Phospholipids as modulators of hepatic recognition of chylomicron remnants

Observations with emulsified lipoprotein lipids

Jayme BORENSZTAJN* and Thomas J. KOTLAR

Department of Pathology and the Feinberg Cardiovascular Research Institute, Northwestern University Medical School, Chicago, IL 60611, U.S.A.

The lipids extracted from chylomicrons, chylomicron remnants generated *in vivo* and hepatic-lipase-treated chylomicrons were emulsified by sonication. These emulsified particles retained the capacity of the native lipoproteins to be differentiated by the liver *in vivo*, i.e. only the particles derived from remnant and hepatic-lipase-treated chylomicron lipids were efficiently taken up by the liver. To investigate the role of phospholipids in this differentiation process, the phospholipids of all three lipoprotein preparations were separated from the remaining lipids by silicic acid chromatography. The phospholipid-free lipid fraction of chylomicrons was then emulsified with the phospholipids derived from each of the three lipoprotein preparations. Only the particles emulsified with phospholipids derived from remnants and hepatic-lipase-treated chylomicrons were efficiently taken up by the liver *in vivo*. These results support the proposition that phospholipids modulate the hepatic differentiation between chylomicrons and remnants *in vivo*.

INTRODUCTION

One of the main functions of chylomicrons is the transport of dietary cholesterol from the intestine to the liver. This process is of major importance for the regulation of plasma cholesterol levels. The dietary cholesterol may cause the suppression of hepatic cholesterol synthesis (Nervi *et al.*, 1975; Nervi & Dietrich, 1976; Cooper, 1977; Andersen *et al.*, 1977), thus decreasing the amount of endogenous cholesterol that is secreted into the plasma, and it may suppress the expression of hepatic low-density-lipoprotein receptors, and thus affect plasma low-density-lipoprotein concentration (Brown *et al.*, 1981). Chylomicrons themselves are not normally taken up by the liver. In order to transport their cholesterol into hepatocytes, these lipoproteins must first undergo compositional changes in the vascular bed, a process that requires the combined actions of lipoprotein lipase and hepatic lipase (Borensztajn *et al.*, 1988). These changes include a decrease in the triacylglycerol core of the particles, the loss of specific surface apoproteins, and the partial loss of surface phospholipids (Mjøs *et al.*, 1975; Tall *et al.*, 1979). The end-products of these compositional changes are the cholesterol-rich chylomicron remnants which, unlike intact chylomicrons, are readily recognized and taken up by liver cells.

The differences in composition between chylomicrons and remnants which enable the liver to distinguish between these two particles have been the subject of several studies (Felts *et al.*, 1975; Shelburne *et al.*, 1980; Pattnaik & Zilversmit, 1980; Windler *et al.*, 1980a,b; Nilsson *et al.*, 1981; Quarfordt *et al.*, 1982; Van Berkel *et al.*, 1983; Arbeen & Rifci, 1984; Windler & Havel, 1985; Levy *et al.*, 1985; Redgrave *et al.*, 1988; Windler *et al.*, 1988; Sultan *et al.*, 1989). Work from our laboratory has demonstrated that the hydrolysis *in vitro* of chylomicron phospholipids with either phospholipase A₂ or hepatic lipase transforms these lipoproteins into remnant-like particles that can be readily removed from circulation by the liver, and which can compete with endogenous remnants for uptake by hepatocytes (Borensztajn *et al.*, 1980, 1988; Borensztajn & Kotlar, 1981). On the basis of these observations, we proposed that discrimination between chylomicrons and remnants by the liver is modulated by the differences in phospholipid composition of the particles. In

this paper we present results of experiments using emulsions of lipoprotein lipids which provide further evidence for this proposal. We conclude that such emulsions may be used to identify specific phospholipid determinants of hepatic recognition and uptake of chylomicron remnants.

MATERIALS AND METHODS

Preparation of lipoproteins

Rat lymph chylomicrons doubly labelled with [1,2-³H]cholesterol and [1-¹⁴C]palmitic acid were prepared as previously described (Borensztajn *et al.*, 1988). In purified chylomicrons < 1 % of the ¹⁴C label was presented in the phospholipid fraction. The division of the ³H label was 75 % in cholesteryl ester and 25 % in unesterified cholesterol. Preparation of chylomicron remnants in functionally hepatectomized rats, treatment of chylomicrons with heparin-releasable rat hepatic lipase and monitoring of triacylglycerol hydrolysis by loss of ¹⁴C label were as previously reported (Borensztajn *et al.*, 1988).

