Rapid switch of hepatic fatty acid metabolism from oxidation to esterification during diurnal feeding of meal-fed rats correlates with changes in the properties of acetyl-CoA carboxylase, but not of carnitine palmitoyltransferase I

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The effects of the ingestion of a meal on the partitioning of hepatic fatty acids between oxidation and esterification were studied *in vivo* for meal-fed rats. The time course for the reversal of the starved state was extremely rapid and the process was complete within 2 h, in marked contrast with the reversal of the effects of starvation in rats fed *ad libitum* [A. M. B. Moir and V. A. Zammit (1993) *Biochem. J.* **289**, 49–55]. This rapid reversal occurred in spite of the fact that, in the liver of the meal-fed animals before feeding, a similar degree of partitioning of fatty acids in favour of oxidation was observed as in 24 h-starved rats (previously fed *ad libitum*). This suggested that the lower degree of ketonaemia observed in meal-fed rats before a meal is not due to the inability of acylcarnitine formation to compete successfully with esterification of fatty acids to the glycerol moiety. Investigation of the possible mechanisms that could contribute towards the rapid switching-off of fatty acid oxidation revealed that this was correlated with a very rapid rise and overshoot in hepatic malonyl-CoA concentration, but not with any change in the activity, or sensitivity to malonyl-CoA, of the mitochondrial overt carnitine palmitoyltransferase (CPT I). The

role of these two parameters in the reversal of fasting-induced hepatic fatty acid oxidation was thus the inverse of that observed previously for refed 24 h-starved rats. The rapid increase in [malonyl-CoA] was accompanied by an immediate and complete reversion of the kinetic characteristics (K_a for citrate, expressed/total activity ratio) of acetyl-CoA carboxylase to those found in the post-meal animals, again in contrast with the time course observed in refed 24 h-starved rats [A. M. B. Moir and V. A. Zammit (1990) *Biochem. J.* **272**, 511–517]. The rapidity with which these changes occurred was specific to the partitioning of acyl-CoA; the meal-induced diversion of glycerolipids towards phospholipid synthesis and the acute inhibition of the fractional rate of triacylglycerol secretion occurred with very similar time courses to those observed upon refeeding of 24 h-starved rats. The results confirm the central role played by differences in the dynamics of changes in hepatic malonyl-CoA concentration, and CPT I sensitivity to it, in determining the route through which ingested glucose is converted into hepatic glycogen upon refeeding of starved rats which had perviously been meal-fed or fed *ad libitum*.

INTRODUCTION

In previous publications [1,2] we presented a rationalization of the observed concerted changes in the kinetic parameters of hepatic acetyl-CoA carboxylase and overt mitochondrial carnitine palmitoyltransferase (CPT I) upon refeeding of starved rats. The central feature of this postulate is that a measure of flux through CPT I persists during the early stages of refeeding and sustains mitochondrial acetyl-CoA concentrations at levels that would enable continued activation of pyruvate carboxylase (and hence gluconeogenesis [3,4]) and of pyruvate dehydrogenase kinase [5]. The latter effect results in the continued inhibition of pyruvate dehydrogenase [6] and the restriction of the activation of lipogenesis upon refeeding of starved rats [7].

That CPT I continues to compete successfully with acylglycerol formation for the common substrate, long-chain acyl-CoA, during the first 5–7 h of refeeding of 24 h-starved rats was directly demonstrated for awake rats *in vivo* [8], by selective labelling of hepatic fatty acids [9]. We also showed that, during this period, refeeding is associated with only a partial restoration of hepatic malonyl-CoA concentrations towards those found in the fed state [9]. Evidently, the combination of only partial elevation of malonyl-CoA concentration and the continued diminished sensitivity of CPT I to malonyl-CoA inhibition [1,8]

results in the sustained ability of the liver to synthesize acylcarnitines, although at a level dictated by the curtailed availability of acyl-CoA substrate.

