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Chylomicron-remnant uptake by freshly isolated hepatocytes

Effect of heparin and of hepatic triacylglycerol lipase

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Chylomicron remnants labelled biologically with [^{3}H]cholesterol were efficiently taken up by freshly isolated hepatocytes during a 3 h incubaton in Krebs bicarbonate medium. Their [^{3}H]cholesteryl ester was hydrolysed (74% net hydrolysis), and 0.1 mM-chloroquine could partially inhibit this hydrolysis, provided that hepatocytes were first preincubated for 2 h 30 min at 37 °C. This hydrolysis was also measured in preincubated cells with remnants double-labelled (^{3}H and ^{14}C) on their free cholesterol moiety; [^{3}H]cholesterol arising from [^{3}H]cholesteryl ester hydrolysis was recovered in the free [^{3}H]cholesterol pool. A dose–response study showed saturation of remnant uptake at 180 μg of remnant protein/10^7 cells. Heparin (10 units/ml) increased remnant uptake by 63% ($P < 0.01$), [^{3}H]cholesteryl ester accumulation in the cell pellet by 110% ($P < 0.025$) and hepatic lipase activity secreted in the medium by 2.4-fold ($P < 0.01$) and by 3.3-fold ($P < 0.01$) at the end of the preincubation and incubation periods respectively. Addition of 100 munits of semi-purified hepatic lipase preparation/flask stimulated remnant uptake by 44–69%, and [^{3}H]cholesteryl ester accumulation in the presence of chloroquine by 2.1-fold ($P < 0.025$). When hepatic lipase was incubated solely with the remnants, it decreased their triacylglycerol and phospholipid contents by 24% and 26% respectively. Thus freshly isolated hepatocytes may be used to study chylomicron-remnant uptake. Hepatic lipase, which seems to underly the stimulating effect of heparin, facilitates remnant uptake *in vitro*, and this could be mediated by at least one (or both) of its hydrolytic properties.

INTRODUCTION

Remnant particles which result from the rapid extrahepatic lipolysis of chylomicrons are taken up very efficiently by the liver through a receptor-mediated process (Cooper, 1977; Windler *et al.*, 1980*a*,*b*; Noël & Dupras, 1983; Arbeeny & Riffici, 1984). After binding to the so-called apo E receptor located on the parenchymal cells (Funke *et al.*, 1984), and perhaps also to the apo B,E receptor (Jensen *et al.*, 1987; Windler *et al.*, 1988), these remnants are internalized, transported to the Golgi–lysosome region of the hepatocyte and to the secondary lysosomes (Jones *et al.*, 1984), where their different components are degraded. *In vitro*, chylomicron-remnant catabolism has been repeatedly studied in hepatocyte monolayers (Florén & Nilsson, 1977*a*,*b*, 1978, 1979; Lippiello *et al.*, 1985; Lenich & Ross, 1987; Jensen *et al.*, 1987), but only rarely in freshly isolated hepatocytes, which seem to present a defect in remnant processing, at least for the protein moiety of the particle. In this respect, degradation of iodine-labelled VLDL remnants has been found to be as low as 4–8% of cell-associated radioactivity (Van Berkel *et al.*, 1981); moreover, the lysosomotropic drug chloroquine does not exert its known inhibitory effect on degradation of chylomicron-associated apo CIII or apo E (Van Berkel *et al.*, 1983). On the other hand, a 2 h preincubation of hepatocytes after the collagenase isolation procedure allowed an enhanced uptake of remnant cholesteryl ester (Nilsson & Åkesson, 1975; Nilsson, 1977). Apparently such a lag period reflects the time required by isolated hepatocytes to recover their functional responsiveness.

The present study was aimed first to test whether the preincubation of hepatocytes would restore chylomicron-remnant catabolism, i.e. the lysosomal degradation of their cholesteryl ester moiety. This degradation was estimated by the accumulation of the ester in the presence of chloroquine, which blocks its hydrolysis in the lysosomes. The remnants used were either single-labelled with ^{3}H on their free and esterified cholesterol, or double-labelled with ^{3}H and ^{14}C on their free cholesterol while cholesteryl ester was ^{3}H labelled solely. Secondly, we studied the effect of heparin in the preincubated hepatocytes for the following reasons: this compound was previously reported to increase chylomicron-remnant uptake by suspended hepatocytes (Nilsson & Åkesson, 1975; Nilsson, 1977); however, its action was not explained at that time. Since hepatic triacylglycerol lipase was later shown to be secreted by hepatocytes *in vitro* (Jansen *et al.*, 1979) and to be stabilized by heparin (Schoonderwoerd *et al.*, 1981; Leitersdorf *et al.*, 1984; Rustan *et al.*, 1986), we wished to investigate whether the stimulatory effect of heparin on chylomicron-remnant uptake could involve hepatic lipase. Indeed, the physiological significance of this enzyme on the latter process remains at present unknown. For this purpose, semi-purified preparations of the enzyme were incubated with the hepatocytes. The results obtained here show that hepatic lipase increases chylomicron-remnant uptake.

Abbreviation used: apo, apolipoprotein.

