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Lecithin–cholesterol acyltransferase activity in normocholesterolaemic and hypercholesterolaemic roosters: modulation by lipid apheresis

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Abstract. Lipid apheresis, a recently described procedure for the elimination of lipid but not apolipoproteins from plasma, was applied to normocholesterolaemic and hypercholesterolaemic roosters. Lipid apheresis resulted in an immediate reduction in plasma unesterified cholesterol concentration, which was sustained for 150 min. The reduction in unesterified cholesterol concentration was higher in the normocholesterolaemic animals than in the hypercholesterolaemic animals. Lipid apheresis induced changes in the ratio of plasma unesterified to total cholesterol in normocholesterolaemic animals but not in hypercholesterolaemic animals. In hypercholesterolaemic animals, lecithin–cholesterol acyltransferase (LCAT) activity was not affected by lipid apheresis, whereas in normocholesterolaemic animals LCAT activity was acutely reduced for 150 min after lipid apheresis. Saturated LCAT kinetics occurred in the hypercholesterolaemic animals but not in the normocholesterolaemic animals. LCAT obeyed Michaelis–Menten kinetics. After lipid apheresis, there was a pool of unesterified cholesterol that was available as substrate for LCAT to a greater extent in hypercholesterolaemic animals than in normocholesterolaemic animals. These observations may have important implications for lipid apheresis as a treatment for atherosclerosis.

Keywords. Atherosclerosis, hypercholesterolaemia, lecithin–cholesterol acyltransferase (LCAT), lipid apheresis, rooster.

Introduction

Atherosclerosis and its primary clinical manifestation, coronary heart disease (CHD), is the major cause of death in developed countries. CHD is a growing problem in the developing countries. It is generally acknowledged that CHD is a multifactorial disease [1]. Evidence from epidemiological and clinical trials in different populations links hypercholesterolaemia to the chain of events that lead to atherosclerosis [2,3]. On the assumption of a causal relation between hypercholesterolaemia and CHD, preventive and therapeutic strategies have been developed to reduce plasma cholesterol levels [1]. Studies have demonstrated that lowering of high plasma cholesterol levels reduces CHD morbidity and mortality [3,4]. If the appropriate lipid-lowering effect is not achieved by diet and other lifestyle measures then pharmacological intervention must be considered [5]. If these conventional therapies fail to control hyperlipidaemia-induced atherosclerosis, then surgical procedures, angioplasty or extracorporeal removal of lipoproteins may be considered.

We recently reported a new procedure for the extracorporeal removal of lipids, but not apolipoproteins, from plasma [6]. The attributes of this procedure as opposed to low-density lipoprotein (LDL) apheresis have been described previously [6]. Essentially, lipid apheresis (LA) results in an acute reduction in circulating plasma cholesterol levels with no effect on apolipoproteins or biochemical or haematological parameters. This effect lasts much longer in normocholesterolaemic animals than in hypercholesterolaemic animals. It has been suggested that this differential response is due to extraplasmatic (outside the plasma compartment) cholesterol pools that are dynamically mobilizable by the apolipoproteins in the treated plasma [6,7]. Following LA, conspicuous changes in the high-density lipoprotein (HDL) and very high-density lipoprotein (VHDL) fractions have also been observed [7].

It has been speculated that apolipoproteins in HDL and VHDL [8] interact with peripheral cell membranes to form lipid–protein complexes with recruitment of membrane cholesterol, thus playing a role in reverse cholesterol transport and the prevention of atherosclerosis [9]. LCAT is an enzyme that is involved with the esterification of unesterified cholesterol, which may be of cellular origin. Thus, the objective of this study was to examine the relationship between the activity of LCAT and its substrates, lecithin (phosphatidylcholine) and unesterified cholesterol, in normocholesterolaemic and hypercholesterolaemic roosters that had been subjected

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to LA. The rooster was chosen for this study for a variety of reasons as discussed previously [6], but essentially because it can spontaneously develop a human-like atherosclerotic plaque when fed a cholesterol diet [10].

