The effects of insulin deficiency on the plasma clearance and exchange of high-density-lipoprotein phosphatidylcholine in rats

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Triolein/cholesteryl oleate/cholesterol/phosphatidylcholine emulsions designed to model the lipid composition of chylomicrons were injected intravenously into control and streptozotocin-treated insulin-deficient rats. As previously described for lymph chylomicrons, the emulsion triolein was hydrolysed and phosphatidylcholine was transferred to the plasma high-density lipoproteins (HDL). This mechanism was used to introduce a phospholipid label into HDL *in vivo*. The subsequent clearance of phospholipid radioactivity from the plasma of insulin-deficient rats was significantly slower than in controls ($P < 0.025$). Plasma clearance was similarly slower in insulin-deficient rats after injection of HDL that was previously labelled with radioactive phospholipids. After injection, the phospholipid label redistributed rapidly between the large-particle fraction of plasma lipoproteins (very-low- and low-density lipoproteins), and the lighter and heavier fractions of HDL. Compared with control rats, in insulin-deficient rats less of the phospholipid label was distributed to the lighter HDL fraction and more to the heavier HDL fraction, and this difference was not due to changes in activity of lecithin:cholesterol acyltransferase or in the apparent activity of phospholipid transfer protein. In insulin-deficient rats the changes in HDL phospholipid clearance and exchange appeared to be secondary to the associated hypertriglyceridaemia and the related changes in distribution of phospholipids between classes of plasma lipoproteins.

INTRODUCTION

Plasma HDL concentrations are inversely related to the incidence of atherosclerotic diseases and to plasma triacylglycerol concentrations in normal [1,2] and hypertriglyceridaemic man [3,4]. HDL are secreted by the liver [5] and intestine [6] as nascent particles, which then fuse with lamellar phospholipids, unesterified cholesterol and proteins liberated during lipoprotein lipase-mediated hydrolysis from the surfaces of triacylglycerol-rich lipoproteins [7,8]. Subsequent activity of LCAT produces mature plasma HDL.

The metabolism of the protein constituents of HDL has been described previously, but the metabolism of HDL phospholipids is poorly defined. HDL become enriched in phospholipids during alimentary chylomicronaemia in man [9–11] and after injection of lymph chylomicrons in rats [12,13]. In rats treated with Triton WR1339 to inhibit lipoprotein lipase, the transfer of emulsion phospholipid radioactivity to HDL was decreased by about 72 % compared with controls [14]. The transfer of surface material, including phospholipid, from triacylglycerol-rich lipoproteins to HDL is substantial [13], and provides a means for investigations of HDL dynamics in intact animals.

Previously, when emulsions with lipid compositions similar to those of natural chylomicrons were injected, we found that labelled phospholipids were efficiently transferred from the emulsions to plasma HDL [15,16]. The introduction of label into HDL by this mechanism facilitated the measurements of the turnover of HDL phospholipids. In the present study emulsions were injected into control rats and into rats made insulin-deficient by treatment with streptozotocin. The metabolism of the protein moiety of HDL has been studied in diabetic rabbits [17], rats [18] and man [19], but the plasma clearance of HDL phospholipids has not been studied previously in diabetes.

MATERIALS AND METHODS

Preparation of emulsions

Emulsions of the required compositions were prepared by sonication and purified by ultracentrifugation [16,20]. Triolein (70 mg), cholesteryl oleate (3 mg), cholesterol (2 mg) (all from Nu-Chek Prep, Elysian, MN, U.S.A.) and 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC; 25 mg) (from Avanti Polar Lipids, Birmingham, AL, U.S.A.), each more than 99 % pure, were dispensed from stock solutions into vials, followed by 30 μ Ci of dipalmitoyl phosphatidyl[*N*-methyl- 3 H]choline or 5 μ Ci of 1-palmitoyl-2-[1- 14 C]oleoyl phosphatidylcholine (Amersham, Sydney, Australia). Solvents were then evaporated under a stream of nitrogen before overnight desiccation to remove any residual solvent.

