Effects of insulin treatment of diabetic rats on hepatic partitioning of fatty acids between oxidation and esterification, phospholipid and acylglycerol synthesis, and on the fractional rate of secretion of triacylglycerol *in vivo*

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1. The hypothesis that insulin treatment of streptozotocin-diabetic rats does not alter acutely the ability of acylcarnitine synthesis to compete successfully for cytosolic long-chain acyl-CoA [Grantham and Zammit (1988) *Biochem. J.* **249**, 409–414], was tested *in vivo* by using the technique of selective labelling of hepatic fatty acids in awake unrestrained rats. In the same animals, the partitioning of hepatic fatty acids between acylglycerol and phospholipid synthesis, and of newly labelled triacylglycerol between secretion into the plasma and retention in the liver, was also studied. 2. In untreated diabetic animals, the ratio of fatty acid oxidation to esterification was double that found in normal fed animals, whereas there were no differences in the values of the above-mentioned parameters of glycerolipid metabolism. Thus the insulin status of the rats only has chronic effects on specific aspects of fatty acid metabolism in the liver. 3. Treatment of diabetic rats with insulin resulted in no change in

the oxidation/esterification ratio for the first 5 h after the start of insulin administration. Thereafter, there were reciprocal changes in the $^{14}\text{CO}_2$ expired (an index of oxidation) and ^{14}C label recovered in hepatic and plasma glycerolipids. However, the pattern of partitioning observed in normal fed rats was still not re-established after 8 h of insulin treatment. 4. There was a small and transient decrease in the fractional rate of triacylglycerol secretion by the liver at the start of insulin treatment and an increase in the proportion of labelled fatty acid that was utilized for phospholipid synthesis such that phospholipid labelling as a proportion of that of total glycerolipids was doubled after 8 h of insulin treatment. 5. The data are discussed in relation to the roles of insulin in mediating acute changes in hepatic fatty acid metabolism and very-low-density-lipoprotein triacylglycerol secretion by the liver.

INTRODUCTION

Insulin is able to modulate hepatic fatty acid metabolism acutely through its actions on several key enzymes, including phosphatidate phosphohydrolase, CTP:phosphocholine cytidyltransferase and acetyl-CoA carboxylase (see [1–3] for reviews). The activity of the last-mentioned enzyme exerts an indirect influence on the rate of fatty acid oxidation, as its product, malonyl-CoA, inhibits the mitochondrial carnitine palmitoyltransferase I (CPT I), the enzyme that catalyses the first reaction that commits the long-chain acyl moiety to oxidation [4]. However, in previous studies on the reversal of states characterized by low insulin concentrations and high fatty acid oxidation rates in the liver (e.g. starvation and streptozotocin-diabetes in the rat), we observed that the activity and kinetic characteristics of CPT I are only gradually returned to normal values after normalization of insulin concentrations or after treatment with pharmacological doses of insulin [5,6]. Thus administration of insulin to diabetic rats, although rapidly reversing the absolute ketogenic rate in the liver (presumably through the rapid lowering of plasma non-esterified fatty acid (NEFA) concentration and a decrease in the proportion of mitochondrial acetyl-CoA diverted towards ketogenesis through inhibition of 3-hydroxy-3-methylglutaryl-CoA synthase activity [7,8]), did not result in a significantly decreased expression of maximal CPT I activity even after 12 h, and the sensitivity of CPT I to malonyl-CoA was unchanged for at least 3 h after the

start of insulin treatment [6]. However, such a gradual effect of insulin on CPT I has not been observed by others after insulin treatment of streptozotocin-diabetic rats or of genetically diabetic animals [9].

If CPT I is refractory to insulin action for several hours after initiation of insulin treatment, this would imply that the ability of the enzyme to compete for the cytosolic pool of long-chain acyl-CoA should persist during this period, even though the absolute flux through the reaction catalysed by CPT I is much diminished (see [10]). In the present study, we have applied the technique of selective labelling of hepatic fatty acids *in vivo* [11] to monitor the partitioning of hepatic fatty acid between acylcarnitine formation (and thus oxidation) and esterification to glycerolipids during insulin treatment of diabetic rats, so as to test *in vivo* the hypothesis that the capacity of the liver to oxidize fatty acids is undiminished during this refractory period. In addition, this approach also allowed us to study the effects of insulin repletion on the fractional rate of secretion, from the liver, of newly synthesized triacylglycerol and the partitioning of the glycerolipid flux between phospholipid and triacylglycerol formation.

