Effect of lipid composition on lipoprotein lipase activity measured by a continuous fluorescence assay: effect of cholesterol supports an interfacial surface penetration model

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The breakdown of normal substrates by lipases requires an interfacial binding step prior to hydrolysis. Interfacial binding and subsequent hydrolysis will be affected by the lipid components and hence physical properties of the substrate surface. In order to investigate in detail the effect of lipid structure on the activity of lipoprotein lipase (LPL), triolein-containing emulsion particles of defined composition have been used as substrates. In addition, lipase activity has been measured using a continuous fluorescence displacement assay that monitors the release of long-chain fatty acids as an alternative to normal radiochemical assays. Using this fluorescence assay, rates of hydrolysis of triolein were the same as when using a standard radiochemical assay under identical conditions. Activation by apolipoprotein CII was very similar by both methods; however, the extent of activation (2–3-fold) was less than has been reported previously using different assay conditions. In order to investigate the effect of cholesterol on LPL activity, emulsion particles were prepared

in which the cholesterol/egg-phosphatidylcholine ratio was increased up to a 1:1 molar ratio. A pronounced stimulatory effect of cholesterol was observed under these assay conditions, with up to a 5-fold increase in rate compared with emulsion particles without cholesterol. Since high molar ratios of cholesterol are reported to exclude triacylglycerol from the phospholipid surface [Spooner and Small (1987) *Biochemistry* **26**, 5820–5825], these results are not consistent with a mechanism involving LPL hydrolysis of surface triacylglycerol. Instead, they support an interfacial penetration model, allowing the enzyme's active site direct access to triacylglycerol in the lipoprotein core. Perturbation of the surface phospholipid monolayer of the emulsion particle as a result of hydrolysis by *Naja naja* phospholipase A₂ resulted in a 10-fold activation of LPL, providing further support for an interfacial penetration model. The stimulatory effect of apolipoprotein CII was not modulated by modification of the interface with cholesterol.

INTRODUCTION

Chylomicrons and very-low-density lipoproteins are triacylglycerol (TG)-rich lipoproteins which function as carriers of metabolic fuel from the intestine and liver respectively to peripheral tissues. Structurally, lipoproteins are biological emulsion droplets with additional peripheral and integral apolipoproteins. The TG core represents between 90 and 55 % of total lipid weight, and is emulsified by a phospholipid monolayer representing about 10–15 % of total lipid weight. This monolayer is made up mainly of phosphatidylcholine (PC), with up to 18 % of phospholipid as sphingomyelin. Additionally, free cholesterol and cholesteryl esters (up to 6 % and 15 % of total lipid weight respectively) are distributed between the core and the interface of these particles. Most apolipoproteins are bound superficially to the lipid interface, with the exception of apolipoprotein B, which is an integral part of the lipoprotein. The exact lipid and protein composition depends on the lipoprotein species under consideration.

Lipoprotein lipase (LPL) is a key enzyme in lipoprotein metabolism [1,2]. It hydrolyses core TG and thus modifies lipoprotein structure. The released fatty acids are then taken up into tissues and either stored as TG or used as an energy source. The restructured lipoproteins resulting from LPL activity have been implicated in the aetiology of cardiovascular disease [2]. The physiological and pathological implications of LPL catalytic

activity and consequent lipoprotein profile highlight the importance of understanding the molecular basis of the regulation of lipoprotein lipolysis.