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EXPERIMENTAL

Chylomicron-remnant preparation

Lymph was obtained from the thoracic duct (Bollman *et al.*, 1949) of male 400 g Wistar rats (Janvier, Le Genest, France). A dose of 500 μCi of [^{3}H]cholesterol and 40 mg of unlabelled cholesterol in 2 ml of corn oil was introduced by an introduodenal catheter, and lymph was collected the following night over ice, with final concentrations of the following preservatives: 1 mM-EDTA, 0.01% NaN_3 and 0.01% merthiolate. Radiolabelled chylomicrons were separated by chromatography on a 2%-agarose column (Sata *et al.*, 1970). Remnants were subsequently obtained by injecting 50 mg of chylomicron triacylglycerol per rat into the left jugular vein of male 400–450 g Wistar rats fasted for 18 h and then functionally eviscerated (Windler *et al.*, 1980*a*); 30 min later, rats were bled by abdominal aortic puncture and remnants isolated by ultracentrifugation of the plasma made up to $d = 1.019$ with KBr, for 10^8 $g_{av.}$-min at 10 °C. Remnants were extensively dialysed and used for experimentation within the ensuing 24 h. Owing to this procedure, both free and esterified cholesterol of the remnants were ^{3}H-labelled.

Double-labelled remnants

In order to obtain double-labelled remnants, ^{3}H-single-labelled remnants were additionally labelled *in vitro* with [^{14}C]cholesterol, the extent of labelling being 20% of remnant free [^{3}H]cholesterol (in d.p.m.). [^{14}C]Cholesterol was dissolved in 1 ml of ethanol and mixed with 2% (w/v) defatted bovine serum albumin, in 0.1 M-phosphate buffer, pH 7.4. Ethanol was evaporated under N_2. Plasma containing ^{3}H-labelled remnants was then added and further incubated for 4 h at 37 °C in the presence of 0.8 mM-5,5′-dithiobis-(2-nitrobenzoic acid) to inhibit lecithin–cholesterol acyltransferase activity. Remnants were re-isolated by ultracentrifugation and extensively dialysed against 0.9% NaCl/1 mM-EDTA and finally against 0.9% NaCl/10 mM-Tricine, pH 7.4.

Hepatocyte isolation and incubation procedures

Cells from male 300 g Wistar rats were prepared by the procedure of Seglen (1972), as modified in our laboratory (Kalopissis *et al.*, 1981). After four washes (each 45 s at 70 *g*) with Seglen's medium to eliminate Kupffer and endothelial cells, hepatocytes were further purified by Percoll-density-gradient centrifugation in order to increase the proportion of viable cells and eliminate cell debris (Dalet *et al.*, 1982). Cell viability determined by exclusion of 0.25% Trypan Blue was 92 ± 1.13% ($n = 14$). Hepatocytes [$(10$–$12) \times 10^6$ cells] were introduced into plastic Nalgen flasks in 3 ml of Krebs bicarbonate medium (pH 7.45) containing 1.5% (w/v) defatted bovine serum albumin, 20 mM-glucose, 20 mM-Tricine, minimal essential medium amino acid mixture, 100 units of penicillin/ml and 100 μg of streptomycin/ml. Hepatocytes were first preincubated for 2 h 30 min at 37 °C in a shaking water bath. Remnants were added after this preincubation and were further incubated for the indicated periods (1, 2 or 3 h). Chloroquine and/or heparin was present in the flasks during the preincubation period as indicated. Each flask was saturated with O_2/CO_2 (19:1) at the start of both preincubation and incubation periods.

Lipid analysis

At the end of the incubation, cells were immediately separated from the medium by centrifugation (1 min at 500 *g*; 4 °C) and washed four times with ice-cold 0.15 M-NaCl/1 mM-Tricine (pH 7.4). Medium and cell lipids were extracted with chloroform/methanol (2:1, v/v) (Folch *et al.*, 1957). Free cholesterol and cholesteryl ester were separated by t.l.c. on silica-gel plastic plates with light petroleum (b.p. 35–60 °C)/diethyl ether/acetic acid (70:30:1, by vol.) as solvent system. They were detected with I_2 vapour, eluted and quantified (Rudel & Morris, 1973); amount of cholesteryl ester was taken to be $1.67 \times$ cholesterol mass. Their radioactivity was measured in a liquid-scintillation counter (Beckman LD-6800), and standards were run under the same conditions.

Hepatic triacylglycerol lipase activity in the incubation medium

At the end of the preincubation (2 h 30 min) and of the entire incubation (2 h 30 min + 3 h), hepatic lipase activity was measured in the medium, with an emulsion of [^{14}C]trioleoylglycerol and neutral acacia gum as substrate, as fully described by Jansen *et al.* (1978) in the presence of 1 M-NaCl. Enzyme activity is expressed in munits (nmol of fatty acids released/min, at 30 °C) per 10^6 cells.

Preparation of semi-purified lipase

The enzyme was partially purified from post-heparin liver perfusates of six rats as desribed by Schoonderwoerd *et al.* (1983). Pooled perfusates were applied to a Sepharose 4B column to which heparin (Sigma grade I) was covalently bound; 1% defatted bovine serum albumin and 0.02% Triton N101 were added to stabilize the eluted enzyme, which was extensively dialysed against Krebs bicarbonate medium for 2 h before use. Enzyme preparation of about 500 munits/ml were used.

