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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) hydrolyses 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 immobilizied PLA_2. The efficacy of reactors were tested *in vivo* with hypercholesterolemic rabbits. We have also initiated some

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studies to understand the mechanism of uptake of PLA_2-LDL by the liver.

2. Experimental procedures

2.1. Materials

NAD^+ and 3α-hydroxysteroid dehydrogenase were obtained from Sigma (St Louis, MO). *Crotalus atrox* PLA_2 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 300–500 mg dl^{-1}). After the rabbits were anesthesized, a vinyl catheter (1.14 × 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 PLA_2 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 10 units ml^{-1} heparin and 0.05% rabbit serum albumin of pH 7.4 and the rabbits were injected with heparin (250 units kg^{-1} body mass). All samples for biochemical determination were taken from the entry port with a syringe. Phosphatidylcholine (PC) and lysophosphatidylcholine (LPC) were separated by thin layer chromatography (TLC) and quantitated as described by Barlett [10]. The rabbits were fully conscious during the perfusion.

2.2.2. PLA_2 immobilization

PLA_2 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 M NaCl, 0.07 M 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 PLA_2

The catalytic activity of imobilized enzyme was quantitated by the method described by Nieuwenhuizen *et al.* [14]. One unit of PLA_2 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* PLA_2 was iodinated with IODO-BEADS (Pierce). The ^{125}I-PLA_2 was dialysed against 50 mM MOPS buffer pH 7.4 and immobilized as unlabelled PLA_2. The immobilized PLA_2-beads were packed in a column (0.7 × 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 anesthesized 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 entered 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 PLA_2 or heat inactivated PLA_2, 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

PLA_2 modifies some LDL phospholipids. It was reported that LDL modified with PLA_2 was rapidly cleared from the blood pool. The fractional catabolic rate of PLA_2-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 PLA_2 reactor to modify extracorporeally plasma low density lipoproteins. *C. Atrox* PLA_2 was immobilized on agarose beads activated with tresyl chloride or *N*-hydroxysuccinimide. Immobilized PLA_2 was stable; no detectable loss of enzymatic

activity was observed after storage at 4 °C for 2–3 months; 90% of the enzymatic activity was retained after one day.

Previous experiments have indicated that the extracorporeal system should include a hollow fibre plasma separator and a PLA_2-reactor [19]. The rabbits were treated for 90 min. The effectiveness of the treatment was verified *in vivo* by using reactors containing agarose and agarose-enzyme conjugates (1.5–3 g wet mass). As quantitated by TLC (10) the PLA_2 reactors modified 80–95% of the plasma phospholipids after a 60–90 min treatment. Control experiments showed no phospholipid modification. The plasma cholesterol concentrations were monitored for 3 h following the beginning of treatment. The average decrease in plasma cholesterol concentration was 15–20% at the end of treatment (90 min) and $32 \pm 10\%$ at 3 h (Fig. 1). In contrast, in the control studies no significant drop in plasma cholesterol was observed ($P < 0.05$).

Treatment efficacy was tested by using reactors with varying enzymatic activities. It was found that increasing the enzymatic activity resulted in an increased reduction in total cholesterol after 90 min treatment (Fig. 2). The further decrease in plasma cholesterol

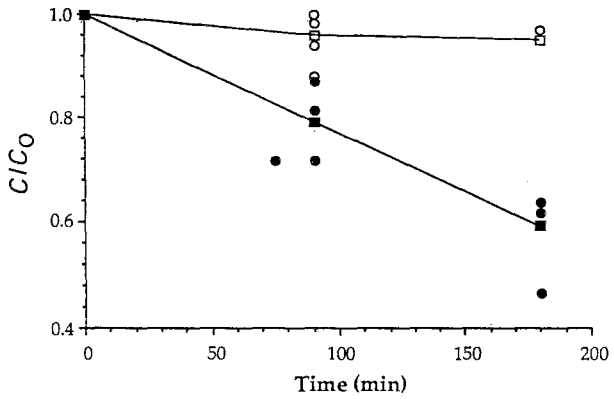

Figure 1 Normalized plasma cholesterol concentration curve C/C_0 for rabbits treated with PLA_2-reactor (○) against rabbits treated with control reactors (●).