Preparation of emulsions

The lipids of chylomicrons, remnants and hepatic-lipase-treated chylomicrons were extracted in chloroform/methanol (2:1, v/v) by the method of Folch *et al.* (1957). The aqueous phase was discarded, and portions (1 mg of lipoprotein cholesterol) of the lipid-containing organic phase was dried first under N₂ and then under vacuum for 2 h at room temperature. Then 5 ml of 0.15 M-NaCl was added to the dried lipids, and the mixture was sonicated with a Heat Systems sonicator (Heat Systems Ultrasonics, Farmingdale, NY, U.S.A.) with a standard microtip. The sample, immersed in ice/water, was sonicated for a total of 15 min with a 2 s burst every 5 s at output setting 4. For certain experiments, the extracted lipoprotein lipids were separated into phospholipids and non-phospholipid fractions (Shel-tawy & Dawson, 1969), which were recombined before sonication. Lipid fractionation was carried out on a 1.4 cm × 2.5 cm column of silicic acid (Bio Sil A, 100–200 mesh; Bio-Rad, Rockville Centre, NY, U.S.A.). Total lipoprotein lipids extracted as above were dried under N₂, dissolved in chloroform and applied to the column. Non-phospholipid lipids were eluted with

* To whom all correspondence should be addressed.

50 ml of chloroform, and phospholipids were then eluted with 50 ml of methanol. The recovery of the lipids eluted from the silicic acid columns was monitored by measurement of ^{14}C and ^3H label for the phospholipid-free fraction ($> 95\%$) and by chemical determination for the phospholipid fraction ($> 90\%$). After sonication the emulsions were applied to 2% agarose columns (1.5 cm $\times$ 50 cm) (Bio-Gel A-50m, 50–100 mesh; Bio-Rad) and eluted with 0.15 M-NaCl. The particles eluted with the void volume were used for the experiments. All emulsions, which were stable for at least 48 h, were used within 2 h of preparation.

Hepatic uptake of lipoproteins and emulsions

The hepatic uptake of lipoproteins and emulsions was determined as previously described (Borensztajn *et al.*, 1988). Briefly, male Sprague-Dawley rats (120–150 g) fed *ad libitum*, were anaesthetized with sodium pentobarbital and partially hepatectomized by ligating the blood vessels supplying the median and caudate liver lobes. The right lateral lobe, accounting for about 20% of the total liver weight, retained its circulation intact. The uptake of the various lipoproteins and emulsions by the intact liver lobe was determined by infusing the labelled preparations, against the blood flow, directly into the portal vein at a rate of 1 ml/min for 30 s. At the end of the infusion, carried out with the aid of a peristaltic pump (Gilson, Middleton, WI, U.S.A.), 30 s was allowed to elapse before the circulation was cut off and the lobe was perfused with 0.15 M-NaCl for 30 s through the portal vein in order to wash out entrapped particles. The whole lobe was then taken for measurement of radioactivity incorporation.

Other procedures

Lipoprotein cholesterol and phospholipids were measured by the methods of Abell *et al.* (1952) and Bartlett (1959) respectively. Triacylglycerols were measured by the method of Bucolo & David (1973) with a Technicon Auto Analyzer (Technicon Corp., Tarrytown, NY, U.S.A.). Analytical electrophoresis of apoproteins was carried out on SDS/10% (w/v) polyacrylamide gels (Laemmli, 1970). For electron microscopy, the lipoproteins and emulsions were negatively stained with a 2% (w/v) solution of phosphotungstic acid. The particles were photographed, enlarged to magnification $\times 55000$ and their diameters measured. The uptake of the various particles by the liver was monitored by measuring the incorporation of their [^3H]cholesterol as previously described (Borensztajn *et al.*, 1980).

RESULTS AND DISCUSSION

Treatment of chylomicrons with phospholipase A_2 or hepatic lipase *in vitro* has proved useful in identifying the role of phospholipids in modulating hepatic differentiation of chylomicrons and remnants (see the Introduction). However, this method causes hydrolysis of the several classes of phospholipids on the surface of the chylomicrons (Tall *et al.*, 1979; Borensztajn

et al., 1980). Thus the increased hepatic uptake which results from phospholipase treatment *in vitro* cannot be ascribed to a particular phospholipid alteration. Lipid emulsions of defined composition offer an alternative method for investigating the role of specific phospholipids in hepatic recognition of remnants. Because other authors have shown that the hepatic uptake of emulsions is affected by the proportions (Maranhao *et al.*, 1986; Mortimer *et al.*, 1989) and molecular species (Bennet Clark & Derksen, 1987; Robins *et al.*, 1988; Redgrave *et al.*, 1988; Lenzo *et al.*, 1988) of the lipids incorporated, we opted to employ solely lipids extracted from lipoproteins in their original proportions. Such emulsions might be expected to resemble intact lipoprotein particles with respect to hepatic recognition.