LPL is interfacially activated; this means that binding to a lipid/water interface is a prerequisite for enzyme catalysis [3,4]. Therefore, by analogy, LPL enzyme activity must be modified in part by the surface lipid composition that creates the lipoprotein interface. Crystallographic studies of the related enzyme pancreatic lipase have provided an insight into this activation process. In this case, productive interfacial binding allows an active-site lid to be displaced, allowing the substrate access to the active site [5,6]. This method of activation has been extended by analogy to hydrolysis by LPL, although there is no direct evidence for this mechanism. Additionally, LPL is activated by apolipoprotein CII (ApoCII), which is a cofactor for the reaction. *In vitro* there is no absolute requirement for this protein; however, lack of ApoCII *in vivo* results in hypertriglyceridaemia [7,8]. It has been suggested that the mechanism of action of ApoCII is to promote interfacial binding of the enzyme without modifying catalysis, a function similar to that of colipase in the activation of pancreatic lipase [9,10]. However, this theory has been contradicted [11], and there are no obvious structural similarities between the two cofactor proteins.

There is little information on the effects of minor structural lipid components, such as cholesterol and sphingomyelin, on LPL catalytic activity, although their contribution to lipid

Abbreviations used: ApoCII, apolipoprotein CII; DAUDA, 11-(5-dimethylaminonaphthalenesulphonyl)undecanoic acid; FABP, fatty-acid-binding protein; LPL, lipoprotein lipase; PC, phosphatidylcholine; PLA₂, phospholipase A₂; ppPLA₂, porcine pancreatic PLA₂; SUV, small unilamellar vesicle; TG, triacylglycerol.

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interfacial properties might prove important in TG metabolism, at least in theory. Cholesterol has been reported to inhibit LPL activity [12,13], as well as decreasing membrane fluidity [14]. Of particular importance, NMR studies have suggested that incorporation of cholesterol into the phospholipid bilayer of vesicles results in exclusion of triolein from the surface [14], and thus provide an explanation for the earlier reports of LPL inhibition by cholesterol. Some studies on lipoprotein clearance have corroborated the inhibitory properties of cholesterol on LPL activity [13,15].

In the present paper the effects of adding cholesterol to triolein/egg-PC emulsion particles on lipoprotein lipase activity and its activation by ApoCII have been investigated. The results highlight a stimulatory effect of cholesterol in this LPL assay system, which is consistent with a mechanism whereby the enzyme is able to perturb the phospholipid monolayer and allow core TG direct access to the active site.

EXPERIMENTAL

Enzymes and reagents

Bovine milk LPL, *Naja naja* phospholipase A₂ (PLA₂) and pancreatic lipase were purchased from Sigma. Porcine pancreatic PLA₂ (ppPLA₂) was obtained from Boehringer. Human ApoCII was purchased from Calbiochem (La Jolla, CA, U.S.A.). Egg PC was obtained from Avanti; triolein and cholesterol were from Sigma. Radioactive lipids were bought from Amersham. DAUDA [11-(5-dimethylaminonaphthalenesulphonyl)undecanoic acid] was obtained from Molecular Probes. Recombinant rat liver fatty-acid-binding protein (FABP) was prepared by methods previously described [16].

Substrate preparation

Emulsion particles

Emulsion particles were prepared according to a established procedure [17]. Briefly, trioleoylglycerol and egg PC in a ratio of 4:1 (w/w) were mixed while in chloroform solution and then dried under nitrogen. In some instances, various amounts of free cholesterol were included with the above lipids. Additionally, 100 μ Ci of [³H]triolein was added, as well as 0.5 μ Ci of [¹⁴C]cholesterol if this lipid was being used. The dried lipid film was further vacuum-dried overnight at 4 °C. A 1 ml aliquot of 1.1 g/ml (2.78 M) NaCl was added and the mixture was left to rehydrate for 10 min. The solution was sonicated for 15 min in cycles of 20 s on and 15 s off (output 80–90 W) with a Heat Systems XL-2020 sonicator, while in iced water. The emulsified particles were purified using a discontinuous salt density gradient with 1.065 g/ml, 1.020 g/ml and 1.006 g/ml NaCl solutions [18]. Initially, the sonicate was underlayered in 3 ml of 1.006 g/ml NaCl and centrifuged for 10 min at 12000 *g* and 20 °C in a Beckman J2-21 centrifuge to remove unemulsified triolein. The bottom 1 ml layer was then underlayered into the NaCl gradient using the three solutions mentioned above and further centrifuged for 1 h at 48000 *g* and 20 °C. The emulsion particles migrated to the top of the gradient and were carefully removed.

