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EVIDENCE THAT REVERSE CHOLESTEROL TRANSPORT OCCURS IN VIVO AND REQUIRES LECITHIN-CHOLESTEROL ACYLTRANSFERASE

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The transport of cholesterol from extrahepatic tissues into plasma (reverse cholesterol transport) and the possible requirement for lecithin:cholesterol acyltransferase was examined in the rat. One hour after removal of the liver plasma cholesterol ester concentrations were significantly increased by 20%, whereas free cholesterol concentrations were unchanged. The lecithin:cholesterol acyltransferase inhibitor, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was administered to eviscerated rats. It inhibited plasma lecithin:cholesterol acyltransferase activity by 90% which in turn totally prevented the increase in plasma cholesterol ester concentrations. In addition, heat-inactivated plasma from DTNB-treated eviscerated rats was 50% more reactive toward a standard source of lecithin:cholesterol acyltransferase compared to plasma from control or untreated eviscerated rats. These data suggest that in the rat a reactive lecithin:cholesterol acyltransferase substrate is formed extrahepatically. Together with lecithin:cholesterol acyltransferase, this reactive substrate removes cholesterol from peripheral tissues.

The majority of cholesterol in plasma is esterified and is found in the core of lipoproteins. Lecithin:cholesterol acyltransferase (EC 2.3.1.43) is responsible for the esterification of cholesterol that takes place in plasma [1]. Nascent lipoproteins secreted by the liver and intestine also contain cholesterol esters produced from intracellular acyl-CoA:cholesterol acyltransferase (EC 2.3.1.26) [2]. In addition to maintaining a significant portion of plasma cholesterol in the esterified form, lecithin:cholesterol acyltransferase has been shown to have profound effects upon cholesterol and lipoprotein metabolism, e.g., in patients who have a genetic loss of functional lecithin:cholesterol acyltransferase [3,4]. Lecithin:cholesterol acyltransferase deficiency results in altered plasma lipoprotein structure and composition as well as in the accumulation of cholesterol in several tissues leading to the development of foam cells and atherosclerosis [3,4].

Since a significant proportion of lipoproteins are taken up, and degraded by extrahepatic tissues [5] which do not have mechanisms to degrade the cholesterol, it is assumed that there exist mechanisms which allow peripheral cellular cholesterol to be returned to plasma. Glomset [6] suggested that HDL together with lecithin:cholesterol acyltransferase may serve such a purpose. Studies using cell culture models have yielded conflicting data as to the requirement of lecithin:cholesterol acyltransferase for 'reverse cholesterol transport'. Whereas, Ray et al. [7] and Fielding and Fielding [8] show evidence for lecithin:cholesterol acyltransferase requirement, Stein et al. [9] and Daniels et al. [10] report opposite results.

Abbreviations: VLDL, very low density lipoproteins; HDL, high density lipoproteins; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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As suggested by Daniels et al. [10] 'negative results in selected in vitro systems,...do not exclude the possibility that lecithin:cholesterol acyltransferase may play a role in reverse cholesterol transport in vivo.' In vivo models of reverse cholesterol transport have not yet been described.

We previously reported that removal of the liver (to prevent hepatic clearance of lipoproteins) caused the accumulation of apolipoprotein E and cholesterol ester in a particle thought to be HDL_1 [11]. Moreover, total serum cholesterol concentrations were found to be increased in eviscerated rats [11] suggesting that cholesterol can be transported from peripheral tissues into plasma in vivo i.e. reverse cholesterol transport. In the present study we further examine the eviscerated rat model to determine the role of lecithin:cholesterol acyltransferase in reverse cholesterol transport in vivo.

[1α,2α(n)-^{3}H] Cholesterol, (spec. act. 58 Ci/mmol) was obtained from Amersham and purified by thin-layer chromatography as described elsewhere [12]. Cholesterol, 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) and bovine serum albumin were purchased from Sigma Chemical Co., St. Louis, MO. Stigmasterol was from Koch-Light Lab., Colnbrooke, U.K. Male Wistar rats 270–370 g, were fed chow and water ad libitum. The animals weere fasted 16–18 h before the experiments were started about 9:00 a.m. Rats were anesthetized with ether before the abdominal cavity was opened. Control animals were opened, internal organs were manipulated and then the abdominal cavity was closed with sutures. In the hepatectomized rats the gastrointestinal tract, including pancreas and spleen, was removed from distal esophagus to rectum. The hepatic artery and the portal vein were ligated. All animals regained consciousness and were able to move freely after surgery. Body temperatures were maintained using a heating lamp. One hour after surgery was completed, rats were anesthetized with ether and exsanguinated by puncture of the abdominal aorta. Blood was drawn with EDTA added (1 mg/ml) and plasma was obtained after centrifugation. The plasma samples were kept cold (+4°C) in order to inhibit lecithin:cholesterol acyltransferase activity. To some control and eviscerated animals, 150 and 110 μl, respectively, of 0.1 M DTNB dissolved in 0.1 M sodium phosphate buffer adjusted to pH 7.4, were injected intravenously. Free cholesterol and total cholesterol were determined by gas-liquid chromatography as described [12] after lipid extraction with 20 vol. of chloroform/methanol (2:1, v/v) [13].