Mixtures of pure lipids were emulsified by sonication in 150 mM-NaCl in 10 mM-Hepes buffer (pH 7.4). The lipids were sonicated in 8.5 ml of the NaCl buffer at 55–56 °C (monitored by a thermocouple in the vessel), with the atmosphere above the mixture purged with nitrogen to prevent lipid oxidation. Sonication was for 20 min by using a 1 cm probe at a continuous output of 90–110 W with a Vibra-Cell high-intensity ultrasonic processor (Sonics and Materials Inc., Danbury, CT, U.S.A.). The density of the crude emulsion was increased to 1.21 g/ml by adding solid KBr. Then 4 ml portions were placed at the bottom of two centrifuge tubes, and 2.5 ml of NaCl solutions of densities 1.065, 1.020 and 1.006 g/ml were sequentially layered above. The tubes were centrifuged in the SW41 rotor of a Beckman L8-70M ultracentrifuge for 22 min at 24 200 g (r_{av} 11 cm) and 20 °C. The large coarsely emulsified particles were removed from the top of the gradient and replaced with 1.006 g/ml solution. This was followed by a second centrifugation at 71 100 g for 20 min. The emulsion particles which floated to the surface were removed and

Abbreviations used: HDL, high-density lipoproteins; LCAT, lecithin:cholesterol acyltransferase (EC 2.3.1.43); LDL, low-density lipoproteins; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; VLDL, very-low-density lipoproteins.

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made up to 3 ml in 0.15 M-NaCl solution, and then analysed and injected into rats within 1 day. Oxidation was prevented by addition of GSH (50 µg/ml) and storage under nitrogen. The emulsions contained triolein ($84.2 \pm 1.2\%$), cholesteryl oleate ($2.6 \pm 0.4\%$), cholesterol ($1.5 \pm 0.3\%$) and phospholipid ($11.7 \pm 0.6\%$) (means $\pm$ S.E.M. of 7 preparations). The particle diameter was 149 ± 1.5 nm, measured by laser light scattering.

Metabolism of injected emulsions

Male Wistar rats weighing approx. 300 g were obtained from the Animal Resources Centre (Willetton, W. Australia) and fed on a commercial rat pelleted diet containing approx. 5% fat. Rats were made insulin-deficient by intravenous injection of streptozotocin (50 mg/kg body wt.) dissolved in 0.01 M-citrate buffer, pH 4. Control rats were injected with 0.01 M-citrate buffer, pH 4. At 21 days after injection, control and insulin-deficient rats were anaesthetized and cannulated in the left carotid artery and the left jugular vein for blood sampling and injections respectively. The animals were not fasted, but were allowed free access to food and water until experimentation commenced. After surgery, the rats were placed in small individual cages [21] and allowed to recover from the effects of anaesthesia for 2–4 h. Hydration of the rats and patency of the cannulas were maintained by injections of small volumes of 0.15 M-NaCl solution. Heparin was not used. The dose of lipid injected was 2–5 mg containing approx. 500 000 d.p.m., in an emulsion of volume 0.35–0.4 ml. Blood samples of 1 ml were then taken at 10, 20, 40, 60, 90 and 120 min after injection of an emulsion. Over the 2 h period of study, the total volume removed amounted to approx. 20% of the blood volume. Ellman's reagent [5,5'-dithiobis-(2-nitrobenzoic acid)] was added to the blood samples to inhibit LCAT activity. After separation by centrifugation, radioactivity in the plasma was measured by liquid-scintillation spectroscopy by taking 30 µl samples and adding 5 ml of ReadySolv EP (Beckman). Immediately after completion of the clearance study, 20 mg of sodium pentobarbitone was injected, a blood sample (5 ml) was taken by heart puncture, and the liver and spleen were removed for extraction of lipids [22] with chloroform/methanol (2:1, v/v) and radioactivities in the purified lipid extracts were measured in 15 ml of scintillant.

Separation of HDL

To separate and subfractionate HDL, 6 ml of a sucrose solution of density 1.020 g/ml was placed in the left chamber and a solution of density 1.21 g/ml was placed in the right chamber of a gradient mixer. Solution from the right chamber was then pumped into a polyvinyl alcohol-coated [23] centrifuge tube, resulting in the flow of less dense solution from the left chamber to mix with the denser solution in the right chamber. In this manner a continuous sucrose gradient was formed of density of 1.050–1.21 g/ml. Solid NaBr (110 mg) was dissolved in 0.4 ml of rat plasma before the plasma was placed with a Pasteur pipette under the gradient. The tube was heat-sealed and then centrifuged in a Beckman VTi 65.1 vertical rotor at 363 000 g (r_{av} 7.7 cm) for 4 h at 20 °C. The bottom of the tube was punctured with an 18-gauge needle and 20 successive 1 ml fractions were removed. Radioactivity was determined in a 500 µl sample, and each fraction was analysed by agarose-gel electrophoresis and for cholesterol and protein. The refractive index of each fraction was measured with an Abbé refractometer (model 105; Yagami International Corp., Nagoya, Japan). Densities were obtained from the relations between refractive index, sucrose concentration and density.

