Selective labelling of hepatic fatty acids *in vivo*

Studies on the synthesis and secretion of glycerolipids in the rat

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1. We describe a method for the selective labelling of hepatic fatty acids in the rat *in vivo*. It relies on (i) the rapid and preferential uptake of cholesteryl ester from chylomicron and/or very-low-density-lipoprotein remnants by the liver [Holder, Zammit & Robinson (1990) *Biochem. J.* 272, 735–741] (without prior exchange of the ester to other lipoproteins in the plasma), and (ii) the very short half-life of the cholesteryl ester in the liver. The ^{14}C -labelled fatty acid moiety generated by cholesteryl ester hydrolysis was shown to be utilized by the liver for glycerolipid synthesis in a very similar pattern to that demonstrated for exogenous fatty acids by isolated cultured hepatocytes in previous studies. 2. Starvation (24 h) was shown to decrease the proportion of fatty acid utilized for glycerolipid synthesis, but to result in a proportionately smaller effect on incorporation into phospholipid. This was accompanied by a decrease in the fraction of synthesized triacylglycerol that was secreted by the liver. 3. Streptozotocin-diabetes did not affect the phospholipid/triacylglycerol ratio, but resulted in a small, but significant, decline in the fraction of triacylglycerol secreted by the liver. 4. In both starved and diabetic animals fatty acid esterification to the glycerol moiety constituted a smaller proportion of the total disposal of label. 5. These findings appear to validate the present method for the selective labelling of liver fatty acids *in vivo* in a non-invasive manner. Other possible uses for the method are suggested.

INTRODUCTION

Glycerolipid synthesis is a major route of fatty acid metabolism in rat liver. The tissue utilizes fatty acids synthesized *de novo* only to a very minor extent for esterification to the glycerol moiety [1,2], and the bulk of the fatty acid used for the synthesis of triacylglycerol and phospholipids comes from pre-formed sources. These include circulating non-esterified fatty acids [3], stored hepatic triacylglycerol (after hydrolysis) [4], and fatty acids delivered to the liver in lipoproteins [5]. In recent years there has been a major improvement in our understanding of the source and fate of fatty acids for glycerolipid synthesis, through work carried out partly on isolated hepatocytes and partly *in vivo* [6–8]. However, a direct comparison between the characteristics of fatty acid metabolism *in vitro* and *in vivo* has only been possible for newly synthesized fatty acids (and glycerol), through the use of labelled precursors *in vitro* (e.g. [^{14}C]lactate) and of injection of $^3\text{H}_2\text{O}$ *in vivo*. The latter results in the incorporation by the liver of ^3H into the glycerol and fatty acid moieties of glycerolipids, whose secretion or retention in the liver can then be monitored (see [9] for review).

A similar approach *in vivo* for the study of metabolism of pre-formed fatty acids has hitherto not been possible because injection of labelled fatty acids into animals results in uptake, and thus non-selective metabolism, by different tissues with consequent complication of the interpretation of the data obtained. Moreover, a recent study [10] has indicated that the apparent fate of injected [^3H]oleic acid in the liver was dependent on its site of injection. The need for a method to study the metabolic fate of hepatic pre-formed fatty acids *in vivo* is highlighted by observations [11] which indicate that the distribution of incorporation of such fatty acids between triacylglycerols and phospholipid by isolated hepatocytes is highly dependent on the concentration of exogenous fatty acid chosen for study. It would therefore be desirable to have a method whereby the pool of pre-formed fatty acids of the liver is selectively labelled *in vivo*.

In this paper we describe such a method. It is based on our previous observations [12] of the rapid and preferential uptake of

cholesteryl ester by the liver from very-low-density lipoproteins (VLDL) injected into rats. We have made use of these observations to optimize the selectivity of the direction of cholesteryl ester towards the liver by injecting into rats apolipoprotein (apo) C-poor remnants of apo E-rich lipoproteins (i.e. chylomicron and VLDL remnants) labelled with cholesteryl [^{14}C]oleate. Using this method we show that about 90 % of the injected label is taken up by the liver extremely rapidly and that the cholesteryl ester is hydrolysed (with a short half-life) to provide the specific labelling of hepatic fatty acids, the incorporation of which into glycerolipids could be monitored.

