Lipoprotein Lipase-catalyzed Hydrolysis of p-Nitrophenyl Butyrate

INTERFACIAL ACTIVATION BY PHOSPHOLIPID VESICLES*

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The mechanism of action of bovine milk lipoprotein lipase (LpL) was studied with a water-soluble substrate of p-nitrophenylbutyrate (PNPB). The calculated maximal velocity (V_{max}) and Michaelis constant (K_m) values at 37 °C were 2.8 μmol of p-nitrophenol released/min/mg of LpL and 0.52 mM, respectively. The addition of phospholipid vesicles enhanced the rate of PNPB hydrolysis by LpL. In the presence of dipalmityl phosphatidylcholine (DPPC) vesicles at 37 °C, the V_{max} and K_m values were 8.8 μmol of p-nitrophenol released/min/mg of LpL and 0.55 mM, respectively, indicating that the enhancement of LpL activity was due to an increase in the V_{max}. The phospholipid-induced enhancement of LpL activity for PNPB was not correlated to the increase in the concentration of PNPB associated with the phospholipid vesicles.

The effects of phospholipid vesicles on LpL activity for PNPB were influenced by the phase transition temperature (T_c) of the lipid. At 17 °C, dimyristoyl phosphatidylcholine (DMPC) vesicles (T_c, 24 °C) and DPPC vesicles (T_c, 41 °C) both caused an 800% increase in LpL activity. At 33 °C, the increase in activity by DMPC and DPPC were 190% and 800%, respectively. At 42 °C, neither DMPC nor DPPC affected enzyme activity. Diether DMPC (T_c, 27 °C) and sphingomyelin (T_c, 37 °C), two lipids which are not substrates for LpL, also caused an 800% increase in the activity of the enzyme for PNPB at 17 °C. In the presence of both DMPC vesicles and DPPC vesicles, the temperature dependence of LpL activity for PNPB was nearly identical with that of DMPC vesicles. When the enzymic reaction was performed at 26 °C in the presence of DMPC vesicles and then DPPC vesicles were added, PNPB hydrolysis by LpL was enhanced. These results support the hypothesis that LpL interacts with lipid interfaces to increase the catalytic activity toward a water-soluble substrate. Furthermore, for interfacial activation, LpL prefers the lipid interface in the gel phase to that in the liquid-crystalline phase. A decrease in the enhancing effect of lipids in the liquid-crystalline phase on LpL activity toward PNPB suggests that interfacial activation is less effective and/or that the phospholipid is the preferred substrate for LpL.

Lipoprotein lipase (EC 3.1.1.34) catalyzes the hydrolysis of triglycerides transported in the circulation by chylomicrons and very low density lipoproteins (Ref. 1, for review). LpL also has phospholipase A_1 activity and hydrolyzes lipoprotein- phospholipids (2-4) and sonicated phosphatidylcholine vesicles (5, 6). For maximal activity for these substrates, the enzyme requires apolipoprotein C-II (7, 8). ApoC-II is a 78-amino-acid protein present in triglyceride-rich lipoproteins and high density lipoproteins (9).

An understanding of the sequence of events in the LpL-catalyzed hydrolysis of lipoprotein-lipids is complicated by the nature of the structure of the lipoprotein particles. The triglyceride-rich lipoproteins consist of a central core of neutral lipids, triglycerides and cholesteryl esters, and an outer monolayer of proteins, phospholipids, and unesterified cholesterol (10). The first probable step in the catalysis of lipoprotein lipids by LpL is the binding of the enzyme to the phospholipid monolayer; apoC-II is not required for this interaction (8). LpL also binds to triglyceride emulsions (11) and phospholipid vesicles (12) in the absence of apoC-II. The purpose of the present study was to determine the effects of the binding of LpL to phospholipid on the catalytic activity of the enzyme. To determine these effects, we have utilized sonicated phospholipid vesicles and a water-soluble substrate, p-nitrophenylbutyrate. The rationale behind the use of PNPB is based on the fact that LpL catalyzes the hydrolysis of short chain fatty acids, such as tributyrin, p-nitrophenylacetate, and PNPB (13-15), and that apoC-II is not required for these substrates (13, 15). The results of the present studies show that in the absence of apoC-II, phospholipid vesicles enhance the activity of LpL for PNPB, suggesting that the interaction of the enzyme with a lipid surface is associated with interfacial activation. The extent of interfacial activation is greatly affected by the incubation temperature such that the gel phase of the phospholipid is more effective than the liquid-crystalline phase.