This method achieved a complete separation of HDL from the VLDL + LDL fractions of plasma in a 4 h centrifugation. Because

we wished to know if transfer might favour particular subfractions of HDL, the method was designed to divide HDL into multiple subfractions according to flotation. The possible conversion of smaller into larger fractions by the action of LCAT would also be detectable. It should be noted, however, that with more prolonged centrifugation for 8 h all HDL migrated into the HDL-2 density region (1.08–1.12 g/ml), and size analysis of the separated subfractions by gradient-gel electrophoresis showed that all were of similar diameter.

Preparation of labelled heavier HDL and lighter HDL

Dipalmitoyl phosphatidyl[N-methyl- 3 H]choline (Amersham) was dispensed into vials containing 20 mg of POPC. Solvents were then dried under a stream of nitrogen before overnight desiccation to remove any residual solvent. The lipid was dispersed in 8.5 ml of 150 mM-NaCl in 10 mM-Hepes buffer (pH 7.4) by sonication for 45 min at 37 °C as described above. The suspension was centrifuged at 1200 g (r_{av} 12 cm) for 1 h to remove titanium debris and then passed over a 38 cm $\times$ 2.4 cm column packed with cross-linked Sepharose 4B (Pharmacia). There were three major peaks corresponding to multilamellar, bilamellar and unilamellar vesicles when examined by electron microscopy. The fractions containing the bilamellar vesicles were concentrated by vacuum ultrafiltration and injected into rats through a cannula placed in a jugular vein. After 15 min circulation in the rat, 20 mg of sodium pentobarbitone was injected and a blood sample (8 ml) was taken by heart puncture. Clotting was prevented with EDTA (final concn. approx. 5 mM) and plasma was obtained by centrifugation. Density ranges corresponding to lighter HDL (1.080–1.11 g/ml) and heavier HDL (1.12–1.16 g/ml) were isolated from plasma by the technique described above for the separation of HDL. The fractions were dialysed against 0.15 M-NaCl/10 mM-Tris/HCl (pH 7.4)/0.01% EDTA. The fractions were then concentrated by vacuum ultrafiltration for injection into rats as described above for the emulsions, except that blood samples (1 ml) were taken 10, 20, 40 and 60 min after injection. Ellman's reagent was added to the blood samples to inhibit LCAT activity.

Preparation of the $d > 1.21$ fraction

Rat plasma was increased in density to 1.21 g/ml by dissolving 5.91 g of NaBr in 20 ml of plasma, then placed into centrifuge tubes before overlaying with 19 ml of solution of density 1.21 g/ml. After centrifugation at 361 000 g (r_{av} 6.6 cm) for 15 h at 4 °C in a fixed-angle 70Ti rotor, the bottom of the tube was punctured with an 18-gauge needle, and 20 ml of the infranatant was collected and dialysed against 0.15 M-NaCl/10 mM-Tris/HCl (pH 7.4), before concentration by vacuum ultrafiltration to a final volume of 5 ml.

LCAT activity *in vivo*

Emulsions labelled with 1-palmitoyl-2-[1- 14 C]oleoyl phosphatidylcholine were used to measure LCAT activity *in vivo*. Emulsion containing 10^6 d.p.m. was injected as above, and blood samples (1 ml) were collected at 10, 20, 40, 60, 90 and 120 min. Ellman's reagent was added to inhibit further LCAT activity. After separation of the HDL fractions from plasma, the lipids were extracted from each fraction and separated by t.l.c. on 0.2 mm-thick layers of silica gel in the solvent system light petroleum (b.p. 40–60 °C)/diethyl ether/formic acid (90:10:1, by vol.). The phospholipid, cholesteryl ester and triacylglycerol bands were scraped from the plate and measured for radioactivity in 5 ml of scintillant. The rate of formation of radioactive cholesteryl oleate with time in the HDL fractions was used as an index of LCAT activity *in vivo*.