Lipoprotein composition

Protein concentrations were determined (Lowry *et al.*, 1951) on lipoprotein samples. After extraction (Folch *et al.*, 1957) of these samples, the amounts of phospholipids (Bartlett, 1959), triacylglycerols (assay kit from Boehringer, no. 148270) and free and esterified cholesterol (Rudel & Morris, 1973) were measured. Apolipoproteins were identified by SDS/polyacrylamide-gel electrophoresis in 8.25% polyacrylamide, after delipidation of chylomicron or remnant fractions with diethyl ether/ethanol (3:1, v/v) at −20 °C and staining with Coomassie Blue.

Expression of radioactivity data

The values (d.p.m.) were divided by the specific radioactivity measured in native remnants and converted into nmol of free or esterified cholesterol. Cell total [^{3}H]cholesterol is the sum of free and esterified [^{3}H]-cholesterol measured in the cell pellet. It represents remnant total [^{3}H]cholesterol uptake. Since inside the cells the [^{3}H]cholesteryl ester is partially hydrolysed after remnant uptake, the measured free [^{3}H]cholesterol is the sum of remnant free [^{3}H]cholesterol and of the cholesterol arising from [^{3}H]cholesteryl ester hydrolysis. Percentages of net hydrolysis were therefore calculated in accordance with Florén & Nilsson (1977*a*), from the decrease in

radioactivity (as d.p.m.) of cholesteryl ester and the increase in that of free cholesterol:

$$= 100 - \frac{\% \text{ cholesterol as ester after incubation} \times 100}{\% \text{ cholesterol as ester before incubation}}$$

In the experiment with double-labelled remnants, the amount of [^{14}C]cholesterol in the cell pellet indicates the uptake of remnant free cholesterol alone, whereas the free [^{3}H]cholesterol is enhanced owing to [^{3}H]cholesteryl ester hydrolysis. The amount of the latter was then calculated (in nmol) as follows:

Cholesteryl ester hydrolysed = [^{3}H]cholesterol − [^{14}C]cholesterol

Statistics

All experiments were repeated at least three times, and each determination was performed in duplicate flasks. Results are expressed as means ± S.E.M. with the numbers of experiments in parentheses. Statistical significance of differences was calculated with Student's *t* test, or with the paired *t* test when indicated.

Chemicals

Collagenase was from Boehringer (Meylan, France); fat-free bovine serum albumin (fraction V), chloroquine, heparin (grade I and grade II) and CNBr-activated Sepharose were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Ultrogel A2 was from IBF Products (Villeneuve La Garenne, France), [1,2(n)-^{3}H]cholesterol from Amersham International (Amersham, Bucks., U.K.), and [^{14}C]cholesterol from CEA (Gif sur Yvette, France). All other chemicals were reagent grade.

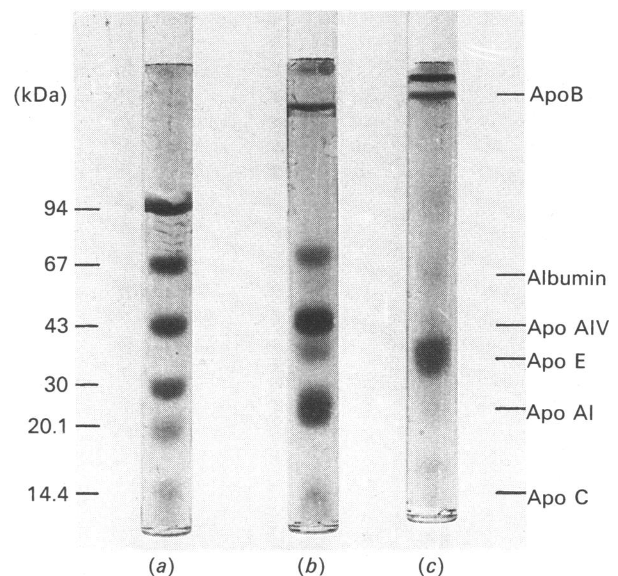

Fig. 1. SDS/polyacrylamide-gel electrophoresis of chylomicrons and chylomicron remnants

A sample containing 50 μg of delipidated lipoprotein was applied to each position and subjected to polyacrylamide-gel electrophoresis in 8.25% gels; (*a*) protein standards of known molecular mass (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soya-bean trypsin inhibitor and α-lactalbumin respectively, downwards); (*b*) lymph chylomicrons; (*c*) chylomicron remnants obtained from eviscerated rats as described in the experimental section.

RESULTS

Characterization of chylomicrons and their remnants

The composition of isolated chylomicrons, expressed as percentages of total mass, was: 92% triacylglycerol, 5.4% phospholipid, 0.47% free cholesterol, 0.68% cholesterol ester and 0.2% protein. Remnants obtained from eviscerated rats contained, as percentages of total mass, 82% triacylglycerol, 7% phospholipid, 2.8% free cholesterol, 3.3% cholesteryl ester and 5.1% protein. These remnants were relatively enriched in free and esterified cholesterol and protein, but were depleted in triacylglycerol. Cholesteryl ester represented 45.8% of total cholesterol on a molar basis and 65–75% of the total ^{3}H radioactivity.

