. Data which conflict with those of Bieseigel were obtained when intracellular lipid mass was measured as an indication of TRL uptake under these conditions. Clearly, there is a significant increase in TRL uptake in the presence of LPL. However, *Rhizopus arrhizus* lipase, which is not structurally homologous to LPL (47,48), produced significant increases in both intracellular triacylglycerol and cholesterol ester, showing that binding and internalization of TRL does not require LPL to be present.

Experiments performed by Cianflone (submitted for publication) using heat inactivated LPL showed no increases in either intracellular triacylglycerol or cholesterol ester when intracellular lipids were quantified as a measure of TRL uptake. This suggests that LPL is required for lipolysis of TRL particles prior to their uptake by HepG2 cells. In contrast, there was an increase in both these intracellular lipids when TRL was lipolyzed in vitro and re-isolated prior to being

added to the cells in the absence of LPL, suggesting again, that although LPL is required for lipolysis, which may lead to changes in structure of the lipoprotein particle, its presence as a ligand is not essential.

The lipase from *Pseudomonas* bacteria was also used in the present studies. In contrast to the *Rhizopus arrhizus* lipase, the presence of the *Pseudomonas* lipase resulted in a large intracellular accumulation of triacylglycerol within the HepG2 cells, but there was no accumulation of cholesterol ester. This lipase possesses a rather high phospholipase activity as well as triacylglycerol hydrolase activity (49). When a high amount of *Pseudomonas* lipase activity was added to TRL particles in vitro, it was found that the particles could not be re-isolated. It was also observed that when the lipoprotein particle was destroyed in the presence of *Pseudomonas* lipase, there was no resulting cholesterol ester accumulation within the cells. This suggests that particle integrity is required for uptake of a remnant cholesterol ester enriched particle and that uptake may be through a receptor mechanism. The large increase in the intracellular triacylglycerol accumulated in the presence of *Pseudomonas* lipase could be due to fatty acids produced from both TRL triacylglycerol hydrolysis and hydrolysed membrane phospholipids.

If LPL does not play the structural role that has been ascribed to it what then is the functional ligand for TRL uptake? Apolipoprotein E (apoE) is a component of both LDL and TRL. As is the case with receptor uptake of LDL, which is mediated by apoB, it has been suggested that apoE is one of the

proteins involved in the receptor mediated uptake of TRL. Studies using monoclonal antibodies to different epitopes on apoE indicate that an arginine-rich cationic region of the apoE molecule may be recognized by a cell surface receptor (59). Experiments have also shown that TRL uptake in hepatocytes is saturable and that it can be enhanced by the addition of apoE (60). Following lipolysis of TRL by LPL, there may be a conformational change in apoE which exposes a cationic region of the protein. It may be partly for this reason that lipolysis is a prerequisite for uptake of these lipoproteins by cells. However, TRL-associated apoE is not the only apoE available as this protein is also known to occur at the surface of HepG2 cells (50). Since all forms of apoE are highly glycosylated, proteoglycans at the cell surface or the carbohydrate on the cell surface of apoE may facilitate TRL remnant uptake through affinity interactions.

Results presented in this thesis indicate that inhibition of TRL remnant uptake by HepG2 cells is possible since cholesterol ester accumulation was decreased with no effect on triacylglycerol accumulation (Figures 19-22). It has been shown previously that glycoproteins such as lactoferrin and asialofetuin significantly inhibit uptake of TRL particles in the hepatocyte as measured by binding of iodinated particles to the extracellular matrix (54). Using ligand blotting, lactoferrin and TRL have been shown to bind to a receptor of the same size (24). The fact that binding of TRL to the cells surface was competitively inhibited by the presence of lactoferrin leads to the speculation that the receptor for both TRL and lactoferrin may be identical. This inhibitory effect is believed to

occur since lactoferrin contains a region rich in arginine residues (54) which is homologous to a region found in apoE. The lactoferrin may, therefore, contain a region which mimics the apoE binding site and effectively competes with apoE for binding to the receptor. It has also been suggested that the asialoglycoprotein receptor may remove TRL directly from the circulation and that uptake could, therefore, be competed with by ligands to this receptor, such as asialofetuin (55). In our experiments, we found that intracellular accumulation of cholesterol ester was essentially completely inhibited in the presence of either lactoferrin or asialofetuin. However, since lactoferrin does not act as a ligand to the asialoglycoprotein receptor (55), it remains unclear whether TRL uptake is specific to either the asialoglycoprotein or the LRP receptor.