LCAT activity *in vitro*

LCAT activity was determined by the method of Wallentin & Vikrot [24]. A toluene solution containing 5 μ Ci of [4- 14 C]-cholesterol was added to 1 ml of bovine serum albumin (50 g/l) in 0.2 M-phosphate buffer, pH 7.4, and toluene was evaporated from the mixture with nitrogen during stirring for 15 min. Samples of plasma (100 μ l) and Ellman's reagent (0.01 M; 20 μ l) were pipetted into each of three glass tubes (two samples and one blank) and mixed for 30 min at 37 °C. Then 30 μ l of the albumin-stabilized [14 C]cholesterol was added before incubation at 37 °C for 4 h. The reaction was started by addition of 20 μ l of 0.1 M-mercaptoethanol. The reaction was stopped in tubes after 10, 20, 30 or 40 min by adding 5 ml of chloroform/methanol (2:1, v/v), followed by 2.5 ml of water. The chloroform phase was evaporated under nitrogen and the extracted lipids were separated by t.l.c. The cholesteryl ester bands were scraped into vials and the radioactivities measured in 5 ml of scintillant.

Phospholipid exchange *in vitro*

Labelled bilamellar vesicles were injected into control and insulin-deficient rats, and after 15 min labelled HDL was isolated from the plasma. The labelled HDL was then incubated with unlabelled plasma from control and insulin-deficient donor rats. After incubation, the mixtures were subfractionated by ultracentrifugation as described above and fractions were counted for radioactivity. All incubations were for 10 min in a shaking water bath at 37 °C.

In a second set of experiments, labelled HDL was incubated with unlabelled whole plasma from control donors in the presence of added amounts of the chylomicron-like emulsion. Parallel experiments were done where labelled HDL and plasma were obtained from insulin-deficient donors. In other incubations the $d > 1.21$ fraction obtained from control and insulin-deficient donors replaced whole plasma. After the incubations the lipoproteins were separated as above, and the $d < 1.065$ fractions were counted for radioactivity to determine the extent of phospholipid exchange and transfer to the VLDL + LDL fraction.

In a third set of experiments, labelled HDL and plasma from either control or insulin-deficient donors were incubated with 40 μ l emulsion. One incubation tube was immediately cooled to 0 °C, whereas duplicate tubes were incubated at 37 °C for 30 min or 60 min respectively and then cooled to 0 °C. Separation was as above, except that samples were ultracentrifuged at 0 °C. In other incubations the $d > 1.21$ fractions replaced whole plasma.

Chemical analysis

The extracted lipids from the emulsions were separated by t.l.c. The triacylglycerol, cholesteryl ester and free cholesterol bands were scraped from the plate for assay of triacylglycerol by

the chromotropic acid method [25], and for free and esterified cholesterol by the *o*-phthalaldehyde procedure [26]. Protein assay was by the procedure of Lowry *et al.* [27]. Phospholipid was measured directly in emulsion suspensions and in total lipid extracts of plasma and HDL [28]. The size of emulsion particles was determined by electron microscopy after staining with osmium tetroxide [29] and by laser light scattering using a BI-90 particle sizer (Brookhaven Instruments Corp., Ronkonkoma, NY, U.S.A.).

RESULTS

Table 1 shows that the insulin-deficient rats were hyperglycaemic and hypertriglyceridaemic. The plasma insulin was markedly decreased, and weight gain was less than for controls. The plasma phospholipid increased, but plasma cholesterol concentrations were unchanged in the insulin-deficient rats. HDL phospholipid was significantly increased by about 40 % compared with control rats.

Removal from plasma of labelled phospholipids

The removal rates of labelled phospholipid from plasma of control and insulin-deficient rats were compared after injection either as a component of chylomicron-like emulsions or as HDL from donor rats, labelled *in vivo* as described in the Materials and methods section. After injection of labelled emulsions, as shown in Fig. 1, there was an initial rapid phase in which about 40 % of the phospholipid radioactivity disappeared from plasma within the first 10 min after injection. This rapid phase was consistent with previous findings of removal by the liver of 37 % of phospholipid radioactivity as partially lipolysed remnant particles 10 min after injection of chylomicrons [30]. From 10 min onwards there was a slower monoexponential decrease of radioactivity, which continued for at least 2 h. The fractional removal rates calculated from the second phase are compared in Table 2. There was a markedly slower rate of removal of phospholipid label from the plasma of insulin-deficient rats compared with controls ($P < 0.025$). As shown in Table 2, emulsion radioactivities recovered in the liver and spleen 2 h after injection were similar in controls and insulin-deficient rats.

