Interaction of purified bovine milk lipoprotein lipase (LpL) with sonicated vesicles of dipalmitoyl phosphatidylcholine in the gel phase is associated with an increase in the rate of the LpL-catalyzed hydrolysis of p-nitrophenyl butyrate. There is a 6-fold increase in \( V_{\text{max}} \). Apolipoprotein C-II, the activator protein for LpL, inhibits the LpL-catalyzed hydrolysis of p-nitrophenyl butyrate. With 0.5 mol \% tritiated oleoylglycerol present in the dipalmitoyl phosphatidylcholine vesicles and in the presence of 20 mM Ca\(^{2+}\), the rate of p-nitrophenyl butyrate hydrolysis is decreased reciprocally compared to trioleoylglycerol hydrolysis and is dependent on apolipoprotein C-II. These results suggest that apolipoprotein C-II enhances the activity of LpL by increasing the affinity of the active site of LpL for triacylglycerol.

Lipoprotein lipase (EC 3.1.1.34) catalyzes the hydrolysis of plasma lipoprotein triacylglycerol, phosphatidylcholine, and phosphatidylethanolamine (see Refs. 1–4 for review). In addition to lipoprotein lipids, LpL\(^{1}\) also catalyzes the hydrolysis of water-soluble short chain fatty acyl ester substrates such as p-nitrophenyl acetate (5) and PNPB.\(^{2}\) For maximal hydrolysis of long chain fatty acyl esters, the enzyme requires apoC-II, a protein constituent of triglyceride-rich lipoproteins and high density lipoproteins (2); apoC-II does not enhance the hydrolysis of water-soluble substrates or of short chain triacylglycerols such as tributyrlylglycerol (6, 7). Recently, we have demonstrated that the LpL-catalyzed hydrolysis of PNPB is enhanced by phospholipid vesicles (8). The extent of enhancement was dependent on the physical state of the lipid and was greatest with lipids in the gel phase. For example, with sonicated vesicles of DPPC and at 26 °C, the rate enhancement was 8-fold.

The purpose of the present report was to determine the effect of the activator protein, apoC-II, on the LpL-catalyzed hydrolysis of PNPB in the presence of DPPC vesicles. In addition, tritiated oleoylglycerol was incorporated into the DPPC vesicles in order to determine whether LpL prefers to hydrolyze the hydrolysis of a long chain triacylglycerol in the presence of apoC-II or the water-soluble substrate which does not require the activator protein.

MATERIALS AND METHODS

DPPC was purchased from Applied Science Laboratories. PNPB and heparin (porcine intestinal mucosa, 169.9 units/mg) were purchased from Sigma. Trifluorinated oleoylglycerol (60 mL/mmol) was obtained from New England Nuclear.

LpL was purified to homogeneity from bovine skimmed milk by chromatography on heparin-Sepharose as described by Kinnunen (9). The purified enzyme had a specific activity of 30 mmol of released oleic acid/mg of protein/h using trifluorinated oleoylglycerol emulsified with Triton X-100 (10). ApoC-II was isolated from triglyceride-rich lipoproteins by gel filtration of the delipidated proteins on Sephadex G-75 followed by ion exchange chromatography on DEA-Septachel at 4 °C in 6 mM urea (11). To prepare DPPC vesicles, phospholipid (10 mg) was dissolved in chloroform, evaporated under a stream of ultrapure N\(_2\), and lyophilized for 0.5 min. Phospholipid dispersions were prepared by adding 5 mL of 0.9% NaCl, 0.01 M Tris-HCl, pH 7.2. The lipid dispersion was sonicated at 42 °C for 15 min using a cell disruptor (Heat Systems Ultrasonics, Inc. model W-225R). The sonicated vesicles were subjected to ultracentrifugation at 15 °C in a Beckman type 50 rotor for 1 h at 60,000 rpm. After ultracentrifugation, the top 1.0 mL was removed by aspiration and discarded. The middle 4.0 mL was then removed and used in the experiments described below. DPPC vesicles containing 0.5 mol \% trifluorinated oleoylglycerol were prepared exactly as described above; lipid vesicles were prepared daily and used immediately. Phospholipid phosphorus was determined by the method of Bartlett (12). The LpL-catalyzed hydrolysis of PNPB was determined by monitoring the increase in absorbance at 400 nm or by extraction of p-nitrophenol as described previously (8). \(^{14}\)C Oleic acid was extracted from the reaction mixture by the method by Belfrage and Vaughan (13). The hydrolysis of PNPB and trifluorinated oleoylglycerol was linear for the indicated incubation times.