TRL may be targeted to the cell surface through its affinity for cell-surface proteoglycans. The HepG2 extracellular matrix is known to be highly glycosylated, with glycosaminoglycans being most abundant (50). Heparin, which is itself a glycosaminoglycan can compete for binding sites on the hepatocyte cell surface (50) In fact, our experiments indicate strongly that heparin does interfere with the accumulation of intracellular cholesterol ester, while intracellular accumulation of triacylglycerol is unaffected (Figure 20).

The compound, 4-methylumbelliferyl-β-D-xyloside, is a specific inhibitor of proteoglycan biosynthesis. This substance was also used to probe the role of proteoglycans in TRL uptake. Most importantly, there was a decrease in accumulation of cholesterol ester in the cells grown in the presence of this

substance, but it had no effect on triacylglycerol accumulation. When LDL was added as a control, there was no inhibition in the accumulation of cholesterol ester (results not shown). LDL is taken up via a well-characterized receptor, by a mechanism which does not involve proteoglycans (58), and our results are consistent with TRL uptake being facilitated by proteoglycan interaction.

It is known that many of the apoproteins (certainly apoB and apoE) are glycosylated and the carbohydrates may also be involved in mediating particle uptake. This supports the idea that the TRL particles must retain integrity for uptake, and that uptake is mediated by one of the surface apoproteins. LPL may not act as a specific ligand for receptor interaction, but may facilitate anchoring between glycosaminoglycans at the cell membrane surface and lipoprotein, and aid in receptor recognition of lipoprotein surface proteins. This mechanism has been shown to be essential for other receptor-ligand interactions such as that between basic fibroblast growth factor or thrombin (61,62).

Figure 27 shows the proposed mechanism of TRL uptake based on our experimental observations. LPL acts on the particle and releases free fatty acids. The remnant particle is then taken up via a receptor mediated mechanism, with proteoglycans providing a bridging mechanism between TRL ligand and cellular interaction, to facilitate subsequent uptake by the LRP receptor. In addition, the data presented in this thesis also suggest that fatty acid uptake occurs independently of cholesterol ester uptake. Even when particle integrity was destroyed, fatty acids were still liberated resulting in an increase in the