Materials and methods

Experimental animals

The roosters used in this study were of White Leghorn Hiline strain and were obtained as 1-day-old chicks. All roosters from 8 weeks of age were transferred into individual cages. Water and feed were unrestricted. At 8 weeks of age, four control birds were fed a commercial poultry ration for 31 days and another group of four birds was fed on the same diet but supplemented with 2·6% cholesterol for a period of 31 days. All four animals of each group were then subjected to LA (see below). All animals were kept off their feed for 3 h following reinfusion of their autologous blood. The animals were conscious throughout the entire experiment. The care of the animals was in accordance with the ethical standards of the University of Queensland.

Lipid–apheresis procedure

The LA procedure was performed as described previously [6]. Briefly, approximately 25% of the calculated blood volume was collected from a brachial vein of the animal with a 21-gauge needle and syringe. The total blood volume was estimated as 8% of the body weight [11]. The blood was collected in heparinized tubes and immediately centrifuged at $900 \times \boldsymbol{g}$ for 5 min at room temperature. The plasma was separated from the blood cells. The blood cells were kept in the tubes at room temperature. The plasma was delipidated for 20 min with a mixture of butanol–diisopropyl ether (DIPE) (25:75, v/v) in a ratio of one volume of plasma to two volumes of butanol–DIPE mixture (organic phase) [12]. After extraction, the mixture was centrifuged at $900 \times \boldsymbol{g}$ for 2 min at room temperature to separate the plasma and organic phase. The organic phase (upper layer) was removed, free of plasma phase, by careful aspiration with a Pasteur pipette under vacuum. Traces of butanol in the plasma phase were washed out with two volumes of diethyl ether (DEE) for 2 min by end-over-end rotation at $9 \times \boldsymbol{g}$. The mixture was then centrifuged at $900 \times \boldsymbol{g}$ for 2 min to separate the plasma and ether phase. The ether phase was subsequently removed by aspiration with a Pasteur pipette. Residual ether was removed by evacuation with a water pump aspirator at 37 °C. This procedure yielded delipidated plasma free of solvent. The plasma samples were not treated with antioxidants or preservatives. The delipidated plasma was remixed with the original blood cells, then reinfused through a brachial vein back into the identical donor animals. The duration of the entire procedure (that is removal of blood from the animal to reinfusion of treated blood back to the animal) was approximately1 h (range 50–70 min).

Laboratory measurements

Measurements of plasma levels of total cholesterol, unesterified cholesterol and choline-containing phospholipids were determined by enzymatic methods [13,14] using reagents from Boehringer Mannheim and a Cobas-Bio centrifugal analyser (Hoffman-La Roche, Zurich, Switzerland). LCAT activity was determined essentially as described previously [15,16]. LCAT activity was inhibited by 5,5-dithiobis 2-nitrobenzoic acid (DTNB) during the 4-h equilibration of labelled and unlabelled cholesterol. This equilibration time was shown to be optimal. The enzyme reaction was initiated by the addition of excess mercaptoethanol and the samples were incubated for 16 h. The reaction was rectilinear for 16 h. The reaction was terminated by the addition of 20 volumes of chloroform–methanol (2:1, v/v) and lipids extracted according to Folch *et al.* [17]. The lipids were then separated by thin-layer chromatography and the fractions comprising unesterified and esterified cholesterol were scraped off and radioactivity determined by liquid scintillation. LCAT specific activity was expressed as μmol cholesterol ester (CE) formed per litre of plasma per hour ($\mu mol\, L^{-1}\, h^{-1}$). The LCAT 'fractional rate', i.e. the fraction of labelled CE to total CE formed, was also calculated.

Statistical analyses

Comparisons between the various times after reintroduction of delipidated autologous blood were made with respect to the percentage change from baseline for the following variables: unesterified cholesterol, LCAT activity and ratio of unesterified to total cholesterol.

