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HYDROLYSIS OF A FLUORESCENT PHOSPHOLIPID SUBSTRATE BY PHOSPHOLIPASE A_2 AND LIPOPROTEIN LIPASE

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The fluorescent phospholipid 1-acyl-2-[6-[(7-nitro-2,1,3benzoxadiazol-4-yl) amino]-caproyl]phosphatidylcholine (C_6-NBD-PC) was used as a substrate for porcine pancreatic phospholipase A_2 (PA_2) and bovine milk lipoprotein lipase (LpL). Hydrolysis of C_6-NBD-PC by either enzyme resulted in a greater than 50-fold fluorescence enhancement with no shift in the emission maximum at 540 nm; Ca^{++} was required for PA_2 catalysis. Identification of the products of hydrolysis showed cleavage at the sn-1 and sn-2 positions for LpL and PA_2, respectively. For PA_2, but not for LpL, there was a marked enhancement of enzyme catalysis at lipid concentrations above the critical micellar concentration of the lipid. Furthermore, apolipoprotein C-II, the activator protein of LpL for long-chain fatty acyl substrates, did not enhance the rate of catalysis of the water-soluble fluorescent phospholipid for either enzyme.

LpL catalyzes the hydrolysis of triacylglycerols, phosphatidylcholine, phosphatidylethanolamine and fatty acyl ester substrates, such as p-nitrophenyl acetate (Refs. 1-2, for review); the enzyme is specific for the sn-1 position of phospholipids. For maximal rates of hydrolysis of long-chain fatty acyl esters, LpL requires apoC-II (3), a protein constituent of plasma triacylglycerol-rich lipoproteins and high density lipoproteins. The purpose of the present study was to determine the effect of LpL on the fluorescent phospholipid C_6-NBD-PC and to compare its properties to PA_2, an enzyme specific for the sn-2 position. In addition, we have examined the effect of

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Abbreviations used: C_6-NBD-PC, 1-acyl-2-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]-caproyl]phosphatidylcholine; LpL, bovine milk lipoprotein lipase; PA_2, porcine pancreatic phospholipase A_2; apolipoprotein C-II, apoC-II; CMC, critical micellar concentration.

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apoC-II on enzyme catalysis and the state of aggregation of the fluorescent phospholipid on the activity of both enzymes towards this substrate.

MATERIALS AND METHODS

C_6-NBD-PC was obtained from Avanti Biochemical Corp. and was found to be pure (Rf=0.38) by thin layer chromatography on Silica gel 60 F-254 (EM Reagents) in a solvent system of chloroform:methanol:water (65:25:1, v/v). Porcine pancreatic PA_2 (600 units/mg) was obtained from Sigma and was used without further purification. Heparin (porcine intestinal mucosal, 169.9 units/mg) was purchased from Sigma.

Lipoprotein lipase was purified from bovine skimmed milk by chromatography on heparin-Sepharose 4B (2.5 mg heparin/ml gel) as described by Kinnunen (4). ApoC-II was isolated from triacylglycerol-rich lipoproteins of subjects with familial endogenous hypertriglyceridemia with fasting chylomicronemia (type V hyperlipoproteinemia) as described previously (5). Fluorescence measurements were performed at 24°C with a Perkin-Elmer MPF-44A or 650-10S ratio recording spectrofluorometer.

RESULTS

Effect of PA_2 and LpL on C_6-NBD-PC fluorescence

The addition of PA_2 or LpL to C_6-NBD-PC caused a >50-fold fluorescence enhancement with no shift in the wavelength of its emission maximum (540 nm). With the experimental conditions shown in Fig. 1, the time course of the fluorescence increase was half-maximal in approximately 3 min for both enzymes. Ca^{++} ion was required for PA_2 but not for LpL activity, and apoC-II had no effect on the rates of catalysis by either enzyme. The fluorescence enhancement was strictly dependent on enzyme concentration (Fig. 2); heat-inactivated LpL and phospholipase C or D produced no fluorescence changes (data not shown).