If this postulate is valid, then this role for acylcarnitine formation and the associated maintenance of mitochondrial acetyl-CoA concentration in enabling the expression of the ‘glucose paradox’ (see [10]) must be lost much more rapidly in meal-fed rats. In these animals, trained to feed for a short period daily (2–3 h in different studies), refeeding after the diurnal fast results in a prompt cessation of gluconeogenesis such that most (> 80%) of the glycogen synthesized in the liver is derived through the direct pathway [11,12]. There is a concomitant hyper-activation of lipogenesis [13] and of PDH [14]. These observations suggest that the mechanisms that sustain CPT I activity in the liver, during the periods of starvation that these animals undergo daily, must be reversed rapidly during the meal. If continued activity of CPT I is genuinely involved in the operation of the glucose paradox in the normal starved-to-refed transition, then it follows that there should be marked contrast in this aspect of hepatic metabolism between normal starved-refed rats and meal-fed rats.

Monitoring of the partitioning of hepatic fatty acids between oxidation and esterification *in vivo* [8] is ideally suited to determine the time course with which the reversal of the ‘starved’ state

occurs in meal-fed rats on consumption of a meal. In the present paper we have performed such experiments, and also used the technique to address the question raised in [14] as to whether the relatively low ketonaemia observed in meal-fed rats before their meal (i.e. after 21 h starvation) is due to the inability of the oxidative pathway to compete successfully with esterification, or whether this is due to a lower overall rate of fatty acid metabolism by the liver.

The data show that there are marked differences in the response of oxidation/esterification partitioning during refeeding between meal-fed and 24 h-starved fed rats [8]. The mechanisms responsible for these differences are shown to include the different roles played by acute changes in the kinetic characteristics of CPT I and acetyl-CoA carboxylase in the two physiological conditions. In addition, the changes in glycerolipid synthesis and secretion by the liver during a meal are also described.

MATERIALS AND METHODS

Animals

Meal-fed animals were female Wistar rats (180–200 g) which were trained to receive food for 3 h daily (09:00–12:00 h). They were maintained on this regime for 4–6 weeks before being used. In a representative set of rats, after approx. 10 days on this regime the food intake stabilized at about 11.4 ± 0.5 g/day (compared with an intake of 13.4 ± 0.6 g/day for rats fed *ad libitum* maintained under otherwise identical conditions). Rats used to perform monitoring of hepatic fatty acid metabolism *in vivo* were fitted with indwelling jugular catheters 6–7 days before the experiments. The cannulae were flushed with citrated saline twice daily. During the post-operative recovery period the daily food intake gradually increased to 9.0 ± 0.4 g on the day before the experiment.

Lipoprotein donor rats were males (400–500 g) which were given 10 % fructose in the drinking water for 48 h before being used.

Preparation and infusion of labelled lipoproteins

ApoC-poor remnant lipoproteins ($d < 1.015$) were prepared and labelled with cholesteryl [$1\text{-}^{14}\text{C}$]oleate and [3H]cholesteryl oleoyl ether as described previously [8]. A sample (0.25–0.30 ml) of lipoprotein solution was injected into animals through the jugular cannula at the start of the period to be monitored. The animals were then placed in a chamber (1.5 l) through which air was drawn at the rate of 5 l/min. Where appropriate, rats had access to food while in the chamber and water was available throughout. At 15 min after injection of label, 0.8–1.0 ml of a solution (10 %) of Triton WR1330 was injected (100 mg/kg). Collection of exhaled $^{14}\text{CO}_2$ and measurement of the incorporation of ^{14}C into liver and plasma glycerolipids were performed as described previously [8] at the intervals indicated. After preliminary experiments to determine the time courses of these processes, routine experiments were performed for successive periods of 60 min after injection of label (see [8]).

Measurement of malonyl-CoA concentrations

Rats were anaesthetized with pentobarbitone (60 mg/kg) and allowed to stabilize for 20 min before the abdominal cavity was opened and the left lateral lobe of the liver was freeze-clamped. Malonyl-CoA concentrations were measured in HClO_4 extracts of the frozen tissue by the method of Singh et al. [15].

