DEGRADATION OF CHYLOMICRON REMNANT CHOLESTERYL ESTER BY RAT HEPATOCYTE MONOLAYERS. INHIBITION BY CHLOROQUINE AND COLCHICINE.

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Received November 30,1976

SUMMARY

Rat hepatocytes in monolayer cultures take up and degrade cholesteryl ester of isolated chylomicron remnants. The cholesteryl ester of native chylomicrons was metabolized at a slower rate. The uptake of cholesteryl ester was decreased by the presence of serum. The hydrolysis of cholesteryl ester but not the uptake or binding of chylomicron remnants by the cells was inhibited by chloroquine, which is known to inhibit the lysosomal degradation of protein and of low density lipoproteins by fibroblasts. Colchicine, which inhibits the hydrolysis of chylomicron cholesteryl ester after the uptake by the liver in vivo, had the same effect in hepatocyte monolayers.

INTRODUCTION

The chylomicron remnants (1,2) which are formed during the action of lipoprotein lipase are degraded by the hepatocytes (3) by a mechanism involving microtubular functions (4) and possibly a cell surface receptor that can be destroyed by proteolytic enzymes (5, 6). For the further study of chylomicron remnant degradation a convenient model system is needed. Both the perfused liver (7,8) and hepatocytes in suspension (5,6,9) have been used to study chylomicron and chylomicron remnant catabolism. Hepatocytes in suspension take up or bind remnant particles more efficiently than native chylomicrons, but the hydrolysis of the CE after the uptake is slow (5). Cells in suspension or in monolayer culture offer, however, obvious practical advantages compared to the perfused liver. In the present study hepatocytes in monolayer cultures (10) efficiently metabolized CE of isolated chylomicron remnant particles. The effects of colchicine and of chloroquine on the CE degradation were examined to test whether microtubules and lysosomal enzymes may participate in the process.

Abbreviations used are: VLDL = very low density lipoproteins; LDL = low density lipoproteins; CE = cholesteryl ester; C = free cholesterol; TAG = triacylglycerol; LCAT = lecithin acyltransferase (E.C. 2.3.1.43.); t.l.c. = thin layer chromatography; g.l.c. = gas liquid chromatography.

MATERIALS AND METHODS

All radioactive compounds were obtained from Radiochemical Centre, Amersham, Bucks, U.K. Male white Sprague-Dawley rats were obtained from Anticimex AB, Stockholm, Sweden. Thoracic duct cannulations were performed according to Bollman et al. (11), as described earlier (6). The rats were fed 0.75 ml corn oil containing 50 μCi [^{14}C]cholesterol and 100 μCi [^{3}H]palmitic acid or 0.75 ml corn oil containing 200 μCi [^{3}H]cholesterol and 50 μCi [^{14}C]linoleic acid. The fat was given in three doses over 4 hrs. Lymph was collected and stored at +4°C with 2.0 mM disodium EDTA present (12). The distribution of radioactivity between particles with $S_f > 400$ and $S_f < 400$ was determined according to Minari and Zilversmit (13). Chylomicrons were prepared by the same procedure. Chylomicron remnants were prepared by injecting chyle into functionally hepatectomized rats (1). After 30 minutes blood was drawn from the abdominal aorta using Na_2 EDTA as an anticoagulant. The plasma was adjusted to d=1.063 by adding a stock solution (d=1.35) of KBr and NaCl. It was layered under saline (d=1.006) and centrifuged in an MSE 3 x 5 ml swinging bucket rotor at 27,000 rpm (70,000 g_{av}) for 2 1/2 hours. Chylomicron remnants ($S_f > 200$) were collected under sterile conditions and were used within 12 hours. Serum labelled with unesterified cholesterol was prepared according to Nilsson and Zilversmit (14).

Leibovitz L-15 medium, fetal calf serum and HEPES (N-2-hydroxyethylpiperazin-N'-ethane sulfonic acid) were obtained from Flow laboratories, Irvine Ayrshire U.K. Acid soluble calf skin collagen, cycloheximide and chloroquine were obtained from Sigma Chemical Co, Saint Louis, Miss., U.S.A., colchicine from BDH Chemicals, Poole, U.K. and porcine insulin from Novo Industri A/S, Bagsvaerd, Denmark.