The data provide evidence that the method reproduces *in vivo* the observations made with isolated hepatocytes *in vitro*, and that consequently it provides a valid technique for monitoring the fate of intrahepatic fatty acids *in vivo*. It is shown that starvation and diabetes induce important changes in glycerolipid synthesis and secretion from pre-formed fatty acids *in vivo*. Further uses to which the technique could be usefully applied are discussed.

MATERIALS AND METHODS

Animals

Wistar rats were used. They were fed *ad libitum* on a standard chow diet (56 % carbohydrate, 19 % protein, 3 % fat, all by wt.; Special Diet Services, Edinburgh, U.K.) and maintained under a 12 h-light/12 h-dark regime. Male rats weighing 400–600 g were used as a source of lipoproteins. They were given a solution of 10 % (w/v) fructose to drink 48 h before exsanguination, during which period they were either fed normally or starved. No difference in the efficiency of the lipoproteins selectively to deliver labelled cholesteryl ester to the liver was observed between those obtained from post-absorptive fed rats or starved animals, and all the data have been combined. Female rats (170–190 g) were used for selective labelling of hepatic fatty acids *in vivo*. They were either fed normally ('fed') or starved for 24 h or made diabetic (blood glucose > 25 mM) by injection with streptozotocin (65 mg/kg body wt.) 4–5 days before the experiment.

Preparation of apo C-poor remnants

For each preparation two or three male rats were anaesthetized and functionally hepatectomized by ligation of the hepatic portal vein and the hepatic artery. The coeliac, superior- and inferior-mesenteric arteries were also ligated. A solution of heparin (100 units/kg body wt. delivered in 0.4–0.5 ml of saline) was injected via a femoral vein and, after 10 min, the rats were exsanguinated through the aorta. The blood collected was further incubated at 37 °C for 30 min after addition of 1 mg of BSA, 3 mg of benzamidine, 1 µg of leupeptin and 1.2 mg of EDTA per ml. The plasma fraction was adjusted to a density of 1.019 g/ml with KBr (to obtain an intermediate-density fraction, but to exclude low-density-lipoprotein) and centrifuged at 105 000 *g* for 18 h at 12 °C. The top 1 ml, containing the apo C-depleted lipoproteins, was collected and dialysed against several changes of 0.15 M-NaCl containing 1.3 mM-EDTA and 0.3 mM-thimerosal, followed by dialysis against EDTA-free medium. The lipoproteins were labelled with cholesteryl [1-¹⁴C]oleate (50 µCi/mg of protein) by incubation with human lipoprotein-deficient serum in the presence of proteinase inhibitors and 0.65 mM-glutathione as described previously [12]. In some experiments they were simultaneously labelled with cholesteryl [³H]oleoyl ether (50 µCi/mg protein). After adjustment of the density to 1.019 g/ml, the labelled lipoprotein preparations were separated by centrifugation (as above), harvested and dialysed exhaustively against the same media described above. Immediately before use they were filtered (0.45 µm pore size). The protein content and the amount of radioactivity were also measured. Separation of the constituent lipids by t.l.c. ascertained that > 98 % of the radioactivity was routinely associated with the band corresponding to cholesteryl ester.

Injection of lipoproteins into animals

Rats were anaesthetized (50 mg of pentobarbitone/kg body wt.) and, after 10 min, they were injected with 0.3 ml of lipoprotein solution (containing 20–30 µg of protein) via a femoral vein. Body temperature was maintained with the use of heat lamps. At the times indicated, their abdominal cavity was opened, a blood sample (3 ml) was rapidly taken from the aorta and the left lateral lobe of the liver was freeze-clamped with tongs cooled in liquid nitrogen. The rest of the liver, the spleen, heart and samples of adipose tissue and hind-leg muscle were also taken and frozen in liquid nitrogen until analysis.