EXPERIMENTAL PROCEDURES

Lipids—DMPC, DPFC, palmitoyl palmityoyleoyl PC, DPPE, egg PC, and bovine brain SpM were purchased from Applied Science Laboratories. PNPB was purchased from Sigma. The diether of DMPC was a generous gift of Dr. G. de Haas (State University of Utrecht). The purity of each lipid was checked by thin layer chromatography in chloroform:methanol:acetic acid:water (90:30:2:8) and in hexane:ethanol:acetic acid (90:10:1). Preparation of LpL—LpL was purified to homogeneity from bovine skimmed milk by affinity chromatography on heparin-Sepharose.

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as described previously (16, 17); the enzyme was stored at -70 °C in 50% glycerol. The specific activity of LpL was 30 nmol of released oleic acid/mg of protein/h using tri[14C]oleoyl glycerol as a substrate. The reaction mixture contained 0.33 mg of trilinolein (Sigma), 0.067 μCi of tri[14C]oleoyl glycerol (New England Nuclear) (60 mCi/mmol), 5 mg of fatty acid-free bovine serum albumin (Sigma, grade V), 0.02% Triton X-100, 1 μg of human apoC-II, and an appropriate amount of enzyme. Final volume of 0.25 ml of 0.1 M Tris-HCl, pH 8.6; released [14C]oleic acid was extracted by the method of Belfrage and Vaughan (18).

Hydrolysis of PNPB—A stock solution of PNPB was prepared by dissolving 20.9 mg in 2 ml of acetonitrile. The reaction mixtures contained the indicated concentration of PNPB, LpL (0 μg), heparin (10 μg, Sigma, porcine intestinal mucosa, 169.9 units/mg) in a final volume of 1.0 ml of 0.1 M sodium phosphate, pH 7.2, containing 0.9% NaCl; the final concentrations of acetonitrile in all reaction mixtures was 15% (v/v). The hydrolysis of PNPB was determined by monitoring the increase in absorbance at 400 nm continuously using a non-enzyme incubation mixture as a blank or by measuring the absorbance after extracting p-nitrophenol from the reaction mixture. In the latter technique, the enzyme reactions were terminated by the addition of 3.25 ml of methanol:chloroform:heptane (1:0.9:0.7, v/v); the mixtures were then shaken vigorously for 10 s and centrifuged at 1,500 × g for 5 min. After warming at 40 °C for 3 min, the supernatant fractions were removed and the absorbance at 400 nm, the maximal absorbance wavelength, was determined against a blank sample which was prepared in the absence of enzyme. The molar extinction coefficient of released p-nitrophenol was determined as 12,000, with a recovery of 90%.

Preparation of Phospholipid Vesicles—To prepare phospholipid vesicles, the lipids were dissolved in chloroform, evaporated under a stream of N2, and lyophilized for 15 min. Phospholipid dispersions were then prepared by adding 0.9% NaCl, 0.1% Tris-HCl, pH 7.2, to give 2 mg of phospholipid/ml. The lipid was suspended in the buffer by sonication and was sonicated above the phase transition temperature of each lipid. Each lipid phase mixture was then removed from the sonicate by ultracentrifugation at 150,000 × g for 1 h at 15 °C; phospholipid vesicles were prepared daily and stored at room temperature.

RESULTS

Hydrolysis of PNPB by LpL—The LpL-catalyzed hydrolysis of PNPB is shown in Fig. 1A. The release of p-nitrophenol was linear up to a LpL concentration of 5 μg/ml (Fig. 1A). At an enzyme concentration of 5 μg/ml, the release of product was linear for 13 min (Fig. 1B).