RESULTS

The time course of the LpL-catalyzed hydrolysis of PNPB is shown in Fig. 1. The addition of DPPC vesicles to the incubation was associated with an immediate 4-fold increase in the rate of PNPB hydrolysis by LpL and is consistent with previous findings (8). The addition of apoC-II (Fig. 1, curve B) to give an apoC-II concentration of 4 µg/mL caused an 80% inhibition in the rate of hydrolysis.

To determine the effects of apoC-II on the kinetic parameters for the LpL-catalyzed hydrolysis of PNPB, rates were determined at various concentrations of PNPB. In these experiments, the incubation mixtures contained a constant amount of DPPC (50 µg) and LpL (5 µg) and variable amounts, as indicated, of PNPB. As shown in Fig. 2, apo C-II caused an increase in the apparent \( K_a \) with no effect on the maximal velocity (\( V_{\text{max}} \)); the calculated \( V_{\text{max}} \) was 8.33 µmol of product released/min/mg of LpL. In the absence of apoC-II, the apparent \( K_a \) was 0.58 µmol. With 0.2, 1.0, or 5.0 µg/mL of apoC-II, the apparent \( K_a \) values were 0.90, 1.37, or 3.17 µmol, respec-
tively. Thus, apoC-II caused a 6.4-fold increase in the apparent 

In the next experiment, 0.5 mol % tritiated oleoylglycerol was 
incorporated into the DPPC vesicles and the effect of apoC-II 
on the rate of hydrolysis of PNPB and tritiated oleoylglycerol 
was determined. To complex the released oleic acid, 20 mM 
CaCl₂ was included in the incubation mixture as described by 
Bengtsson and Olivecrona (14). As shown in Fig. 3, apoC-II 
had a reciprocal effect on the rate of hydrolysis of PNPB and 
tritiated oleoylglycerol. With increasing concentrations of apoC-
II, the rate of hydrolysis of PNPB decreased and there was a 
corresponding increase in the rate of triacylglycerol hydroly-
sis.

Fig. 1. Effect of sonicated vesicles of DPPC and apoC-II on 
the LpL-catalyzed hydrolysis of PNPB. The incubation mixture 
contained 1.25 µmol of PNPB, 1% (v/v) acetonitrile in a final volume 
of 2.2 ml of 0.1 M sodium phosphate, pH 7.2. At 1 min, 100 µl of LpL 
(45 µg/ml) and 10 µl of heparin (2 mg/ml) were added. At 3 min, 25 
µl of DPPC vesicles (4 mg/ml) were added. At 5.5 min, 10 µl of apoC-
II (1 mg/ml, curve B) were added; incubation mixture A received 10 
µl of 0.1 M sodium phosphate, pH 7.2. The release of p-nitrophenol 
was monitored continuously by following the increase in the absorbance at 400 nm using a no-enzyme incubation mixture as blank. The temperature was 26 °C.

Fig. 2. Effect of apoC-II on the LpL-catalyzed hydrolysis of 
PNPB. The reaction mixture contained the indicated amount of 
PNPB, 1% (v/v) acetonitrile, heparin (10 µg), LpL (5 µg), DPPC 
vesicles (50 µg), and the indicated micrograms of apoC-II (numbers on the right) in a final volume of 1.0 ml of 0.1 M sodium phosphate, 
ph 7.2. After incubation for 7 min at 30 °C, released p-nitrophenol 
was extracted from the mixtures as described under “Materials and 
Methods” and its concentration was determined by the absorbance at 
400 nm (5). The Lineweaver-Burk plots were constructed from the primary data.