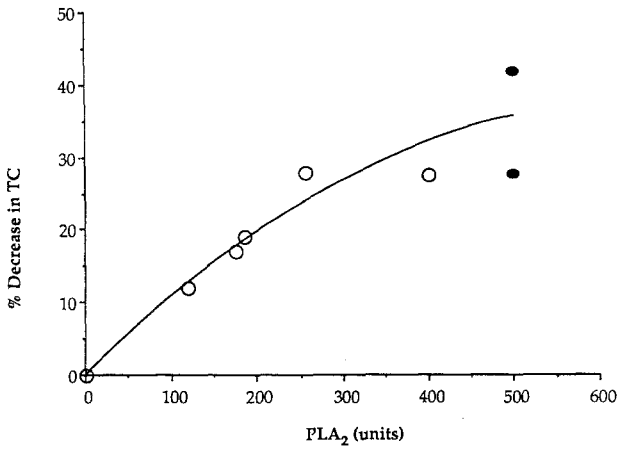

Figure 2 Decrease in plasma cholesterol concentration in hypercholesterolemic rabbits against reactor activity on completion of 90 min treatments. (○) Extracorporeal PLA_2-circuit and (●) intravenous infusion of PLA_2.

concentrations after treatment may be attributed to metabolism of PLA_2-modified LDL.

Blood samples at the end of the treatment revealed no significant changes in white and red blood cell counts, platelets or hematocrit. The concentrations of albumin, uric acid, bilirubin, and liver enzymes remained within normal ranges. Plasma free hemoglobin levels were less than 0.1–0.2 g dl^{-1}. The level of high density lipoproteins, measured after LDL or PLA_2-LDL precipitation with heparin/manganese chloride [20] showed no significant change after the treatment.

As an approach to understanding the metabolism of PLA_2-LDL *in vivo*, we have studied the fate of PLA_2-LDL. Biodistribution studies have indicated that PLA_2-LDL accumulated primarily in the liver [18]. The liver also plays an important role in bile acid metabolism. Liver is the sole organ that converts cholesterol to bile acids, and it secretes bile acids vectorially into bile. Bile is composed primarily of bile salts, cholesterol, phospholipids (predominantly PC), protein, bilirubin, and organic salts. We have therefore determined the changes in bile composition following modification of LDL in plasma. Modification of LDL was obtained by intravenous infusion of soluble PLA_2 (200–500 units, at a constant flow rate over a 1 h period) into hypercholesterolemic rabbits (NZW and WHHL). Ninety percent of plasma PC was hydrolysed to LPC after the injection of PLA_2 (data not shown). No change in PC content was observed in rabbits that received injections of heat inactivated PLA_2.

3.1. Lecithin and bile salts secretions

Following modification of LDL in plasma an increase in bile salts and phospholipids released was observed (Figs 3 and 4). Bile phospholipid composition was 90–95% LPC as compared with more than 90% before injection of active PLA_2 (Fig. 5), while phospholipid total mass increased by 10%. In control experiments, no LPC was detected in the bile. Modification of PC in plasma coincided with the release of LPC in the bile. Thus PLA_2 infusion induced a radical change in biliary phospholipid composition. Therefore, most LDL phospholipids are secreted through the bile as lysophospholipids. Transport, utilization, and biliary secretion of LPC was studied in rats by Angelico *et al.* [21]. In their studies LPC was rapidly cleared from the plasma by the liver and was either released in the bile or rapidly acylated with free fatty acids by microsomal enzymes. This mechanism may prevent elevated concentrations of LPC, a haemolytic lipid, in the plasma. LPC is a good detergent that will solubilize cholesterol released in the bile, thus preventing gallstone formation.

3.2. Cholesterol secretion

The rate of cholesterol release into the bile was also measured. We first noticed a significant difference in cholesterol release rate between the two rabbit models (WHHL and NZW rabbits) used in this study. The

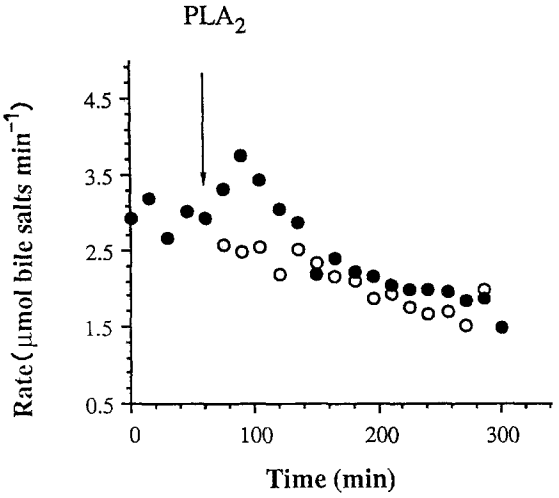

Figure 3 Rate of bile salt released in rabbits that received infusion of PLA_2 (●) against control rabbits that received inactivated PLA_2 (○). The enzyme was infused beginning at 60 min for a 1 h period.

