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PHOSPHOLIPASE ACTIVITY OF BOVINE MILK LIPOPROTEIN LIPASE ON PHOSPHOLIPID VESICLES: INFLUENCE OF APOLIPOPROTEINS C-II AND C-III

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SUMMARY

The effect of human plasma apolipoproteins C-II and C-III on the hydrolytic activity of lipoprotein lipase from bovine milk was determined using dimyristoyl phosphatidylcholine (DMPC) vesicles as substrate. In the absence of apoC-II or C-III, lipoprotein lipase has limited phospholipase activity. When the vesicles were preincubated with apoC-II and then phospholipase activity determined, there was a time dependent release of lysolecithin; activity was dependent upon both apoC-II and lipoprotein lipase concentrations. The addition of apoC-III to DMPC did not stimulate phospholipase activity. We conclude that apoC-II has an activator effect on the phospholipase activity of lipoprotein lipase and that the mechanism is beyond that of simply altering the lateral compressibility of the lipid.

INTRODUCTION

Lipoprotein lipases are the enzymes involved in the hydrolysis of triglycerides transported in human plasma chylomicrons and very low density lipoproteins (VLDL). At least two lipoprotein lipases are present in post-heparin plasma (Ref. 1 for review). One of the enzymes is localized on the vasculature endothelial lining whereas the other is of hepatic origin. The characteristic feature of the extra-hepatic enzyme is that it is stimulated by apolipoprotein C-II (apoC-II) (2,3), a 78 amino acid residue peptide (4) present in chylomicrons, VLDL and high density lipoproteins (HDL). Another apoprotein, apoC-III, has been shown by a number of investigators (5-8) to inhibit triacylglycerol hydrolysis by lipoprotein lipase *in vitro* in the presence of apoC-II. In addition to triglyceride hydrolysis, lipoprotein lipase from both post-heparin plasma and milk hydrolyzes the primary acyl bond of phosphatidylethanolamine and phosphatidylcholine in chylomicrons, VLDL and artificial triglyceride-phospholipid substrates (9-12); HDL phospholipids are not hydrolyzed (13). Using an

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isolated perfused rat heart and phosphatidylethanolamine-coated triglyceride particles as substrate, Groot et al. (12) reported that the phospholipase activity of heparin-releasable lipoprotein lipase was dependent on apoC-II and that apoC-III did not inhibit the apoC-II activation. The mechanism by which apoC-II enhances the activity of lipoprotein lipase toward triglycerides and phospholipid is unknown. To understand the mechanism of action of the enzyme on phospholipids, we have utilized sonicated vesicles of dimyristoyl phosphatidylcholine (DMPC) as substrate and purified lipoprotein lipase from bovine milk. The results of these studies show that lipoprotein lipase has phospholipase activity which is dependent on apoC-II.

MATERIALS AND METHODS

Lipoprotein lipase. Bovine milk lipoprotein lipase was purified to homogeneity by affinity chromatography on heparin-Sepharose as described by Kinnunen (14). The enzyme preparation had a specific activity of 87 mmol fatty acid released/h/mg protein using glycerol [^{14}C]triolein in 0.02% Triton X-100 as substrate. The enzyme was stored in 50% glycerol, 10 mM Tris-HCl, pH 7.4, 1 M NaCl, at -70°C, and thawed at -20°C prior to its addition to the reaction mixtures.

Preparation of phospholipid vesicles. Dimyristoyl phosphatidylcholine (Sigma) was purified on Silica gel and yielded a single species upon two-dimensional TLC on Silica gel 60 F-254 (EM Reagents) in chloroform:methanol:25% ammonia:water, 180:108:11:11 and chloroform:methanol:acetic acid:water, 180:80:24:4. To prepare phospholipid vesicles, the DMPC was dissolved in chloroform, evaporated under a stream of N_2, and lyophilized for 15 min. Phospholipid dispersions were then prepared by adding a standard buffer (0.1 M NaCl, 0.1 M Tris-HCl, pH 7.4) to give 10 mg DMPC/ml. The lipid was suspended in the buffer by mechanical agitation and was sonicated at 25°C for 30 min using a Heat Systems Ultrasonics, Inc. Cell Disrupter (Model W-225R). Large phospholipid structures were then removed from the sonicate by ultracentrifugation at 150,000 x g for 1 h at 15°C. The vesicles were prepared daily and stored at room temperature

Isolation of apolipoproteins. ApoC-II and apoC-III (with 2 residues of sialic acid) were isolated from VLDL which was obtained from plasma of fasting individuals with endogenous type IV hyperlipoproteinemia, as described previously (4). The isolated proteins were homogeneous as determined by isoelectric focussing (15). [^{125}I]apoC-II was prepared by the method of Roholt and Pressman (16).