Our findings suggest that the liver of the diabetic rat treated with insulin takes 3–5 h to start to revert to the pattern of partitioning between fatty acid oxidation and esterification observed in the normal fed animal. Moreover, insulin is shown to affect directly only marginally the fractional rate of hepatic secretion of triacylglycerol in diabetic animals.

Abbreviations used: CPT I, carnitine palmitoyltransferase I; NEFA, non-esterified fatty acid; VLDL, very-low-density lipoprotein.

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Table 1 Partitioning of [^{14}C]fatty acid label between oxidation and esterification products and between phospholipids and triacylglycerol retained in the liver or secreted in the plasma, during successive 60 min periods, as indicated, in the course of insulin treatment of diabetic rats (see the Materials and methods section)

Values are expressed as a percentage of the total net cholesteryl oleate hydrolysis that occurred in the liver during the same period, and are means ($\pm$ S.E.M.) for n separate rats as indicated in parentheses. The duration of insulin treatment before the injection of label is indicated.

Rats/treatment	Cholesteryl 1- ^{14}C oleate metabolized in liver (% of injected dose)	Products recovered (% of cholesteryl 1- ^{14}C oleate metabolized)				
		^{14}C Glycerolipids				$^{14}\text{CO}_2$
		Liver		Plasma		
		Triacylglycerol	Phospholipids	Triacylglycerol	Phospholipids	
Control diabetic ($n = 9$)	81.7 $\pm$ 4.1	7.9 $\pm$ 0.9	4.3 $\pm$ 0.3	16.2 $\pm$ 1.1	0.2 $\pm$ 0.1	22.4 $\pm$ 2.7
Insulin 1 h ($n = 4$)	82.7 $\pm$ 4.5	10.3 $\pm$ 0.9	7.3 $\pm$ 1.1	13.1 $\pm$ 1.1	0.2 $\pm$ 0.1	30.5 $\pm$ 0.6
Insulin 3 h ($n = 6$)	82.8 $\pm$ 1.9	7.8 $\pm$ 0.3	6.1 $\pm$ 0.9	15.3 $\pm$ 2.9	0.2 $\pm$ 0.1	33.6 $\pm$ 2.0
Insulin 5 h ($n = 4$)	88.7 $\pm$ 1.2	10.6 $\pm$ 1.2	9.5 $\pm$ 2.1	18.4 $\pm$ 2.4	0.7 $\pm$ 0.5	27.1 $\pm$ 2.8
Insulin 7 h ($n = 6$)	86.5 $\pm$ 2.8	12.9 $\pm$ 1.4	13.3 $\pm$ 2.1	18.5 $\pm$ 1.8	0.3 $\pm$ 0.1	21.1 $\pm$ 2.7

MATERIALS AND METHODS

Animals

Female Wistar rats weighing 180–200 g before the start of experimental treatment were used and fed on a normal laboratory chow diet [12]. At 6 days before the experiment, each animal was fitted with a jugular cannula and, 24 h later, injected with 65 mg of streptozotocin/kg body wt. (intraperitoneally) and given glucose in its drinking water for a further 24 h. Severity of the diabetes induced was checked 4 days later. Only rats with blood glucose > 20 mM were considered diabetic.

Monitoring of hepatic fatty acid metabolism *in vivo*

The preparation of apolipoprotein-C-poor very-low-density lipoprotein (VLDL) and chylomicron remnants and their labelling with cholesteryl 1- ^{14}C oleate were performed as described previously [11–13]. On the day of the experiment, diabetic rats received insulin (15 units/kg body wt.) at zero time and their blood glucose was monitored hourly. Further doses of insulin were administered, as needed, to maintain blood glucose below 10 mM. Injection of lipoproteins was performed either at the same time as the start of the insulin treatment (0–1 h) or at hourly intervals thereafter, as indicated. The animals were placed in individual metabolic chambers and injected intravenously through the chronically implanted cannula with Triton WR 1339 [0.5 ml of a 10% (w/v) solution per 100 g body wt.] 15 min after injection of label [11]. The CO_2 exhaled by the animals was trapped [11] and their urine was collected for 1–3 h, as indicated (see the Results section). At the end of the experimental period, animals were anaesthetized with pentobarbitone, a blood sample was obtained from the abdominal aorta and a liver sample was freeze-clamped by using tongs cooled in liquid nitrogen. The rest of the liver was rapidly frozen in liquid nitrogen. The contents of the urinary bladder were withdrawn into a syringe and pooled with the urine collected. Lipid extraction and separation of different lipid classes from liver and plasma samples were performed as described previously [12]. The validity of the comparisons with data from previous experiments [12,13] was