Hepatocytes were prepared by a collagenase procedure (15), using the conditions of Seglen (16). The apparatus has been described earlier (17). The perfusate was buffered with 40 mM HEPES. Sterilized equipment and aseptic technique was used during operation. Hepatocytes were cultured in primary monolayers (10,18) according to Lin and Snodgrass (19). The culture medium was Leibovitz L-15 containing 28 mM HEPES, pH 7.4, 1 mM sodium succinate, 0.5 μg insulin, 100 μg penicillin and 100 μg streptomycin or 50 μg gentamicin per ml. Collagen coated (19) 60 mm Petri dishes (Falcon No. 1007) were used. 3-8 x 10^6 suspended hepatocytes were plated in each dish in a volume of 2.5 ml. The dishes were placed in humidified air at 37°. During the first 24 hours the medium contained 5% fetal calf serum. Medium was changed after 4 and after 21-24 hours. The hepatocytes were then incubated with chyle lipoproteins and chylomicron remnants in humidified air at 37° while shaking 25 cycles/min. Each cell preparation was checked at several time intervals by phase contrast microscopy. Trypan blue viability of the cells in suspensions was better than 90 %. After 24 hours the cells had arranged in monolayers of trabecular cell aggregates as described by others (10,19) and all the cells except a very small percentage that was not firmly attached to the dish excluded trypan blue. At 25-28 hr of culture the efficiency of plating was 51.5 ± 3.9% (n=8, means ± S.E.M.).

After the incubations with lipoproteins the medium was collected and the cells were washed twice with 0.5 ml 0.85% NaCl. They were scraped off with a rubber policeman during repeated additions (4 x 1 ml) of methanol:water 2:1 (v/v). Lipids were extracted according

TABLE I. Metabolism of chylomicron remnants and native chyle in hepatocyte monolayers. Effect of serum.

Time hr	Lipoprotein	Serum	% CE cellassociated	% CE hydrolysed	TAG/CE in medium
0	chyle	0	-	-	0.91
4	"	0	10.6	11.6	0.94
12	"	0	21.8	37.1	1.12
24	"	0	33.2	45.0	1.20
4	"	+	2.2	1.6	0.91
12	"	+	3.6	4.0	0.81
24	"	+	6.9	10.7	0.84
0	Remnants	0	-	-	0.33
4	"	0	23.2	38.4	0.57
12	"	0	42.8	57.0	0.73
24	"	0	49.8	58.1	0.71
4	"	+	6.7	6.0	0.36
12	"	+	8.8	20.9	0.41
24	"	+	16.1	35.3	0.45

Native chyle or chylomicron remnants were incubated with or without 15% fetal calf serum as described under Methods. The lipoproteins were added in a volume of 100 or 200 µl to each dish to a final volume of 2.5 ml medium. The added chyle contained 11.6 µg TAG and 0.36 µg cholesterol (434 cpm ^{14}C as CE and 154 cpm ^{14}C as C), 72% of CE in particles with $S_f > 400$. The chylomicron remnants contained 3.1 µg TAG and 0.36 µg cholesterol (512 cpm ^{14}C as CE and 76 cpm ^{14}C as C). Each dish contained 1.3 mg cell protein. The figures are means of two or three incubations.

to Bligh and Dyer (20). Lipid classes were separated by t.l.c. and radioactivity was determined as described earlier (17). Chyle cholesterol was determined (21) after saponification of the lipid extract (22). Triacylglycerols were determined by g.l.c. of the fatty acid methylesters (23). Three dishes in each experiment were used for determination of cellular protein (24). The protein content per dish in different experiments varied between 1.3 and 2.8 mg. Corrections were made for blank value obtained by scraping a collagen coated but cell free Petri dish (≈ 60 µg protein).