Phospholipase activity. The activity of lipoprotein lipase toward DMPC vesicles was assayed under a variety of conditions of temperature, apoprotein concentrations, and inhibitors, as described in the legends to the Figures and Table. In all experiments the reactions were initiated by the addition of the enzyme and were terminated by the addition of 600 µl MeOH and 300 µl $CHCl_3$, followed by the lipid extraction (17). The lipid was redissolved in $CHCl_3$ and spotted on Silica gel TLC plates. The plates were developed one-dimensionally in chloroform:methanol:water (70:30:4), and the lecithin and lysolecithin spots visualized with I_2 staining; the lipids were scraped from the plates and phospholipid determined (18).

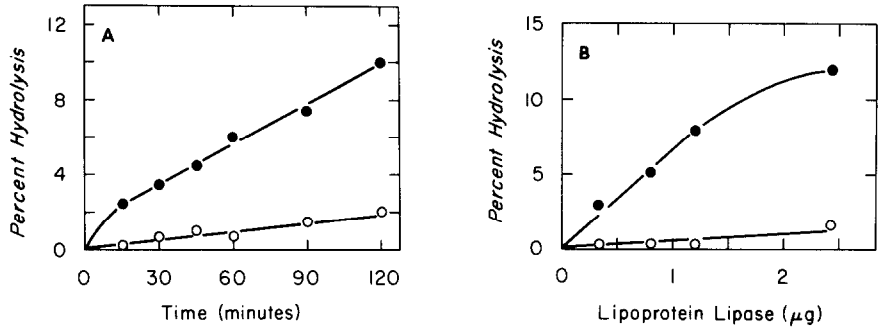

FIGURE 1. Time and enzyme dependence of hydrolysis of dimyristoyl phosphatidylcholine (DMPC) in the absence (-o—o-) and presence (-●—●-) of apoC-II. (A) Each reaction mixture contained in a final volume of 0.2 ml of 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4, the following additions: DMPC vesicles (700 μg), apoC-II where appropriate (0.8 μg) and fatty acid-free bovine serum albumin (5 mg). Each reaction mixture was preincubated at 23.4°C for 15 min prior to the addition of lipoprotein lipase (1.4 μg). At the indicated reaction times, the mixtures were extracted and the % hydrolysis determined as described in the text. (B) The reaction conditions were the same as those described in (A), with the exception of variable amounts of enzyme and a final incubation time of 90 min.

RESULTS

In the absence of any apoprotein cofactor, phospholipase activity of bovine milk lipoprotein lipase against DMPC was negligible (Fig. 1) at all enzyme to substrate concentrations tested. The addition of apoC-II (4 μg/ml) increased enzyme activity. The reaction was time and enzyme dependent.

The stimulation of the phospholipase activity of lipoprotein lipase by apoC-II was concentration dependent, as shown in Table I. Approximately 15% hydrolysis was observed with 1.6 μg of apoC-II, compared with 35% and 50% hydrolysis with 20 and 90 μg, respectively. In the next experiment, apoC-III was tested for its ability to activate. Table I shows that apoC-III slightly stimulated lipoprotein lipase activity. However, the possibility that apoC-II may contaminate the apoC-III preparation to 1% and thus account for the slight stimulation, cannot be discounted.

When apoC-III was added to mixtures of apoC-II, apoC-III had no appreciable effect at low ratios on the apoC-II activated phospholipase activity (Table I). However, high apoC-III levels (60 μg apoC-III to 20 μg apoC-II) inhibited the apoC-II activated lipase activity by approximately 20%.