Single unilamellar vesicles (SUVs)

The appropriate cocktail of lipids was mixed and dried down under nitrogen from the chloroform solution. The lipid film was resuspended in methanol to a lipid concentration of 1 mg/ml. To form SUV particles, 50 μ l of this solution was injected with a Hamilton syringe into 10 ml of assay buffer (0.1 M Tris/HCl,

0.1 M NaCl, 5 mM CaCl₂, pH 8) giving a final lipid concentration of 50 μ g/ml.

Chemical analysis

Phospholipid incorporation into the emulsion particles was measured by the method of Bartlett [19]. The incorporation of triolein was initially assessed using Sigma enzymic kit which measured glycerol spectrophotometrically. The data were later compared with values obtained by measuring [³H]triolein incorporation. Results were comparable and therefore radioactive measurement was routinely used. Cholesterol incorporation was determined by the measurement of [¹⁴C]cholesterol.

Fluorescence assay

All assays (1 ml) were performed in 0.1 M Tris/HCl, pH 8, containing 0.1 M NaCl and 1 μ M DAUDA cocktail as previously described [20], with the following modifications. In certain experiments either 1 or 5 mM CaCl₂ or 2 mM EDTA was added to the buffer. The appropriate volume of emulsion particle preparation was added to the cocktail to give, unless otherwise stated, a final triolein concentration of 10 nmol/ml. The substrate concentration was modified by varying the volume of emulsion particle solution. FABP was added at 0.01 mg/ml final concentration. The principle of the assay is the displacement of the highly fluorescent fatty acid probe DAUDA from FABP by long-chain fatty acids released as a result of lipase activity. The maximal initial rate of decrease in fluorescence is a measure of the initial rate of lipolysis, and was achieved within 30 s after addition of enzyme. All assays were calibrated by addition of up to 5 μ l of 0.2 mM oleic acid in methanol. Assays were performed in triplicate at 37 °C and routinely contained [³H]triolein (0.045 μ Ci), together with [¹⁴C]cholesterol (7.5 nCi) if appropriate.

Radiochemical assay

The incubation (1 ml) used in the fluorescence assay was immediately added to 0.5 ml of methanol containing 0.1 μ M oleic acid. After mixing, 1 ml of chloroform was added and the solution was vortexed for 2–3 min. The organic chloroform layer was subsequently removed and dried under nitrogen. The lipid was redissolved in 50 μ l of 1:1 (v/v) chloroform/methanol, applied to a TLC silica gel plate and developed using cyclohexane/ethyl acetate (3:2, v/v). The lipid position was visualized with iodine, the silica gel removed and the radioactivity measured in a liquid scintillation counter.

RESULTS

Use of emulsion particles

Emulsion particles have been used extensively as model systems for normal lipoproteins [17,21,22]. They allow accurate and easy control of the interface, as well as being metabolized in the same way as TG-rich lipoproteins *in vivo* [15,23]. Therefore with emulsion particles it is possible to manipulate the composition of the LPL substrate in order to evaluate the effects of the various lipid components on particle metabolism.

Assay characterization

A continuous fluorescence displacement assay has been described that allows the measurement of enzymes that catalyse the release of long-chain fatty acids [20,24]. A particular advantage is that

Table 1 Comparison of LPL activity in the presence and absence of ApoCII using the fluorescence and radiochemical assay systems

Enzyme rate was determined over a time period of 30 s using both fluorescence and radiochemical assay systems in the presence of 0.01 mg/ml FABP to bind released oleic acid. Each 1 ml incubation contained 100 ng of LPL and, where required, 50 ng of ApoCII. Experiments were performed at 37 °C and with emulsion particle concentrations of 10 nmol of triolein/ml. Data represent means $\pm$ S.E.M. of six measurements using two different batches of emulsion particles prepared from the same proportions of triolein and PC. The final weight ratio of triolein/PC was 2.7:1 and 3.9:1 for these two batches of emulsion particles.