In insulin-deficient rats phospholipid was increased in plasma and HDL (Table 1); therefore the slow fractional clearance of phospholipid label could have been explained by an expansion of the pool size. By using the clearance rates given in Table 2 and assuming complete mixing with the whole plasma phospholipid pool, turnovers of 1.68 and 1.91 mg/min were calculated for control and insulin-deficient rats respectively. If complete mixing with the HDL pool only were assumed, then turnovers of HDL phospholipid would be very similar for controls and insulin-deficient rats, at 0.58 and 0.62 mg/min respectively.

For comparison with the removal of phospholipid label from

Table 1. Plasma triacylglycerol, cholesterol, plasma and HDL phospholipid, glucose, insulin and body weight

Rats were matched for body weight (296 ± 9 g) at the time of injection of streptozotocin, and the weights shown were measured 3 weeks later. The results are means $\pm$ S.E.M. for the numbers of rats in parentheses: *significantly different from controls ($P < 0.005$ by Student's *t* test); †significantly different from controls ($P < 0.01$ by Student's *t* test).

Rats	Triacylglycerol (mg/dl)	Cholesterol (mg/dl)	Phospholipid (mg/dl)	HDL phospholipid (mg/dl)	Glucose (mg/dl)	Insulin (μ -units/ml)	Body wt. (g)
Control	42.3 ± 4.4 (28)	61.1 ± 3.2 (28)	129.3 ± 12.0 (6)	44.6 ± 4.1 (6)	179 ± 10.2 (26)	83.4 ± 11 (11)	433 ± 10 (26)
Insulin-deficient	$103.7 \pm 9.7^*$ (18)	68.2 ± 6.3 (18)	$191.3 \pm 9.1^*$ (6)	$62.4 \pm 3.7^\dagger$ (6)	$425 \pm 30.6^*$ (17)	$5.8 \pm 1.2^*$ (8)	$355 \pm 14^*$ (18)

Fig. 1. Removal of phospholipid label from plasma after intravenous injection of triolein/cholesteryl oleate/cholesterol/phospholipid emulsions into unanaesthetized control and insulin-deficient rats

By 10 min 40% of the radioactivity had been cleared from the plasma; then followed a slower second phase. In the second phase, the rate of removal of phospholipid label from the plasma of insulin-deficient rats was significantly less than in controls ($P < 0.025$). Results are means $\pm$ S.E.M. from 8 control (□) and 6 insulin-deficient (■) rats.

Fig. 2. Distribution of phospholipid label in plasma after injection of labelled emulsion

Plasma was fractionated by centrifugation in density gradients into $d < 1.065$ fraction, lighter HDL (d 1.08–1.11) and heavier HDL (d 1.12–1.16). The data are means $\pm$ S.E.M. of samples taken between 10 and 120 min after injection of labelled emulsions in 8 control (□) and 6 insulin-deficient (■) rats. In insulin-deficient rats there was less label in the lighter HDL fraction, and more in the heavier HDL fraction, compared with controls: *significant difference ($P < 0.001$).

plasma after injection of emulsions, pre-labelled heavier HDL or lighter HDL was injected and plasma radioactivity was measured over 60 min. Table 2 shows that the clearance of phospholipid label from heavier HDL was significantly faster than that of label from emulsion, but again clearance from plasma was slower in insulin-deficient than in control rats. The removal rate of label from whole plasma of insulin-deficient rats after injection of labelled lighter HDL also appeared slower when compared with controls, but this difference was not statistically significant. As shown in Table 2, the organ recoveries of phospholipid radioactivity in the liver and spleen after injection of the heavier HDL and the lighter HDL were similar.

Redistribution of plasma phospholipid label

Concurrent with the overall disappearance of phospholipid from plasma, there was a rapid redistribution of labelled phospholipid between lipoprotein fractions. By 10 min after injection of labelled emulsion, the phospholipid radioactivity was distributed between emulsion (< 1.065 g/ml), lighter

(1.080–1.11 g/ml) and heavier (1.12–1.16 g/ml) HDL fractions in proportions which remained much the same for the ensuing 110 min, despite the disappearance during that time of 45% and 40% of injected label from the plasma of control and insulin-deficient rats respectively. In insulin-deficient rats less of the HDL label was in the lighter HDL fraction, and more was in the heavier HDL, than in controls, whereas the $d < 1.065$ fraction contained a similar proportion of total label in both groups, as shown in Fig. 2.