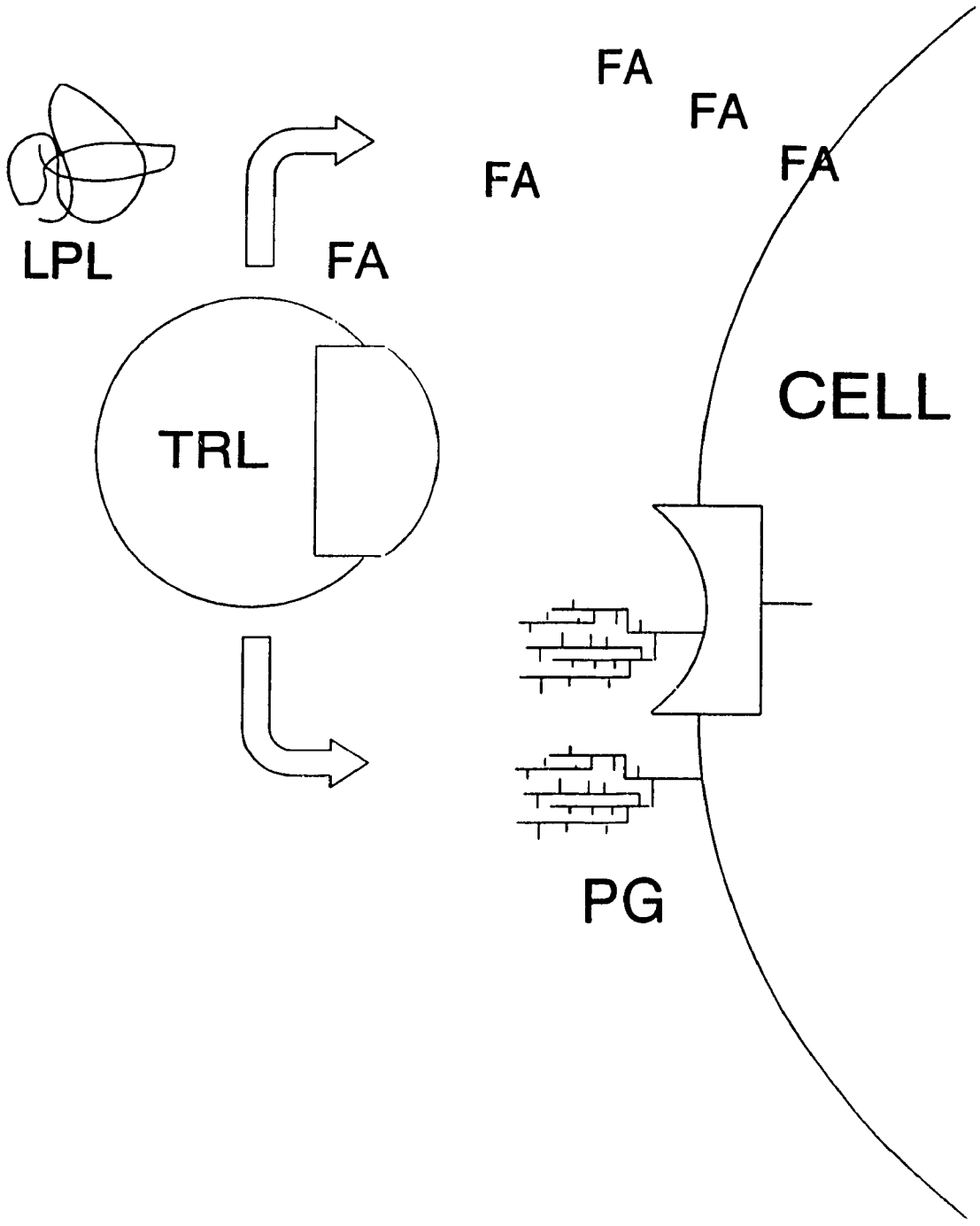

Figure 27. Proposed mechanism for triacylglycerol-rich lipoprotein uptake
TRL, triacylglycerol-rich lipoprotein, FA, fatty acid,
LPL, lipoprotein lipase, PG, proteoglycans.

intracellular triacylglycerol accumulation.

It has been proposed that the LRP receptor internalizes TRL particles. This issue is still under investigation, since it has been demonstrated that this receptor is also capable of binding several other ligands such as α-2-macroglobulin and lactoferrin (23,24). The LRP receptor seems a likely candidate for the TRL receptor, however, since it is composed of four transmembrane subunits which bear significant structural homology to the LDL receptor.

The liver is the main metabolic site for cholesterol. Although TRL must go through the liver in order to be cleared from the bloodstream, it must in turn be resecreted as a lipoprotein particle for transport and storage of the triacylglycerol in the adipose tissue. Hepatically secreted lipoproteins contain a lipid core, an outer phospholipid monolayer and apoproteins required for function such as apoB, which is required for uptake by the LDL receptor. ApoB is known to be transcribed in far greater quantities than is necessary to package the amount of lipids which are metabolized by the liver (63). Studies have demonstrated that only 20-40% of the apoB synthesized is actually secreted. The remainder is degraded by cytosolic and endoplasmic reticulum proteases. The limiting factor in the secretion of hepatic lipoproteins, which has been the subject of much debate, therefore is thought to be lipid. Because the liver has a great capacity for recycling fatty acids, it has been suggested, up until now, that only triacylglycerol regulates secretion of apoB-containing lipoproteins from the liver.