Substrate	LPL activity (μ mol of oleic acid/min per mg)			
	Fluorescence assay		Radiochemical assay	
	LPL	LPL + ApoCII	LPL	LPL + ApoCII
PC only	2.73 $\pm$ 0.66	4.50 $\pm$ 0.51	3.65 $\pm$ 0.83	4.95 $\pm$ 0.65
+ 4.5% Cholesterol	4.66 $\pm$ 0.51	14.92 $\pm$ 1.08	—	—
+ 9% Cholesterol	14.40 $\pm$ 1.06	26.94 $\pm$ 1.31	—	—
+ 12% Cholesterol	13.45 $\pm$ 2.28	20.53 $\pm$ 1.81	15.43 $\pm$ 2.15	22.01 $\pm$ 3.11

Figure 1 Effect of LPL concentration on the rate of hydrolysis of emulsion particles

Oleic acid (OA) released by increasing concentrations of LPL enzyme in the presence (▲) or absence (■) of 50 ng/ml ApoCII was measured at 37 °C and in the absence of Ca^{2+} . The concentration of substrate used corresponded to 10 nmol of triolein/assay. Values represent means $\pm$ S.E.M. of six measurements using two different batches of PC/triolein control emulsion particles prepared with the same proportions of triolein and PC. The final weight ratio of triolein/PC was 2.7:1 and 3.9:1 for these two batches of emulsion particles.

this assay allows the use of natural lipid substrates rather than fluorescent lipids. As expected, the assay provided a sensitive method for the measurement of LPL activity; however, in view of the indirect nature of the assay, it needed to be verified by comparison with traditional radiochemical methods.

Initially, LPL specific activity was measured using both fluorescence displacement and radiochemical assays. The values obtained for these assays showed no significant difference between the two systems (Table 1). These values, which are from assays using low substrate concentrations, are in line with other published data for experiments performed in the absence of ApoCII [9,22].

The effect of enzyme concentration on initial rate was determined using the fluorescence assay (Figure 1). The lower limit of detection using these assay conditions was 10 ng/ml, and a reasonably linear relationship was achieved up to 100 ng/ml

Figure 2 Effect of substrate concentration on hydrolysis by LPL

Oleic acid (OA) released by 50 ng/ml LPL in the presence (●) or absence (□) of 50 ng/ml ApoCII was measured at 37 °C and in the absence of Ca^{2+} . Values represent means $\pm$ S.E.M. of six measurements using two different batches of emulsion particles prepared with the same proportions of triolein (TO) and PC (see the legend to Figure 1).

LPL. Assays were routinely performed at a final LPL concentration of 100 ng/ml.

In previously published methods albumin was used rather than FABP to bind the released fatty acids. Possible differences between the behaviour of these two proteins were assessed using a radiochemical assay; albumin interferes with the fluorescence assay, as it also binds the fluorescent probe [20]. LPL specific activity did not show significant differences when comparing FABP and albumin (results not shown). Hence the fluorescence assay was shown to be an accurate measure of triolein hydrolysis by LPL under these assay conditions.

When the substrate concentration was varied between 0.01 and 0.1 mM triolein there was minimal change in the initial rate (Figure 2), indicating that the substrate concentration was approaching saturation over the range used for these assay conditions. This result is at variance with other work [9] and must reflect a much higher affinity of the LPL for the emulsion particle under these assay conditions. Variations in calcium concentration also resulted in non-significant differences in LPL activity (results not shown).