Within-group comparisons were made using Wilcoxon's signed-rank test. Adjusted mean percentage changes were obtained using an analysis of covariance on the individual percentage changes with treatment as a model effect and baseline value as a covariate. Adjusted mean percentage changes were similar to the equivalent unadjusted values and therefore the difference in unadjusted mean percentage changes was considered an appropriate estimate of the difference between baseline value and treated value.

The comparability of the treatment groups of baseline and post-infusion levels of delipidated autologous blood resulting in the reduction of plasma lipids was assessed by means of analysis of variance on the ranked values of the reduction in plasma lipid levels with delipidated blood infusion as a factor.

Two-sided tests were used for all statistical comparisons of LA treatment. All probability values were rounded to two decimal places and the statistical significance of LA treatment or dietary treatment comparisons was declared if the rounded probability values (P) was less than or equal to 0·05.

Table 1. Effect of control and high cholesterol diets on plasma total-cholesterol, unesterified cholesterol and LCAT levels

Component	Control diet ($n = 4$)	High-cholesterol diet ($n = 4$)
Total cholesterol (mmol L^{-1})	3·53 ± 0·80	15·0 ± 4·78*
Unesterified cholesterol (mmol L^{-1})	1·33 ± 0·32	5·84 ± 2·00*
Ratio: unesterified cholesterol–total cholesterol	0·40 ± 0·18†	0·42 ± 0·13†
LCAT fractional rate‡	0·16 ± 0·09	0·06 ± 0·01*
LCAT activity (μmol L^{-1} h^{-1})	279·5 ± 152·8	327·1 ± 40·5

* $P < 0{\cdot}001$ when compared with the corresponding control.
† The ratio for each individual animal was calculated and the mean of these data determined.
‡ The fraction of labelled to total cholesterol esterified by LCAT.

Results

Effect of high-cholesterol diet on plasma cholesterol concentrations and LCAT activities

Roosters fed on a high-cholesterol diet for 31 days became hypercholesterolaemic. The plasma total and unesterified cholesterol concentrations increased four-fold (Table 1). Consequently, the ratio of unesterified cholesterol to total cholesterol remained constant. The enzymatic percentage esterification of unesterified cholesterol to cholesterol ester by LCAT (fractional rate) decreased approximately 60% in the hypercholesterolaemic plasma compared with normocholesterolaemic controls. If the total pool of unesterified cholesterol was taken into consideration (LCAT activity) then there were no differences in LCAT activity when hypercholesterolaemia was compared with normocholesterolaemia (Table 1).

Effect of infused delipidated autologous plasma on plasma unesterified cholesterol over a period of 16 h

Infusion of delipidated autologous plasma (approximately 25% of plasma volume) resulted in a rapid reduction in plasma unesterified cholesterol concentrations (Fig. 1). The percentage changes induced by LA in normolipidaemic and hypercholesterolaemic animals were immediate and sustained for up to at least 150 min. The mean percentage reduction in the plasma unesterified cholesterol concentration was as high as 60% for normolipidaemic controls and up to 40% for hypercholesterolaemic animals. Sixteen hours after LA, the plasma concentration of unesterified cholesterol returned to pretreatment values in both groups of animals.

Effect of infused delipidated plasma on ratios of unesterified cholesterol to total cholesterol

LA of normocholesterolaemic animals resulted in significantly ($P < 0{\cdot}05$) decreased ratios of unesterified to total cholesterol (Table 2). In the case of the hypercholesterolaemic animals the ratios remained remarkably constant and were not significantly different from the pre-LA level (Table 2).