Measurement of CPT I activity

The isolation of liver mitochondria and measurement of CPT I activity (at 37 °C) in the presence of 35 μM palmitoyl-CoA and different concentrations of malonyl-CoA were performed as in [8]. Maximal activity was measured with 300 μM palmitoyl-CoA.

Measurement of acetyl-CoA carboxylase activity in liver cytosol

This was performed as described by Moir and Zammit [2]. Briefly, animals were anaesthetized (see above) and the left lateral lobe of the liver was rapidly cold-clamped (without freezing) and homogenized in fluoride-containing medium [16]. Cytosolic acetyl-CoA carboxylase was precipitated by making the post-mitochondrial fraction 35 % saturated with respect to $(\text{NH}_4)_2\text{SO}_4$. The precipitate, which contained all the cytosolic acetyl-CoA carboxylase, was resuspended and used to measure carboxylase activity before and after activation by exogenous protein phosphatase (2 units) and 20 mM citrate, as described previously [2]. Enzyme activity was measured at 30 °C. The assay medium contained 100 mM Tris/acetate (pH 7.4), 0.5 mM EDTA, 10 mM MgSO_4 , 2.5 mM ATP, 150 μM acetyl-CoA, 1 mM dithiothreitol, 10 mg of BSA/ml and 150 μM $\text{NaH}^{14}\text{CO}_3$ (2.5 Ci/mol). Different citrate concentrations (up to 20 mM) were obtained as described in [2]. The computation of the K_a for citrate and the fraction of carboxylase in the active form (i.e. expressed/total activities) was also performed as in [2].

Materials

The sources of biochemicals and radiochemicals were as described in [2,8]. The sources of [$1\text{-}^{14}\text{C}$]butyryl-CoA and rat liver fatty acid synthase and protein phosphatase have also been described [2,8].

RESULTS AND DISCUSSION

Partitioning of hepatic fatty acids between oxidation and esterification

In Figure 1(a) changes in the incorporation of [^{14}C]oleate (released by intrahepatic hydrolysis of cholesteryl [$1\text{-}^{14}\text{C}$]oleate) into total glycerolipids (plasma and liver) are shown for successive 1 h periods during the first 7 h after the commencement of a meal. Food was presented to the animals for the first 3 h of this period. It is apparent that in the pre-meal animal (i.e. after 21 h starvation) the proportion of [^{14}C]fatty acid used for esterification was very similar to that in the liver of 24 h-starved animals (Figure 1a). There was no change during the first 1 h of intake of the meal (rats routinely consumed 4–6 g of food during the first 1 h), but subsequently there was an abrupt and total reversion to the value observed for fed rats *ad libitum*. This change was entirely completed during the second 1 h period of the meal. Figure 1(b) shows that this rapid change was mirrored by an equally rapid and complete attainment of the 'fed' state with respect to proportion of the [^{14}C]fatty acid flux into oxidation products ($^{14}\text{CO}_2$ and [^{14}C]ketone bodies, which are presumed to give rise to $^{14}\text{CO}_2$ in the periphery; see [8]). The remarkable feature of these two time courses is the rapidity with which they occurred when compared with the very gradual changes in the two parameters observed during refeeding of 24 h-starved rats which had previously been fed *ad libitum* [8]. Consequently the ratio ($^{14}\text{CO}_2$ exhaled/ ^{14}C incorporated into glycerolipids) switched between the 'starved' and 'fed' states within 2 h (Figure 1c). By contrast, in the normal starved-to-fed transition this ratio

Figure 1 Partitioning of hepatic fatty acids between oxidation and esterification products monitored *in vivo* during a meal

Hepatic fatty acids were selectively labelled (see the Materials and methods section) and the incorporation of ^{14}C label into (a) total glycerolipids (liver plus plasma) and (b) exhaled $^{14}\text{CO}_2$ (derived directly from the liver and from ^{14}C ketone-body oxidation) were monitored over successive 1 h periods. In (c) the ratios between the two parameters for successive 1 h periods are given. Values ($\pm$ S.E.M.) are for 4 separate determinations. Food was presented to the animals for 3 h (thick axis line) after a 21 h fast for 4 weeks before, and during, the experiment. Bars S and F represent the values obtained for animals fed *ad libitum* before (F) and after (S) a 24 h fast (values taken from [8]).

was not entirely back to 'fed' levels even 7 h after the start of refeeding [8].

