Archives internationales de Physiologie et de Biochimie, 1981, **89**, 57-62

Reçu le 5 novembre 1980.

Morphological and compositional changes of rat plasma triglyceride-rich lipoproteins incubated with adipose tissue

BY

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(3 figures)

Triglyceride-rich rat lipoproteins (mainly VLDL), purified by ultracentrifugation and dialysis, were incubated for 120 min in the presence of epididymal fat-pad pieces from fed rats in media containing heparin. During this process, the lipoproteins were depleted of triglycerides and proportionally enriched with proteins, causing their increased density. After incubation, electron microscopic study of the triglyceride-rich lipoproteins revealed partially degraded structures, and some particles had atypical discoid flattened form and marked lamellar structure. These findings are in agreement with those of BLANCHETTE-MACKIE & SCOW (1976) in triglyceride hydrolysed chylomicrons and confirm their observation of monolayers formed by accumulated lipolytic products that move by lateral diffusion, giving rise to a spiral fold between the core triglycerides and the aqueous space.

Introduction

The very low density lipoproteins (VLDL) constitute the main transport vehicle of plasma triglycerides of endogenous synthesis in Mammals (SMITH *et al.*, 1978). It has been proposed that these lipoproteins consist of a core of triglycerides, with minor amounts of cholesterol esters, enclosed by a surface film of phospholipids, cholesterol and proteins (GUSTAFSON, 1966; SATA *et al.*, 1972). The metabolism of VLDL is similar to that of chylomicrons (BERGMAN *et al.*, 1971; EISENBERG & RACHMILEWITZ, 1975; MJØS *et al.*, 1975; DECKELBAUM *et al.*, 1977), the other triglyceride-carrier lipoproteins, and requires the hydrolysis of triglycerides by the enzyme system lipoprotein lipase which is mainly localized near the vascular bed (ROBINSON, 1970; SCOW, 1970; BLANCHETTE-MACKIE & SCOW, 1971) in extra-hepatic tissues (adipose tissue, heart, muscle and mammary gland) (SCOW *et al.*, 1976). After hydrolysis of their triglycerides, VLDL *in vivo* are converted to a lipoprotein of higher density designated as an "intermediate lipoprotein" or as "remnants" (HAVEL, 1965; BILHEIMER *et al.*, 1972; EISENBERG *et al.*, 1973). In studies with VLDL in perfused rat hearts (DORY *et al.*, 1978) or after their i.v. administration to evisce-

rated rats (MJØS *et al.*, 1975), it has been shown that remnants from plasma VLDL have a flattened structure with membrane-like borders around a partially emptied interior. Further studies of this type, performed under different experimental conditions, should improve our understanding of the entire process of plasma VLDL catabolism. In the present work we investigated the ultrastructural changes in rat VLDL after their incubation *in vitro* with adipose tissue pieces in the presence of heparin. In these conditions, the heparin enhances the release of lipoprotein lipase into the medium (PERSSON *et al.*, 1966; STEWART & SCHOTZ, 1974) allowing efficient contact of the enzyme with its substrate.

Materials and Methods

Female Wistar rats weighing 180-200 g and fasted for 3 h were bled from the aorta under ether-O_2 anaesthesia. Blood was collected on solid EDTA-Na_2 and plasma was rapidly separated by centrifugation at 1 000 × *g* for 20 min at 4 °C. Fresh plasma aliquots were centrifuged at 143 000 × *g* in a 40.3 Beckman rotor for 18 h at 20 °C under 0.15 M NaCl with a precise density of 1.006, adjusted by using an Abbe refractometer. The top portion of the tube, containing the VLDL and chylomicrons still present in the initial plasma, was cut and the lipoproteins were purified by dialysis three times against 0.9 % NaCl-1 mM EDTA-Na_2 and finally against 0.9 % NaCl only for a total of 18 h at 4 °C. These purified lipoproteins were called triglyceride-rich lipoproteins and contained mainly VLDL although separation of possible contaminant chylomicrons was not attempted. The purified lipoproteins coming from an initial 50 ml volume of plasma were brought to a final volume of 14 ml with 0.9 % NaCl. Aliquots of this triglyceride-rich lipoprotein suspension (0.5 ml) were incubated for 120 min with or without pieces of epididymal fat pads (about 20 mg/vial) from fed male rats (180-190 g) placed in 1 ml Krebs-Ringer bicarbonate buffer, pH 7.4, and supplemented with bovine albumin purified according to the method of CHEN (1967), with glucose and heparin in amounts to yield a concentration of 0.8 %, 4 mM and 3 IU/ml, respectively. Krebs-Ringer bicarbonate components were adjusted to give the final normal concentration in the medium (UMBREIT *et al.*, 1964) after addition of the triglyceride-rich lipoprotein saline suspension. Incubations were carried out under O_2/CO_2 (95/5 %) atmosphere at 37 °C and shaken as previously reported (HERRERA & AYANZ, 1972). The incubations were terminated by removing the adipose tissue pieces. The media (with a density of 1.01) were rapidly centrifuged at 143 000 × *g* for 18 h. After removing the top portion of the tubes, the bottoms were adjusted to a density of 1.21 with solid NaBr and centrifuged for 24 h at 143 000 × *g*, at 20 °C. Aliquots of the final top and bottom fractions and of the different samples were used for lipid extraction (FOLCH *et al.*, 1957) and phospholipids were removed from the lipid extracts with activated silicic acid in chloroform. Saponification was performed in aliquots of phospholipid-free lipid extracts dried under N_2 with 1 M ethanolic KOH for 1 h at 100 °C after which the cooled samples were treated with 0.18 M $MgSO_4$, centrifuged at 1 000 × *g* for 15 min, and glycerol was measured (GARLAND & RANDLE, 1962) in the supernatants. Protein concentration was also evaluated (WANG & SMITH, 1975) in aliquots of the lipoprotein fractions. The lipoproteins were examined by electron microscopy following negative staining with phosphotungstic acid at pH 7.0 (BLANCHETTE-MACKIE & SCOW, 1976) in a Philips electron microscope (model EM 301).