After injection of labelled heavier HDL, an equilibrium distribution of phospholipid radioactivity was reached within 10 min and persisted until the end of the experiment at 60 min. The distribution can be compared in Fig. 3 with that when the HDL pool was labelled indirectly by injection of labelled emulsion (Fig. 2). In Fig. 3(a) the labelled phospholipid was injected as lighter HDL, and in Fig. 3(b) as heavier HDL. In both cases there was less label in the lighter HDL fraction and more in the VLDL + LDL ($d < 1.065$) fraction in insulin-deficient rats compared with controls.

Table 2. Removal from plasma and organ recovery of phospholipid label after injection of emulsion, heavier HDL or lighter HDL in control and insulin-deficient rats

Fractional removal rates were calculated over the period of 20–120 min after injection of emulsion and over the period 10–60 min after injection of the HDL fractions. Organ uptakes were measured after killing at 120 min for the emulsion studies, and after 60 min for the HDL studies. Results are means $\pm$ S.E.M. and n is the number of rats in each group: *significantly different from control rats ($P < 0.025$); †significantly faster than phospholipid clearance after injection of emulsion ($P < 0.001$); ‡significantly different from control rats ($P < 0.05$).

Material injected	Rats ... <i>n</i>	Fractional removal rate (min ⁻¹)		Organ uptake (% of injected dose)			
		Control	Insulin-deficient	Control		Insulin-deficient	
				Liver	Spleen	Liver	Spleen
Emulsion	8	0.013 $\pm$ 0.001	6 0.010 $\pm$ 0.001*	35.1 $\pm$ 3.13	0.6 $\pm$ 0.08	36.5 $\pm$ 4.04	0.5 $\pm$ 0.06
Heavier HDL	9	0.019 $\pm$ 0.001†	7 0.015 $\pm$ 0.001‡	27.4 $\pm$ 1.2	0.9 $\pm$ 0.10	26.2 $\pm$ 2.7	0.7 $\pm$ 0.07
Lighter HDL	7	0.016 $\pm$ 0.001	4 0.014 $\pm$ 0.003	27.6 $\pm$ 1.7	1.1 $\pm$ 0.09	27.1 $\pm$ 2.3	0.6 $\pm$ 0.10

Fig. 3. Distribution of label in plasma after injection of HDL labelled with radioactive phospholipid

The data are means $\pm$ S.E.M. of samples taken between 10 and 60 min after injections: *significant differences ($P < 0.001$). (a) Findings when the labelled phospholipid was injected as lighter HDL in 7 control ($\square$) and 4 insulin-deficient ($\blacksquare$) rats; (b) findings when injected as heavier HDL in 9 control and 7 insulin-deficient rats. In both cases there was less label in the lighter HDL fraction, and more in the VLDL + LDL ($d < 1.065$) fraction, in insulin-deficient rats compared with controls.

LCAT activity *in vivo*

Whether labelled phospholipid was introduced intravenously on emulsion particles, as heavier HDL or as lighter HDL, less in proportion was distributed to the lighter HDL in insulin-deficient rats, raising the possibility of defective conversion of heavier HDL into lighter HDL owing to defective LCAT activity. To test this possibility, rats were injected with emulsion containing radioactive POPC labelled in the oleoyl moiety at the glycerol 2-position. As Fig. 4(a) shows, in control rats little cholesteryl oleate was found 10 min after injection, but increasing amounts were found at later times. By 40 min more labelled cholesteryl oleate was found in the plasma of insulin-deficient rats than in controls, and the difference persisted for 2 h. By 2 h approx. 3% of the injected radioactivity was present as cholesteryl oleate in the plasma of control rats. As shown in Fig. 4(b), in insulin-deficient rats more labelled cholesteryl oleate was found in the heavier HDL fraction than in the lighter HDL fraction, whereas in controls the ratio was reversed. In insulin-deficient rats the ratio of heavier/lighter HDL decreased progressively with time, whereas in controls the ratio increased slightly with time. Emulsion radioactivities recovered in the liver were similar to those shown in Table 2, and most label in liver persisted as phospholipid, with $< 0.03\%$ found as cholesteryl oleate in either control or insulin-deficient rats.