It was initially found that apoB secretion increases in a concentration

dependent manner with increasing amount of lipid accumulated in the cells (Figure 24). Therefore, increased lipid availability clearly increases the apoB secreted from the liver. Since accumulation of intracellular lipids can be independently manipulated as seen in the results (Section 3.1), the effects of triacylglycerol and cholesterol ester on apoB secretion may be separated. Cholesterol ester accumulation was either reduced by inhibiting remnant particle uptake or increased by adding cholesterol-rich LDL, which greatly increased the cholesterol ester content without affecting the triacylglycerol content of the cell. Addition of fatty acids alone can produce up to three-fold increase in intracellular triacylglycerol without greatly affecting the levels of cholesterol ester accumulated. When LDL is added to the cells the apoB levels increase dramatically which indicates a role for cholesterol ester in the synthesis and regulation of apoB containing lipoproteins. Our results indicate that apoB secretion increases with increasing intracellular cholesterol ester accumulation, but is insensitive to triacylglycerol accumulation (Figure 26). Similar results were obtained by Craig and her coworkers who also used HepG2 cells (64). However, no selective inhibition of triacylglycerol synthesis and accumulation was attempted in the present study and such inhibition should be the focus of future studies.

Published data (31,32) have been conflicting as to which of the two major core lipids regulates apoB secretion from the liver. When fatty acid alone was added in our experiments, there was a small increase in cholesterol ester mass

as well as a substantial increase in triacylglycerol mass. A small but significant increase above basal levels in apoB secreted was also observed revealing that elevated intracellular triacylglycerol levels cause some increase in apoB secretion (Table II). Previous studies done on HepG2 cells in our lab using a competitive inhibitor of ACAT (Figure 4) and lovastatin, an inhibitor of hydroxymethylglutaryl-CoA reductase required for cholesterol biosynthesis, show that inhibition of cholesterol ester or cholesterol synthesis in the presence of fatty acid also significantly affected apoB secretion (65). During the same study, 2-bromooctanoate, which prevents acylation by DGAT to yield triacylglycerol (Figure 3) was also used, but inhibition of triacylglycerol synthesis did not have as great an effect on apoB secretion as inhibition of cholesterol ester and cholesterol synthesis. These results also agree with studies done by Tanaka on rabbit hepatocytes which show that apoB secretion closely follows cholesterol ester synthesis (66). More recently, in vivo studies performed by Huff on a miniature pig model (67) using ACAT inhibitors over several days have added data showing decreases in apoB-containing lipoprotein secretion following inhibition of cholesterol ester formation. This again suggests that cholesterol ester plays a significant role in modulating the synthesis of lipoprotein particles by the liver.

There are, however, conflicting data which suggest that it is, in fact, triacylglycerol and not cholesterol ester which regulates apoB secretion by liver cells. Ginsberg (68) and coworkers have reported increases in intracellular

triacylglycerol accumulation in liver cells with the addition of fatty acid alone. The increases that were observed in triacylglycerol accumulation paralleled a significant increase in apoB secretion. There was no observable change in cholesterol ester synthesis with the addition of fatty acid alone, however. These contradictory data may be the result of very different experimental conditions. These experiments were performed over very short time periods (3 hours) and the incorporation of radiolabeled fatty acid tracer into triacylglycerol and cholesterol ester was measured and not total intracellular lipid mass. It was found in our experiments, that significant changes in ACAT activity could be detected in the presence of its inhibitor. Since the overall changes in cholesterol esterified were so small, however, there was no observed change in total mass of the cholesterol ester when this was measured, even following an 8 hour time period (unpublished results).

Further experiments performed by Ginsberg and coworkers using Triacsin D showed substantial decreases in triacylglycerol synthesis as well as apoB secretion from HepG2 cells (69). Both incorporation of radiolabeled tracer and lipids mass were measured, but only changes in the triacylglycerol levels were found. Triacsin D, however, is an inhibitor of acyl CoA synthetase and will non-specifically inhibit all acylation reactions. Studies done during the development of this family of inhibitors by the original investigators clearly showed that in addition to inhibiting triacylglycerol synthesis, Triacsin D also inhibits cholesterol ester synthesis, phospholipid synthesis, protein acylation and may even affect

Golgi transport, and therefore all lipoprotein and protein secretion.