Other methods

Perfusion of isolated rat livers and preparation of parenchymal and non-parenchymal liver cells were performed as described in [13]. Extraction of lipids from tissues and separation of the individual lipid classes by t.l.c. were performed as described previously [12]. Blood glucose concentrations were measured in HClO₄ extracts as described previously [14]. Protein was determined as in [15] with BSA as standard. SDS/PAGE was performed as in [12].

Chemicals

The sources of most chemicals were as described previously [11]. In addition, cholesteryl [³H]oleoyl ether was from Amersham International (Amersham, Bucks., U.K.). Triton WR 1339 and heparin was from Sigma Chemical Co. (Poole, Dorset, U.K.).

RESULTS AND DISCUSSION

The rationale behind the development of the present method for the selective labelling of hepatic fatty acids *in vivo* is two-fold. First, advantage is taken of the fact that rat plasma is deficient in

cholesteryl ester transfer protein [15,16]. Consequently, labelled cholesteryl ester injected with the lipoproteins will remain associated with the injected lipoproteins and will not exchange to other lipoprotein classes before uptake by the liver. Second, because the labelled cholesteryl ester is incorporated *in vitro* into apo C-poor lipoproteins (chylomicron and VLDL remnants generated by incubation with lipoprotein lipase released into the same plasma from which they were obtained), no cholesteryl ester is taken up by lipoprotein lipase-containing peripheral tissues upon injection of the lipoproteins into the experimental animals. Consequently, labelled cholesteryl ester will be directed specifically to the liver, where, as we have shown previously [12], there is a very rapid uptake of the label, some of which occurs preferentially to the uptake of the whole lipoprotein particle. The precise mechanism by which this rapid uptake of cholesteryl ester is mediated is still to be determined. In particular, it is not known whether the lipoproteins require to be recognized by the apo B,E (LDL) [17] or the putative chylomicron-remnant [18] receptors. Nevertheless it is noteworthy that the lipoproteins injected were rich in apo E, as the method used to generate the post-heparin plasma fraction was specifically designed to minimize the number of centrifugation steps required, as it is known that repeated centrifugation results in the progressive loss of apo E from lipoproteins [19]. SDS/PAGE of apoproteins obtained from plasma of rats that were functionally hepatectomized and treated with heparin confirmed that this procedure resulted in the loss of virtually all apo C-type proteins, with the retention of apo E (result not shown). Moreover, experiments in which we perfused isolated livers *in vitro* with the labelled lipoproteins confirmed the findings of [14] that the cholesteryl esters in VLDL and chylomicron remnants are taken up primarily (> 95 %) by the parenchymal cells of the liver.

The proportion of the injected cholesteryl oleate that was specifically directed to the liver could not be experimentally determined, because of the very rapid hydrolysis and metabolism of the liberated labelled fatty acid moiety. Dual-labelling of the injected lipoproteins with cholesteryl [1-¹⁴C]oleate and cholesteryl [³H]oleoyl ether enabled us to obtain a value for this parameter in preliminary experiments: 92.6 ± 3.9 % (S.E.M.) for fed rats (*n* = 8) and 88.6 ± 2.8 % for 24 h-starved rats (*n* = 7).

Table 1. Distribution of ¹⁴C label in plasma and extrahepatic tissues 15 min after intravenous injection of fed or 24 h-starved rats with apo C-poor lipoproteins (*d* < 1.019) labelled with cholesteryl [1-¹⁴C]oleate

Brown adipose tissue was obtained from the intrascapular region. White adipose tissue was from the periovarian depot. Mixed muscle samples were obtained from the hind leg. Total plasma and whole-body white adipose and muscle mass per animal were calculated from published values [32], [33]. Differences due to depot or muscle type are therefore not reflected. The tissue values have not been corrected for plasma content, and are therefore likely to be overestimates. The numbers of separate animals used are shown in parentheses.