Lecithin:cholesterol acyltransferase activity was measured using standard procedures [14]. To estimate substrate reactivity a modification of the method of Glomset and Wright [15] was used. This involved using native plasma (20 μl) from control animals as the enzyme source and 180 μl of heat-inactivated (59°C for 60 min) plasma from each animal as the substrate source. 60 μl of 0.1 M sodium phosphate buffer, pH 7.4, 40 μl of 0.1 M mercaptoethanol and 30 μl of albumin-stabilized emulsion of [^{3}H]cholesterol [16] were then added and incubated in a shaking waterbath for 6 h at 37°C. A modification of the method of Stokke and Norum [16] was used to measure the net plasma cholesterol esterification. 100 μl of plasma was incubated with 30 μl of albumin-stabilized [^{3}H]cholesterol. To these incubations 20 μl of 0.1 M mercaptoethanol or 20 μl of isotonic saline was added to examine if the in vivo inhibited lecithin:cholesterol acyltransferase could be reactivated. The incubations were performed at 37°C for 120 min. Using both enzyme assays the formation of cholesterol ester occurred in a linear manner throughout the time of the assay.

One hour after evisceration total plasma cholesterol ester concentrations were increased by 20% ($p < 0.05$) (Table I). The increase in plasma cholesterol ester concentrations was specific and not caused by hemoconcentration since the concentration of plasma free cholesterol was unaffected (Table I). In addition, we have previously shown that evisceration does not affect the concentrations of serum albumin, apolipoproteins, or hematocrit [11]. The increased cholesterol ester and unchanged free cholesterol concentrations resulted in an increase in the percent of plasma cholesterol which was esterified from 71% (control) to 75% after evisceration ($p < 0.01$). Since the liver and intestines were removed, the increased amount of plasma cholesterol esters are probably synthesized by lecithin:cholesterol acyltransferase.

To examine the role of lecithin:cholesterol

TABLE I

EFFECT OF DTNB AND EVISCERATION ON PLASMA CHOLESTEROL CONCENTRATIONS

The animals were treated as described in the method section. Free cholesterol and total cholesterol were determined by gas-liquid chromatography. The data represent the means ± S.D. of duplicate samples from five animals in each group. Statistical evaluation was performed with Wilcoxon's test.

Animal groups	Free cholesterol (mM)	Cholesterol ester (mM)	Cholesterol ester (% total cholesterol)
Control	0.29 ± 0.02	0.70 ± 0.03	71.0 ± 2.1
Evisceration	0.28 ± 0.05	0.84 ± 0.10 [a]	75.0 ± 0.6 [a]
Evisceration + DTNB	0.32 ± 0.03	0.70 ± 0.06 [b]	69.0 ± 2.9 [b]

[a] $p < 0.05$ compared to control.
[b] $p < 0.05$ compared to eviscerated.

acyltransferase in producing the increase of plasma cholesterol ester after evisceration, rats were treated with the lecithin:cholesterol acyltransferase inhibitor DTNB. Preliminary results showed that when injected into eviscerated rats, DTNB did not affect VLDL lipolysis (i.e. plasma triacylglycerol concentrations were similarly reduced by 90% in both non-treated and DTNB-treated eviscerated rats). DTNB did not alter plasma free or esterified cholesterol concentrations in control rats (data not shown). However, when injected into eviscerated rats, DTNB totally blocked the increase in plasma cholesterol ester concentrations (Table I).

To document that DTNB did, in fact, inhibit the in vivo activity of lecithin:cholesterol acyltransferase, its activity was measured in fresh plasma. In eviscerated rats, DTNB almost totally (90%) inhibited lecithin:cholesterol acyltransferase activity (Table II, first column). When injected into control rats, DTNB inhibited lecithin:cholesterol acyltransferase activity by 50% (data not shown). Other studies showed that the effect of DTNB was totally reversible, i.e. addition of mercaptoethanol to plasma obtained from control and eviscerated rats treated with DTNB increased lecithin:cholesterol acyltransferase activity to values which were similar to their respective non-DTNB-treated controls (data not shown). These data show for the first time that DTNB inhibits lecithin:cholesterol acyltransferase activity in vivo.