![Fig. 1A](image1.png)

**FIG. 1.** LpL-catalyzed hydrolysis of PNPB. **A,** effect of LpL concentration on PNPB hydrolysis. The incubation mixtures contained 0.5 mM PNPB, 1% (v/v) acetonitrile, heparin (10 μg), and the indicated amount of LpL in a final volume of 1.0 ml of a standard buffer containing 0.10 M sodium phosphate, pH 7.2, and 0.9% NaCl. The reaction mixtures were incubated at 37 °C for 7.0 min. The released p-nitrophenol was extracted as described under "Experimental Procedures" and the concentration was determined by the absorbance at 410 nm. B, time course of PNPB hydrolysis by LpL. Incubation mixtures contained 0.5 mM PNPB, 1% (v/v) acetonitrile, heparin (10 μg), and LpL (5 μg) in a final volume of 1.0 ml of standard buffer. The reaction mixtures were incubated at 37 °C for the indicated times; released p-nitrophenol was determined as described in A.

![Fig. 2](image2.png)

**FIG. 2** (left). Effect of phospholipids on the LpL-catalyzed hydrolysis of PNPB. The incubation mixtures contained 0.5 mM PNPB, 1% (v/v) acetonitrile, heparin (10 μg), LpL (5 μg) in a final volume of 1.0 ml of standard buffer. After incubation at 37 °C for 4 min, 10% (v/v) dispersions (C) or DPPC vesicles sonicated for either 1 min (△), 4 min (□), or 16 min (■) were added to the incubation mixture. At the indicated times, released p-nitrophenol was determined as described in Fig. 1A.

**FIG. 3** (right). Effect of various phospholipids on the LpL-catalyzed hydrolysis of PNPB. The incubation mixtures contained 0.5 mM PNPB, 1% (v/v) acetonitrile, heparin (10 μg), LpL (5 μg), and the indicated amounts of various phospholipids in 1 ml of standard buffer. DPPC (●), diether DMPC (X−X), DMPC (○−○), egg PC (Δ−Δ), palmitoyl palmityloleoyl PC (×−×), bovine brain SpM (C−C), and DPPPE (■−■). Incubations were performed at 37 °C. After 7 min, released p-nitrophenol was determined as described in Fig. 1A.

**Effects of Phospholipids on the Hydrolysis of PNPB by LpL**—The effects of DPPC on the LpL-catalyzed hydrolysis of PNPB is shown in Fig. 2. The addition of multimamellar liposomes of DPPC to the reaction mixture caused only a slight increase in the rate of catalysis of PNPB. However, the addition of sonicated vesicles of DPPC increased the rate approximately 5-fold. In the presence of DPPC the release of the product was linear for 6 min. Fig. 3 shows the effects of various phospholipids on the rate of hydrolysis of PNPB by LpL. In these experiments, sonicated vesicles of phospholipids were preincubated with LpL for 2 min at 37 °C. PNPB was then added to the incubation mixture and the rate of hydrolysis was determined after 7 min. At 37 °C, DPPC gave the greatest enhancement of enzyme activity, there being a 5-fold increase with 50 μg of lipid. The enhancement of activity for other choline-phospho-
lipids tested (50 μg) was as follows: egg PC (210%), palmitoyl palmitoleoyl PC (190%), DMPC (150%), diether DMPC (260%), and SpM (225%). However, the ethanolamine phospholipid DPPC gave only a 125% increase in activity, suggesting that the polar head group plays a role in activation.

**Effects of DPPC on Enzyme Kinetic Parameters**—To determine the effects of DPPC on the kinetic parameters for the LpL-catalyzed hydrolysis of PNPB, the rates of hydrolysis were determined at various concentrations of PNPB, as shown in Fig. 4A. Lineweaver-Burk double reciprocal plots are shown in Fig. 4B. The effects of DPPC on the kinetic parameters of the LpL-catalyzed hydrolysis of PNPB at concentrations < 0.7 mM were to increase the maximal velocity (V_max) of the reaction with minimal effect on the Michaelis constant (K_m). The calculated V_max and K_m values in the absence of a phospholipid vesicle were 2.0 μmol of product released/min/mg of LpL and 0.52 mM, respectively. In the presence of vesicles of DPPC, (50 μg), the values were 8.8 and 0.55. Thus, DPPC increased the V_max approximately 4.2-fold at 37 °C.