Fig. 3. Reciprocal effect of apoC-II on the LpL-catalyzed 
hydrolysis of PNPB and tritiated oleoylglycerol. The incubation 
mixtures, in triplicate, contained PNPB (0.25 pmol), DPPC (100 µg) 
containing 0.5 mol % of tritiated oleoylglycerol, LpL (0.11 µg), CaCl₂ 
(10 µmol), and the indicated amounts of apoC-II in a final volume 
of 0.5 ml of 25 mM sodium barbiturate, pH 7.6, containing 0.9% NaCl. 
After incubation for 20 min at 30 °C, 3.25 ml of methanol: 
chloroform:heptane (1:0.9:0.7, v/v) and 1.0 ml of potassium borate, 
ph 10.5, were added to each incubation mixture. The mixtures were 
shaken vigorously for 10 s and centrifuged at 1500 × g for 5 min. After 
washing at 42 °C for 3 min, the supernatant fractions were removed. 
One-ml of the supernatant fraction was taken to dryness in a scintil-
ation vial with a stream of nitrogen and oleic acid radioactivity was 
determined. p-Nitrophenol was determined by absorbance at 400 nm. 
The molar extinction coefficient of released p-nitrophenol in the 
extracted upper phase was 12,000, with a recovery of 95%. The results 
are expressed as the mean ± S.E.

Fig. 4. Dixon plot of the reciprocal of the initial velocities of 
Fig. 3 for the LpL-catalyzed hydrolysis of PNPB versus apoC-
II concentration, as described in Equation 1 of the text. The 
displayed linear fit was generated by linear least squares analysis. 
Inset, replot of the slopes of the Lineweaver-Burk plots of Fig. 2.
As shown previously (8), interfacial activation of LpL by DPPC in the gel phase is associated with an increase in the \(V_{\text{max}}\) of PNPB hydrolysis, with no change in \(K_m\). The present study shows that apoC-II inhibits the DPPC-stimulated LpL-catalyzed hydrolysis of PNPB. Furthermore, when the DPPC vesicles contain 0.5 mol \% trioleoyglycerol, the addition of apoC-II results in a corresponding enhancement of the LpL-catalyzed hydrolysis of trioleoylglycerol.

The kinetics pattern for DPPC-stimulated LpL-catalyzed hydrolysis of PNPB at various apoC-II concentrations is consistent with competitive inhibition by apoC-II (Fig. 2). The simplest mechanism for the observed inhibition by apoC-II is:

\[
\begin{align*}
E-DPPC + S & \quad \xrightarrow{k_1} ES-DPPC \quad \xrightarrow{k_{-1}} E-DPPC + \text{product} \\
+CH & \quad \xrightarrow{k_{CI}} K_{\text{CI}} \\
E-CII-DPPC & \quad \xrightarrow{k_{cat}} \text{product}
\end{align*}
\]

SCHEME 1

In this mechanism, E-DPPC is the complex formed by LpL and the DPPC vesicles; ES-DPPC is the Michaelis complex of E-DPPC and S, the substrate PNPB, and E-CII-DPPC is the binary complex formed between LpL and apoC-II at the vesicle surface. The dissociation constant of the LpL:apoC-II complex is \(K_{\text{CI}}\), and the stoichiometry of the complex is 1:1 (LpL:apoC-II). In addition, the LpL:apoC-II complex has no affinity for PNPB, and hence is a dead end complex with respect to PNPB hydrolysis. Finally, E-DPPC is considered the only form of free enzyme since Shirai et al. (15, 16) have shown that under the conditions of the experiment, all enzyme is bound to the DPPC vesicles.

The steady state equation, in Lineweaver-Burk double-reciprocal form, for the dependence of initial velocity on PNPB concentration is:

\[
V_{-1} = \frac{1}{V_{\text{max}}} = \frac{K_m}{V_{\text{max}}} \left(1 + \frac{[\text{CII}]}{K_{\text{CI}}} \right) \frac{1}{[S]}
\]

where \(K_m = (k_{cat} + k_{-1})/k_1\), and \(V_{\text{max}} = k_{cat} E_L\) (total LpL concentration). According to this equation, the slopes of the Lineweaver-Burk plots for LpL-catalyzed hydrolysis of PNPB depend on the concentration of apoC-II, and therefore the plot constructed from the slopes and shown in the inset to Fig. 4 allows one to calculate \(K_{\text{CI}}\). The dissociation constant of the LpL:apoC-II complex formed at the vesicle surface. This analysis gives \(K_{\text{CI}} = 0.13 \pm 0.01 \mu M\). Furthermore, the linearity of the plot supports the two mechanistic assignments made earlier (17). (a) The stoichiometry of the LpL:apoC-II complex is 1:1. If it were 1:2 (LpL:apoC-II), for example, the velocity equation would contain a \([\text{CII}^2 \text{term} and the plot would be nonlinear and quadratic in apoC-II concentration. (b) The LpL:apoC-II complex formed at the vesicle surface is catalytically inactive toward PNPB. If this complex retains PNPB hydrolysis activity, though diminished, the plot would be linearically inactive toward PNPB. If this complex retains PNPB hydrolysis activity, though diminished, the plot would be linear.