regularly tested by the random inclusion of normal fed or starved rats within the groups of animals studied in order to check that the behaviour of the lipoproteins as carriers of the labelled cholesterol ester was reasonably uniform.

Other methods

At the time of the experiment, blood glucose was measured on 5 μl samples withdrawn from the cannula, by using an Exactech blood glucose monitor (Medisense Britain Ltd., Birmingham, U.K.). Values at the end of the experiment were confirmed subsequently by using a spectrophotometric assay [14]. Plasma volume for each animal was calculated from body weight according to the formula given in [15], which has been verified experimentally in a previous study in our laboratory [16].

Materials

The sources of animals and chemicals were as described previously [11–13]. In addition, streptozotocin was from Sigma Chemical Co. (Poole, Dorset, U.K.).

Statistical analysis

Statistical significance of differences was analysed by using the non-paired Student t test.

RESULTS

Parameters of hepatic fatty acid metabolism in diabetic rats

The use of remnant-associated cholesteryl 1- ^{14}C oleate to generate a pulse of [^{14}C]oleate selectively in the liver (as described previously [11–13]) enables the quantification of the partitioning of the intrahepatically generated label between the different fates of fatty acid metabolism, although it does not allow the determination of absolute fluxes through the various pathways. The simultaneous labelling of the carrier lipoproteins with the non-hydrolysable [^3H]cholesteryl ether enables the estimation of the degree of hepatic selectivity achieved in each individual

Figure 1 Time course of the recovery of $^{14}\text{CO}_2$ in air exhaled by rats after injection of label

Normal rats (□), untreated diabetic rats (●) or diabetic rats treated with insulin for 1 h before and for the duration of the experiment (○) were injected with labelled remnants as described in the Materials and methods section. The $^{14}\text{CO}_2$ expired was collected and the cumulative total is shown. Values are expressed as a percentage of the total net cholesteryl 1- ^{14}C oleate metabolized in the liver of the individual animals. They are means ($\pm$ S.E.M.) for three separate animals each.

animal. Routinely, this value was 85–90% in the present experiments, and no statistically significant difference was observed between the different groups of animals (results not shown). The fraction of the total cholesterol [^{14}C]oleate injected that was metabolized in the liver (see Table 1) was derived by subtraction of the label that remained associated with the hepatic cholesterol ester fraction at the end of the 60 min experimental period from the total delivered to the liver (i.e. [^3H]cholesteryl ether). The fraction of label metabolized tended to be higher in animals treated with insulin for 5–7 h, but this difference was not statistically significant (cf. also [12,13]). In any event, the interpretation of the data should not be affected by this minor difference, as only relative fluxes are considered from data expressed as a percentage of the label metabolized in the liver of each individual animal.

The fraction of label metabolized in the liver that was recovered as $^{14}\text{CO}_2$ in the exhaled air in fed diabetic rats during the first 60 min after injection was $22.4 \pm 2.7\%$ ($n = 9$). As with normal fed rats, this represented only a fraction (approx. 0.45) of the maximal amount of $^{14}\text{CO}_2$ that could be recovered, in parallel experiments, from diabetic rats monitored for 3 h (see Figure 1) and is taken as an index of the oxidative metabolism of fatty acids in the liver [12,13]. However, in the case of diabetic rats, the amount exhaled as $^{14}\text{CO}_2$ yielded an underestimate of hepatic capacity for fatty acid oxidation, because in untreated diabetic animals $4.9 \pm 0.6\%$ of the label was recovered in the urine 60 min after injection of label. This was presumed to reflect the renal filtration of labelled ketone bodies into the urine because of the hyperketonaemia displayed by these animals. Therefore, in Figure 2 the combined values for ^{14}C in $^{14}\text{CO}_2$ and urine are given. When this correction is applied, the oxidation/esterification ratio for untreated diabetic animals was $27.3 \pm 1.5\%$. This is similar to that previously obtained for normal 24 h-starved rats [12], and double that obtained for normal fed rats [12]. The amount of label recovered in the urine decreased to $< 1\%$ during the first 1 h of insulin treatment. When an estimate was obtained of the amount of ^{14}C label present as ketones and acylcarnitines in the blood (by measuring acid-