SDS/polyacrylamide-gel electrophoresis (Fig. 1) shows that remnants had more apo E as compared with the native chylomicrons and virtually no apo AI and apo AIV, as previously reported (Nilsson *et al.*, 1981; Lippiello *et al.*, 1985; Arbeeny & Rifici, 1984). B and C apolipoproteins were poorly detected by using SDS/polyacrylamide (8.25%)-gel electrophoresis and only traces of albumin were detected. Although remnant composition was well reproducible from one preparation to another, a remnant population, such as that isolated after 30 min of hydrolysis *in vivo*, is without doubt heterogeneous, which provides a reason for data dispersion despite the standardized isolation conditions.

Uptake of remnants and responsiveness to chloroquine of hepatocytes, either preincubated or not

The total [^{3}H]cholesterol measured in the cell pellet at 37 °C represents remnant uptake (binding and internalization). At 4 °C, the radioactivity determined in the pellet amounted to 7–10% of the radioactivity at 37 °C, and should be considered as a measure of binding alone; this indicates that during the incubation most of the cell-associated radioactivity represents remnant particles which were actually internalized in the hepatocytes.

As shown in Table 1, the total [^{3}H]cholesterol increased linearly and similarly during the 3 h of incubation whether the cells were preincubated or not, and regardless of the presence or absence of chloroquine in the medium. In contrast, [^{3}H]cholesteryl ester in the cell pellets increased very little during incubation (Figs. 2*a* and 2*b*). When percentages of net hydrolysis were calculated, it appeared that about 40% of the ester taken up by the cells was hydrolysed after 1 h and 74% after 3 h of incubation at 37 °C, which may explain the lack of cholesteryl ester accumulation in both non-preincubated and preincubated hepatocytes. When chloroquine was added at the start of the incubation, the amount of [^{3}H]cholesteryl ester was almost unchanged in the non-preincubated hepatocytes but was increased by 2-fold in the preincubated cells ($P < 0.05$), and by 3-fold ($P < 0.005$) when the drug was already present during the preincubation (Fig. 2*b*).

[^{3}H]Cholesteryl ester hydrolysis, as measured with double-labelled remnants in preincubated hepatocytes

Double-labelled remnants were used to quantify the degree of intracellular [^{3}H]cholesteryl ester hydrolysis, as given by the difference between [^{3}H]- and [^{14}C]-cholesterol (Fig. 3). This difference increased linearly with time, and

Table 1. Total [^{3}H]cholesterol in non-preincubated and preincubated hepatocytes after remnant uptake

Hepatocytes (10^7 cells/flask) were used either after collagenase isolation (non-preincubated cells) or after preincubation for 2 h 30 min at 37 °C. Remnants (60 μg of protein), corresponding to 90 nmol and 65.3 nmol of free and esterified cholesterol respectively, were added to the cells and further incubated for the indicated times. Chloroquine (0.1 mM) was added at the start of either the incubation or the preincubation. Values, expressed as nmol/10^6 cells, are means ± S.E.M. for three independent experiments. Statistically significant differences from values obtained at 1 h are indicated by: $*P < 0.05$; $***P < 0.01$.

	Incubation time	Total [^{3}H]Cholesterol (nmol/10^6 cells)		
		No chloroquine	+chloroquine	+chloroquine in the preincubation
Non-preincubated cells	1 h	1.25 ± 0.110	1.09 ± 0.087	–
	2 h	2.21 ± 0.087***	2.01 ± 0.191*	–
	3 h	3.26 ± 0.149***	2.65 ± 0.222***	–
Preincubated cells	1 h	1.28 ± 0.084	1.08 ± 0.054	1.51 ± 0.226
	2 h	2.09 ± 0.205*	1.84 ± 0.104***	2.22 ± 0.227*
	3 h	2.96 ± 0.249***	2.56 ± 0.110***	3.33 ± 0.328***

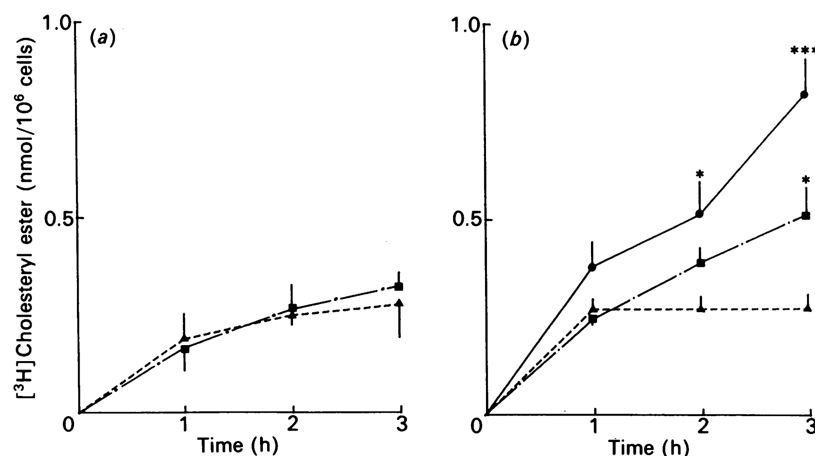

Fig. 2. Time-course study of cell [^{3}H]cholesteryl ester after remnant uptake by non-preincubated and preincubated hepatocytes