We also examined the possibility that a reactive substrate for lecithin:cholesterol acyltransferase is formed extrahepatically and thus would accu-

TABLE II

EFFECT OF DTNB AND EVISCERATION ON PLASMA CHOLESTEROL ESTERIFICATION

Lecithin:cholesterol acyltransferase was determined as percent of labeled free cholesterol esterified per hour with two different methods (Stokke-Norum and Glomset-Wright) as stated in the methods section. The data obtained with the Glomset-Wright method indicate the reactivity of substrate (heat-inactivated plasma) to a standard amount of plasma lecithin:cholesterol acetyltransferase. The results given represent means ± S.D. of duplicate samples from five animals in each group; unit μmol/l per h.

Animal groups	Modified Stokke-Norum	Glomset-Wright plasma reactivity
Control	55.0 ± 12.2	12.7 ± 2.6
Evisceration	41.6 ± 10.9	14.3 ± 1.6
Evisceration + DTNB	5.8 ± 3.3 [a,b]	20.1 ± 2.4 [a,b]

[a] $p < 0.01$ compared to control.
[b] $p < 0.01$ compared to eviscerated.

mulate in DTNB-treated rats. Heat-inactivated plasma from each animal was used as a source of lecithin:cholesterol acyltransferase. The results (Table II, second column) show that plasma obtained from DTNB-treated eviscerated rats had a 50% increase in reactivity to a standard amount of plasma lecithin:cholesterol acyltransferase. The increased reactivity of plasma from DTNB-treated rats was not due to increased free cholesterol availability since its concentration was unaffected.

Previous studies by others show that DTNB does not affect hepatic function (perfused livers [17]) nor does it impair the function of cultured fibroblasts [8]. These, in vivo studies show that DTNB clearly did not affect the survival or general health of the rats, i.e. DTNB-treated rats behaved the same as non-DTNB-treated rats. Furthermore, DTNB did not affect VLDL lipolysis, i.e., serum triacylglycerols were reduced by 90% in both treated and untreated eviscerated rats. However, DTNB almost completely inhibited lecithin:cholesterol acyltransferase activity which prevented the increase in plasma cholesterol ester concentration which occurs after evisceration.

In previous work the finding that evisceration caused total plasma cholesterol concentrations to increase suggested that a flux exists in vivo, whereby extrahepatic tissue cholesterol is transported into plasma [11]. The present experiments show that all of the cholesterol which accumulates in plasma is in the esterified form and that lecithin:cholesterol acyltransferase is required for this process.

It was first proposed by Schumaker and Adams [18] and later elaborated by Tall and Small [19] that VLDL lipolysis may be intimately linked to lecithin:cholesterol acyltransferase plasma cholesterol ester production. Upon loss of core triacylglycerol via lipoprotein lipase, the surface of VLDL becomes redundant and forms lamellar phospholipid structures. These disc shaped particles, like nascent HDL secreted by DTNB-treated rat livers [17], are thought to react with lecithin:cholesterol acyltransferase to produce HDL spheres. Norum, Glomset and coworkers [3,4] have found abnormal HDL disc particles in plasma of lecithin:cholesterol acyltransferase-deficient patients. These HDL disc particles were found to be more reactive substrates for lecithin:cholesterol acyltransferase compared to spherical HDL [3,4]. The plasma of DTNB-treated eviscerated rats, which is a better substrate for lecithin:cholesterol acyltransferase (Table II), probably also contains the disc-shaped HDL particles. Reaction of disc-shaped HDL particles with lecithin:cholesterol acyltransferase would require additional cholesterol to form the core cholesterol esters. This cholesterol could be derived from other lipoproteins or peripheral tissue cholesterol. Previous studies have shown that apolipoprotein E-enriched HDL_1 accumulates in eviscerated rats [11], suggesting that HDL_1 might be the metabolic end product of lecithin:cholesterol acyltransferase metabolism which occurs in plasma.

Apolipoprotein E is likely to be involved in hepatic high-affinity uptake [20–22]. Since a rat lacks a plasma cholesterol ester exchange protein, HDL_1 may function to return cholesterol to the liver directly or indirectly. In other animals which do posess a plasma cholesterol ester exchange protein, cholesterol esters may be transferred from HDL_1 to lower density lipoprotein particles [23].

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