Fig. 4. Formation of cholesteryl oleate by the action of LCAT in plasma and HDL fractions after injection of emulsion containing radioactive POPC labelled in the oleoyl chain at the glycerol 2-position

More labelled cholesteryl oleate was formed in the insulin-deficient rats than in control rats. Panel (b) shows that control rats had a lower ratio of label as cholesteryl oleate in heavier HDL than in lighter HDL, which increased with time, compared with insulin-deficient rats, where the ratio was much higher and decreased with time. Results are means $\pm$ S.E.M. from 3 controls ($\square$) and means $\pm$ range from 2 insulin-deficient rats ($\bullet$).

LCAT activity *in vitro*

Measured as described in the Materials and methods section, the fractional esterification rate tended to be faster in the plasma of insulin-deficient rats, 30.6 ± 5.1 nmol/h per ml ($n = 6$) compared with control rats, 21.1 ± 2.7 ($n = 6$), but the difference was not statistically significant at $P < 0.05$ by either parametric or non-parametric procedures.

Transfer of phospholipid label from heavier HDL *in vitro*

After intravenous injection labelled phospholipid was rapidly exchanged between triacylglycerol-rich particles in the $d < 1.065$ fraction, heavier HDL and lighter HDL. The transfer was investigated *in vitro* as described in the Materials and methods section. The results of incubating labelled heavier HDL with plasma from control and insulin-deficient rats and with saline are summarized in Fig. 5. After control incubations for 10 min in the absence of plasma (i.e. in 0.15 M-NaCl) most of the label was recovered in the HDL fractions. The apparent transfer of about one-third of heavier HDL label to lighter HDL may be an artifact of re-isolation of heavier HDL. After addition of plasma from either insulin-deficient rats or control rats, considerably more label was transferred to the VLDL + LDL ($d < 1.065$) fraction. Transfer of label to the VLDL + LDL fraction was significantly greater with insulin-deficient than with control plasma.

In insulin-deficient plasma, because of hypertriglyceridaemia (Table 1) the larger pool of unlabelled phospholipid available on

Fig. 5. Distributions of phospholipid label in plasma after incubations of control plasma (□), insulin-deficient plasma (■) or 0.15 M-NaCl (▨) with labelled heavier HDL

After incubations with 0.15 M-NaCl less than 2% of label was redistributed to the VLDL + LDL ($d < 1.065$) fraction. Incubations with plasma from insulin-deficient rats produced more transfer to the VLDL + LDL fraction than with control plasma. Results are means $\pm$ S.E.M. from 4 incubations of each type: *significant difference ($P < 0.001$).

the surfaces of triacylglycerol-rich lipoproteins may have explained the increased exchange with unlabelled heavier HDL phospholipid. As a test for this possibility, emulsion was added to the incubations. When increasing volumes of emulsion were added, more labelled phospholipid was transferred to the $d < 1.065$ fraction (results not shown). The lipoprotein-free fraction of plasma ($d > 1.21$) mediated less transfer than did whole plasma.

The rates of transfer of phospholipid label from heavier HDL were measured in incubations of labelled heavier HDL with a fixed volume of emulsion suspended in whole plasma or lipoprotein-free plasma ($d > 1.21$). More label was transferred to the $d < 1.065$ fraction in incubations with insulin-deficient plasma than with control plasma, and transfer increased with increasing time of incubation up to 60 min (results not shown). The effect disappeared when $d > 1.21$ preparations were used instead of whole plasma, providing additional evidence that the increased transfer in plasma from insulin-deficient rats was due to the increased amounts of triacylglycerol-rich lipoproteins, and not to increased activity of a transfer protein.

DISCUSSION

We recently demonstrated that labelled phosphatidylcholine was efficiently transferred to plasma HDL from injected emulsions [15]. The introduction of label into HDL by this physiological mechanism facilitated the measurements of the turnover kinetics of HDL phospholipids, with results similar to direct measurements with HDL labelled in phosphatidylcholine [31] or in $C_{18:0}$ -containing sphingomyelin [32]. We have now compared the metabolism of HDL phospholipids in insulin-deficient rats with that in normal control rats. On the basis of our previous findings [15,16] we selected an emulsion stabilized with POPC and labelled with dipalmitoyl phosphatidylcholine, since POPC was a reasonable model for physiological phospholipid species, and the label, being more rapidly removed from plasma than isologous label, would undergo less transfer to cell membranes and to other lipoproteins. Rats were chosen because

of the absence of HDL-3 [33] and the absence of cholesteryl ester transfer proteins activity against emulsion cholesteryl oleate [34].