Relationship between lecithin and unesterified cholesterol

There were linear relationships between unesterified cholesterol concentrations and lecithin concentrations before and at various times after LA apheresis in the hypercholesterolaemic and normocholesterolaemic animals (Fig. 2). Although the mean concentrations of plasma cholesterol were markedly different between the groups, the mean lecithin concentrations remained essentially unchanged (mean ± SD: control, 2·50 ± 0·45 mmol L^{-1}; hypercholesterolaemic, 3·01 ± 0·47 mmol L^{-1}) and within the same ranges for both groups (Fig. 2). Subsequently, the ratio of unesterified cholesterol to lecithin was approximately fivefold

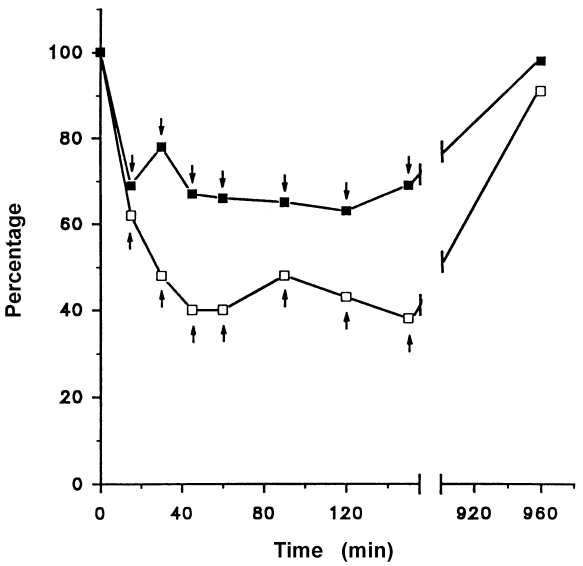

Figure 1. Changes in plasma unesterified cholesterol concentration after lipid apheresis. The plasma unesterified cholesterol concentrations of roosters on control diet (□) and hypercholesterolaemic diet (■) were determined before and at various times after reinfusion of lipid apheresed blood. The results are expressed as percentage changes. The data from the two groups of animals were pooled to determine statistical differences. Arrows indicate significant differences ($P < 0{\cdot}05$) of those values compared with the prelipid apheresis values (100%).

Table 2. Effect of lipid apheresis on absolute concentrations of plasma unesterified cholesterol, total cholesterol and the ratio of unesterified to total cholesterol

	Control ($n = 4$) Cholesterol (mmol L^{-1})			Hypercholesterolaemic ($n = 4$) Cholesterol (mmol L^{-1})		
Time after LA (min)	Unesterified	Total	Unesterified Total	Unesterified	Total	Unesterified Total
0	1·33 ± 0·32	3·53 ± 0·80	0·40 ± 0·18	5·84 ± 2·00	15·00 ± 4·78	0·42 ± 0·13
15	0·79 ± 0·12*	3·31 ± 0·63	0·25 ± 0·07*	4·07 ± 1·24*	11·30 ± 3·40	0·37 ± 0·12
30	0·59 ± 0·14*	3·00 ± 0·85	0·22 ± 0·12*	4·42 ± 1·36*	12·90 ± 1·36	0·34 ± 0·09
45	0·50 ± 0·06*	2·76 ± 0·56	0·19 ± 0·05*	3·80 ± 0·56*	10·50 ± 2·47	0·38 ± 0·11
60	0·51 ± 0·18*	2·83 ± 0·89	0·18 ± 0·03*	3·86 ± 0·84*	9·90 ± 2·49	0·37 ± 0·04
90	0·78 ± 0·18*	2·76 ± 0·77	0·34 ± 0·19*	3·65 ± 0·78*	9·80 ± 2·71	0·39 ± 0·10
120	0·65 ± 0·34*	2·63 ± 0·85	0·28 ± 0·19*	3·51 ± 0·61*	9·96 ± 2·31	0·36 ± 0·07
150	0·53 ± 0·26*	2·50 ± 0·28	0·22 ± 0·13*	3·82 ± 0·96*	9·76 ± 1·71	0·39 ± 0·07

*$P < 0·05$ when compared with the zero time point (pre-LA). Data represent means ± SD.

higher in the hypercholesterolaemic group (1·37 ± 0·10) than in the normocholesterolaemic group (0·26 ± 0·71).