Emulsion particles as a substrate for other lipases

The activities of other lipase enzymes with this substrate were compared with LPL activity. Pancreatic lipase, a related TG lipase, showed a long lag phase of about 100 s and 8-fold lower specific activity compared with LPL (Figure 3). This result contrasts dramatically with the immediate high activity expressed by this enzyme on simple triolein dispersions [24], where the enzyme is not required to interact with a phospholipid monolayer coat.

The ability of secretory PLA_2 s to hydrolyse the outer phospholipid monolayer was also investigated (Figure 3). *N. naja* venom PLA_2 showed the highest specific activity of all the enzymes tested, while pp PLA_2 activity was comparable with that of pancreatic lipase (Table 2). The human secretory non-pancreatic PLA_2 was inactive against PC, as previously demonstrated with pure PC vesicles [25].

The reaction profile of the phospholipases was distinct from

Figure 3 Fluorescence displacement traces for hydrolysis of triolein/PC control emulsion particles by various lipases

Each trace shows the decrease in fluorescence with time as a result of fatty acid release by 100 ng of lipase enzyme. Assays (1 ml) contained 10 nmol of triolein, 1 μ M DAUDA, 0.01 mg of FABP and 5 mM Ca^{2+} . Blanks contained no enzyme. Results are representative of three similar incubations carried out for each experiment. The assay is based on competitive binding; the fluorescence response is increasingly non-linear with respect to fatty acid release above 50% displacement. Abbreviations: hsnp-PLA₂, human secretory non-pancreatic PLA₂; PL, pancreatic lipase; ppPLA₂, *N. naja* venom PLA₂.

Table 2 Effect of cholesterol incorporation into emulsion particles on the hydrolytic activity of various lipase enzymes

Enzyme rates were determined using 10 nmol of triolein/ml in the presence of 5 mM Ca^{2+} and at 37 °C. Each 1 ml incubation contained 100 ng of the appropriate enzyme. Data represents means $\pm$ S.E.M. of six measurements using two different batches of emulsion particles prepared from the same proportions of triolein and PC. The final weight ratio of triolein/PC was 2.8 and 4:1 for these two batches of emulsion particles. Abbreviations: PL, pancreatic lipase; *Nn*PLA₂, *N. naja* PLA₂; *hnps*PLA₂, human non-pancreatic secretory PLA₂.

Enzyme	Lipase activity (μ mol of oleic acid released/min per mg)			
	PC only	+ 4.5% Cholesterol	+ 9% Cholesterol	+ 12% Cholesterol
LPL	2.94 $\pm$ 0.14	7.49 $\pm$ 0.42	15.68 $\pm$ 2.95	14.55 $\pm$ 2.54
PL	0.38 $\pm$ 0.14	2.79 $\pm$ 0.10	2.07 $\pm$ 0.11	2.05 $\pm$ 0.27
<i>Nn</i> PLA ₂	104.6 $\pm$ 22.7	73.8 $\pm$ 1 2.5	101.5 $\pm$ 7.1	90.0 $\pm$ 16.2
ppPLA ₂	0.48 $\pm$ 0.17	8.51 $\pm$ 0.63	7.83 $\pm$ 0.42	16.64 $\pm$ 2.35
<i>hnps</i> PLA ₂	0.03 $\pm$ 0.05	0.03 $\pm$ 0.05	0.07 $\pm$ 0.03	0.18 $\pm$ 0.01

that of LPL (Figure 3). The fluorescence trace showed hydrolysis by phospholipases, most clearly seen with *N. naja* PLA₂, to be a rapid but short-lived reaction that corresponded to hydrolysis of the outer monolayer. In contrast, comparable amounts of LPL showed a lower rate of activity but a longer time span, thus releasing a higher overall amount of oleic acid. A second addition of *N. naja* PLA₂ resulted in no further hydrolysis, suggesting that the substrate had been depleted. However, if LPL was added at this stage the hydrolysis rate was about 10-fold higher than that with LPL alone (Figure 4). This stimulation as a result of *N. naja* PLA₂ treatment did not appear to be due to a change in vesicle size, as no change in light scattering was detected, whereas LPL hydrolysis of these particles did cause a reduction in light scattering. The additive effects of *N. naja* PLA₂ and LPL imply that the two enzymes hydrolyse different lipids, and this substrate