Figure 2 Partitioning of ^{14}C label between oxidation and esterification products of hepatic fatty acid metabolism during insulin treatment of streptozotocin-diabetic rats

In (a) the proportions of label recovered as $^{14}\text{CO}_2$ (●) or plasma and liver glycerolipids (▲) are given as a function of the duration of insulin treatment. The bars indicate the respective values for normal fed animals (data from [12]) (OP, oxidation products in exhaled air and in urine; GL, glycerolipids in liver and plasma). In (b) changes in the ratio between the two parameters are shown for each time point. See the legend to Figure 1 for other details.

soluble radioactivity), it was found that, even in the untreated diabetic rats, this represented less than 1% of the injected dose, reflecting the very high turnover of label in the circulation.

We also monitored the incorporation of [^{14}C]oleate into liver and plasma lipids, in order to assess the partitioning of the incorporation of label between triacylglycerol and phospholipid as well as the fractional rate of secretion of these two major classes of lipids. As found previously [11–13], only a very small proportion of the labelled phospholipid was secreted into the plasma, confirming that the acyl-CoA pool labelled *in vivo* through hydrolysis of the administered cholesteryl 1- ^{14}C oleate behaves in the same way as that labelled by fatty acids delivered to the liver exogenously (see [11]). In contrast, a sizeable proportion of the newly labelled [^{14}C]triacylglycerol was always rapidly secreted, such that the fractional rate of secretion (i.e. the secreted/total [^{14}C]triacylglycerol) over the 60 min experimental period could be calculated. In untreated diabetic rats, this ratio (see below) was very similar to that found in normal fed rats (see [12,13]), indicating that long-term insulin deficiency does not intrinsically affect this parameter.

Similarly, the partitioning of label between phospholipid and total triacylglycerol (retained and secreted) was very similar to

Figure 3 Effect of insulin treatment of streptozotocin-diabetic rats on (a) the fractional rate of hepatic triacylglycerol secretion and (b) the proportion of ^{14}C label incorporated into phospholipid as a fraction of total glycerolipid

Details were as in Figure 1. Values that are statistically significant differ from those obtained for untreated animals (zero time) are indicated by * ($P < 0.05$) and ** ($P < 0.01$).

that found in normal fed animals (0.16 ± 0.01 for diabetic and 0.14 ± 0.02 for normal rats [12]), again indicating that insulin deficiency does not have a direct, chronic, effect on this ratio.

Effect of insulin treatment

Insulin treatment resulted in a rapid decline in the blood glucose concentration from an average of $27.9 (\pm 1.0, \text{S.E.M.}, n = 23)$ to $6.4 \pm 0.9 \text{ mM}$ ($n = 4$). Thereafter, glycaemia was maintained at near-normal values ($< 10 \text{ mM}$) by additional doses of insulin as required.

(i) Partitioning of fatty acid between oxidation and esterification

The ratio of oxidation products recovered in the first 60 min after injection of label (corrected as described above) as a fraction of the total ^{14}C label recovered in glycerolipids (plasma and liver) remained unchanged during the first 4 h of insulin treatment (Figure 2). Starting from the period spanning the 6th hour of insulin treatment, we observed a statistically significant decline in this ratio, which became more pronounced after 7–8 h of insulin treatment. This decline was due to reciprocal changes in the amounts of label recovered in $^{14}\text{CO}_2$ and ^{14}C -labelled

glycerolipids (Figure 2). It is therefore evident that changes in these parameters only occurred after a lag of several hours after the start of insulin treatment and the normalization of blood glucose levels.

