Phospholipase A$_2$-catalysed modification of plasma low density lipoproteins caused reduction of hypercholesterolemic rabbit plasma cholesterol in vivo*

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Phospholipase A$_2$ (PLA$_2$) hydrolys certain phospholipids of low density lipoproteins. PLA$_2$-treated LDL is known to be rapidly cleared from plasma. A prototype plasma filter containing immobilized *Crotalus atrox* PLA$_2$ on agarose beads was developed. After a 90 min treatment with the extracorporeal device, plasma cholesterol concentration in cholesterol-fed NZW rabbits decreased by 32%. The decrease was dependent on the enzymatic activity in the plasma filter. The decrease in plasma cholesterol of hypercholesterolemic rabbits that were treated with control reactors (agarose beads only) was 5%. White and red blood cell counts and platelets remained unchanged during the treatment. Plasma cholesterol reduction (25-40%) was also obtained following intravenous injection of active PLA$_2$ to modify plasma lipoproteins. PLA$_2$ infusion created a radical change in biliary composition. Bile phospholipid composition was 90-95% lysophosphatidylcholine as compared with more than 95% before injection of active PLA$_2$. Phospholipid and bile salts total mass increased by 10%. While biliary secretion rate of protein increased by 10%, biliary secretion rate of cholesterol remained unchanged. This technique is specific for lipoproteins, does not require any fluid replacement of sorbent regeneration, and offers a potential new approach for lowering serum cholesterol and LDL levels.

1. Introduction
Coronary heart disease (CHD) accounts for more deaths annually than any other disease, including all forms of cancer combined [1]. Observational epidemiologic studies have established that the higher the total plasma cholesterol and low density lipoproteins (LDL) levels, the greater the risk that CHD will develop [2, 3]. Coronary heart disease arises from coronary arterial obstruction by atherosclerotic lesions. An early event in the formation of these lesions involves cholesterol accumulation, presumably from plasma low density lipoproteins.

Total plasma or LDL-cholesterol can be reduced by diet, drugs, or by direct removal of LDL from the blood. Drug therapies may have potential severe side effects which limits their use [4]. Plasmapheresis, the removal of the patient's plasma with replacement by donor albumin, has been a successful therapy [5, 6]. However, the cost and potential risk of transmission of contagious disease makes this technique less practical. Specific removal of LDL can be accomplished using affinity chromatography [7, 8]. This technique usually requires a two-column system. One column removes the LDL from the plasma while the other column is being regenerated. The limited capacity of the adsorbents makes these techniques cumbersome and expensive. Over the past few years therapies making use of immobilized enzymes have been investigated. We have studied the potential of LDL removal from plasma by modification with immobilized phospholipase A$_2$ (PLA$_2$). PLA$_2$ are a class of ubiquitous enzymes that hydrolyse the sn-2 fatty acyl ester bond of phospholipids generating free fatty acids and lysophospholipids. PLA$_2$ have been shown to hydrolyse some LDL phospholipids. Since recent studies have shown that PLA$_2$-LDL are rapidly removed from the plasma compartment, we have developed an extracorporeal reactor containing immobilized PLA$_2$. The efficacy of reactors were tested in vivo with hypercholesterolemic rabbits. We have also initiated some

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lysophosphatidylcholine (LPC) were separated by thin layer chromatography (TLC) and quantitated as described by Barlett [10]. The rabbits were fully conscious during the perfusion.

2. Experimental procedures

2.1. Materials

NAD⁺ and 3α-hydroxysteroid dehydrogenase were obtained from Sigma (St Louis, MO). Crotalus atrox PLA₂ was purified from the venom (Miami Serpentarium, Salt Lake City, UT) as reported by Hachimori et al. [9]. New Zealand white (NZW) rabbits were purchased from Hazelton (Denver, PA) and Watanabe heritable hyperlipemic (WHHL) rabbits from NIH (Bethesda, MD). Other reagents were of reagent grade from local suppliers.