Figure 4 Fluorescence displacement traces for the hydrolysis of triolein/PC control emulsion particles by a combination of *N. naja* PLA₂ and LPL

Each trace shows the decrease in fluorescence with time as a result of fatty acid release by 100 ng of *N. naja* PLA₂ (N-PLA₂) followed by 100 ng of either LPL or *N. naja* PLA₂. Assays (1 ml) contained 10 nmol of triolein, 1 μ M DAUDA, 0.01 mg of FABP and 5 mM Ca^{2+} . Blanks show the fluorescence trace in the absence of enzyme. Results are representative of three similar incubations carried out for each experiment. The assay is based on competitive binding; the fluorescence response is increasingly non-linear with respect to fatty acid release above 50% displacement.

selectivity was further supported by analysing *N. naja* PLA₂ activity using the radiochemical assay system. This assay system showed that the venom PLA₂ did not hydrolyse [³H]triolein, although non-esterified fatty acid had been detected by iodine staining after TLC. It has been shown by others [26] that the PLA₁ activity of LPL is minimal when presented with triolein-containing emulsion particles.

The greatly enhanced LPL activity when added to emulsion particles previously treated with *N. naja* PLA₂ indicates that interfacial perturbation facilitates triolein hydrolysis by LPL. The enhanced rate may be due to increased access of the enzyme to the core triolein. This conclusion is supported by the fact that calibration of the rapid fall in fluorescence indicated that about 10–15 mol% of the triolein had been hydrolysed, a far greater value than the 2–3 mol% initially present in the surface monolayer [14]. Pancreatic lipase activity is also activated by pre-treatment with *N. naja* PLA₂, but only about 2-fold; however, the observed lag phase is dramatically reduced.

Effect of ApoCII on LPL activity

It is well known that ApoCII is an activator of LPL both *in vivo* and *in vitro*, although the precise mechanism of activation is unclear. Using emulsion particles, Tajima et al. [9] reported a maximal 20-fold activation of the LPL enzyme rate by ApoCII when the molar ratio of phospholipid/ApoCII was 2000:1. However, under our assay conditions, ApoCII caused a maximal activation of LPL hydrolysis of normally between 2- and 3-fold (Figure 1) using emulsion particles with a molar ratio of phospholipid/ApoCII of 500:1. The stimulation by ApoCII was the same using both the fluorescence and the radiochemical assay systems. The radiochemical assay was performed using either FABP or albumin. Activation by ApoCII was not dependent on preincubation of the ApoCII with either LPL or substrate (results not shown).

Activation was not dependent on the amount of enzyme (Figure 1) or substrate (Figure 2) present. ApoCII concentrations

Figure 5 Effect of ApoCII concentration on LPL activity

Oleic acid (OA) released by 50 ng/ml LPL in the presence of various concentrations of ApoCII was measured at 37 °C and in the absence of Ca^{2+} . Assays (1 ml) contained 10 nmol of triolein, 1 μM DAUDA and 0.01 mg of FABP. Values are means $\pm$ S.E.M. of six measurements using two different batches of PC/triolein control emulsion particles prepared from the same proportions of triolein and PC (see the legend to Figure 1).

greater than 10 ng/ml gave similar levels of activation (Figure 5). Routinely, 50 ng/ml ApoCII was used to study its effects under the various substrate conditions. The use of emulsion particles with different lipid compositions did not modify the way in which ApoCII activates hydrolysis by LPL (Table 1).

Previous work by others [9,17,22,26] routinely used high concentrations of both heparin and calcium. However, in our system neither heparin nor the addition of calcium or EDTA had a significant effect on LPL activity or its activation by 50 ng/ml ApoCII (results not shown).