Equation 1 shows that, when injudicious velocities are determined at a single PNPB concentration but varying apoC-II concentration, \(V_{-1}\) is a linear function of apoC-II concentration. Figure 4 shows such a plot, a Dixon plot (17), constructed from the data of Fig. 3 for the apoC-II inhibition of LpL-catalyzed hydrolysis of PNPB. The linearity of the Dixon plot in Fig. 4 lends further support to the mechanism of Scheme 1. In addition, Fig. 3 shows that the apoC-II inhibition of the LpL-catalyzed hydrolysis of PNPB in the presence of DPPC vesicles containing 0.5 mol \% trioleoyglycerol is accompanied by a corresponding increase in the LpL-catalyzed hydrolysis of trioleoylglycerol. This reciprocal effect of apoC-II on LpL-catalyzed PNPB and trioleoylglycerol hydrolysis strongly suggests that the same LpL-apoC-II interaction is responsible for apoC-II inhibition of PNPB hydrolysis and activation of trioleoylglycerol hydrolysis. Furthermore, the apoC-II complexes shown in Figs. 1 and 2 demonstrate that triacylglycerol need not be present for apoC-II inhibition of DPPC-stimulated LpL-catalyzed PNPB hydrolysis to be expressed. It thus appears that apoC-II inhibits DPPC-stimulated LpL-catalyzed hydrolysis of PNPB by forming a 1:1 complex with the enzyme in which the formation of the active site of LpL is changed. In this altered conformation, LpL is no longer a catalyst for PNPB hydrolysis. One way that apoC-II inhibition of LpL-catalyzed hydrolysis of PNPB may be expressed is that in its altered conformation the LpL active site has a much higher affinity for DPPC monomers that are contained in the vesicle surface. This suggestion is supported by the fact that DPPC is a substrate, though poor, of LpL. However, apoC-II inhibits the LpL-catalyzed hydrolysis of water-soluble p-nitrophenyl ester even in the absence of phospholipid. This inhibition also appears to involve LpL:apoC-II complexes, with a dissociation constant in the range of 0.26 to 0.83 \mu M. An alternate possibility, that apoC-II sterically blocks access of PNPB to the active site, is precluded by the fact that apoC-II simultaneously inhibits LpL-catalyzed PNPB hydrolysis and stimulates LpL-catalyzed trioleoylglycerol hydrolysis. It is therefore reasonable to suggest that the LpL conformational change that causes inhibition of PNPB hydrolysis when the enzyme interacts with apoC-II is the molecular dynamic event responsible for apoC-II stimulation of trioleoylglycerol hydrolysis.

The analysis of kinetic data presented in this paper suggests that apoC-II inhibits the DPPC-stimulated LpL-catalyzed hydrolysis of PNPB and that apoC-II reciprocally stimulates the LpL-catalyzed hydrolysis of trioleoylglycerol contained in DPPC vesicles through the formation of 1:1 LpL:apoC-II complexes. This stoichiometry has previously been suggested by Chung and Scasno (18) for apoC-II stimulation of the rat heart LpL-catalyzed hydrolysis of trioleoylglycerol monolayers, and by Fielding and Fielding (19) for apoC-II enhancement of the rat postheparin plasma LpL-catalyzed hydrolysis of trioleoylglycerol emulsified with dioleoylphosphatidylcholine. Moreover, the dissociation constant determined herein for the LpL:apoC-II complex of 0.13 \mu M is in reasonable agreement with the corresponding dissociation constants estimated by Bengtsson and Olivecrona (20) for the bovine milk LpL-catalyzed hydrolysis of emulsified trioleoylglycerol (0.038 \mu M) and monooleoylglycerol (0.34 \mu M), and with the dissociation constants determined by Smith et al. (21) for interaction in the absence of substrate of bovine milk LpL and daisylated apoC-II fragments, which are in the range of 8.22 to 4.0 \mu M.