Tissue	Rats ...	Label (% of injected dose per animal)	
		Fed	24 h-starved
Plasma		3.7 ± 0.6 (9)	3.2 ± 1.0 (4)
White adipose		4.3 ± 1.0 (5)	2.2 (2)
Brown adipose		0.1 (2)	0.1 (2)
Total muscle		4.9 ± 1.0 (4)	4.3 (1)
Heart		1.9 (2)	—
Spleen		3.1 ± 0.4 (5)	7.1 (2)

Fig. 1. Rate of disappearance of ¹⁴C label from the cholesteryl ester pool in the liver of rats

Normal fed (●) or 24 h-starved (○) or streptozotocin-diabetic (△) rats were injected with cholesteryl [¹⁻¹⁴C]oleate-labelled lipoproteins as described in the Materials and methods section. At the indicated times, their livers were freeze-clamped and used to prepare extracts of total lipid. The amount of ¹⁴C associated with cholesteryl ester was determined after separation of the different lipid classes by t.l.c. The values for zero time are taken from experiments in which [³H]cholesteryl ester was also incorporated into the injected lipoproteins (see the text). See Table 2 for the numbers of separate animals used.

This uptake was complete within 5–10 min (results not shown). The remainder of the radioactivity could be accounted for by minor association with the other tissues sampled (Table 1). The amount of total lipoprotein injected (20–30 μg of protein) into each rat was extremely low, so that the associated labelled cholesteryl ester and ether were only injected in tracer amounts and would not have affected the mass of these compounds reaching the liver.

There was a very rapid net hydrolysis of the cholesteryl ester delivered to the liver (cf. [12]), which occurred with a similar half-life (approx. 18 min) for the ester in the liver of rats in all three (patho)physiological states studied (Fig. 1). After 65 min the amount of label still associated with cholesteryl ester in the liver was less than 10% of the injected dose. The amount of radioactivity associated with the fatty acid fraction in liver extracts was low (3–7%) throughout the 65 min period of the experiments, indicating that fatty acid moieties were used up rapidly upon their formation through cholesteryl ester hydrolysis.

Fig. 2. Time courses of incorporation of ¹⁴C from cholesteryl [¹⁻¹⁴C]oleate into glycerolipids in plasma (a) and liver (b) of injected rats

Results for fed (●), 24 h-starved (○) and diabetic (□) animals are shown. The numbers of separate animals used were as shown in Table 2.

The liver does not release unesterified fatty acids into the circulation, and so any fatty acid label generated intrahepatically after cholesteryl ester hydrolysis is withheld within the tissue.

As described previously [12], no significant amounts of label generated intrahepatically appeared in the plasma by 15 min after injection (Fig. 2a), even though half the ¹⁴C label was no longer associated with cholesteryl ester. This was as expected from the fact that lipoprotein assembly and secretion from pre-formed fatty acids take 30–40 min in rat liver [20]. Consequently, we injected Triton WR 1339 (which prevents utilization of the secreted lipoproteins and enables their accumulation in the circulation) at either 15 min (in most of the experiments) or 35 min (in initial experiments) after injection of label. Very similar results were obtained with either protocol, and the data are combined (Fig. 2a).

Table 2. Incorporation of ¹⁴C label into triacylglycerol, phospholipids and total glycerolipids in plasma and liver of Triton-treated rats 65 min after injection with apo C-poor lipoproteins (*d* < 1.019) labelled with cholesteryl [¹⁻¹⁴C]oleate

Differences between fed rats and animals in the two other conditions that are statistically significant (*P* < 0.01) are shown by an asterisk.