Relationship between fluorescence changes in C_6-NBD-PC and enzyme catalysis

To provide direct evidence for a relationship between the fluorescence increase in C_6-NBD-PC and enzyme catalysis, the reaction products were separated by thin layer chromatography. In the results shown in Fig. 3, PA_2 or LpL were added to an incubation mixture containing C_6-NBD-PC. At the indicated times samples were removed, the enzyme reaction terminated, and its products separated and quantitated. With hydrolysis of C_6-NBD-PC by these enzymes, the amount of C_6-NBD-PC in the aqueous phase decreased concomitantly with an increase in the fluorescent reaction products, NBD-hexanoic acid (Fig. 3A) or lyso NBD-PC (Fig. 3B), in the organic phase. For both enzymes, the

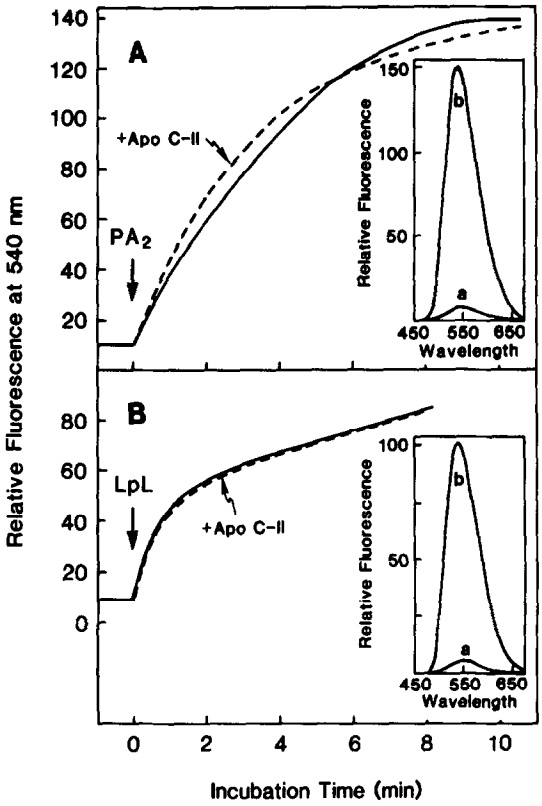

Figure 1: Effect of (A) porcine pancreatic PA_2 and (B) bovine milk LpL on C_6-NBD-PC fluorescence. Each reaction mixture at 24°C contained C_6-NBD-PC (5×10^{-6}M) and either no apoC-II (solid line) or 2 μg/ml apoC-II (dashed line) in 1.0 ml of 10 mM Tris-HCl, pH 7.4, 100 mM KCl; the PA_2 incubation mixture contained 2 mM $CaCl_2$. At the indicated time, 10 μg of PA_2 or 0.3 μg of LpL was added and fluorescence was monitored continuously as a function of time. Fluorescence excitation was at 470 nm and emission at 540 nm. The insets show the fluorescence spectra of (a) the initial reaction mixture and (b) after 15 min of enzyme catalysis.

fluorescence increase in the original reaction mixture closely paralleled both the decrease in C_6-NBD-PC in the aqueous phase and the appearance of products in the organic phase. The release of NBD-hexanoic acid by LpL was higher than expected from its specificity for the primary acyl bond and is most probably due to migration of the C_6-NBD-PC fatty acyl group from the sn-2 to the sn-1 position, followed by enzyme catalysis.

Effect of substrate concentration on the PA_2 and LpL-catalyzed hydrolysis of C_6-NBD-PC

C_6-NBD-PC begins to form aggregate structures in the range of 2×10^{-7} M (6); below this concentration the lipid exists as soluble monomers. The

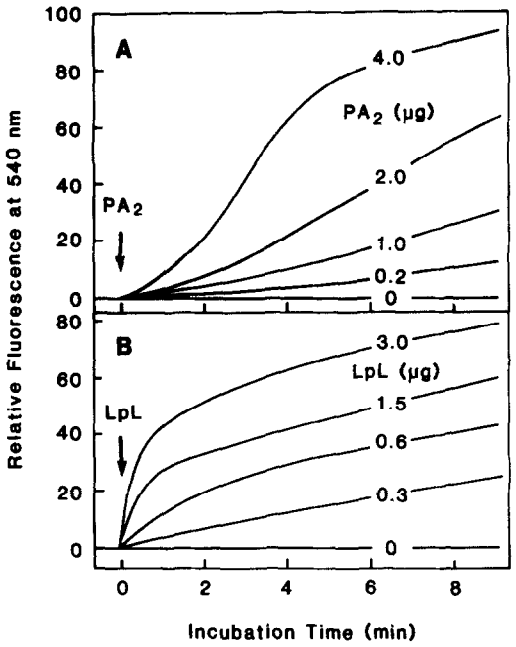

Figure 2: Effect of enzyme concentration of (A) porcine pancreatic PA_2 or (B) bovine milk LpL on C_6-NBD-PC fluorescence. Each reaction mixture contained C_6-NBD-PC (5×10^{-6} M) in 1.0 ml of 10 mM Tris-HCl, pH 7.4, 100 mM KCl; the PA_2 incubation mixture contained 2 mM $CaCl_2$. At zero time, the indicated amount of PA_2 or LpL was added and fluorescence was monitored continuously as described in Fig. 1.