(ii) Fractional rate of triacylglycerol secretion

The amount of triacylglycerol-associated label that accumulated in the plasma (as a result of Triton WR 1339 administration; see the Materials and methods section) as a proportion of total [^{14}C]triacylglycerol (liver plus plasma) was $67.3 \pm 2.6\%$ in untreated diabetic rats. There was a small decrease in this ratio (to $55.9 \pm 2.7\%$; $P < 0.05$) at the start of insulin treatment, which was rapidly reversed such that no significant change in the value of this parameter was observed (Figure 3a), even when the oxidation/esterification ratio changed markedly (between 5 and 8 h).

(iii) Partitioning of label between phospholipid and triacylglycerol

The basal value of 0.16 ± 0.01 ($n = 9$) for the ratio of label in phospholipid/total glycerolipid (PL/AG) in diabetic-rat liver was the same as that in normal fed animals (see above). However, by contrast with the fractional rate of secretion of triacylglycerol, this parameter increased gradually during insulin repletion, although the effect was modest and did not attain statistical significance until 5 h after the start of insulin treatment (Figure 3b). After 8 h of insulin treatment, the PL/AG ratio was doubled to an average value of 0.29 ± 0.03 ($n = 6$). This value was similar to, although still lower than, that achieved very rapidly (within 2 h) upon refeeding of 24 h-starved rats (0.33 ± 0.03 ; see [12]) or meal-fed animals (0.41 ± 0.20 ; see [13]). Moreover, the effect of sustained insulin treatment was not reversed for as long as the treatment was continued (8 h), in contrast with the phasic nature of the effect observed during the ingestion of meals [12,13].

DISCUSSION

Two main questions were addressed in the present study. The first concerned the inference made from enzyme kinetic studies on CPT I in insulin-treated diabetic rats [6], that insulin repletion does not result in the immediate re-establishment of the normal pattern of partitioning between fatty acid oxidation and esterification. CPT I remains over-expressed for more than 24 h after the start of insulin treatment, and its sensitivity to malonyl-CoA remains fully depressed even after 3 h of insulin repletion [6], and thereafter only returns to normal over a period of several hours [6]. This suggests that the hepatocyte should retain the ability to synthesize long-chain acylcarnitine from cytosolic acyl-CoA for the duration of this period of apparent refractoriness to the effects of insulin. Evidence from studies on rats subjected to long-term hyperinsulinaemic/euglycaemic clamps [16] and on perfused liver preparations [17] and hepatocytes [18,19] isolated from diabetic or anti-insulin-serum-treated rats given insulin have already provided support for this suggestion. The present data directly demonstrate the effect, and its consequences, *in vivo*, i.e. under conditions that include the continuously changing hormonal and substrate conditions that such a metabolic perturbation involves. No significant changes in the partitioning of fatty acid label towards oxidation or esterification occurred for up to 4 h after the start of insulin treatment. Thereafter, there were steady reciprocal changes in the two parameters. However, even after 8 h the respective values of the two parameters had still not achieved those found in the liver of normal fed animals (Figure 2). This phenomenon is

reminiscent of that observed upon refeeding of 24 h-starved rats, in which the capacity of acylcarnitine synthesis to compete successfully for long-chain acyl-CoA was found to be lost only very gradually over a period of more than 8 h after the start of refeeding [12]. Those observations have led to the suggestion of the existence of a fatty acid–glucose ‘paradox’ [20]. This envisages that a minimal rate of fatty acid oxidation commensurate with continued synthesis of glucose 6-phosphate could be maintained in the liver during the starved-to-refed transition, in spite of rapidly declining concentrations of NEFA in the circulation. The phenomenon ensures that during this metabolic transition fatty acid oxidation and synthesis of glycosyl units are integrated by physiological increases in insulin concentrations as well as other effectors of liver metabolism (see [20,21]). In the non-physiological situation represented by the treatment of diabetic rats with pharmacological doses of insulin, the absolute rates of hepatic fatty acid oxidation and of glyconeogenesis are likely to be curtailed much more rapidly than in starved–refed animals, not least because of the much more rapid suppression of substrate availability for the two processes. (In insulin-treated animals, once adipose-tissue lipolysis has been depressed by insulin, there are no alternative sources of NEFA, whereas in the starved–refed animals NEFA will also reach the liver through the ‘leak’ of fatty acid into the portal circulation [22] and as a result of the release of the products of chylomicron-triacylglycerol hydrolysis by lipoprotein lipase [23].) However, it is evident that the capacity of hepatic acylcarnitine synthesis to compete for acyl-CoA substrate continues to follow the physiological response pattern, namely one that involves a delay of several hours before hepatic fatty acid oxidative capacity is decreased.

