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Apolipoprotein B exhibits phospholipase A_1 and phospholipase A_2 activities

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Low density lipoproteins (LDL) as well as isolated apolipoprotein B (ApoB) have been shown to exhibit phospholipase A_2 (PLA_2) activity toward phospholipids containing an oxidized or short fatty acyl chain at position 2. Some of these studies employed the fluorescent analogue of phosphatidyl choline (PC), C_6-NBD-PC, containing NBD-caproic acid (C_6-NBD-FA) at position 2 as a substrate, representative of short fatty acyl chains. The release of NBD-caproic acid from position 2 is attributed to PLA_2-catalysed hydrolysis. However, this fatty acid can be released also by other enzymatic pathways. In the present study we examined, and ruled out, other enzymatic pathways which may be responsible for the hydrolysis of fatty acids from position 2 of phospholipids. On the other hand, we found that LDL as well as isolated ApoB hydrolyse C_6-NBD-FA from both carbon 1 and carbon 2 of these phospholipids, thus exhibiting independent and simultaneous activities of phospholipase A_1 and phospholipase A_2.

Apolipoprotein B; Low density lipoprotein; Phospholipase A_1; Phospholipase A_2

1. INTRODUCTION

Low density lipoproteins (LDL) have been shown to contain phospholipase A_2 (PLA_2) activity as it hydrolyses oxidized or short fatty acids from the 2 position of phospholipids [1,2]. This activity is attributed to the apolipoprotein B (apoB) in the LDL particle as it is also exhibited by isolated ApoB [2]. Similar activity is exhibited also by LDL towards an exogenous substrate, C_6-NBD-PC, a fluorescent analogue of phosphatidylcholine which contains NBD-caproic acid (C_6-NBD-FA) at the 2 position [3]. Thus it has been postulated that the hydrolysis of C_6-NBD-PC by LDL reflects the activity toward short or oxidized fatty acyl chains at position 2 of LDL phospholipids [1]. The definition of this enzymatic hydrolysis as PLA_2 activity was based on determination of the fatty acid released from the 2 position. However, the fatty acid from this position may be released not only by a direct action of PLA_2, but also by alternative enzymatic pathways. These are mainly phospholipase C (PLC) followed by diglyceride lipase (DGL), or phospholipase A_1 (PLA_1) followed by lysophospholipase (LPase) [4], as depicted in Fig. 1. The present study was undertaken to examine the enzymatic pathways through which LDL, as well as isolated ApoB (delipidated LDL) hydrolyses C_6-NBD-PC, as representative of phospholipids containing short or oxidized fatty acyl chains.

We have found that LDL as well as isolated ApoB do not exhibit PLC or LPase activities, but exhibit independent activities of both PLA_2 and PLA_1 toward C_6-NBD-PC.

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Abbreviations. LDL, low density lipoproteins; ApoB, apolipoprotein B; C_6-NBD-PC, 1-acyl-2-(*N*-4-nitrobenzo-2-oxa-1,3-diazole)-aminocaproylphosphatidylcholine; C_6-NBD-FA, NBD-caproic acid; C_6-NBD-DAG, C_6-NBD-diacylglycerol; C_6-NBD-LPC, C_6-NBD-LysoPC; GPC, glycerophosphorylcholine; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C; LPase, lysophospholipase.

2. MATERIALS AND METHODS

2.1. *Preparation of LDL*

Human LDL was isolated from donor's blood by fractional centrifugation [5].

ApoB was isolated from human LDL by delipidation with acetone–butanol as previously described [2].

2.2. *Preparation of phospholipase A_1*

Rat brain PLA_1 was prepared from the lysosomal fraction as previously described [6].

2.3. *Preparation of C_6-NBD-diacylglycerol (C_6-NBD-DAG)*

C_6-NBD-PC in aqueous phase (650 μM) was interacted with 0.5 mg of phospholipase C (*Clostridium perfringens*; Sigma, St. Louis, MO) in 0.5 ml of Tris buffer at pH 7.4 for 1 h. The reaction mixture was chromatographed on silica thin-layer developed in C/M/H_2O (65:35:5). C_6-NBD-DAG was detected by comparison to known markers and extracted from the silica in chloroform. Its concentration was determined by its fluorescence intensity compared to standard solutions to C_6-NBD-FA in the same solvent.