The Lineweaver-Burk plots shown in Fig. 4B were not linear at PNPB concentrations > 0.7 mM. At approximately 1 mM, PNPB becomes insoluble and small lipid droplets form. However, we have no evidence for the formation of micelles of PNPB. This conclusion is based on the fact that there were no detectable spectral shifts of rhodamine 6G or bromophenol blue in the presence of PNPB up to 1.7 mM.

To determine the effects of lipid structure on the kinetic parameters, V_max and K_m were determined for the LpL-catalyzed hydrolysis of PNPB in the absence and presence of DMPC at temperatures below, at, and above the phase-transition temperature of the lipid. As shown in Table I, the physical state of the lipid has a negligible effect on K_m. On the other hand, V_max values showed an increase of 5.4- and 1.6-fold by the addition of DMPC vesicles at 17 and 32 °C, respectively.

**Effects of Temperature on the LpL-catalyzed Hydrolysis of PNPB and Phospholipids**—In the experiments described above, the reactions were carried out at 37 °C. Fig. 5 shows the temperature dependence of the phospholipid-enhanced LpL-catalyzed hydrolysis of PNPB. In the absence of phospholipid, the activity of LpL for PNPB increased up to 42 °C (Fig. 5A). Egg PC and palmitoyl palmitoleoyl PC increased the activity up to 200% at all temperatures studied (Fig. 5B).

At temperatures below 34 °C, DPPC enhanced enzyme activity > 8-fold. Between 34 and 42 °C, the enhancement decreased; at 42 °C negligible enhancement of activity occurred. SpM vesicles also increased the LpL-catalyzed hydrolysis of PNPB at 18 °C by 8-fold (Fig. 5C). However, in contrast to DPPC, the activation by SpM decreased linearly from 18 to 42 °C. The effect of vesicles of DMPC or diether DMPC on the LpL-catalyzed hydrolysis of PNPB is shown in Fig. 5D. At 18 °C both lipid vesicles gave an 8-fold enhancement of PNPB hydrolysis. With DMPC there was a sharp decrease in PNPB hydrolysis between 18 and 33 °C. The temperature dependence of diether DMPC was remarkably different from DMPC. With diether DMPC, the enhancing effect decreased gradually between 18 and 42 °C and was equal to DMPC at only 42 °C.

The temperature dependencies shown in Fig. 5 suggest some relationship between the structure of the lipids and their ability to enhance the activity of LpL. Therefore, we have determined the transition temperature of each lipid by fluorescence polarization of DPH-labeled phospholipid vesicles. Fig. 6 shows a transition temperature of 27 °C for vesicles of diether DMPC and 37 °C for bovine brain SpM. The transition temperatures for DMPC and DPPC vesicles were 24 and 41 °C, respectively (Fig. 7). Thus, DPPC, SpM, DMPC, and diether-DMPC enhanced the activity of LpL for PNPB by almost the same extent below 20 °C, where all of these lipids are in the gel phase. At temperatures above the transition temperature, the enhancement of LpL activity for PNPB was diminished. The lack of temperature dependence of egg PC and palmitoyl palmitoleoyl PC on the enhancement of LpL for PNPB may be explained by the liquid-crystal state of these lipids at all temperatures studied.