Effect of infused delipidated plasma on LCAT activity

LA did not cause any changes in the LCAT activity in the hypercholesterolaemic animals. However, in the case of normocholesterolaemic animals, there was an immediate reduction in plasma LCAT activity (Fig. 3). This reduction was highly significant and was sustained for up to 150 min after LA. Sixteen hours after LA, LCAT activity had returned to its pretreatment value.

Effect of the absolute concentrations of unesterified cholesterol on LCAT activity following LA

Before and at various times after LA, the mean plasma unesterified cholesterol concentrations in the normocholesterolaemic animals ranged from 1·33 to 0·50 mmol L^{-1}, whereas in the hypercholesterolaemic animals the mean plasma unesterified cholesterol concentration ranged from 5·84 to 3·51 mmol L^{-1} (Table 2). There was a highly significant ($r = 0·84$; $P < 0·001$) linear correlation between the observed LCAT activity and unesterified cholesterol in the plasma of the normocholesterolaemic animals. In contrast, there was no

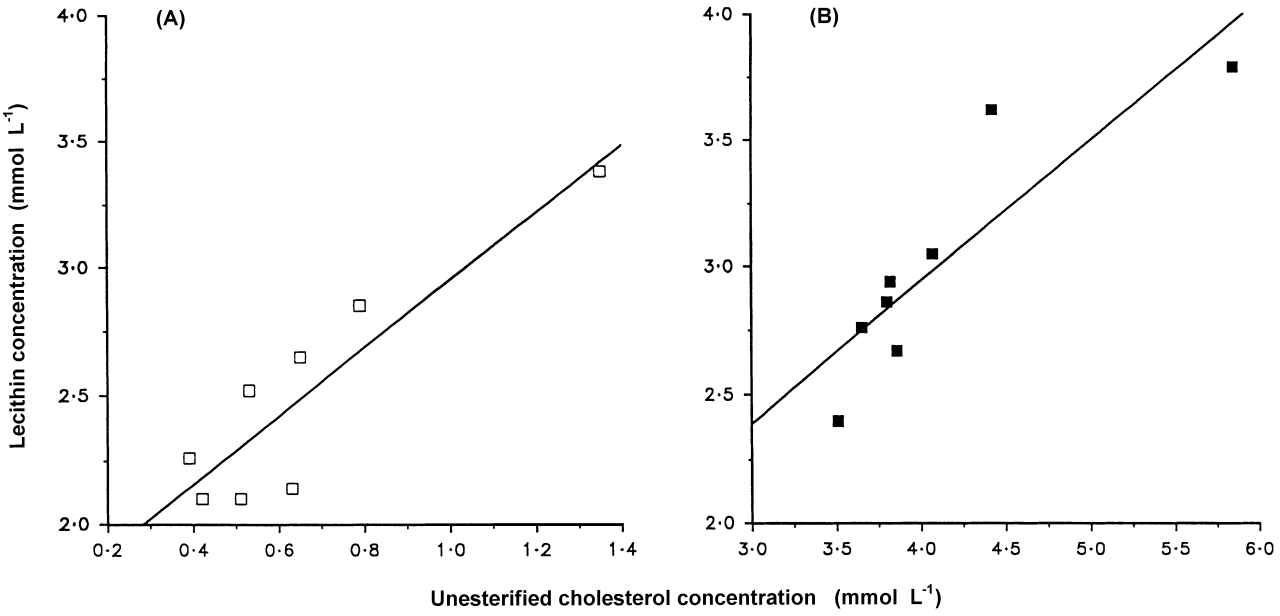

Figure 2. Relationship between lecithin and unesterified cholesterol before and at various times following lipid apheresis in hypercholesterolaemic (■) and normocholesterolaemic (□) roosters. Results are expressed as mean values at each time point. The plasma lecithin concentrations were in the same range for both groups of animals (see Results). There were highly significant ($P < 0·001$) linear correlations between lecithin concentrations and unesterified cholesterol concentrations in the hypercholesterolaemic group ($r = 0·88$) and normocholesterolaemic group ($r = 0·90$) of animals.