Hepatocytes (10^7 cells/flask) were either directly incubated at 37 °C (*a*) or first preincubated for 2 h 30 min at 37 °C and then incubated for the indicated times (*b*). Incubation conditions were the same as in Table 1. Chloroquine (0.1 mM) was absent (▲), or present solely during incubation (■) or already present during the preincubation (●). Values, expressed as nmol/10^6 cells, are means ± S.E.M. for three independent experiments. Statistically significant differences compared with the control (without chloroquine) are indicated: $*P < 0.05$, $***P < 0.01$.

corresponded to 26.3% of the ester being hydrolysed at the end of the incubation. Chloroquine present during the preincubation inhibited ester hydrolysis by 87%. When the percentages of net hydrolysis were calculated (Florén & Nilsson, 1977*a*) and compared for either single- or double-labelled remnants of the same preparation, it was observed that net hydrolysis was 2-fold less with the double-labelled remnants (29.2%, versus 60% with the single-labelled), suggesting that the double-labelling procedure, requiring 5,5′-dithiobis-(2-nitrobenzoate), might have somehow modified the physical properties of the remnants. Therefore only single-labelled remnants were subsequently used, and experiments were performed solely with preincubated cells, which responded well to chloroquine.

Dose–response curves of remnant uptake and of [^{3}H]cholesteryl ester accumulation in preincubated hepatocytes

A representative experiment, presented in Fig. 4, shows a typical saturation profile, with saturation levels of chylomicron remnants reached at 180 μg of remnant protein/flask. Chloroquine did not significantly modify the total [^{3}H]cholesterol uptake, but clearly inhibited the ester hydrolysis, as shown by the accumulation of [^{3}H]cholesteryl ester.

Effects of heparin on remnant uptake and on [^{3}H]cholesteryl ester accumulation in preincubated hepatocytes

Addition of 10 units of heparin/ml (Table 2), increased the total [^{3}H]cholesterol uptake by 63% and by 62%, and the [^{3}H]cholesteryl ester accumulation by 115% and 110%, in the absence or the presence of chloroquine, respectively.

The stimulating effect of heparin is thus well in keeping with the increased disappearance of remnant cholesteryl ester from the medium, as was previously observed in suspended hepatocytes (Nilsson & Åkesson, 1975; Nilsson, 1977).

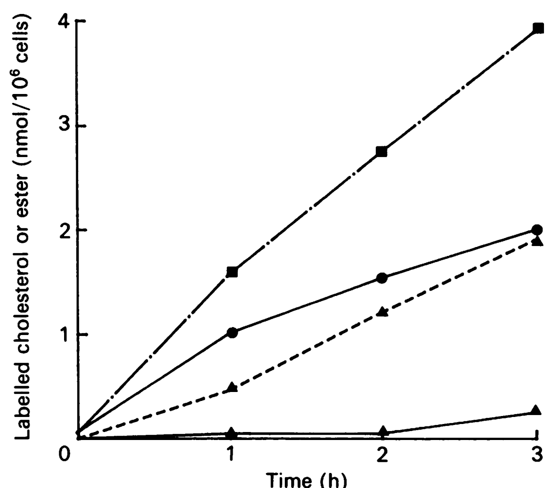

Fig. 3. Time-course study of cell free [^{3}H]cholesterol, free [^{14}C]cholesterol and hydrolysed [^{3}H]cholesteryl ester after uptake of double-labelled remnants by preincubated hepatocytes

Hepatocytes (10^7 cells/flask) were preincubated for 2 h 30 min, then further incubated with remnants labelled endogenously with [^{3}H]cholesterol and *in vitro* with [^{14}C]-cholesterol as described in the Experimental section. Remnants (60 μg of protein) corresponding to 60.8 nmol and 43.04 nmol of free and esterified cholesterol respectively were added per flask. Values from duplicate determinations of free [^{3}H]cholesterol (■) and free [^{14}C]cholesterol (●) are given as nmol/10^6 cells. [^{3}H]-Cholesteryl ester hydrolysis is expressed as the difference between [^{3}H]- and [^{14}C]-cholesterol (nmol), and it was calculated when 0.1 mM-chloroquine was absent (▲---▲) or present (▲——▲) during the preincubation of the cells.