**Effects of Mixtures of DMPC and DPPC on the LpL-catalyzed Hydrolysis of PNPB**—In this series of experiments we wished to determine the preferred lipid structure for LpL using mixtures of DMPC and DPPC. In the first experiment, DMPC-DPPC vesicles were prepared by co-sonicating DMPC and DPPC (1:1 molar ratio). As is shown in Fig. 8, DMPC-DPPC vesicles enhanced the activity of LpL for PNPB up to 28 °C; the enhancement was approximately to the same extent as DMPC alone. However, above 30 °C, the mixed phospholipid vesicle was much less effective. The temperature dependence of PNPB hydrolysis in the presence of the DMPC-DPPC vesicles was between that for pure DMPC and DPPC vesicles. As is shown in Fig. 7, the transition temperature of the DMPC-DPPC vesicles (32 °C) was also between the two phospholipids.

In the next experiment, phospholipid vesicles of DMPC and DPPC were mixed together (1:1 molar ratio) and their effects on LpL activity determined. Fig. 8 shows that the mixed vesicle system enhanced PNPB hydrolysis. The temperature dependence of this enhancement was nearly identical to that

<table>
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<th>K_m (mm)</th>
<th>V_max (μmol/min/mg LpL)</th>
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Interfacial Activation of Lipoprotein Lipase

Fig. 5. Effect of temperature on the enhancement of the LpL-catalyzed hydrolysis of PNPB by phospholipids. A, the reaction mixtures contained 0.5 mM PNPB, 1% (v/v) acetonitrile, heparin (10 pg), and LpL (5 pg) in 1.0 ml of standard buffer. The reactions were carried out at various temperatures as indicated. The rate of hydrolysis of PNPB was determined in the linear portion of the curves. Released p-nitrophenol was determined as described in Fig. 1A. B, the reaction conditions were identical to A with the exception that LpL was preincubated with egg PC (○) or palmitoyl palmitoleoyl PC (△) for 2 min at the indicated temperatures. The reaction mixtures were then initiated by the addition of PNPB. The relative activity of LpL for PNPB in the presence of phospholipid vesicles is compared to their activities in the absence of phospholipid vesicles and is expressed as the per cent activation.

C, effect of temperature on the LpL-catalyzed hydrolysis of PNPB in the presence of DPPC (○) or SpM vesicles (△). The incubation conditions were identical to those described in B. D, effect of temperature on the LpL-catalyzed hydrolysis of PNPB in the presence of DMPC (○) or diether DMPC (△). The reaction conditions were identical to those described in B.

DMPC or DPPC vesicles was 0.126 and 0.109 mM, respectively. At 16 °C, a temperature at which both lipids are in the gel phase, the concentration of PNPB was 0.112 or 0.103 mM, respectively. These results show that the increased rates of hydrolysis of PNPB were not due to a difference in the concentration of PNPB associated with the phospholipid vesicles.

To confirm the preference of LpL for DPPC in the gel phase to the liquid-crystal phase of DMPC, DMPC and DPPC vesicles were added to the reaction mixtures in different chronological sequences and the rates of hydrolysis of PNPB were determined. As shown in Fig. 9 (curve C), the addition of DPPC vesicles to the reaction mixture at 26 °C increased the rate of PNPB hydrolysis by LpL; the further addition of DMPC vesicles had no effect on the rate. In the reverse experiments, DMPC was first added to the incubation mixture and then DPPC was added. As is shown in Fig. 9 (curve B), DPPC enhanced the rate of PNPB hydrolysis to the same

Fig. 6. Temperature dependence of the fluorescence polarization values of diether DMPC (○) and SpM (△) labeled with DPH. The experimental conditions and procedures for fluorescence polarization are described under "Experimental Procedures."

Fig. 7. Temperature dependence of the fluorescence polarization. Values of DMPC (○), DPPC (△), and DMPC-DPPC (1:1 molar ratio, △) labeled with DPH were determined. The experimental details are described under "Experimental Procedures."
were added as indicated.