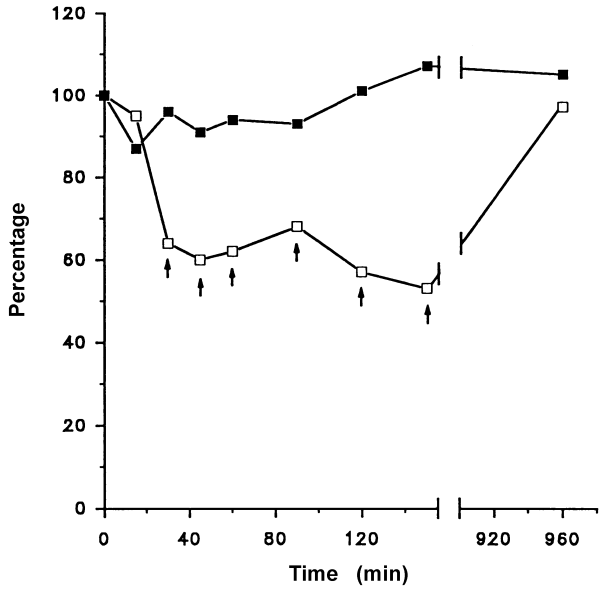

Figure 3. Changes in plasma LCAT activity after lipid apheresis. The plasma LCAT activities (μmol L^{-1} h^{-1}) of roosters on control diet (□) and hypercholesterolaemic diet (■) were determined before and at various times after reinfusion of lipid apheresed blood. The results are expressed as percentage changes. The data from the two groups of animals were pooled to determine statistical differences. Arrows indicate significant differences ($P < 0{\cdot}05$) in those values compared with the prelipid apheresis values (100%).

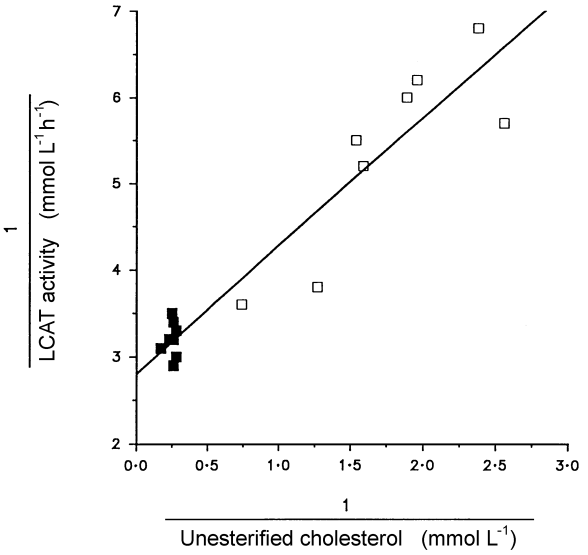

Figure 4. Double-reciprocal plot of LCAT activity and unesterified cholesterol concentration. The plasma LCAT activities (μmol L^{-1} h^{-1}) of roosters on control diet (□) and hypercholesterolaemic diet (■) were determined before and seven times after reinfusion of lipid apheresed blood. Results are the mean values at each time point. For the plot ($r = 0{\cdot}95$) the calculated K_m and V_{max} are 1·89 mmol L^{-1} and 676 μmol L^{-1} h^{-1} respectively. In calculating K_m and V_{max}, a number of assumptions were made (see Results).

correlation between such parameters in the hypercholesterolaemic animals ($r = 0{\cdot}12$).