Effect of cholesterol incorporation into emulsion particles on LPL activity

The effect of incorporating cholesterol into emulsion particles at 4.5 %, 9 % and 12 % of total lipid weight, or 0.25:1, 0.75:1 and 1.25:1 molar ratios with respect to phospholipid, was investigated. Addition of increasing amounts of cholesterol did not modify the phospholipid/triolein ratio in emulsion particles. This suggested that the size of the emulsion particles did not vary, and this was confirmed by visualization under the electron microscope. These particles showed an average diameter of approx. 100 nm, and were all spherical in shape (Figure 6).

The specific activity of LPL was increased in a concentration-dependent manner by the presence of cholesterol (Table 2). A physiological concentration of 4.5 % was enough to cause a 2.5-fold increase in activity. The activating effect was confirmed using 12 % cholesterol in the radiochemical assay system (Table 1). Addition of ApoCII resulted in activation levels (about 2-fold) which were comparable with those obtained with control substrate particles (Table 1). Thus cholesterol does not appear to modify the mode of action of ApoCII in this system.

The other TG lipase, pancreatic lipase, was activated 10-fold by the presence of cholesterol in terms of specific activity, thus being comparable with LPL stimulation. However, pancreatic lipase was more sensitive to the presence of cholesterol, since the effect was concentration-independent over the range used in this study. Additionally, a decrease in the lag phase was observed. When the phospholipase enzymes were tested, human secretory non-pancreatic PLA_2 remained essentially inactive, and *N. naja*

Figure 6 Electron micrographs of emulsion particles without (top) and with (bottom) 12% cholesterol

Samples of 25 μl of emulsion particles were stained with an equal volume of 2% osmium tetroxide for 5 min. After pelleting the emulsion particles to remove the osmium tetroxide, the pellet was resuspended in 20 μl of 1% sodium tungstate. A 5 μl aliquot of the suspension was then loaded on to carbon-formyl-coated copper grids and viewed under a Philips 300 transmission electron microscope (80 kV). The mean diameter (nm) of the emulsion particles was estimated as approx. 100 ± 25 ($\pm$ S.E.M.). This value was not significantly different between the two emulsion particle preparations.

PLA_2 specific activity was not modified. However, pp PLA_2 was activated in a concentration-dependent manner, with a maximal 6-fold increase in activity in the presence of 12 % cholesterol.

Pretreatment of cholesterol-containing substrate with *N. naja* PLA_2 did not further stimulate LPL activity. Therefore it would appear that facilitation of triolein catalysis by LPL perturbation of the phospholipid interface can be due either to addition of cholesterol or to lysophospholipid production, but the two effects are not additive. In contrast, the rate of hydrolysis by pancreatic lipase, using cholesterol-containing substrate, was enhanced 2-fold by pretreatment with *N. naja* PLA_2 , and there was a significant reduction in the lag phase.

Assays using SUVs

The effect of cholesterol on ppPLA₂ activity described above was inconsistent with previously published data using SUVs that suggested inhibition of enzyme action [25], and this discrepancy needed to be resolved.

Egg PC SUVs were used as a substrate to analyse the effects of substrate structure on lipase activity. Changing the method of substrate presentation to SUVs resulted in a 13-fold increase in ppPLA₂ specific activity compared with the control emulsion particle substrate. On the other hand, LPL activity was about 10-fold lower in SUVs than when using control emulsion particles. These results were consistent with LPL having about 5–10% PLA₁ activity in the absence of TG substrate. Addition of cholesterol (0.5:1 molar ratio) to the PC-based vesicles reduced ppPLA₂ activity by about 3-fold compared with that in pure PC vesicles, confirming our original observations [25]. The very low LPL activity seen with SUVs did not allow the potential inhibitory effect of added cholesterol to be quantified. Thus the activation of LPL by the presence of cholesterol in the emulsion particles is due to a change in the structure of the substrate.