were determined continuously by monitoring the increase in the absorbance at 400 nm with a no-enzyme incubation mixture as a blank. The results of the present studies show that the binding of LpL to a lipid interface in the gel phase causes an increase in the catalytic activity of the enzyme for a water-soluble substrate. The activities of a number of other lipolytic enzymes are affected by interfaces (23, for review). Sarda and Desnuelle (24) demonstrated that purified pancreatic lipase had minimal activity toward triacetin in a monomeric state. However, when the solubility limit of triacetin is exceeded there is a sharp increase in enzymic activity (24). Glass beads also increase the activity of pancreatic lipase (25). Peterson et al. (26) showed that the activity of porcine pancreatic phospholipase A₂ depend on substrate concentration. At concentrations above the critical micelle concentration, the activity of the enzyme dramatically increases (26). The results of the present study showing that phospholipid vesicles enhance the LpL activity for PNPB suggest that LpL also belongs to the class of lipolytic enzymes whose activity is affected by interfaces. Substrate aggregation alone did not increase the activity of LpL. At concentrations of >1.0 mM, PNPB formed lipid droplets. However, the lack of spectral changes of rhodamine 6G or bromphenol blue shows that the aggregates were not micellar structures. These results suggest that the interfacial activation of LpL requires a specific structural property for the interface.

The mechanism by which phospholipid vesicles enhance the activity of LpL for PNPB could arise either because the binding of the enzyme to the phospholipid interface increases the catalytic power of the enzyme or because the phospholipid alters the properties of the PNPB substrate. Evidence against the latter explanation was partly provided by equilibrium dialysis experiments. The amount of PNPB associated with DPPC vesicles was no greater than that to DMPC vesicles at 30 °C, a temperature at which the DMPC vesicles showed that LpL also belongs to the class of lipolytic enzymes whose activity is affected by interfaces. Substrate aggregation alone did not increase the activity of LpL. At concentrations of >1.0 mM, PNPB formed lipid droplets. However, the lack of spectral changes of rhodamine 6G or bromphenol blue shows that the aggregates were not micellar structures. These results suggest that the interfacial activation of LpL requires a specific structural property for the interface.
results that a decrease in the enhancing effect of lipid vesicles in the liquid-crystalline phase may be due to weak association of the interface recognition site of the enzyme with the lipid surface.

Another possible explanation for the decrease in the enhancing effect of phospholipids in the liquid-crystalline state is that the affinity of the catalytic site of LpL for PNPB might decrease in the liquid-crystalline state relative to the gel state. This speculation is partially based on the observed differences in the temperature dependence of phosphatidylcholine as compared to the nonsubstrate phospholipids. As shown in Figs. 5 and 8, the enhancement of activity by DMPC, DPPC, and DMPC-DPPC vesicles decreases sharply at the Tc of the lipid. On the other hand, with diether DMPC and SpM, which are not substrates for LpL, the decrease in the enhancement of activity was much more gradual and was minimal at temperatures above the Tc. The sharp break in the temperature dependence for the LpL-catalyzed hydrolysis of PNPB is possibly related to the fact that the affinity of the catalytic site of the enzyme for phosphatidylcholine is greater in the liquid-crystalline state. Even though the affinity of the catalytic site for the liquid-crystalline phase of phosphatidylcholine might be higher than for the gel phase, it is still relatively weak, especially in the absence of apoC-II, such that LpL prefers the gel phase of DPPC to the liquid-crystalline phase of DMPC, as discussed above.

The preference for LpL for the gel phase of lipid is consistent with a recent report on phospholipase A2 by Menashe et al. (28). When phospholipase A2 was preincubated with a phospholipid substrate below the transitional temperature of the lipid and then assayed at high temperature, no lag period was observed. On the other hand, the time course of hydrolysis exhibited a distinct lag period when the enzyme was first mixed with DPPC vesicles above the phase transition temperature. Menashe et al. (28) concluded from their results that the organization of substrate and enzyme was most rapid when the phospholipid was in the gel state.

In summary, we conclude that the catalytic power of LpL for a water-soluble substrate increases by the association of the enzyme with a phospholipid surface, preferentially in the gel phase. With these conditions and in the absence of apoC-II, the catalytic site of the enzyme is not properly directed to the phosphatidylcholine molecule. One possible mechanism by which apoC-II increases the activity of LpL for water-insoluble substrates is to direct the catalytic site to the substrate or to stabilize an enzyme-substrate complex after the enzyme binds to the lipid interface.

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