Figure 4 illustrates a double-reciprocal plot of LCAT activity and unesterified cholesterol concentration in both the normolipidaemic and hypercholesterolaemic animals before and at various times after LA. From this Lineweaver–Burk plot, a V_{max} of 676 μmol L^{-1} h^{-1} and a K_m of 1·89 mmol L^{-1} were obtained. The highly significant linear correlation ($P < 0{\cdot}0001$) and the high linear correlation coefficient ($r = 0{\cdot}95$) of the plotted parameters indicate that LCAT obeyed Michaelis–Menten kinetics. In determining K_m and V_{max} from Fig. 4 a number of assumptions were made. Firstly, the concentration of LCAT protein present in plasma samples obtained from the same animal before and after LA treatment is constant. This assumption is reasonable given that new synthesis of LCAT protein is unlikely to occur immediately after LA. LCAT is not known to be an acute phase reactant. Secondly, as the specific activities of LCAT (substrate-saturating conditions) in pre-LA plasma samples from the normocholesterolaemic and hypercholesterolaemic groups were not statistically different (Table 1), it can be assumed that the amount of LCAT protein is similar in both groups. Thirdly, the LCAT activity returned to pre-LA values 16 h after LA treatment (Fig. 3). Furthermore, if we assume that the amount of LCAT protein is *not* similar in both groups and recalculate the K_m and V_{max} excluding the hypercholesterolaemic results, values of K_m and V_{max} are obtained that are similar to those in which all values are included (i.e. $K_m = 1{\cdot}54$ mmol L^{-1}; $V_{max} = 613$ μmol L^{-1} h^{-1}; $r = 0{\cdot}85$).

Discussion

The present study has investigated the effect of a recently described novel LA procedure [6] on LCAT activity, an important enzyme involved in reverse cholesterol transport. LCAT activities in pre-LA treated roosters are approximately 3·5-fold and 2·7-fold that of human and monkey respectively [15], and the plasma levels of total and unesterified cholesterol of rooster are remarkably similar to human [15]. The rooster is known to develop spontaneously a human-like atherosclerotic plaque when fed a cholesterol diet [10] and is among the species that are susceptible to atherosclerosis based on the substrate specifity of LCAT [18]. Taken together, these findings confirm that the rooster is a suitable model for studies of LCAT activity and its involvement in the pathogenesis and treatment of atherosclerosis.

LA resulted in a marked decrease in plasma unesterified cholesterol in both the normocholesterolaemic and hypercholesterolaemic groups, with a greater reduction in normocholesterolaemic than in the hypercholesterolaemic animals (Fig. 1), confirming previous studies by our group [6]. The greater decreases in unesterified cholesterol in the normocholesterolaemic group after LA suggest that the unesterified cholesterol pool was affected by other event(s). Theoretically, one would have expected a decrease in plasma unesterified cholesterol of 25%, the amount that was removed by extracorporeal

LA. In practice, a reduction in plasma unesterified cholesterol of 62% was observed (Fig. 1 and Table 2). The significant decreases in unesterified cholesterol after LA were not reflected in changes in the ratios of unesterified to total cholesterol for both groups. Ratios were significantly decreased for normocholesterolaemic animals but not for hypercholesterolaemic animals (Table 2).

These alterations in plasma unesterified and total cholesterol concentrations may be explained by the action of LCAT. There was a linear correlation between LCAT activity and unesterified cholesterol concentration in the plasma of the normocholesterolaemic animals, whereas there was no such correlation between these parameters in the hypercholesterolaemic animals. In both groups of animals the concentration of lecithin was similar and not markedly affected by LA, indicating that this substrate was not rate-limiting for LCAT. It would appear that, in the case of the hypercholesterolaemic animals, in which the plasma unesterified cholesterol concentrations were up to several-fold higher than in normocholesterolaemic animals, LCAT was saturated by both substrates, lecithin and unesterified cholesterol (V_{max} conditions). In contrast, the V_{max} of LCAT was not evident for the LA-treated normocholesterolaemic animals (Fig. 4). Therefore, under these conditions the K_m and V_{max} were calculated for LCAT in LA-treated animals and found to be $1{\cdot}89\,mmol\,L^{-1}$ and 676 $\mu mol\,L^{-1}\,h^{-1}$ respectively.