2.2. Procedures

2.2.1. Procedure for in vivo reactor studies

New Zealand white rabbits fed a 0.5% cholesterol diet were used (average initial plasma cholesterol 300–350 mg dl⁻¹). After the rabbits were anesthetized, a vinyl catheter (1.14 x 1.63 mm from Bolab) was inserted in the jugular vein (7 cm) and carotid artery (5–10 cm) to draw blood. The extracorporeal treatments were performed one week after the catheters were implanted. The catheters were connected by a three-way plastic stopcock (entry and exit ports) by a 16-gauge needle (Becton-Dickinson) to the extracorporeal circuit (Masterflex size 14, Cole Palmer). Sepharose 4B-CL beads, onto which PLA₂ was immobilized, were packed into the column to form the active reactor. Control reactors consisted of non-activated Sepharose beads. Before each experiment, the circuit was primed with a sterile isotonic saline solution containing bovine serum albumin as a standard. PLA₂ was immobilized onto tresyl-chloride activated agarose beads as described by Nilsson and Mosbach [11]. PLA₂ was immobilized on agarose beads activated with tresyl chloride or N-hydroxysuccinimide. Immobilized PLA₂-beads were packed in a column (0.7 x 5 cm) in order to mimic in vivo studies. Plasma was pumped through the column in a closed circuit for 2 h at room temperature at a flow rate of 1 ml min⁻¹. Samples were withdrawn at different times. The radioactivity in washing solutions and plasma samples were determined. The fraction of radioactivity precipitable with 10% TCA was determined in each sample.

2.2.2. PLA₂ immobilization

PLA₂ was immobilized onto tresyl-chloride activated agarose beads as described by Nilsson and Mosbach [11] or via N-hydroxysuccinimide activated agarose as reported by Pharmacia [12]. To desorb non-covalently bound enzyme molecules, the support was washed with several volumes of PBS (0.5 μM NaCl, 0.07 μM phosphate, pH 7.0) as described by Mullon et al. [13]. The amount of immobilized enzyme determined from the difference between the initial total protein and the amount recovered in the PBS wash was approximately 1.6 mg ml⁻¹ of beads. The typical enzymatic activity was 60–70 units g⁻¹ of wet beads.

2.2.3. Activity of immobilized PLA₂

The catalytic activity of immobilized enzyme was quantitated by the method described by Nieuwenhuizen et al. [14]. One unit of PLA₂ is defined as the amount of enzyme hydrolysing 1 μmol of phosphatidylcholine min⁻¹ at pH 8.9 and 25°C.

2.2.4. Determination of enzyme leaching

Accurate measurements of enzyme leaching can be obtained with radiolabelled protein. C. atrox PLA₂ was iodinated with IODO-BEADS (Pierce). The 125I-PLA₂ was dialysed against 50 mM MOPS buffer pH 7.4 and immobilized as unlabelled PLA₂. The immobilized PLA₂-beads were packed in a column (0.7 x 5 cm) in order to mimic in vivo studies. Plasma was pumped through the column in a closed circuit for 2 h at room temperature at a flow rate of 1 ml min⁻¹. Samples were withdrawn at different times. The radioactivity in washing solutions and plasma samples were determined. The fraction of radioactivity precipitable with 10% TCA was determined in each sample.

2.2.5. Bile metabolism study

Twenty rabbits (cholesterol-fed NZW and WHHL rabbits) were anesthetized by intramuscular injection of Ketamine (40 mg kg⁻¹) and Xylazine (5 mg kg⁻¹). The abdomen was opened through transverse skin and muscle incisions. The cystic duct was ligated and the common bile duct was cannulated with a polyethylene catheter (Intramedic, Clay Adams, ID 1.1 mm, OD 1.6 mm). A second catheter of similar dimension was inserted in the duodenum. The free ends of both tubes were connected to allow bile to flow to restore the enterohepatic circulation. Bile (0.5–1 ml) was collected every 15 min for determination of flow, bile salts, cholesterol, protein, and phospholipids. The experimental and control rabbits were injected intravenously with active PLA₂ or heat inactivated PLA₂, respectively, through the marginal ear vein over a 1 h period.

2.2.6. Bile components analysis

Bile salts were quantitated enzymatically by the method of Turley and Dietschy [15]. Since no inorganic phosphate is present in bile, the total amount of phospholipids was directly measured by quantitating the amount of phosphorous present as described by Barlett [10]. Cholesterol was quantitated as described previously [16]. Protein concentrations were quantitated by the method of Lowry [17] with bovine serum albumin as a standard.

3. Results and discussion

PLA₂ modifies some LDL phospholipids. It was reported that LDL modified with PLA₂ was rapidly cleared from the blood pool. The fractional catabolic rate of PLA₂-LDL in hypercholesterolemic rabbits (WHHL or cholesterol-fed NZW rabbits) was 6–10 times greater than the catabolic rate of native LDL [18]. We therefore designed a device containing an immobilized PLA₂ reactor to modify extracorporeally plasma low density lipoproteins. C. Atrox PLA₂ was immobilized on agarose beads activated with tresyl chloride or N-hydroxysuccinimide. Immobilized PLA₂ was stable; no detectable loss of enzymatic