In another series of experiments, fed female rats were i.v. injected in the tail with 60 μCi of sodium [9-10 (n)-^{3}H] palmitate (230 mCi/mmol) and 30 μCi [U-^{14}C] glycerol (46 mCi/mmol) (Radiochemical Center, Amersham) dissolved in 8 % puri-

fied bovine albumin. Blood was collected 30 min after the injection and the triglyceride-rich lipoproteins were purified as described above. Portions of these prelabelled lipoproteins were used for lipid extraction (FOLCH *et al.*, 1957) and fractionation as previously described (CARMANIU & HERRERA, 1980). It was found that more than 87 % of the [^{3}H] lipids in these lipoproteins was in the form of esterified fatty acids while more than 97 % of the [^{14}C] lipids was in the form of glyceride-glycerol. Aliquots of these prelabelled lipoproteins having 1×10^4 dpm of each tracer as adjusted initial radioactivity/vial were incubated for 120 min with pieces of epididymal fat pads (18.3 ± 1.3 mg/vial) from fed rats in the presence or absence of heparin (3 IU/ml), in the same conditions as described above. The incubations were terminated by placing portions of the media in chloroform-methanol (2 : 1, v/v) for lipid extraction (FOLCH *et al.*, 1957) and fractionation (CARMANIU & HERRERA, 1980).

Results

Rat triglyceride-rich lipoproteins (mainly VLDL) were incubated *in vitro* for 120 min with heparin, with or without epididymal fat-pad pieces. As shown in Table I, in the absence of tissue, incubation produced minor changes in the ultracentrifugal separation of the triglyceride-rich lipoproteins, and most of them appeared floating in the fraction at a density of 1.01, with only a small amount recovered at $d > 1.01$. At $1.01 > d < 1.21$, the recovered lipoproteins appeared depleted of glycerides of neutral-lipids (presumably triglycerides) and proportionally enriched in proteins, probably as a consequence of the passive loss of triglycerides. When incubations were performed in the presence of the tissue, more than 70 % of the initial glycerides and proteins were lost from the fractions floating at $d > 1.01$ (Table I), while 52 % of the initial proteins appeared in the $1.01 > d < 1.21$ fraction.

TABLE I. *Altered composition of purified triglyceride-rich lipoproteins after 120 min of incubation in the presence of rat epididymal fat-pad pieces.*

Density	% values *vs.* the non-incubated preparation			
	Glyceride-glycerol of neutral lipid	*P*[a]	Proteins	*P*
Incubation without tissue				
$d < 1.01$	76.5 ± 6.4[b]		71.2 ± 9.1	
$1.01 < d < 1.21$	7.0 ± 3.6		15.2 ± 2.6	
$d > 1.21$	4.4 ± 1.3		—	
Incubation with tissue				
$d < 1.01$	27.4 ± 10.4	<0.01	30.0 ± 4.9	< 0.01
$1.01 < d < 1.21$	13.8 ± 3.5	NS	52.1 ± 9.7	< 0.01
$d > 1.21$	3.9 ± 1.4	NS	—	

[a] *P* values correspond to the statistical comparison between the groups incubated in the presence and in the absence of tissue. The unincubated triglyceride-rich lipoproteins contained 3.39 ± 0.14 nmol of glyceride-glycerol of neutral lipid/μg protein.