RESULTS AND DISCUSSION

The present study demonstrates, that rat hepatocytes in monolayer culture take up and hydrolyse the CE of isolated chylomicron remnant particles. When equal amounts of CE were added as remnant particles and as native chyle or chylomicrons, much more CE was taken up and degraded in the incubations with remnants (Table I, Fig. 1). Similar observations have been made with the perfused rat liver, which was shown to take up remnants prepared from radioiodinated chylomicrons much more efficiently than the native chylomicrons

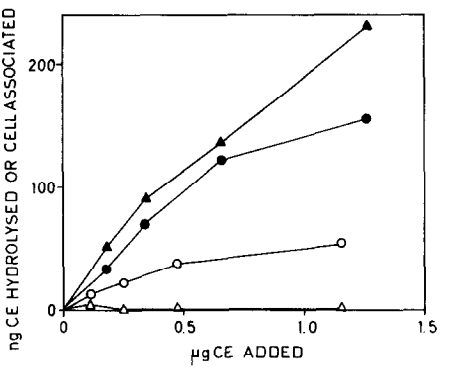

FIGURE 1
Effects of chylomicron and chylomicron remnant concentrations on CE hydrolysis and binding/uptake of CE by the cells. Varying concentrations of chylomicron remnant CE (0.16-1.36 µg CE, 4.3-36.6 µg TAG, cpm ^{14}C as CE 117-1010, cpm ^{14}C as C 103-892) added to dishes containing 2.8 mg cell protein. Different concentrations of chylomicron CE (0.11-1.13 µg CE, 11.3-116.3 µg TAG, cpm ^{3}H as CE 634-6404, cpm ^{3}H as C 1994-20109) added to dishes containing 1.3 mg cell protein. Incubations for 4 hr, means of two or three dishes.

▲——▲ % hydrolysis of added chylomicron remnant CE
●——● % remaining chylomicron remnant CE associated with cells
△——△ % hydrolysis of added chylomicron CE
○——○ % remaining chylomicron CE associated with cells

(8). The present data indicate that the structure of the intact tissue is not necessary for a selective uptake of remnants compared to native chylomicrons. This suggests that an increase in the affinity of the lipoprotein particle for hepatocytes occur during the conversion of chylomicrons to remnant particles, and not only a decrease in size that facilitates the filtration of the particle into the Space of Disse. Such an increase in affinity may be due to the changes in peptide or lipid composition that occur during the formation of remnants (2,8), or to the adhesion of lipoprotein lipase to the particles (7).

The presence of 15% fetal calf serum decreased the uptake and the hydrolysis of both native chyle lipoprotein and of chylomicron remnant CE. The reason for this is not clear, but inhibition of the binding of chylomicrons to suspended hepatocytes by serum has been observed earlier (4,9). The effect may be due to competition between chyle lipoproteins and other plasma lipoproteins for binding sites involved in lipoprotein degradation (25,26) or it may be a

more nonspecific phenomenon. Whether the binding of the remnant particles to the cells occurs to a specific receptor or not, it, however, is undoubtedly succeeded by hydrolysis of the CE portion. In the concentration range of remnants that were used, the mass of CE that was degraded increased with the concentration of remnant particles (Fig. 1).

The cholesteryl ester/cholesterol ratio of the medium was always higher than or equal to that of the added material. There was thus no evidence for hydrolysis of remnant CE in the medium. The added free cholesterol of the remnant particles might be esterified in the cells and by LCAT secreted into the medium. The cholesterol esterification was, however, slow as indicated by experiments in which [^{3}H]cholesterol labelled serum was added. In 4 hr incubations the esterification of free cholesterol never exceeded 1.0%. It can thus cause only a small underestimation of the net hydrolysis of the chyle cholesteryl ester.

Metabolism of chyle glycerolipids

During the preparation of chylomicron remnants from doubly labelled ([^{14}C]cholesterol, [^{3}H]palmitic acid) chyle 49-87% of the triacylglycerol was hydrolysed as indicated by the change in the [^{3}H]triacylglycerol/[^{14}C]cholesteryl ester radioactivity ratio.