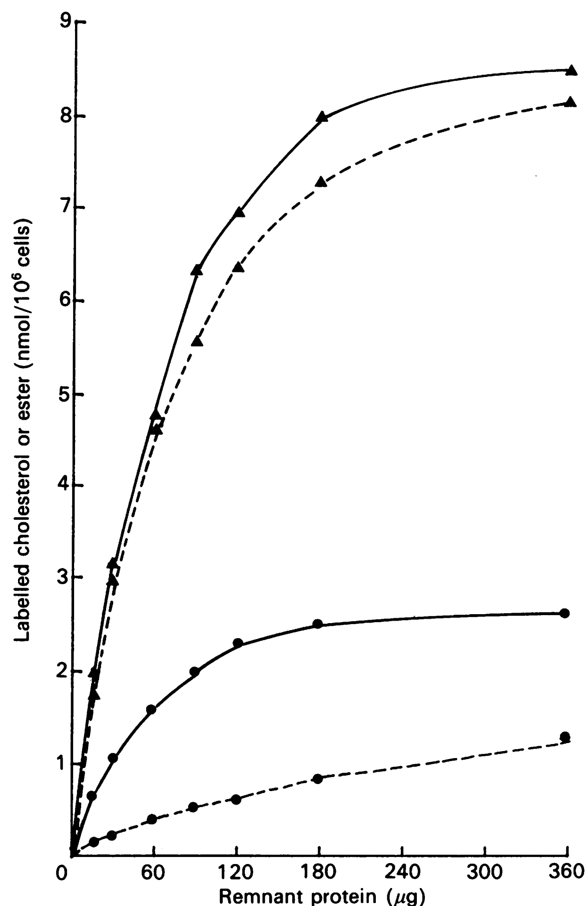

Fig. 4. Dose–response curves of cell total [^{3}H]cholesterol and [^{3}H]cholesteryl ester after remnant uptake by preincubated hepatocytes

Hepatocytes (11.3×10^6 cells/flask) were preincubated for 2 h 30 min at 37 °C in the absence or presence of 0.1 mM-chloroquine. Curves from one experiment, representative of three separate studies, are presented. Remnants (for 60 μg of protein: 102 nmol of cholesterol and 74 nmol of cholesteryl ester) were added at increasing concentrations of protein, and were incubated for 3 h with the cells. Values are given as nmol/10^6 cells for total [^{3}H]cholesterol without (▲---▲) or with (▲——▲) 0.1 mM-chloroquine and for [^{3}H]cholesteryl ester without (●---●) or with (●——●) 0.1 mM-chloroquine.

Hepatic triacylglycerol lipase activity in cell medium at the end of the preincubation and incubation periods

Hepatic lipase activity was measured in samples of the incubation medium after preincubation (2 h 30 min) and at the end of the entire incubation (5 h 30 min) of the hepatocytes. The enzyme activity was very low at the start of the experiment [0.34 ± 0.08 (9) munits/10^6 cells], but increased 4-fold after preincubation [1.48 ± 0.23 (7)] and 5-fold at the end of the incubation [1.78 ± 0.23 (11)]. Heparin increased this activity by 2.4-fold [3.49 ± 0.56 (3)] and by 3.3-fold [5.83 ± 0.37 (5)] at the above times. Addition of remnants did not modify lipase activity (results not shown).

It should be stressed that, before addition of the remnants to the preincubated hepatocytes, hepatic lipase activity was high enough to have a physiological effect, and was even more so in the presence of heparin.

Effect of semi-purified hepatic lipase on remnant uptake, on cell [^{3}H]cholesteryl ester accumulation and on remnant hydrolysis

At the highest initial concentration of semi-purified lipase (100 units/flask), total [^{3}H]cholesterol uptake was increased by 44–69 % whereas [^{3}H]cholesteryl ester was increased by 67–100% the effects being significant only in the presence of chloroquine (Table 3). At lower concentrations (50 munits/flask), the enzyme was most probably inactivated before having any effect.

In a separate experiment, hepatic lipase was incubated during 2 h with the same remnants as above, but in the absence of hepatocytes (Table 4). Decreases of 24% ($P < 0.025$) in triacylglycerol content and of 26% ($P < 0.025$) in phospholipid content of the remnants were found, without modification of their free cholesterol content and with only a minor decrease (6%) in cholesteryl ester. These results indicate that our hepatic lipase preparation displays a dual activity towards chylomicron remnants, i.e. a triacylglycerol hydrolase and a phospholipase activity.

DISCUSSION

The present study shows that freshly isolated hepatocytes were able to remove chylomicron remnants by a receptor-mediated process, as indicated by the

Table 2. Effect of heparin on remnant uptake (total [^{3}H]cholesterol) and on cell [^{3}H]cholesteryl ester in preincubated hepatocytes

Hepatocytes (10^7 cells/flask) were first preincubated for 2 h 30 min at 37 °C with heparin (10 units/ml) and chloroquine (0.1 mM) when indicated. Remnants (60 μg of protein), corresponding to 75.3 nmol and 56.4 nmol of free and esterified cholesterol respectively, were added and the cells were further incubated for 3 h. Values are expressed as nmol/10^6 cells, and are means ± S.E.M. with the numbers of experiments in parentheses. Statistically significant differences with controls are indicated by: $^{**}P < 0.025$; $^{***}P < 0.01$.

	Without chloroquine	With chloroquine
Control		
Total [^{3}H]cholesterol	3.01 ± 0.280 (5)	3.12 ± 0.339 (5)
[^{3}H]Cholesteryl ester	0.25 ± 0.045 (5)	0.76 ± 0.073 (5)
+ Heparin (10 units/ml)		
Total [^{3}H]cholesterol	4.93 ± 0.452 (5)***	5.06 ± 0.433 (3)**
[^{3}H]Cholesteryl ester	0.54 ± 0.153 (4)	1.61 ± 0.297 (3)**

Table 3. Effect of semi-purified hepatic lipase on remnant uptake (total [^{3}H]cholesterol) and on cell [^{3}H]cholesteryl ester in preincubated hepatocytes

Hepatocytes (10^7 cells/flask) were first preincubated for 2 h 30 min at 37 °C with chloroquine (0.1 mM) when indicated. Remnants (30 μg of protein), corresponding to 33 and 22.1 nmol of free and esterified cholesterol respectively, were added and the cells were further incubated for 3 h. Semi-purified hepatic lipase (HL) corresponding to three different preparations was added with the remnants, at initial concentrations of 50 or 100 munits/flask. Values are expressed as nmol/10^6 cells and are means ± S.E.M., with the numbers of experiments in parentheses. Statistically significant differences from control are indicated by: $^{*}P < 0.05$; $^{**}P < 0.025$.