effect of lipid concentration on enzyme catalysis of C_6-NBD-PC is shown in Fig. 4. The rate of C_6-NBD-PC fluorescence enhancement by LpL catalysis increased with higher lipid concentrations (Fig. 4B), but no dramatic enhancement of activity occurred above the CMC. In contrast, and consistent with the well known enhancement of PA_2 activity with aggregated substrates (7), there was a marked increase in fluorescence at C_6-NBD-PC concentrations $>10^{-7}$ M (Fig. 4A). For both enzymes, apoC-II (2 μg/ml) had no effect on the rate or extent of the fluorescence increase at all C_6-NBD-PC concentrations tested (data not shown).

DISCUSSION

The fluorescent phospholipid C_6-NBD-PC undergoes a large fluorescence increase upon hydrolysis either by PA_2 or LpL. Thus, this substrate is an accurate fluorescent indicator of its own hydrolysis even at low enzyme and substrate concentrations; hydrolysis of C_6-NBD-PC (5×10^{-6} M) could be moni-

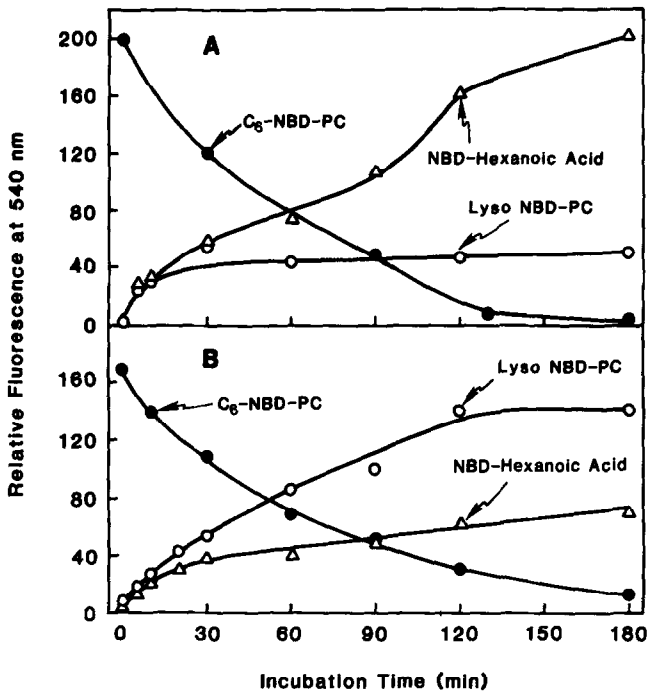

Figure 3: Time course of the (A) porcine pancreatic PA_2 and (B) bovine milk LpL-catalyzed hydrolysis of C_6-NBD-PC. The incubation mixture contained C_6-NBD-PC (5×10^{-6} M) in a final volume of 11 ml of 10 mM Tris-HCl, 100 mM KCl, pH 7.4; the PA_2 incubation mixture also contained 2 mM $CaCl_2$. After addition of LpL (17 µg) or PA_2 (40 µg), 1 ml, at the indicated time points, of each incubation mixture was removed and the enzyme reaction was terminated by the addition of 3.25 ml of methanol:chloroform:heptane (1.42:1.25:1.0, v/v). One ml of the aqueous and organic phase at each time point was taken to dryness, dissolved in 50 µl methanol and spotted on Silica Gel G-high performance thin layer chromatography plates (Analtech, 250 µ thick); the plates were developed in a solvent system of chloroform: methanol:water (65:25:4, v/v). After development, C_6-NBD-PC, lyso NBD-PC and NBD-hexanoic acid were located with a UV-lamp, scraped from the plate and the gel suspended in 2.0 ml of ethanol:water (1:1, v/v). After removing the gel by centrifugation, fluorescence at 540 nm was determined. The symbols correspond to C_6-NBD-PC (-●-●-), NBD-hexanoic acid (-Δ-Δ-) and lyso NBD-PC (-o-o-).

tored by as little as 0.1 µg of either enzyme. Concomitant with the fluorescence increase, C_6-NBD-PC is hydrolyzed by these enzymes to form NBD-hexanoic acid and lyso NBD-PC. Consistent with their known specificities, the major fluorescent product of PA_2 catalysis is NBD-hexanoic acid (cleavage at the sn-2 position), whereas LpL yields lyso NBD-PC (cleavage at the primary fatty acyl bond). Because of its water-solubility and its large fluorescence increase upon hydrolysis, C_6-NBD-PC has allowed us to address two important questions concerning LpL and PA_2 catalysis: One, does the physical form of