Clearance of the emulsion phospholipid radioactivity could be divided into an initial rapid phase of about 10 min, in which about 40% of the label disappeared from plasma, followed by a slower decrease of radioactivity which continued mono-exponentially for at least 2 h. The rapid phase of clearance of phospholipid label could be accounted for by the uptake of remnants by the liver [30]. In both control and insulin-deficient rats at least two-thirds of the plasma label was present in HDL 10 min after injection as emulsion, heavier HDL or lighter HDL. Since most of the phospholipid label was found in HDL fractions, the second phase is probably explained by transfer of label to the HDL fraction [13], followed by the irreversible loss of phospholipid label from the plasma HDL pool by mechanisms that are currently undefined. In the second phase the disappearance of labelled phospholipids from the plasma was slower in insulin-deficient rats than in controls, whether the label was introduced on a triacylglycerol-rich emulsion or as HDL prepared in donor rats (Fig. 1 and Table 2).

Rapid redistribution of label maintained an equilibrium between the three lipoprotein fractions, $d < 1.065$ (VLDL + LDL), lighter HDL and heavier HDL, with only 5–10% of total plasma label in higher-density fractions ($d > 1.16$). The equilibrium distribution of label presumably reflected the fraction of the phospholipid pool with which the label exchanged. From the data of Table 1, plasma triglycerides increased 2.5-fold in insulin-deficient rats; therefore VLDL would account for most of the phospholipid increase in plasma. Thus when injected with labelled heavier HDL or labelled lighter HDL, compared with control rats a larger fraction of radioactivity was found in the triacylglycerol-rich lipoproteins in the hypertriglyceridaemic insulin-deficient rats (Fig. 3). However, when emulsion was injected, the pool of $d < 1.065$ phospholipid in control rats was expanded, abolishing the difference compared with insulin-deficient rats (Fig. 2).

The increased distribution *in vivo* of label to the $d < 1.065$ fraction in insulin-deficient rats could have been due to the increased pool of VLDL or to increased phospholipid transfer activity. As shown in Fig. 5, more label was transferred from heavier HDL in incubations with insulin-deficient plasma compared with controls. Increasing the pool of triacylglycerol by adding increasing volumes of emulsion to incubations *in vitro* led to increased transfer of phospholipid label from heavier HDL to the $d < 1.065$ fraction.

The transfer and exchange of phospholipids between lipoproteins is facilitated by a transfer protein from the $d > 1.21$ fraction of plasma [35,36]. The increased transfer of phospholipid label to the VLDL + LDL ($d < 1.065$) fraction of plasma in insulin-deficient rats was not due to an increase in phospholipid transfer activity, because incubations with the $d > 1.21$ fraction of plasma abolished the consistent differences between control and insulin-deficient rats in the transfer to the $d < 1.065$ fraction found in incubations with whole plasma.

It has been suggested that LCAT plays a key role in the conversion of heavier HDL into lighter HDL via esterification of free cholesterol [37,38]. As shown in Fig. 4, there was no evidence of defective activity of LCAT in insulin-deficient rats, since more cholesteryl oleate was formed in the plasma of insulin-deficient rats than in controls. Studies *in vitro* of the LCAT rate supported this finding, with slightly higher activity in insulin-deficient rats, although the difference failed to reach statistical significance. Furthermore, our studies confirmed that the metabolism of phospholipids by LCAT was slow in comparison with the exchange of phospholipid radioactivity between the fractions.

Only about 1–2% of the phospholipid label was consumed by this pathway in either control [15] or insulin-deficient rats.

Plasma phospholipids exchange rapidly between HDL and other lipoproteins. The equilibrium distribution of label presumably reflects the size of each fraction of the phospholipid pool. In insulin-deficient rats the changes in HDL phospholipid clearance and exchange appear to be secondary to the associated hypertriglyceridaemia, consistent with measurements *in vitro*, where the transfer of phospholipid from HDL to emulsion was proportional to the quantity of added emulsion [34].

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