	Without chloroquine	With chloroquine
Control		
Total [^{3}H]cholesterol	1.82 ± 0.346 (4)	1.45 ± 0.121 (4)
[^{3}H]Cholesteryl ester	0.094 ± 0.031 (3)	0.202 ± 0.011 (3)
+ HL (50 munits)		
Total [^{3}H]cholesterol	2.10 ± 0.327 (3)	1.78 ± 0.298 (3)
[^{3}H]Cholesteryl ester	0.119 ± 0.027 (3)	0.292 ± 0.013 (3)
+ HL (100 munits)		
Total [^{3}H]cholesterol	3.07 ± 0.430 (3)	2.09 ± 0.146 (4)*
[^{3}H]Cholesteryl ester	0.157 ± 0.026 (3)	0.417 ± 0.054 (4)**

Table 4. Remnant composition after hydrolysis *in vitro* by semi-purified triacylglycerol lipase (HL)

Semi-purified HL preparations (initial activity 100 munits) were incubated for 2 h at 37 °C with 400 μg of remnant protein in a final volume of 5 ml. Medium contained 20 mM-Tris, pH 7.4, 2.5% (w/v) serum albumin, 0.3% glycerol, 125 mM-NaCl, 0.83 mM-$CaCl_2$ and 0.62 mM-KH_2PO_4. At the end of the incubation, the medium was adjusted to $d = 1.019$ and remnants were re-isolated by ultracentrifugation. Values are expressed as mg/ml of remnant suspension, and are means ± S.E.M., with the numbers of experiments in parentheses. Statistically significant differences ($^{**}P < 0.025$) were obtained with the paired t test.

	Composition of incubated remnants (mg/ml)	
	Without HL	With HL
Triacylglycerol	4.43 ± 0.434 (5)	3.36 ± 0.343 (5)**
Phospholipid	0.260 ± 0.037 (5)	0.190 ± 0.042 (5)**
Free cholesterol	0.135 ± 0.017 (4)	0.132 ± 0.018 (4)
Esterified cholesterol	0.190 ± 0.027 (4)	0.178 ± 0.022 (4)

saturable uptake curve of these particles by the cells. It also shows that hepatocytes are able to degrade part of the chylomicron remnants in the lysosomes, provided that the cells had first been preincubated at 37 °C for 2 h 30 min under which conditions cholesteryl ester hydrolysis could be inhibited by chloroquine. Our data with the latter drug confirm observations made on hepatocyte monolayers (Florén & Nilsson, 1977*a*,*b*, 1978).

The reason for the absence of response to chloroquine in non-preincubated cells, although uptake of remnants and hydrolysis of cholesteryl ester were comparable with those in preincubated hepatocytes, is not clear at present. It was reported (Oswald & Quarfordt, 1987) that apo E

added to triacylglycerol emulsions increased lipid uptake by hepatocyte monolayers 24 h after plating of the cells, but was without effect on the freshly isolated hepatocytes. The authors suggested that cytoskeletal configuration changes were a major reason for the delay in hepatocyte responsiveness to apo E. In the present study, preincubation of the hepatocytes for 2 h 30 min may similarly restore one (or more) step(s) of the endocytotic process.

Besides, other data (Beisiegel *et al.*, 1988) indicate that liver cells possess at least three proteins that bind apoE-containing lipoproteins with high affinity, one of them being predominantly located in the endoplasmic reticulum. This 59 kDa protein probably serves in the intracellular transport of the chylomicron remnants. Whether the 59 kDa protein or the apo B,E receptor needs to be restored by the preincubation of the fresh isolated hepatocytes is not known at present. The coexistence of at least two pathways for remnant processing could underly the difference in chloroquine responsiveness between preincubated and non-preincubated cells.

Concerning remnant labelling, the '*in vivo*' method was chosen because the resulting [^{3}H]cholesteryl ester in the remnants was more efficiently hydrolysed than when [^{3}H]cholesteryl ester was added by an exchange method *in vitro*. However, the '*in vivo*' method labels both free and esterified cholesterol, and free cholesterol may exchange with hepatocyte membrane cholesterol, thus leading to complex interpretation of the results. Nevertheless, it has been shown for biologically prepared remnants that the exchangeable free cholesterol was already exchanged during their preparation (Carrella & Cooper, 1979). Moreover, when remnants were labelled with [^{3}H]cholesterol and [^{3}H]phosphatidylcholine, both components were taken up at similar rates (Lippiello *et al.*, 1985). These observations thus validate the method of remnant labelling used.