TABLE I

EFFECTS OF APOLIPOPROTEIN C-II, APOLIPOPROTEIN C-III AND MIXTURES THEREOF ON PHOSPHOLIPASE ACTIVITY OF BOVINE MILK LIPOPROTEIN LIPASE[a]

ApoC-II μg	ApoC-III μg	Hydrolysis %
---	---	<1.0
1.6	---	17.0, 14.3
20.0	---	34.9, 35.7
90.0	---	50.0, 49.1
---	1.6	2.1, 3.0
---	20.0	4.9, 5.9
---	90.0	9.5, 10.0
20.0	10.0	32.6, 33.4
20.0	20.0	35.0, 36.0
20.0	30.0	34.4, 33.8
20.0	60.0	26.8, 28.5

[a]Each reaction mixture in duplicate contained in a final volume of 0.4 ml of 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4, the following: DMPC vesicles (1.1 mg); fatty acid-free bovine serum albumin (5 mg); and the indicated amounts of apoC-II or apoC-III. The reaction mixtures were incubated at 23.4°C for 15 min and then bovine milk lipoprotein lipase (2.8 μg) was added. After 90 min at 23.4°C, the % hydrolysis was determined as described in Materials and Methods.

Phospholipase activity of the bovine milk lipoprotein lipase was measured in the presence of different ions and the Ca^{++}-chelating agent, EGTA. No difference in enzyme activity was detected when reaction mixtures (0.4 ml of 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4, containing 5 mg fatty acid-free BSA, 1 mg DMPC, 25 μg apoC-II and 2.8 μg lipoprotein lipase) included 10 mM EGTA or 0.0, 1.5 and 5.0 mM $CaCl_2$. Protamine sulfate, although reported to be an inhibitor of the phospholipase activity of post-heparin lipoprotein lipase (12), had no effect on milk phospholipase activity when assayed at concentrations of either 3 mg/ml or 5 mg/ml. NaCl (1 M) inhibited the enzyme 10-20%.

DMPC vesicles were chromatographed on a Sepharose CL-48 column before and after treatment with bovine milk lipoprotein lipase to detect changes in the vesicular structure caused by hydrolysis (Fig. 2). Figure 2A shows the elution profile of DMPC vesicles alone. Figure 2B shows that [^{125}I]apoC-II binds to DMPC vesicles and at the concentrations used in the experiments did