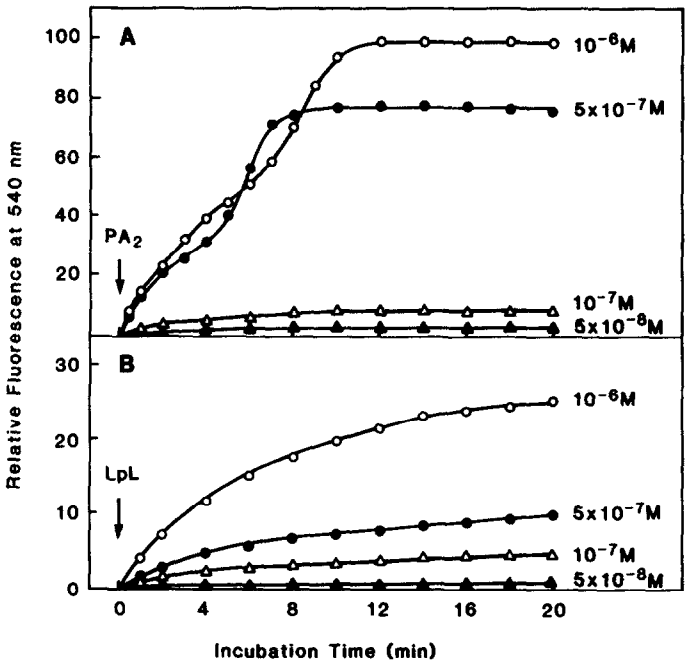

Figure 4: Effect of C_6-NBD-PC concentration on (A) porcine pancreatic PA_2 and (B) bovine milk LpL-catalysis. Each reaction mixture contained the indicated concentration of C_6-NBD-PC in 1.0 ml of 10 mM Tris-HCl, pH 7.4, 100 mM KCl with 2 mM $CaCl_2$ in the PA_2 reaction. At zero time PA_2 (5 µg) or LpL (0.4 µg) was added to the incubation mixture and fluorescence was monitored continously as described in Fig. 1.

the substrate (monomeric or aggregate structures) affect the rate of enzyme catalysis and, two, what is the effect of apoC-II on enzyme activity?

An essential element of PA_2 catalysis is an increase in the efficiency of catalysis by interfaces. Pieterson et al. (7) showed a dramatic increase in the rate of hydrolysis of diheptanoylphosphatidylcholine at lipid concentrations greater than the CMC (>1.6 mM). An increase in PA_2 catalysis above its CMC was also observed with C_6-NBD-PC (Fig. 4A). The advantage of the fluorescent phospholipid is that the phenomenon of activation by interfaces was observed at µM substrate concentrations, thus, avoiding the problem of lipid insolubility. In contrast to PA_2, LpL did not show an increase in catalysis with the aggregated fluorescent phospholipid, a finding consistent with that of Shinomiya et al. (8) with dihexanoylphosphatidylcholine as substrate. These findings suggest marked differences in interfacial activation characteristics of these two enzymes.

Recently, Bengtsson and Olivecrona (9) have shown that the C-apolipoproteins, including apoC-II, stimulate PA_2 activity towards liposomes of dimyristoylphosphatidylcholine. They suggested that the observed enhancement of PA_2 activity results from reorganization of liposome lipid structure and not from a specific interaction of PA_2 and apoC-II. Consistent with their hypothesis (9), we found that apoC-II does not stimulate PA_2 catalysis of the water-soluble fluorescent phospholipid, C_6-NBD-PC. With respect to the effect of apoC-II on LpL catalysis, it is known that LpL requires the activator protein for maximal rates of hydrolysis of phosphatidylcholines with two long (>10 carbon atoms) fatty acyl chains (3). The presence of a fluorescent reporter group at the sn-2 position of phosphatidylcholine (as in C_6-NBD-PC) somehow alters the apoC-II activation of LpL for this substrate, even though the sn-1 position has a 16-carbon fatty acyl chain. Apparently, the presence of the bulky fluorescent probe and/or the length of the fatty acyl moiety in the sn-2 position of C_6-NBD-PC prevents the normal transition-state stabilization observed for long-chain substrates with the addition of apoC-II. Experiments are currently in progress to systematically vary the fatty acyl chain length in the sn-1 and sn-2 positions so as to determine their relative importance for apoC-II activation of LpL.

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