2.4. *Preparation of C_6-NBD-LysoPC (C_6-NBD-LPC)*

The experiments described below showed that the commercially available C_6-NBD-PC is a mixture of about 80% 2-C_6-NBD-PC and

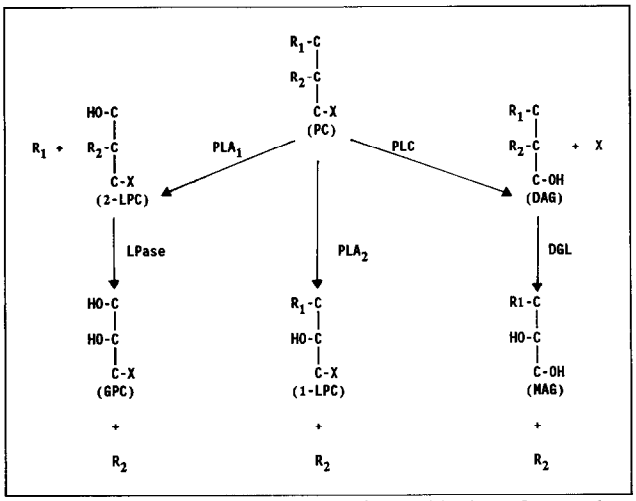

Fig. 1. Enzymatic pathways leading to fatty acid release from carbon 2 (R_2) of phosphatidylcholine. X, phosphorylcholine; PC, phosphatidylcholine; 1-LPC, 1-acyl-lysoPC; 2-LPC, 2-acyl-lysoPC; GPC, glycerophosphorylcholine; DAG, diacylglycerol; MAG, monoacylglycerol; PLA_1, phospholipase A_1; PLA_2, phospholipase A_2; PLC, phospholipase C; LPase, lysophospholipase; DGL, diglyceridelipase.

20% 1-C_6-NBD-PC. This finding was utilized here for preparation of two forms of C_6-NBD-LysoPC.

2.4.1. 1-C_6-NBD-LPC

In control experiments with snake venom PLA_2, as well as pancreatic PLA_2, we noticed that the interaction of these enzymes with the commercially available C_6-NBD-PC produced C_6-NBD-LPC in addition to C_6-NBD-FA, as shown in Fig. 2A. The amount of C_6-NBD-LPC accounted for about 20% of the total substrate. Exchange of fatty acid between carbon 1 and 2 in the course of phospholipid synthesis is a known phenomenon. Thus, this finding suggests that the substrate which is defined as PC having C_6-NBD at the 2 carbon, is not pure but contains about 20% of C_6-NBD linked to carbon 1. This possibility was confirmed by the manufacturer (Walter Shaw, Avanti, Birmingham; personal communication). This finding was utilized to obtain 1-C_6-NBD-LPC by interacting snake venom PLA_2 with C_6-NBD-PC. The reaction mixture was chromatographed on silica thin-layer, C_6-NBD-LPC was detected by comparison to an LPC marker, and extracted in C/M/H_2O (1:3:1).

2.4.2. 2-C_6-NBD-LPC

2-C_6-NBD-LPC was prepared by applying rat brain PLA_1 to C_6-NBD-PC (500 μM in 0.03 M acetate buffer, pH 4.2, containing 1 mg/ml Triton X-100 for 4 h at 37°C) [6]. The reaction mixture was chromatographed on silica thin-layer plates.