During incubation of native chyle or of remnants with cells there was no decrease in this ratio. On the contrary it increased somewhat with time (Table I). There was thus no evidence for triacylglycerol hydrolysis in the medium or during reversible binding of lipoproteins to the cell surface. The increase in the [^{3}H]triacylglycerol/[^{14}C]cholesterylester ratio may be due to secretion of labelled fatty acid as VLDL rather than to a selective uptake of smaller triacylglycerol poor remnants since it was not seen in incubations with colchicine that inhibits VLDL secretion (27,28). More of the fatty acid radioactivity was in polar lipids after incubation, indicating that hydrolysis of triacylglycerols and reincorporation of fatty acids into phospholipids had occurred.

Effects of colchicine

In an earlier study (4) colchicine and vinblastine inhibited the hydrolysis of chylomicron cholesteryl ester after the uptake by the liver in vivo. Similarly, in experiments where postheparin plasma stimulated the catabolism of chylomicron CE by hepatocyte

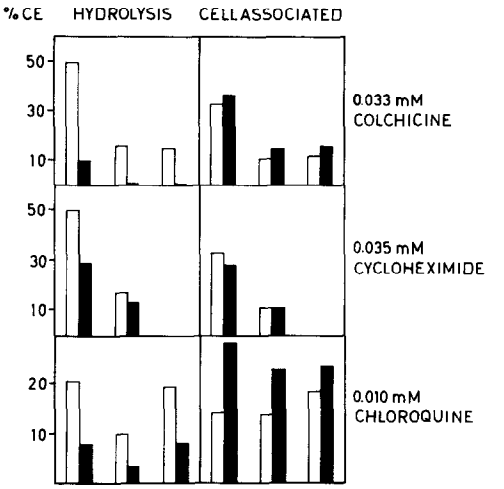

FIGURE 2
Effects of colchicine, cycloheximide and chloroquine on the degradation of chylomicron remnant CE in hepatocyte monolayers. Data from three individual experiments with colchicine, two with cycloheximide and three with chloroquine are shown. Dishes with hepatocytes (1.3-2.8 mg cell protein) were incubated with chylomicron remnants (0.14-1.2 µg cholesterol) for 4 hrs. Open bars: Control dishes. Filled bars: Dishes with inhibitor. Each bar represents means of results from two or three dishes, the difference between individual values being less than 10%.

suspensions the antimicrotubular agents inhibited the CE hydrolysis (5). In the present experiments colchicine inhibited the hydrolysis of chylomicron remnant CE when isolated remnants were incubated with hepatocyte monolayers (Fig. 2). Although colchicine decreased the disappearance of remnant CE from the medium the cellular CE radioactivity was slightly increased (Fig. 2). This suggests that inhibition of the hydrolysis does occur after the uptake by the cells. Cycloheximide caused less inhibition of cholesteryl ester hydrolysis (Fig. 2). This may be secondary to a slightly decreased uptake of cholesteryl ester by the cells as has been shown with suspended hepatocytes (5). Neither colchicine nor cycloheximide increased significantly the reesterification of added nonesterified lipoprotein cholesterol (0.7% increase in reesterification both with 0.033 mM colchicine and 0.035 mM cycloheximide).

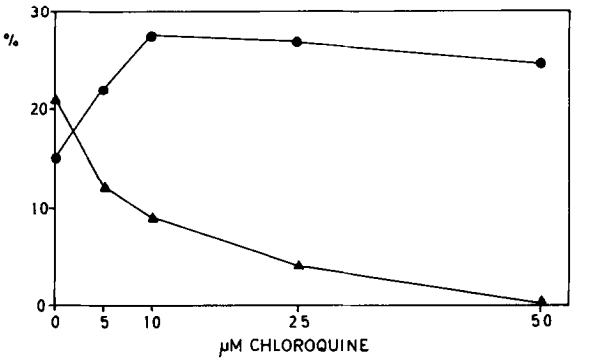

FIGURE 3
Effects of varying concentrations of chloroquine.
Chylomicron remnants were incubated 4 hr without serum with varying concentrations of chloroquine. The cells were preincubated with chloroquine for 2 hr. To each dish was added 19.4 μg TAG and 0.8 μg cholesterol as chylomicron remnants with 300 cpm as [^{14}C]CE and 267 cpm as [^{14}C]C. Each dish contained 1.8 mg cellular protein. Each point represents means of two dishes. One of two similar experiments.
●——● % remaining cholesterylester associated with cells
▲——▲ % cholesterylester of added amount hydrolysed