Currently there are four identifiable steps in reverse cholesterol transport [19,20].

1 Efflux of cholesterol from cell membranes to HDL in the extracellular space. The rate of efflux may depend on the intracellular concentration of total cholesterol, the activity of cytosolic cholesterol ester hydrolase, the properties of the plasma membrane and the binding of HDL-related lipoproteins or isolated apolipoproteins to the cell membrane, and the affinity of membranous cholesterol for these (apo) lipoproteins.

2 Esterification of HDL-cholesterol by LCAT. The extent of esterification may depend on LCAT concentration and LCAT activity and the conformation of HDL particles.

3 Transfer of cholesteryl esters from HDL to other plasma lipoproteins by cholesterol ester transfer protein (CETP).

4 Delivery of the cholesteryl esters to the liver in which a proportion of this cholesterol is eventually excreted through the biliary system as unesterified cholesterol or bile acids.

To date, it has been shown that LA may modulate reverse cholesterol transport by increasing cholesterol efflux. This is achieved because LA results in the formation of apolipoprotein A-1 [12,21], discoidal HDL [22] and pre-beta HDL particles [7]. These HDL particles are regarded as good cholesterol acceptors from cellular cholesterol and may be important components of reverse cholesterol transport [19,20,23–25].

LA may also increase reverse cholesterol transport by removing cholesterol from the body by way of extracting cholesterol directly from plasma which is the essence of the LA procedure [6,7,12,21].

Cholesterol is stored intracellularly in the form of cholesterol ester. These esters can be hydrolysed by the action of cytosolic cholesterol ester hydrolase to form unesterified cholesterol, which is thought to be in equilibrium with cell membrane unesterified cholesterol. Thus, it would be interesting to know how intracellular cholesterol ester hydrolase responds to LA in hypercholesterolaemic animals *in vivo*. In previous *in vitro* studies using this delipidation procedure it was shown that cholesterol ester hydrolase activity increased [22].

In this communication we present evidence that LA has desirable effects on LCAT activities in hypercholesterolaemic and normocholesterolaemic animals. In the hypercholesterolaemic animals there is a much larger pool of total body cholesterol, including intracellular and extracellular plaque cholesterol [26]. In this situation LCAT is working under V_{max} conditions. Under normocholesterolaemic conditions LA does not result in LCAT V_{max} kinetics (Figs 3 and 4). Thus, it may be concluded that, in hypercholesterolaemic animals, LA resulted in a pool of unesterified cholesterol that was available as substrate for LCAT to a greater extent than in normocholesterolaemic animals. The observations in this communication are supported by the following reports.

Unesterified cholesterol from cellular origin can interact with plasma HDL particles [27] and these particles are regarded as good cholesterol acceptors. Unesterified cholesterol in subfractions of plasma HDL are the preferred substrates for LCAT [27]. Different preparations of HDL_3 differ markedly in their reactivity with LCAT. This difference has been shown to be inversely related to lipoprotein particle size [27]. The activity of LCAT depends on the structure apo A-1 at the interface with lipid [28]. Most recent studies have shown that LA induces changes in HDL to pre-beta-like HDL particles [7], and the latter particles are regarded as good acceptors of unesterified cholesterol of cellular origin and the preferred substrates for LCAT. This cascade of events may be of considerable importance in the overall regulation of cholesterol transport from peripheral tissues to the plasma. These events are triggered by LA but not by the conventional LDL apheresis systems whereby whole lipoproteins are removed from the plasma. Thus, LA may have important effects on the reverse cholesterol transport system and implications for the management of atherosclerosis. Indeed, LA has been shown to be a safe and effective procedure in three animal models (roosters, calves and pigs) for the regression of atherosclerosis (manuscripts in preparation). The significance of these observations has encouraged us to proceed to the next stage, this being the application of LA to humans in a clinical setting for possible treatment of atherosclerosis by extracorporeal cholesterol elimination.

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