Rats	Liver (% of injected dose)			Plasma (% of injected dose)			Percentage secreted		
	Triacyl-glycerol	Phospho-lipids	Total glycerolipids†	Triacyl-glycerol	Phospho-lipids	Total glycerolipids†	Triacyl-glycerol	Phospho-lipids	Phospholipid/total glycerolipids (%)
Fed (<i>n</i> = 5)	23.3 ± 2.1	6.2 ± 0.4	31.4 ± 2.2	17.6 ± 2.3	0.2 ± 0.1	18.8 ± 2.4	45.7 ± 1.7	3.3 ± 1.1	13.1 ± 1.2
24 h-starved (<i>n</i> = 6)	8.0 ± 0.6*	3.8 ± 0.2*	11.9 ± 1.2*	4.1 ± 0.8*	0.2 ± 0.1	5.4 ± 0.8*	30.1 ± 4.7*	4.5 ± 1.0	23.8 ± 1.6*
Diabetic (<i>n</i> = 3)	18.7 ± 1.2	4.7 ± 0.7	24.4 ± 1.4*	10.4 ± 0.6*	0.2 ± 0.1	9.8 ± 1.8*	36.1 ± 1.6*	5.6 ± 1.2	14.3 ± 2.0

† Includes mono- and di-acylglycerols.

The time courses for the appearance of the label into intrahepatic glycerolipids are given in Fig. 2(b). A detailed breakdown of the values obtained at 65 min after injection is given in Table 2 for normal fed rats, 24 h-starved and diabetic rats. The main observations were as follows.

(i) The liver of fed rats showed by far the highest proportion of the injected label that was incorporated into glycerolipids (50%). Only about one-third of the label was incorporated into glycerolipids in diabetic rats, and an even smaller proportion (17%) in the liver of 24 h-starved animals. This pattern of incorporation is in accord with the expected greater competition from β -oxidation for fatty precursors in the diabetic and starved states, owing to the enhanced activity of the ketogenic pathway (see [21]).

(ii) Phospholipids accounted for only 13.0% and 14.3% of total glycerolipids synthesized in fed and diabetic rats respectively, but for a significantly ($P < 0.01$) higher proportion (23.0%) in starved animals. This observation suggests that when the rate of esterification of fatty acids by the liver is decreased, phospholipid synthesis is spared, presumably to ensure that adequate liver membrane structure is maintained, as most of the phospholipid is retained within the liver (see below). In [22] it was shown that incorporation of [^3H]glycerol into phospholipids was also much less affected by starvation in isolated hepatocytes and after injection of [^3H]glycerol into rats.

The pattern of partitioning of fatty acids between acylglycerol and phospholipid synthesis in fed animals is similar to that found with cultured rat hepatocytes when these were exposed to concentrations of exogenous oleate close to those that occur in fed animals [8]. In those studies a much higher proportion of fatty acid was used for the synthesis of phospholipids when lactate was the precursor, i.e. when newly synthesized (endogenous) fatty acids were the substrates. However, the contribution of newly synthesized fatty acids towards total glycerolipid synthesis is known to be very small [1,2]. Consequently, the present observations of a dominant synthesis of neutral acylglycerols over that of phospholipids may result from the overwhelming contribution of preformed fatty acids towards total fatty acid incorporation into glycerolipids. It is not possible to ascertain, of course, whether it also results partly from the preferential equilibration of the labelled fatty acids liberated by cholesteryl ester hydrolysis with the preformed fatty acid pool rather than with that of newly synthesized fatty acids, which evidently has distinct characteristics [8]. In quantitative terms, however, the latter pool is very minor (see above).

(iii) Only a very small proportion of the phospholipid synthesized was secreted into the plasma. This observation again confirms the findings obtained with isolated hepatocytes that, when exogenous oleate was used as labelled substrate, only about 2% of phospholipid synthesized was secreted into the medium [8]. By contrast, more than 10% of the phospholipid formed from fatty acids synthesized *de novo* (incorporating ^3H from injected $^3\text{H}_2\text{O}$) *in vivo* were secreted [8], again suggesting that the present method monitors primarily the fate of the quantitatively preponderant pool of fatty acids delivered to the liver or arising from hydrolysis of endogenous triacylglycerol.