[b] Values correspond to means ± SEM of 4 samples/group.

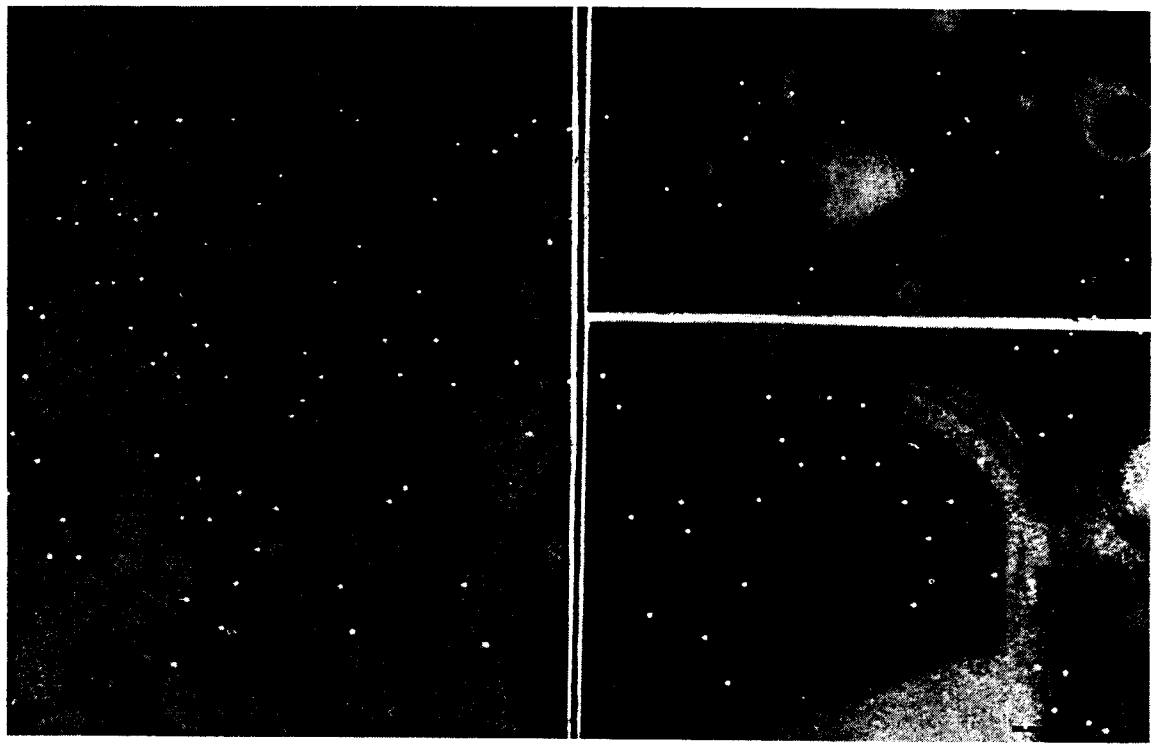

FIG. 1. *Negatively stained, purified triglyceride-rich lipoproteins from rat* (×140 000). Most particles are 150-600 Å in diameter, corresponding to VLDL. There are also large particles (1 000-2 000 Å in diameter) which correspond to chylomicrons. The bar represents 25 nm on all the figures.

FIGS 2 & 3. *Negative stain of the floating fraction at 1.01 > d < 1.21 of triglyceride-rich lipoproteins incubated for 120 min in the presence of rat epididymal fat-pad pieces* : Fig. 2 = ×120 000; Fig. 3 = ×480 000.

The electron microscopic appearance of the lipoprotein preparation before incubation is shown in Figure 1 where compact particles are evident with diameter ranging from 150 to 600 Å corresponding to VLDL. Some chylomicrons are also

TABLE II. *Effect of heparin on prelabelled triglyceride-rich lipoproteins from rat incubated* in vitro *with pieces of epididymal fat pads from fed rats.*

	[^{3}H] Esterified fatty acids (dpm/vial)	[^{3}H] Free fatty acids (dpm/vial)	[^{14}C] glyceride-glycerol (dpm/vial)
Basal controls.....	5 079 ± 439	2 669 ± 145	5 998 ± 389
Heparin (3 IU/vial).....	1 710 ± 289	5 976 ± 604	1 794 ± 153
P	<0.001	<0.001	<0.001

Incubations were carried out for 120 min with pieces of epididymal fat pads (18.3 ± 1.3 mg/vial) in the presence of rat triglyceride-rich lipoproteins prelabelled with ^{3}H in the esterified fatty acids and with ^{14}C in the glyceride-glycerol. Initial radioactivity was adjusted to 1×10^4 dpm for each tracer/vial. Values correspond to means ± SEM of the dpm in each lipid fraction in the media. *n* = 4-8/group. *P* corresponds to the statistical comparison between the heparin and basal control vials.