In the present study we used emulsions prepared from lipids extracted from chylomicrons, chylomicron remnants generated *in vivo* and hepatic-lipase-treated chylomicrons. Table 1 shows the lipid distribution of typical lipoprotein preparations. In agreement with previous observations (Borensztajn *et al.*, 1988), the extent of phospholipid hydrolysis of chylomicrons treated with hepatic lipase, relative to the triacylglycerol hydrolysis, was considerably higher than in the remnants generated *in vivo* by the action of lipoprotein lipase.

Two types of emulsions were prepared from lipoprotein lipids extracted with chloroform and methanol. For the first type, the total lipids were emulsified without any fractionation. For the second type of emulsions, lipids extracted from chylomicrons, hepatic-lipase-treated chylomicrons and remnants generated *in vivo* were separated on silicic acid into two fractions: (a) phospholipids and (b) remaining lipids (phospholipid-free fraction). Before emulsification these two fractions were recombined in the same proportions as the intact particles (Table 1). To each 0.1 mg of cholesterol (phospholipid-free fraction) was added phospholipid (phospholipid fraction) in the amounts of 0.850 mg, 0.182 mg and 0.553 mg for, respectively, chylomicrons, hepatic-lipase-treated chylomicrons and remnants generated *in vivo*. Fig. 1 shows the particle size distribution of intact chylomicrons and remnants, and also of emulsions prepared with their unfractionated lipids. The emulsified particles were considerably larger than the intact lipoproteins. A similar size distribution was observed with emulsified particles prepared with lipids from hepatic lipase-treated chylomicrons (results not shown).

To determine whether the emulsified lipoprotein lipids could serve as models to investigate the determinants of hepatic recognition of remnants, their ability to be taken up by the liver *in vivo* was compared with that of intact lipoproteins. The results obtained are shown in Table 2. In agreement with previous observations (Borensztajn *et al.*, 1988), remnants and hepatic-lipase-treated chylomicrons were recognized and taken up by the liver significantly ($P < 0.001$) more efficiently than chylomicrons (Table 2). All emulsified particles, prepared by sonication of unfractionated (Table 2) as well as fractionated and recombined lipids (Table 2) extracted from chylomicrons, remnants and hepatic-lipase-treated chylomicrons, retained the capacity of the

Table 1. Lipoprotein lipid distribution

Chylomicron remnants and hepatic-lipase-treated chylomicrons were prepared as described in the Materials and methods section. The ratio values are the means of lipid determinations carried out in duplicate.

	Chylomicrons	Remnants	Hepatic-lipase-treated chylomicrons
Triacylglycerol:cholesterol ratio	136.0	50.1	86.5
Phospholipid:cholesterol ratio	8.5	5.53	1.82
Triacylglycerol hydrolysis (%)	—	63	36
Phospholipid hydrolysis (%)	—	35	75

Fig. 1. Size distribution of intact lipoproteins (■) and emulsified lipoprotein lipids (□)

The lipids extracted from chylomicrons and remnants generated *in vivo* were emulsified as described in the Materials and methods section. The intact and emulsified particles were negatively stained, photographed at a magnification of $\times 55000$ ($\times 148500$ for intact remnants) and their diameters measured; 360 particles from each preparation were measured.

intact lipoproteins (Table 2) to be differentiated by the liver. It is important to underscore that the hepatic uptake of emulsions was not an artifact caused by the extraction, fractionation and emulsification of lipoprotein lipids. Emulsions prepared with

unhydrolysed chylomicron lipids were taken up by the liver at the same low rate as intact chylomicrons (Table 2). This finding suggests further that the efficient uptake of emulsified particles prepared with lipids extracted from remnants and hepatic-lipase-treated chylomicrons occurred by a mechanism similar to that for the intact lipoproteins. The liver was able to differentiate between various emulsions which had similar size distributions (Fig. 1), indicating that particle size did not seem to be an important parameter in this process. This conclusion is in agreement with those of previous studies (Sherril & Dietsch, 1978), which reported that large and small chylomicron remnants were recognized and taken up equally well by the isolated perfused rat liver.

During the procedure used for extracting the lipoprotein lipids with organic solvents, the water-soluble components of the extracts (e.g. apoproteins) were discarded. As a result, all the emulsified particles were apoprotein-free, as determined by SDS/polyacrylamide-gel electrophoresis (results not shown). When infused into the bloodstream, all emulsified particles presumably acquired apoprotein E, as well as all other water-soluble apoproteins by transfer from the circulating lipoproteins (Maranhao *et al.*, 1986). Apoprotein E is generally believed to mediate the hepatic uptake of remnants (Mahley *et al.*, 1989). To explain why only the emulsified particles derived from remnants and hepatic-lipase-treated chylomicrons were taken up efficiently by the liver, it might be reasoned that the steric conformation of the acquired apoprotein E on the surface of those particles was such that it could interact with specific binding sites on the surface of hepatocytes. Such a suggestion has previously been made to explain how cells recognize apoprotein E on the surface of triacylglycerol-rich lipoproteins (Krul *et al.*, 1985). If this suggestion is correct, it implies that a specific phospholipid combination on the surface of the lipoproteins is essential for the apoprotein-E-mediated recognition of the particles. Alternatively, the hepatic differentiation of the particles might have been influenced by other lipid components of the emulsion. For example, the cholesterol (Maranhao *et al.*, 1986) and monoacylglycerol (Mortimer *et al.*, 1989) contents of artificial emulsions have been shown to influence markedly their hepatic clearance from circulation. The observation that emulsions prepared from silicic-acid-fractionated lipoprotein lipids were differentiated by the liver (Table 2) allowed us to investigate the role of the phospholipid fraction in this process. For this purpose, 0.850 mg of phospholipids from chylomicrons, remnants or hepatic-lipase-treated chylomicrons were mixed with portions of the phospholipid-free fraction (containing 0.1 mg of cholesterol) obtained from chylomicrons. The proportions of the recombined