(iv) A sizeable proportion (46%) of triacylglycerol synthesized was secreted into the plasma in fed animals by 65 min after injection of label. This is of the same order as the proportion of triacylglycerols secreted into the medium by cultured rat hepatocytes when the triacylglycerol is synthesized from exogenous oleate [8]. Moreover, it was possible to demonstrate that the proportion of triacylglycerols secreted by the liver was significantly higher in normal fed animals than in the liver of either starved or diabetic rats (Table 2). The observation that 36% of the total triacylglycerol synthesized in the liver of

diabetic rats was secreted further suggests that the effects of insulin deficiency *in vivo* on triacylglycerol secretion by the liver may not be directly predictable from the effects of insulin added to hepatocytes in culture or to perfusates of isolated liver preparations. Thus insulin has been variously described as exerting an inhibitory [23,24] or stimulatory [25] effect on the proportion of synthesized triacylglycerol that is secreted by preparations *in vitro*. Interestingly, Gibbons and co-workers [7] have found that the effect of insulin on the proportion of triacylglycerol secreted by cultured hepatocytes is dependent on the duration of exposure of the cells to the hormone *in vitro*, although, overall, insulin removal always resulted in increased secretion of stored triacylglycerol [7]. It would appear from the present data that the overall effect of insulin deficiency *in vivo* (with its attendant changes in other hormonal and metabolic parameters) was to decrease the proportion of synthesized triacylglycerol that was secreted by the liver, although the change was not as large as that observed for starved rats. It is interesting that in [24] it was shown that insulin stimulated VLDL secretion only in isolated livers perfused at high flow rates. Of course, partitioning of label between liver and plasma does not give any information about the absolute rates of triacylglycerol secretion. For example, in diabetes the specific radioactivity of intrahepatic triacylglycerol will be decreased, owing to the accumulation of intracellular triacylglycerol in this state. Previous studies have yielded conflicting data on the effects of diabetes on the accumulation of plasma triacylglycerols after Triton treatment [6,26,27]. In addition, the contribution made by intestinal secretion could be substantial in diabetic rats, as pointed out in [28,29]. Further experiments, in which the specific radioactivities of the triacylglycerol pools in the liver and the plasma after selective labelling of hepatic fatty acids are measured, should resolve the issue of the effect of diabetes on the absolute rates of hepatic triacylglycerol secretion *in vivo*, as use of the present method would allow distinction between the hepatic and intestinal origin of plasma triacylglycerol.

Conclusions

We have demonstrated that the rapid and selective labelling of hepatic fatty acids *in vivo* by incorporation of labelled cholesteryl oleate into apo C-poor remnants of chylomicrons and VLDL particles provides a valid method for the study of the metabolic fate of preformed fatty acids. This was evidenced by the characteristics of the incorporation of label into glycerolipids and their secretion into the plasma. We envisage that this technique will be useful in quantitative studies on the source and fate of fatty acids metabolized by the liver, such as the ones suggested above. It should also enable the study of the metabolism of distinct classes of fatty acids (by utilizing different cholesteryl esters, e.g. of acyl moieties of various degrees of unsaturation) and of different nutritional states. Because of the very rapid hepatic uptake and the short half-life of the cholesteryl ester, the method should also prove valuable, when coupled with the use of awake unrestrained rats, in obtaining detailed time courses of changes in hepatic fatty acid metabolism *in vivo* during well-defined physiological perturbations, in particular, of the partitioning of fatty acids between esterification and oxidation. The major advantages of such a method are its non-invasiveness and its ability to monitor metabolism under physiologically pertinent hormonal and substrate concentrations. It is also noteworthy that the method need not be restricted to the rat. This species presents the experimental advantage of having a very low cholesteryl-ester-transfer-protein activity in the plasma. However, an acute suppression of this activity for the duration of the experiment can be achieved with the use of specific antibodies (see [30,31]).

We thank Professor J. Shepherd (Department of Pathological Biochemistry, University of Glasgow) for the gift of human lipoprotein-deficient serum. This work was funded by the Scottish Office Agricultural and Fisheries Department.

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Received 9 October 1991/4 November 1991; accepted 20 November 1991