Additional data which do not support an effect of triacylglycerol on apoB secretion includes previous work done by Cianflone et al. (70) using glucose as a carbohydrate source. Glucose was shown to cause increased triacylglycerol accumulation either by being converted to fatty acids or incorporated into the triacylglycerol backbone. It was found that increasing triacylglycerol accumulation using glucose caused larger triacylglycerol-enriched apoB-containing lipoproteins to be secreted from the liver, but did not cause concurrent increases in apoB itself. Feeding studies done on both animal and human subjects on high carbohydrate diets showed similar results; the size of secreted lipoproteins was increased due to increased triacylglycerol mass, but the number of apoB particles remained the same (71). This is further evidence that cholesterol ester plays a regulatory role in apoB-containing lipoprotein secretion.

In summary there are several conclusions which can be drawn from the data presented in this work. The first is that lipolysis is a prerequisite to cellular uptake of both fatty acids and cholesterol ester from the TRL particles used in these experiments. Lipolysis by LPL was shown initially to be required for both intracellular lipid accumulation as well as for the subsequent secretion of apoB containing lipoproteins from the hepatocyte. Further experiments showed that even though triacylglycerol hydrolase activity is required for uptake of fatty acids and cholesterol by hepatocytes, this effect is not specific to LPL and can be produced by both a fungal lipase as well as a bacterial lipase. In addition, the

phospholipase activity of the *Pseudomonas* lipase causes an increase in intracellular triacylglycerol accumulation, but not in the accumulation of intracellular cholesterol ester. This may be explained by the fact that TRL particle uptake may require particle integrity, which would allow functionally important proteins to remain at the particle surface. These data further indicate that fatty acids are entering the cell by independent mechanisms. Finally, substances which have affinity for proteoglycans on the cell surface interfere with the uptake of TRL particles, suggesting that proteoglycans may be involved in recognition events at the cellular level which allow the uptake of TRL particles.

The data presented in this work also show that both fatty acid and cholesterol ester have an effect on the apoB-containing lipoprotein secreted by hepatocytes. However, it appears that under the experimental conditions used in this work, fatty acid has a lesser effect than cholesterol ester on apoB secretion. Using inhibitors of cholesterol ester accumulation within the cells, it was also found that decreasing the available cholesterol ester, caused a significant decrease in apoB-containing lipoprotein secretion, further indicating that there is a strong correlation between the two.

5. SUGGESTIONS FOR FUTURE WORK

The data presented in this thesis strongly point to a proteoglycan mediated mechanism of TRL uptake by HepG2 cells. The specific types of proteoglycans involved in mediating this TRL uptake, however, remain to be identified. The experiments presented in this thesis could be extended to use substances which cleave specific types of proteoglycans either at the cell surface or at the lipoprotein surface.

In addition, the structure of the TRL receptor and the ligand which it recognizes remain to be identified. If apoE is required for receptor recognition of TRL, then mutations at potentially important regions of the molecule followed by binding studies would be useful in confirming its role as ligand.

This thesis has also shown that cholesterol ester plays an important role in the assembly and secretion of apoB containing TRL by HepG2 cells. Although HepG2 is a human cell line, this cell model does have certain limitations. HepG2 are only able to secrete approximately 10% of their intracellular lipid stores even though they synthesize their own apoproteins and secrete intracellular lipids in the form of mature lipoproteins. Because of the virtual impossibility of obtaining human liver tissue for culture, the next best thing would be to find a suitable animal model. The hamster provides such a model since it secretes lipoproteins similar to those of humans. Specific enzyme inhibitors of either cholesterol ester hydrolase or ACAT could be used to look at the effect of inhibiting cholesterol

ester formation in these primary hepatocytes.

Finally, there were no data examining the effect inhibiting triacylglycerol accumulation presented in this work. It would be useful to obtain a specific inhibitor of DGAT in order to observe its effect on the secretion of apoB containing lipoproteins in these primary hepatocytes.

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