Remnant uptake by hepatocytes was dependent on the remnant concentration; its value was 20–26% of added remnants with 60 μg of remnant protein, but it attained only 9–10% at saturating amounts (180 μg of remnant protein) (Fig. 4), the latter values being similar to those reported in hepatocyte monolayers (Lippiello *et al.*, 1985). At remnant saturating amounts it was calculated that for a 10 g liver (corresponding to about 1.25×10^9 hepatocytes) cholesterol uptake would be 1.10 mg/h. This value is lower than the maximal uptake *in vivo* measured in the liver after chylomicron injection (Andersen *et al.*, 1977), which was 2 mg of cholesterol/h. Taking into account the relative contribution of the different cell types to remnant uptake (Lippiello *et al.*, 1985), our values would give 1.5 mg of cholesterol/h. Thus the uptake capacity of suspended hepatocytes appears to be in the range of data in the literature.

Our results clearly show that heparin enhanced chylomicron-remnant uptake by preincubated hepatocytes. Although such an effect was previously observed by some authors (Nilsson & Åkesson, 1975; Nilsson, 1977), contradictory data have also been reported. An inhibition by heparin of apo E-induced uptake of triacylglycerol emulsions by isolated hepatocytes was found (Oswald *et al.*, 1986); also, high heparin concentrations (0.4–2 mg/ml) lead to decreased chylomicron-remnant binding to liver plasma membranes (Carrella & Cooper, 1979). One explanation of this discrepancy may lie in the fact that heparin binds to chylomicron remnants at binding sites located on apo E (Weisgraber *et al.*, 1986) and thus prevents their binding to hepatocyte-surface heparan sulphates, which appear also to be involved in the internalization of apo E-rich lipoproteins (Oswald *et al.*, 1986). This effect could then depend on heparin concentrations, molecular species and incubation conditions.

On the other hand, heparin at 20 munits/ml stabilizes hepatic lipase *in vitro* (Schoonderwoerd *et al.*, 1981), and furthermore it increases its release by the hepatocytes (Leitersdorf *et al.*, 1984; Oswald *et al.*, 1986). In our conditions the positive effect of heparin on hepatocyte remnant uptake is likely to be mediated through its stimulation of hepatic lipase. A further reinforcement for this hypothesis is our finding that the addition of semi-purified lipase allowed a significant increase in remnant uptake by hepatocytes. Despite the known rapid inactivation of the enzyme at 37 °C, we were able to demonstrate this effect for the first time in a model *in vitro*. Since under our conditions hepatocytes were exposed to heparin for 2 h 30 min before the addition of remnants, our data favour the hypothesis that heparin improved remnant uptake through its stimulation of lipase release and of its stabilization, rather than merely through its binding to remnant apo E.

An additional argument in favour of hepatic lipase effect on remnant uptake is given by results which we obtained in a different set of experiments (F. Sultan, D. Lagrange, X. Le Liepvre & S. Griglio, unpublished work) in which chylomicron remnants were labelled, this time, with a non-degradable [^{14}C]cholesteryl oleoyl ether. These remnants were first hydrolysed by the enzyme *in vitro*, re-isolated by ultracentrifugation and then incubated with the hepatocytes under the same conditions as in the present work. The uptake of such hydrolysed remnants was increased by 1.96-fold ($P < 0.005$, $n = 4$) after 1 h and by 1.57-fold ($P < 0.001$, $n = 4$) after 2 h of cell incubation, thus showing that hepatic lipase acted on the remnant particles directly.

The exact mechanism(s) whereby hepatic lipase facilitates chylomicron-remnant uptake remain(s) to be established. The lipolytic activities of this enzyme are heterogeneous in nature (Jensen *et al.*, 1982; Belcher *et al.*, 1985) and variously responsive to proteolytic degradation (Belcher *et al.*, 1985; Persoon *et al.*, 1987). Accordingly, we observed that our semi-purified lipase preparations exhibited both triacylglycerol hydrolase and phospholipase activities with regard to remnants. The question as to which of these activities is rate-limiting for remnant uptake is unanswered at present. The phospholipase A_1 activity of hepatic lipase has received increasing interest over the last few years, and this lipase effect on phospholipids of high-density lipoprotein (HDL_2) is well documented (Van Tol *et al.*, 1980; Landin *et al.*, 1984). With respect to chylomicron remnants, of particular interest is the observation that intact chylomicrons treated with commercial phospholipase were taken up as efficiently as remnants by the perfused liver (Borensztajn & Kostlar, 1981). Phospholipids were shown to be involved in apo CII and apo CIII fixation on the chylomicron-remnant surface (Windler *et al.*, 1986). These two apolipoproteins are known to inhibit hepatic remnant removal (Windler *et al.*, 1980*b*). Lysophosphatidylcholine that may be derived from phosphatidylcholine hydrolysis has been suggested to increase apo C-III-0 and apo C-III-3 removal from the

remnant surface (Windler *et al.*, 1986), and this process may subsequently favour the recognition of apo E by the hepatic receptors. Such a mechanism could underly the role of hepatic lipase in remnant uptake, but it necessitates further physicochemical studies.

From a physiological point of view, data obtained with fat-loaded rats showed that injection of anti-(hepatic lipase) antiserum led to plasma accumulation of apo-B_{48}-rich particles similar to chylomicron remnants (Daggy & Bensadoun, 1986). Thus hepatic lipase may play a role in remnant processing by the liver cells *in vitro* as well as *in vivo*.

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