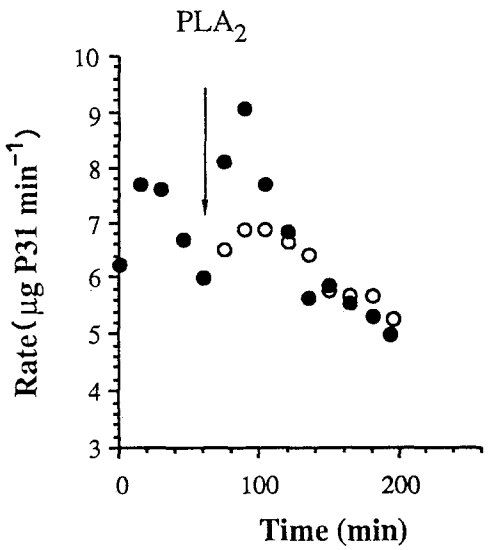

Figure 4 Rate of phosphorous (P31) release in rabbits that received infusion of PLA_2 (●) against control rabbits that received inactivated PLA_2 (○). The enzyme was infused beginning at 60 min for a 1 h period.

basal rates in WHHL and NZW rabbits were on the average 0.5 μg min^{-1} and 8 μg min^{-1}, respectively. The rate of cholesterol release in the bile in rabbits infused with PLA_2 was similar to the basal rate (data not shown). A constant release of cholesterol in the bile will prevent cholesterol gallstone formation.

During the course of the studies with PLA_2 infusion, plasma lipid concentrations were measured. Upon injection of active PLA_2, the average plasma cholesterol decrease was 27% and 32% for WHHL and cholesterol-fed NZW rabbits, respectively. In contrast, upon infusion of inactivated enzyme, the decrease was only 2–6%. The decrease in total cholesterol observed with infusion of soluble PLA_2 fit the data generated with our extracorporeal reactors (Fig. 2). Therefore it may be possible to develop a treatment method for decreasing plasma low density lipoproteins without the use of an extracorporeal reactor by injection or implantation of long-acting

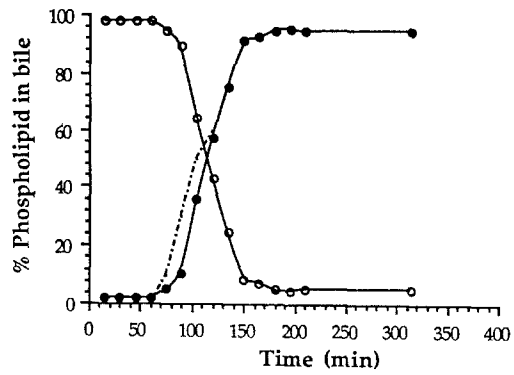

Figure 5 Percentage of PC (○) and LPC (●) released in the bile upon infusion of *C. atrox* PLA_2. The enzyme was infused beginning at 60 min for a 1 h period (–·–·).

PLA_2. Modifications of the soluble enzyme could be used including polyethylene glycol-conjugated PLA_2 or other modifications (which could alter both the lifetime and immunogenicity of the enzyme as well as its haemolytic activity), such as antibody (directed to LDL) bound to PLA_2 for targeting, as well as other injectable enzyme modifications.

3.3. Protein secretion

Protein concentrations in bile are low, of the order of 60–400 $mg\,l^{-1}$ in canine bile and 300–3000 $mg\,l^{-1}$ in human bile [22]. Biliary excretion of proteins was quantitated upon infusion of PLA_2 in rabbits. An increase was observed with time (data not shown). The concentration values ranged from 500 to 4000 $mg\,l^{-1}$, which was similar to human bile. The bile proteins were analysed by polyacrylamide gel electrophoresis. The major band corresponded to albumin. Albumin was the only protein that was positively identified and was the only band whose intensity varied from sample to sample. No PLA_2 was secreted in the bile, which was consistent with our biodistribution data showing that only 0.09% of the injected ^{125}I-PLA_2 radioactivity was recovered in the bile (data not shown). Albumin is the most abundant plasma protein and biliary albumin is derived from the plasma pool. Other bile proteins probably originate from plasma as well, and their concentration appears to be inversely related to their molecular mass [23–25]. The bile contains a variety of lysosomal enzymes, presumably excreted via haepatocyte exocytosis independently of bile acid secretion. It also contains many plasma membrane ectoenzymes. Immunoglobulin A is found in bile in appreciable amounts, especially in the rat. It is taken up by liver cell receptor-mediated endocytosis and secreted into bile by vesicular transport [26]. The observation that albumin seemed to vary over time indicated that albumin served as a protein carrier for the secretion of LPC. Since PLA_2 was not found in the bile it can be concluded that biliary LPC arose from plasma LPC and not biliary PC that was modified in the bile.

Although additional studies must be performed to determine the long-term effect of extracorporeal treatments with PLA_2, the reported studies show that

reduction of plasma cholesterol could be achieved in cholesterol-fed NZW following a short treatment. The decrease is correlated to the amount of enzymatic activity. The study also shows that the uptake of PLA_2-LDL is mediated by the liver. This new technique may be useful in the treatment of severe hypercholesterolemia and provide a potentially new application for the use of immobilized enzyme to remove toxic substances in blood [27–29].

Acknowledgement

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