2.5. *Hydrolysis of C_6-NBD-PC*

C_6-NBD-PC dispersed in Tris buffer (100 mM, pH 7.2) was incubated at 37°C with either LDL, snake venom PLA_2, porcine pancreatic PLA_2 in Tris buffer, or with isolated ApoB in Tris buffer supplemented with 10 mM sodium deoxycholate. For detection of GPC the reaction was carried out in PBS buffer since Tris reacts with the detection spray. The reaction mixture (total of 100 μl) was applied to two separate TLC plates (LK6 Whatman, Clifton, NJ) and developed in two solvent systems: (1) Chloroform/methanol/water (65:35:5) [7] for separation of C_6-NBD-FA (R_f = 0.87), C_6-NBD-PC (R_f = 0.45), C_6-NBD-LPC (R_f = 0.125), and C_6-NBD-DAG (R_f = 0.95); (2) dichloromethane/methanol/water/formic acid (50:50:10:5) for separation of glycerophosphorylcholine (GPC; R_f = 0.13), which in the former system remains at the origin. The developed plates were examined by both fluorescence and one of the following colorimetric sprays: Molybdenum blue spray (Sigma, St. Louis) for detection of apolar phospholipids, or HCl/$HClO_4$/NH_4Mo spray for detection of GPC [8].

3. RESULTS AND DISCUSSION

The fluorescent analogue of phosphatidylcholine, C_6-NBD-PC, forms micelles in aqueous phase in which the fluorescence is diminished due to self-quenching [9]. As previously shown [9], upon addition of LDL, C_6-NBD-PC incorporates into the LDL lipid phase, as is evident from the increase in fluorescence intensity due to increased quantum yield and decreased self-quenching.

In the present study C_6-NBD-PC was interacted with LDL for various durations to obtain partial or complete hydrolysis. As demonstrated in Fig. 2A and Fig. 3 (lane b), under all conditions this interaction yielded only one fluorescent product, NBD-caproic acid (C_6-NBD-FA). No other fluorescent product was detected at any duration of the interaction. The same results were obtained by interaction of C_6-NBD-PC with isolated ApoB (lane c in Figs. 2A and 3).

As noted above, the release of a fatty acid from position 2 of phospholipids may be obtained by either direct action of PLA_2 or the alternative pathways, mainly PLC followed by DGL, or PLA_1 followed by LPase (see Fig. 1).

To examine the possibility that C_6-NBD-FA is produced by PLC followed by DGL, LDL or ApoB were incubated for various durations with C_6-NBD-DAG, obtained by the action of bacterial phospholipase C on C_6-NBD-PC, and the reaction mixture was analysed by thin-layer chromatography (see section 2). No C_6-NBD-DAG was hydrolysed by either LDL or ApoB at any duration. This rules out the action of PLC followed by DGL as the enzymatic pathway which produces C_6-NBD-FA from C_6-NBD-PC.

To examine the possibility that C_6-NBD-FA is produced from C_6-NBD-PC by PLA_1 followed by LPase, both 1- or 2-C_6-NBD-LPC were interacted with LDL or ApoB for various durations, as described in section 2. The reaction mixtures were then analyzed by thin-layer chromatography. Under all experimental conditions neither of these substrates was hydrolysed by either LDL or ApoB. This suggests that the pathway of PLA_1 followed by LPase is not responsible for the release of C_6-NBD-FA from C_6-NBD-PC by LDL.

As noted in section 2 and shown in Figs. 2A and 3 (lanes d and e), the interaction of C_6-NBD-PC with snake venom or pancreatic PLA_2 produces C_6-NBD-LPC in addition to C_6-NBD-FA. This is indeed expected in the light of the finding that this substrate contains about 20% of the NBD-caproic acid at position 1, which PLA_2 cannot hydrolyse. In contrast, as shown in Figs. 2 and 3, the interaction of C_6-NBD-PC with LDL or isolated ApoB, at any duration up to complete hydrolysis of the substrate (Fig. 3), never produced C_6-