Table 2. Hepatic uptake of intact lipoproteins and emulsified lipoprotein lipids

The lipids extracted with chloroform and methanol from chylomicrons, remnants generated *in vivo* and hepatic-lipase-treated chylomicrons (all doubly labelled with [^3H]cholesterol and [^{14}C]labelled fatty acids) were emulsified as described in the Materials and methods section. Two types of emulsions were prepared: (a) with unfractionated lipid mixtures, and (b) and re-mixed phospholipid-free lipids and phospholipid fractions separated by chromatography on silicic acid columns. Intact lipoproteins, emulsions of unfractionated lipids, and emulsions of fractionated and recombined lipids were infused ($10\ \mu\text{g}$ of cholesterol) into the portal vein of partially hepatectomized rats (four rats/group). The cholesterol incorporated into the intact right lateral lobe was determined by [^3H]cholesterol radioactivity, as described in the Materials and methods section. Results are expressed as μg of cholesterol taken up/g of liver (means $\pm$ S.D.).

Type of emulsion	Source of emulsion lipids		
	Chylomicrons	Remnants	Hepatic-lipase-treated chylomicrons
Control (intact lipoproteins)	0.20 ± 0.02	2.65 ± 0.23	2.80 ± 0.62
Unfractionated lipids	0.24 ± 0.11	1.69 ± 0.19	2.45 ± 0.10
Fractionated/recombined lipids	0.16 ± 0.02	1.83 ± 0.08	2.67 ± 0.32

Table 3. Hepatic uptake of emulsified lipoprotein lipids

The phospholipid component of chylomicrons, remnants generated *in vivo*, and hepatic-lipase-treated chylomicrons (all doubly labelled with [^3H]cholesterol and [^{14}C]labelled fatty acids) were separated from the other lipoprotein lipids by silicic acid chromatography as described in the Materials and methods section. Each of the phospholipid fractions was mixed with the phospholipid-free lipid fraction of chylomicrons in the same proportions as intact chylomicrons (8.5 mg of phospholipids/1 mg of cholesterol). After emulsification, the resulting particles were infused into the portal vein of partially hepatectomized rats (four animals/group), and the radioactivity incorporated into the right lateral lobe was measured as described in the Materials and methods section. The results are expressed as μg of cholesterol taken up/g of liver (means $\pm$ S.D.).

Source of emulsion phospholipids	Cholesterol uptake ($\mu\text{g/g}$ of liver)
Chylomicrons	0.3 ± 0.08
Remnants	1.8 ± 0.20
Hepatic-lipase-treated chylomicrons	2.7 ± 0.40

lipids were the same as those of intact chylomicrons (see Table 1). After their emulsification, the capacity of the resulting particles to be taken up by the liver was examined by infusing them through the portal vein of partially hepatectomized rats, as described in the Materials and methods section. Table 3 shows the results obtained. Although all three emulsions were prepared with the same phospholipid-free lipid fraction of chylomicrons, only the particles emulsified with phospholipids derived from remnants and hepatic-lipase-treated chylomicrons were efficiently taken up by the liver. Thus the ability of the emulsions to be differentiated by the liver was determined solely by their emulsifying phospholipids. The observation reinforces our previous conclusion that the ability of liver cells to differentiate between chylomicrons and remnants is determined by the phospholipids on the surface of the particles (Borensztajn *et al.*, 1988).

In conclusion, emulsions prepared with lipids extracted from chylomicrons and remnants behave like the native lipoproteins with respect to their clearance from circulation by the liver. When lipoprotein lipids are fractionated and recombined before emulsification, it is the lipoprotein origin of the phospholipid component which determines the rate of hepatic clearance of the emulsion. By using the procedures described in the present paper, it should be possible to reconstitute lipoprotein-like emulsions of defined phospholipid composition. Such emulsions should be useful in identifying the specific phospholipids which modulate the hepatic differentiation of chylomicrons and remnants.

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