present with diameter ranging from 1 000 to 2 000 Å. When the triglyceride-rich lipoprotein preparation was incubated for 120 min in the presence of epididymal fat-pad pieces and heparin, no morphological changes were observed in lipoproteins of $d < 1.01$. In the lipoproteins of higher density ($1.01 > d < 1.21$), however, important changes are evident : they are smaller in size than those of $d < 1.01$ and have a partially degraded structure (Figs 2 & 3). Some of these particles have an atypical discoid and flattened form and a definite lamellar structure (Fig. 3) with a periodicity of about 50 Å.

To substantiate the effect of heparin enhancing the lipoprotein lipase action on the hydrolysis of lipoprotein-triglycerides, rat triglyceride-rich lipoproteins prelabelled with ^{3}H in the esterified fatty acids and with ^{14}C in the glyceride glycerol were incubated for 120 min in the presence or absence of heparin. The changes in the labelled lipid fractions in the medium are shown in Table II. The presence of heparin produced a significant decrease in both the [^{3}H] esterified fatty acids and [^{14}C] glyceride glycerol and an increase in the appearance of [^{3}H] free fatty acids in the medium.

Discussion

Although the VLDL used in the present study were contaminated with some chylomicrons, it was evident that they were depleted of triglycerides after their incubation *in vitro* in the presence of heparin and epididymal fat-pad pieces. This effect was accompanied by reduction in size of the lipoprotein particles and alteration in shape, as shown by their electron microscopic appearance. These morphological changes are similar to those observed in both chylomicrons and VLDL after their administration *in vivo* to eviscerated rats (Mjøs *et al.*, 1975), incubation *in vitro* in the presence of lipoprotein lipase (Blanchette-Mackie & Scow, 1976) or in perfusion studies (Dory *et al.*, 1978), suggesting that they are caused by the action of adipose tissue lipoprotein lipase on these particles.

In the conditions used in the present study, it was seen that, when epididymal fat-pad pieces were incubated in the presence of heparin and prelabelled triglyceride-rich lipoproteins, there was a marked disappearance of labelled, esterified fatty acids and glyceride-glycerol and appearance of free fatty acids in the medium. This demonstrates that heparin produced an increase in lipoprotein lipase action on the lipoproteins in the medium. The effect is primarily exerted on the hydrolysis of triglycerides which are lost from the particle, causing an important structural change. In the condition *in vivo* it has been shown that, in addition to triglycerides, the "remnant" lipoproteins from both VLDL and chylomicrons are depleted of phospholipids and C apoproteins which are transferred to HDL (Mjøs *et al.*, 1975), probably through a lecithin-cholesterol acyl transferase (LCAT)-mediated mechanism. In the present experimental protocol, lipoproteins were incubated in the absence of either LCAT or HDL and thus the "remnant" particles formed probably contained an excess of polar external components which may have caused their irregular shape, forming greater surface with the same volume. This interpretation is in agreement with the findings of Mjøs *et al.* (1975) in which discoid and flattened chylomicron "remnants" were obtained instead of regular ones in animals with low plasma HDL concentrations.

After incubation, some of the large and medium diameter lipoproteins had apparent "holes" (Figs 2 & 3) or concavities probably caused by lipoprotein lipase action. Blanchette-Mackie & Scow (1973 & 1976) observed this shape in chylomicrons when triglycerides were hydrolysed by the lipoprotein lipase, showing that there were actual aqueous spaces that gave rise to lamellar structures similar to those

observed in the present study. On the basis of previous observations with myelin structures (STOECKENIUS, 1959; STOECKENIUS *et al.*, 1960) and artificial monomolecular films (TRURNIT & SCHIDLOWSKY, 1960), the dark bands of the lamellae may correspond to polar hydrophylic regions while the light ones correspond to non-polar sides. These monolayers are probably formed by the accumulation of lipolytic products that move by lateral diffusion (MCCONNELL *et al.*, 1972) from the site of lipoprotein lipase action to the monolayer lining the aqueous space. These monolayers may produce a spiralling fold within the aqueous space, as already proposed by BLANCHETTE-MACKIE & SCOW (1973 & 1976).

Acknowledgments. — The authors wish to express their gratitude to Drs. BARGALLÓ and LÓPEZ from the *Servicio de Microscopía de la Universidad de Barcelona* for their valuable assistance in the use of the microscope and for their continuous advice; to Dr. CUEVAS for the preparation of the photographs; and to Caroline S. DELGADO for her editorial help.

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