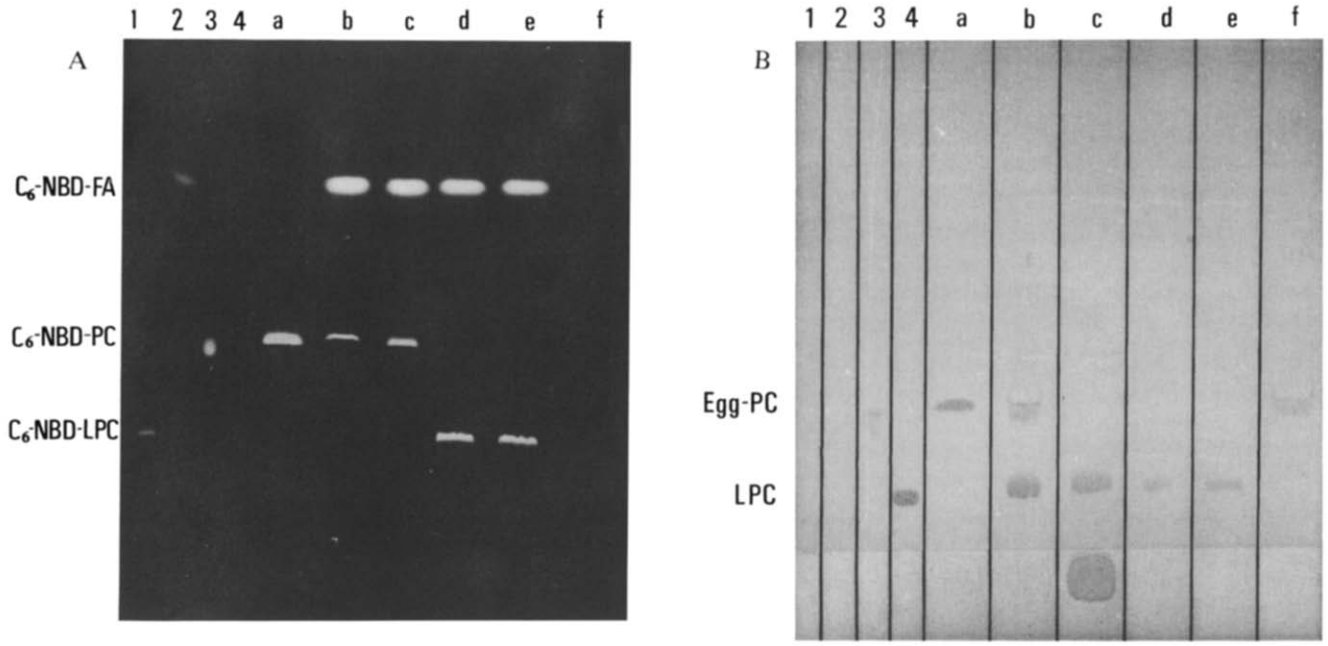

Fig. 2. (A and B) Thin-layer chromatograms of the enzymatic hydrolysis products of C_6-NBD-PC. C_6-NBD-PC (650 μM in final volume of 0.1 ml in Tris buffer at pH 7.4) was interacted for 2 h with the following moieties: (a) buffer alone (blank); (b) LDL, equivalent to 0.1 mg protein; (c) ApoB isolated from 0.3 mg LDL; (d) snake venom PLA_2, 1 μg; (e) pancreatic PLA_2, 1 μg. Lane (f) depicts LDL alone (blank) after incubation of 2 h. The reaction mixtures were then divided into two equal portions, each was applied to a separate thin-layer plate, and developed in C/M/H_2O (65:35:5). Plate A was photographed under UV light, showing the NBD-containing compounds. Plate B was sprayed with Molybdenum blue for detection of apolar phospholipids. Since NBD has strong yellow color, this plate was photographed through a filter, which blocks the yellow color, thus showing the phosphate-containing (blue) compounds. Lanes 1, 2, 3, 4 correspond to markers of C_6-NBD-LPC (1), C_6-NBD-FA (2), C_6-NBD-PC + egg PC (3), LPC (4).

NBD-LPC. Thus, C_6-NBD-FA is hydrolysed from both 1 and 2 positions of the substrate. This might be the result of either of two enzymatic pathways: (1) the action of PLA_2 to release C_6-NBD-FA from carbon 2 followed by LPase releasing C_6-NBD-FA from carbon 1, thus producing glycerophosphorylcholine (GPC); (2) independent action of PLA_1 and PLA_2 releasing C_6-NBD-FA from carbon 1 and 2, thus producing non-fluorescent lysoPC with long fatty acid at the alternate carbon.