One possible explanation for these results is that in the presence of vesicles of DPPC LpL may preferentially hydrolyze PNPB that is bound to the vesicles, and that the rate-determining step may be the binding of PNPB to the vesicles. If such were true, apoC-II could inhibit the DPPC-stimulated LpL-catalyzed hydrolysis of PNPB by preventing PNPB binding to the vesicles. This explanation can be discounted for a number of reasons. (a) Shirai and Jackson (8) showed that stimulation of the LpL-catalyzed hydrolysis of PNPB by vesicles of DPPC was greater than that by vesicles of dimeristeryl phosphatidycholine at 30 °C, although dimeristoyl phosphatidylcholine is bound to the vesicles. This explanation can be discounted for a number of reasons. (a) Shirai and Jackson (8) showed that stimulation of the LpL-catalyzed hydrolysis of PNPB by vesicles of DPPC was greater than that by vesicles of dimeristoyl phosphatidylcholine at 30 °C, although dimeristoyl phosphatidylcholine is bound to the vesicles.

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\[ 2 \text{ K. Shirai, T. J. Fitzharris, H. G. Mautz, J. A. K. Harmon, R. L. Jackson, and D. M. Quinn, manuscript in preparation.} \]

\[ 3 \text{ K. Shirai, T. J. Fitzharris, H. G. Mautz, J. A. K. Harmon, R. L. Jackson, and D. M. Quinn, manuscript in preparation.} \]
phosphatidylcholine vesicles bound more PNPB than did DPPC vesicles. (b) Shirai and Jackson (8) found that DPPC vesicles stimulate the LpL-catalyzed hydrolysis of PNPB by increasing $V_{\text{max}}$ without affecting $K_m$. Since $V_{\text{max}}$ is the maximum velocity of substrate turnover from the LpL-PNPB Michaelis complex, the putative binding step of PNPB to the DPPC vesicles must occur before the rate-determining step measured by $V_{\text{max}}$. (c) ApoC-II inhibits the DPPC-stimulated LpL-catalyzed hydrolysis of PNPB competitively, i.e. $V_{\text{max}}$ is unaffected but $K_m$ is increased. Two ways that apoC-II might increase $K_m$ that are consistent with preventing PNPB binding to DPPC vesicles are by directly binding to PNPB or by binding to the DPPC vesicle surface so that the on-step of PNPB to the vesicles is slowed. The analysis of apoC-II inhibition of the DPPC-stimulated LpL-catalyzed hydrolysis of PNPB determined in the present report yielded $K_{\text{CI}} = 0.13 \mu M$, which we assign to the dissociation constant of the LpLapoCI complex. However, it is (in the more general sense) the concentration of apoC-II required for half-maximal inhibition when [PNPB] < $K_m$. For example, when [PNPB] = 0.13 mM and [apoC-II] = $K_{\text{CI}} = 0.13 \mu M$, the PNPB/apoC-II ratio is 1000:1, so that for 50% inhibition to occur each apoC-II molecule must bind 500 PNPB molecules, or ~7 molecules of PNPB/amine acid. If such were the case, it is difficult to imagine that the resulting complex of apoC-II and PNPB could simultaneously stimulate trioleoylglycerol hydrolysis. Hence, direct binding of apoC-II and PNPB does not appear to explain the apoC-II inhibition of DPPC-stimulated LpL-catalyzed hydrolysis of PNPB. Moreover, Cardin et al. (22) determined a dissociation constant of 6.5 $\mu M$ for the interaction of apoC-II and DPPC vesicles. Since $K_{\text{CI}} = 0.13 \mu M$ is ~50-fold smaller than the dissociation constant measured by Cardin et al. (22), it must reflect some molecular event other than interaction of apoC-II and the DPPC vesicle surface. We suggest that $K_{\text{CI}}$ reflects the LpLapoCI interaction. It thus seems that apoC-II binding to the vesicle surface such that PNPB binding is slowed cannot be the mechanism for apoC-II inhibition of DPPC-stimulated LpL-catalyzed hydrolysis of PNPB. This argument is supported by the numerical similarity of $K_{\text{CI}}$ and the inhibition constants of 0.26–0.83 $\mu M$ determined for apoC-II inhibition of the LpL-catalyzed hydrolysis of PNPB in the absence of lipids.

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