DISCUSSION

The continuous fluorescence assay used here had previously been employed to measure phospholipase activity using phospholipid vesicles or lipid dispersions [16,20,24]. Its main advantages are that it allows accurate initial-rate measurements of the release of long-chain fatty acid and the study of unmodified, physiologically relevant, lipids.

Initially, the data obtained using this system were confirmed by parallel experiments using a standard radiochemical assay. The specific activity of LPL was comparable between the two techniques and with values published previously [9,22]. However, activation by ApoCII was much reduced compared with several previous reports [9,17,27]. The properties of interfacial enzymes are very sensitive to assay conditions, particularly substrate presentation. The fluorescence assay has to employ low substrate concentrations, while liver FABP replaces albumin as the fatty acid acceptor. These differences may explain the somewhat lower activation achieved by ApoCII under these assay conditions.

The precise mechanism by which ApoCII causes activation of LPL has not been established. Structural studies have shown that ApoCII has a lipid binding domain at the N-terminus, as well as a distinct sequence at the C-terminus which is thought to be responsible for LPL activation [28]. Therefore it was suggested that ApoCII activates LPL by facilitating enzyme binding to the interface [9,10]. Under the *in vitro* conditions used, high-affinity binding to the substrate is already indicated, and this may explain why ApoCII is not an efficient activator of LPL. However, *in vivo* lipoproteins contain many other apolipoproteins bound to their surface, and it is possible that ApoCII is required to overcome inhibitory actions of apolipoproteins or to anchor the enzyme at the appropriate part of the interface. The precise nature of the molecular interactions between LPL and lipoproteins *in vivo* has not been elucidated, and both interfacial lipid [11,29] and apolipoprotein B [29] have been implicated, in addition to ApoCII.

The effect of cholesterol on LPL activity in the present study was unexpected. It has been proposed that LPL hydrolyses the small fraction of triolein present in the outer monolayer of lipoproteins [3,4]. Moreover, NMR studies have indicated that cholesterol is able to displace triolein from the phospholipid surface of vesicles, such that at a 1:1 molar ratio of cholesterol/phospholipid there was essentially zero triolein present in the

interface [14]. Earlier work had indicated that a high molar proportion of cholesterol resulted in essentially complete inhibition of LPL activity [12,13], consistent with an interfacial surface mechanism for triolein hydrolysis. However, a more recent study [30] showed minimal effects of cholesterol on emulsion particle hydrolysis. An alternative surface penetration model, in which the LPL would directly access the core TG, has been proposed more recently by Rojas et al. [17] to explain the greatly enhanced hydrolysis of emulsion particles compared with the hydrolysis of the equivalent triolein-saturated liposomes. Moreover, crystallographic studies of pancreatic lipase highlight the displacement of the active-site lid on interfacial binding which is linked to perturbation of the substrate monolayer and could then allow direct access of core TG to the active site [5,6].

In the present study, the ability of *N. naja* PLA₂ to greatly enhance the initial rate of emulsion particle hydrolysis by LPL is consistent with perturbation of the monolayer due to phospholipid hydrolysis enhancing the access of LPL to core TG.

The precise physical effects of cholesterol on phospholipid interfaces remain ill-defined, particularly where complex mixtures of phospholipid species are involved. However, perturbation of the interfacial structural organization by cholesterol could provide a mechanism for enhanced penetration of the interface by LPL and hence increased activity. We therefore suggest that the results of the present study using a novel fluorescence assay system support an interfacial penetration model for LPL activity.

It should be stressed that the studies described here are of a model system, and do not necessarily reflect the conditions experienced by LPL *in vivo*. Nevertheless, this model system does allow a detailed examination of the effect of lipid composition on basic interfacial binding and catalysis, and will be extended to include sphingomyelin, cholesterol esters and TGs of different molecular structures.

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