To distinguish between these possibilities we performed the following procedures: C_6-NBD-PC (60 nmol) was subjected to complete hydrolysis (determined by the disappearance of the C_6-NBD-PC fluorescent band, as in Fig. 3) by LDL or ApoB, as well as snake venom or pancreatic PLA_2 (for control of pure PLA_2). The reaction mixture was chromatographed on silica thin-layer plate, containing a reference lane of GPC (marker), and developed for separation of GPC (see section 2). The amount of GPC used for reference was 20 nmol, which was easily detected by the $HCl/HClO_4$/NH_4Mo spray at R_f = 0.13. This is about one-third of the amount of GPC that would be expected to be produced by complete hydrolysis of the C_6-NBD-PC used in these reactions. Yet, under all treatments no GPC was detected, suggesting that neither LDL nor isolated ApoB hydrolyse LPC. On the other hand, as shown in

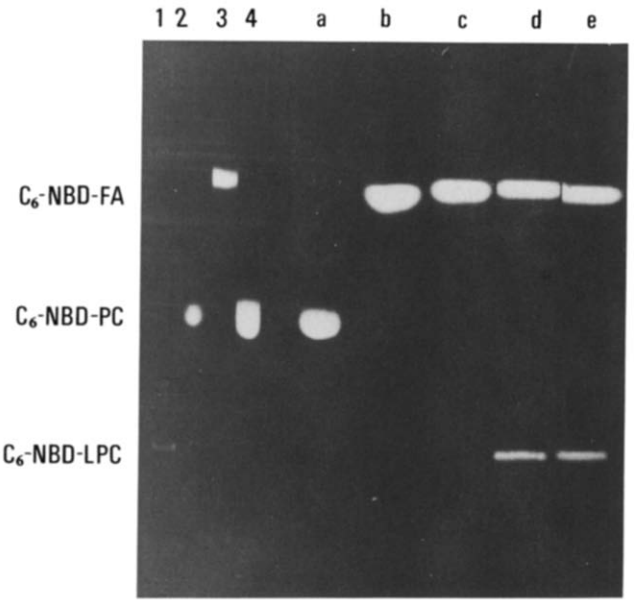

Fig. 3. Thin-layer chromatogram of C_6-NBD-PC fluorescent products after complete enzymatic hydrolysis. The same enzymatic system as in the experiment of Fig. 2A (a–e) were carried out, but the amount of LDL and ApoB was doubled to obtain complete hydrolysis of C_6-NBD-FA from C_6-NBD-PC. Lanes 1, 2, 3 and 4 correspond to the fluorescent markers C_6-NBD-LPC (1), C_6-NBD-PC (2), C_6-NBD-FA (3), and C_6-NBD-PC + LPC (4, to rule out nonenzymatic exchange between these substances).

Fig. 2B, both LDL and ApoB produced the non-fluorescent LPC in addition to C_6-NBD-FA, but not C_6-NBD-LPC (Fig. 2A). This clearly shows that LDL as well as isolated ApoB hydrolyse the NBD-linked caproic acid from carbon 1 and 2 independently, but do not hydrolyse the long chain fatty acid from the alternate carbon.

The results of this study demonstrate that ApoB, either isolated or in LDL, exhibits activity of both PLA_1 and PLA_2 towards the short fatty acyl chain in C_6-NBD-PC. As noted above, LDL as well as isolated ApoB have been reported to act as PLA_2 specific for oxidized or short fatty acyl chain [1,2] as measured by the hydrolysis of C_6-NBD-PC [1]. Since oxidized fatty acyl chain enhances the cellular uptake of LDL via the scavenger pathway, it has been proposed that ApoB, by removing the oxidized fatty acids from the 2 position of the LDL phospholipids, reduces its cellular uptake and thus plays a protective role. Our finding that ApoB exhibits both PLA_1 and PLA_2 activities suggests that ApoB may play this physiological role by removing undesirable fatty acid from both carbons of the LDL phospholipids.

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