The other questions addressed by the present studies were whether partitioning of hepatic fatty acids into different glycerolipid classes, and the fractional rate of secretion of newly synthesized triacylglycerol, were affected by streptozotocin-diabetes and acute insulin treatment of diabetic animals. These studies were aimed at establishing the direct role of insulin in the large changes seen in these parameters upon refeeding of starved animals. It is well established that insulin stimulates triacylglycerol synthesis in all the rat liver preparations *in vitro* that have been tested [24–26], apparently in keeping with the hypertriglyceridaemia that accompanies hyperinsulinaemic states [27]. However, the effect of the hormone on hepatic triacylglycerol secretion (in VLDL) has been more controversial [24–26]. The more recent contributions have provided convincing evidence that insulin acutely inhibits not only triacylglycerol secretion but also net apolipoprotein B synthesis and secretion in culture hepatocytes and isolated perfused rat liver ([28,29]; see [30] for recent review), although a complicating factor has emerged through the demonstration of a stimulatory effect on triacylglycerol secretion after prolonged cultured of hepatocytes with insulin [31].

It was therefore of interest to find that in diabetic rats, in which basal circulating insulin concentrations are very low, the fractional rate of triacylglycerol secretion and the [^{14}C]phospholipid/total [^{14}C]glycerolipid ratio were the same as those found in normal fed animals. This indicated that, whereas the insulin status of the animals had a chronic effect on the oxidation/esterification ratio, it had no similar effect on the partitioning of glycerolipid synthesis or on the fractional rate of triacylglycerol secretion. Teleologically, such a lack of effect of chronic insulin deficiency would be expected from other findings indicating that the rate of hepatic secretion of triacylglycerol in VLDL in fed diabetic animals is not decreased *in vivo* compared with that found in normal fed rats (see, e.g., [32]).

In apparent confirmation of the lack of a direct effect of insulin

on the fractional rate of triacylglycerol secretion, administration of insulin to diabetic rats had a minimal effect on this parameter, even after 8 h of treatment. This is in sharp contrast with the effects of food intake in starved–refed rats [12] or meal-fed rats [13], in which a large acute depression in the proportion of labelled triacylglycerol that is secreted into the plasma is observed. It has been suggested that the post-prandial rise in portal insulin may inhibit VLDL secretion [33], and this appeared to be a plausible explanation for the meal-induced decrease in the fraction of newly synthesized triacylglycerol that is secreted [13]. However, the present data suggest that factors besides a direct effect of insulin may be involved in the observed meal-induced effect. Therefore the hormone may have a permissive rather than a direct role in controlling the fractional rate of hepatic triacylglycerol secretion *in vivo*.

The very similar time courses for the effect of a meal on the fractional rate of secretion of labelled triacylglycerol and on the increase in the labelling of phospholipid relative to that of triacylglycerol (the PL/TGL ratio) suggested that the two effects may be mediated by the same metabolic or hormonal effector [12,13]. The present studies show that injection of insulin into diabetic rats can differentiate between the two effects. Thus, whereas the fractional rate of secretion of newly labelled triacylglycerol was only marginally affected, the PL/TGL ratio increased upon insulin treatment, although at a relatively moderate rate. This modest increase in the PL/TGL ratio suggests that the involvement of the hormone in more physiological conditions may also be indirect. In the non-physiological situation of the streptozotocin-diabetic rat, insulin deficiency may result in the alteration of the entire range of parameters of the hepatic metabolic profile. Thus the inability of the liver to respond acutely to peripheral insulin administration by a decrease in the fractional rate of triacylglycerol secretion may be due to chronic changes in the storage or secretory pathways involved in triacylglycerol metabolism. However, it has been shown recently that the low rate of VLDL secretion by cells prepared from diabetic rats can be reversed by the provision of lipogenic substrates in the culture medium [34], suggesting that the triacylglycerol-synthetic and -secretory machinery is unimpaired in diabetes.

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