Effects of chloroquine

Chloroquine, which accumulates in lysosomes (29) and inhibits lysosomal degradation of protein (30) has been used to provide evidence, that in fibroblasts the degradation of LDL occurs by endocytosis (31). In fibroblast cultures chloroquine does not inhibit the uptake of LDL but its degradation after the uptake. In the present study chloroquine affected the metabolism of chylomicron remnant CE in a similar way. 10 μM chloroquine did not inhibit the uptake of CE by the cells, but caused a marked inhibition of the hydrolysis after the uptake (Figs 2,3). With 50 μM chloroquine present there was virtually no hydrolysis of chylomicron remnant CE, whereas the uptake of CE by the cells was still largely unaffected. The cells thus accumulated unhydrolysed remnant CE. At higher concentrations (> 100 μM) the uptake was also decreased and the cells looked markedly granular in phase contrast microscopy. The cells remained, however, adherent to the plastic dishes even with 0.5 mM chloroquine present.

10 μM chloroquine did not influence the incorporation of $^{3}H_2O$ into cholesterol and glycerolipids. In 4 hr incubation without serum the

incorporation of ^{3}H into cholesterol was 46.7 ± 3.5 (n=3, means ± S.E.M.) ngatom ^{3}H/mg protein without and 42.8 ± 3.2 with chloroquine present. The incorporation into glycerolipids was 114.0 ± 8.4 and 106.9 ± 5.2 ngatom/mg protein with and without chloroquine. These figures are in the same range as found in hepatocyte suspension (32). The incorporation of added [^{3}H]palmitic acid into glycerol lipids was also unaffected by 10-25 μM chloroquine (Florén, Nilsson, unpublished data) as was the esterification of added unesterified lipoprotein [^{3}H]cholesterol. In doses, which caused no changes in biosynthetic rates chloroquine thus inhibited the hydrolysis of chylomicron remnant CE by the hepatocytes. At the same chloroquine concentrations the ratio [^{3}H]triacylglycerol/[^{3}H]phospholipids in the cells rose from 3.7 in control cells to 5.4-8.0. This suggests that chloroquine caused a decrease also in the hydrolysis of triacylglycerols and thereby a decrease in the reincorporation of fatty acids into phospholipids.

The data are compatible with the idea that chylomicron remnants are degraded by endocytosis and lysosomal hydrolysis. A role of the acid cholesterol esterase, which may be identical to the acid lipase (33), and which is known to be present in hepatocytes (34) could then be postulated. This hypothesis is also compatible with the finding that colchicine inhibits the hydrolysis of chylomicron remnant CE after the uptake by the cells. For instance the remnants might be taken up in endocytic vesicles that do not undergo fusion with the primary lysosomes at a normal rate in the presence of antimicrotubular agents (35). Final conclusions about the mechanism of chylomicron remnant degradation in vivo must, however, awaite further studies on the intact animal. Even if the present study indicates that hepatocytes in monolayers can endocytoze remnant particles, other transport processes might operate in the intact liver where the structural relation between different cell types is intact. Electronmicroscopic autoradiographic observations (36) are, however, compatible with the idea that lysosomes participates in the degradation of peptides from VLDL remnants also in vivo.

ACKNOWLEDGEMENT

Miss Gertrud Olsson provided skillful technical assistance. The work was supported by grants from the Swedish Medical Research Council (grant No. 03X-3969), the Medical Faculty, University of Lund, and Albert Påhlssons Stiftelse.

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