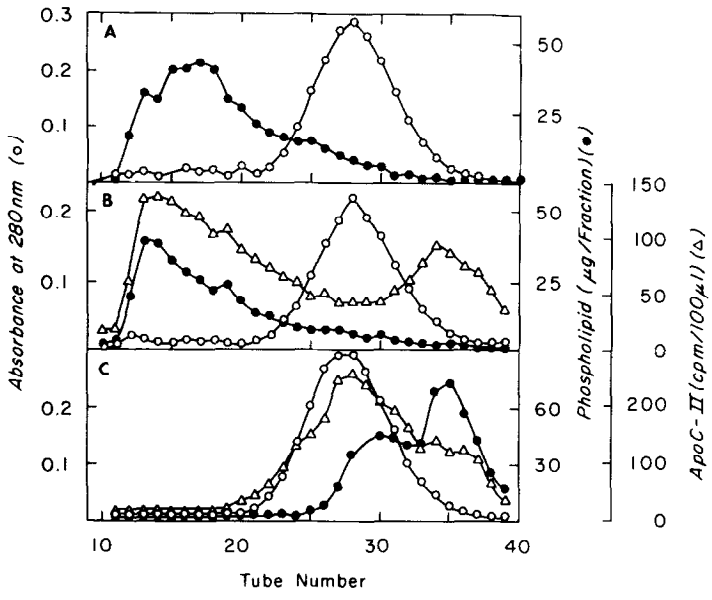

FIGURE 2. Sepharose CL-4B chromatography of DMPC vesicles before (A) and after (B) incubation with apoC-II and (C) apoC-II plus bovine milk lipoprotein lipase. (A) An incubation mixture containing 0.4 ml of 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4, 0.9 mg DMPC vesicles, and 5 mg fatty acid-free bovine serum albumin, was preincubated at 23.4°C for 15 min and then chromatographed at 24°C on a column (1.5 x 25 cm) of Sepharose CL-4B equilibrated with 0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4. The flow rate was 40 ml/hr and one ml fractions were collected. DMPC (—●—●—) and protein (—o—o—) content of each fraction was determined by assaying for phosphorous (18) and absorbance at 280 nm, respectively. (B) The incubation mixture was identical to (A) with the exception of the addition of [^{125}I]apoC-II (40 μg, 681 cpm/μg). The [^{125}I]apoC-II content (—Δ—Δ—) was determined in a Packard Auto Gamma Scintillation Spectrometer. (C) The reaction conditions were identical to (B). After incubation at 23.4°C for 15 min, bovine milk lipoprotein lipase (6.9 μg) was added and the mixture incubated at 23.4°C. After 90 min, the reaction mixture was chromatographed and analyzed as described above. An aliquot of the reaction mixture was extracted and the % hydrolysis was 85%.

not affect vesicular structure. The peak of [^{125}I]apoC-II at fraction No. 34 is unbound apoC-II; in the absence of lipid, 95% of [^{125}I]apoC-II eluted at this position. Figure 2C shows the elution profile of a mixture of DMPC vesicles, [^{125}I]apoC-II, BSA, and lipoprotein lipase after incubation for 90 min. No detectable vesicles were present and the phospholipid was mainly associated with the albumin fraction.

In all of the experiments reported above, the preincubation and incubation temperature was at 23.4°C, the phase transition temperature for DMPC. Since apoC-II and apoC-III are known to bind to DMPC vesicles at the phase transition (19), it was of interest to determine whether the phospholipase activity of

lipoprotein lipase was also maximal at 23.4°C. A 0.4 ml solution (0.1 M Tris-HCl, 0.15 M NaCl, pH 7.4) containing 1.1 mg DMPC and 5 mg BSA was preincubated at 23.4°C for 15 min in the presence and absence of apoC-II (18 µg); lipoprotein lipase (2.8 µg) was then added and the mixture incubated at various temperatures for 90 min. In the presence of apoC-II, incubation of the enzyme at 15, 23.4, 30, and 37°C yielded 2.7%, 19.0%, 28.7%, and 30.4% hydrolysis, respectively. In the absence of apoC-II, the % hydrolysis was 0.9, 1.5, 1.3 and 0.8

DISCUSSION

The results of the present study show that bovine milk lipoprotein lipase hydrolyzes DMPC, but only in the presence of the activating protein, apoC-II. In contrast to its inhibitory effect with triglyceride as substrate (5-8), apoC-III did not significantly inhibit the apoC-II activation of the enzyme with phospholipid as substrate. The latter finding is consistent with the recent report of Groot et al. (12), who showed that apoC-III did not inhibit the activation of post-heparin lipolytic activity by apoC-II in a perfused heart system using a synthetic substrate of both triglyceride and phosphatidylethanolamine. It is of interest that NaCl and protamine sulfate did not affect phospholipase activity. Similarly, Rapp and Olivecrona (20) have shown that heparin and NaCl have only a limited effect using tributyrin as substrate.

It seem reasonable to assume that the mechanism of activation by apoC-II is not due to apoprotein induced changes in the lipid structure, since both apoC-II and apoC-III react with DMPC. Although apoC-II binds to the DMPC at the phase transition temperature (19), the activity of the lipoprotein lipase toward DMPC vesicles is not maximal at this temperature. By comparison, pancreatic phospholipase A_2, an enzyme that does not require a co-factor, is inactive toward phosphatidylcholine vesicles at temperatures above or below the transition temperature (21).

Another possible explanation for the activation of the enzyme is that the apoprotein facilitates the interaction of the lipoprotein lipase with the substrate. However, in other experiments (unpublished data) we have shown that

lipoprotein lipase interacts with DMPC and can penetrate a monomolecular film of diglyceride and triglyceride even in the absence of apoC-II. Thus, it would appear that the mechanism of apoC-II activation is not due to an interaction of the apoprotein with the enzyme which then allows interaction with the lipid. A more likely explanation for the mechanism is that a non-lipid binding portion of apoC-II interacts with the enzyme. The studies of Kinnunen et al. (22) using synthetic fragments of apoC-II, and of Miller and Smith (23) using monolayer techniques, tend to support this conclusion.

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