The high $^{14}\text{CO}_2$ /[^{14}C]glycerolipid ratio in pre-meal animals suggests that the partitioning of fatty acids between oxidation and esterification is similar to that found in 24 h-starved rats and that any decrease in the degree of ketonaemia in meal-fed animals (for an equivalent plasma non-esterified fatty acid concentration) [14] is due to causes other than the inability of

acylcarnitine formation to compete for acyl-CoA substrate (see below). (The apparently higher $^{14}\text{CO}_2$ /[^{14}C]glycerolipid ratio for pre-meal rats (1.09 ± 0.11) compared with 24 h-starved (0.80 ± 0.03) rats resulted partly from the fact that the dynamics of exhalation of $^{14}\text{CO}_2$ were slightly different in meal-fed rats. Thus a greater proportion of total exhaled $^{14}\text{CO}_2$ over 3 h was detected during the first 60 min (79% of the total after 3 h) than that exhaled during the first 60 min by fed or 24 h-starved rats (see [8]). Correction for this factor results in a ratio of the same order as that obtained for 24 h-starved rats.) Therefore, these findings complement those obtained for meal-induced changes in hepatic lipogenic and gluconeogenic fluxes in meal-fed rats (see the Introduction). In particular, they confirm that changes in the ability of the hepatocyte to oxidize fatty acids are central in determining the direction of flux from pyruvate towards either glycogenesis or lipogenesis, as suggested previously [1,2].

Mechanisms involved in switching off fatty acid oxidation

(i) CPT activity and sensitivity to malonyl-CoA

In normal starved-refed animals the maintenance of a high capacity for hepatic fatty acid oxidation is achieved, to a considerable extent, by the continued high maximal activity of CPT I [1] and slow reversion of the enzyme to its malonyl-CoA sensitive ('fed') form [1,8]. It was therefore decided to find out whether these parameters change more rapidly in the meal-fed animal. This was also of more fundamental interest, as the mechanism through which changes in CPT I sensitivity to malonyl-CoA occur is not definitively known. In contrast with observations in [17], changes in sensitivity have been shown mostly to be very gradual [1,18,19], prompting suggestions (supported by experiments *in vitro*) that these changes occur as a result of alterations in lipid ordering of the mitochondrial outer membrane [20].

It was therefore particularly interesting to find (in contrast with [21]) that no change occurred in the sensitivity of CPT I to malonyl-CoA in mitochondria isolated from meal-fed rats before or after a 3 h meal (Figure 2). During this time, the switch from fatty acid oxidation to esterification was fully achieved (see above). This observation highlights another difference between starved meal-fed and rats fed *ad libitum*, namely that, whereas the decrease in sensitivity of CPT I observed for 24 h-starved-refed rats appears to be most closely correlated with diversion of fatty acids away from oxidation [8], this mechanism appears to be totally irrelevant to the switch that occurs in meal-fed rats. However, the IC_{50} value (concentration required for 50% inhibition) for malonyl-CoA inhibition of CPT I in mitochondria from meal-fed rats was more similar to the 'fed' than to the '24 h-starved' value (Figure 2). Consequently, CPT I in the liver of the pre-meal animal is more predisposed to inhibition by any increase in malonyl-CoA concentration that accompanies the intake of the meal, than in the equivalent situation in the 24 h-starved rats.

In addition, the maximal activity of CPT I in mitochondria from meal-fed rats, even before feeding, was significantly lower than in those from 24 h-starved rats (20.2 ± 1.2 versus 35.8 ± 3.8 nmol/min per mg of mitochondrial protein; $n = 4$, $P < 0.001$) and not significantly different from that in mitochondria from rats fed *ad libitum* (19.2 ± 1.4 nmol/min per mg; $n = 4$). Therefore, in terms both of expression of the enzyme protein and of its sensitivity to malonyl-CoA, CPT I in the pre-meal rat (21 h-starved) has characteristics that are much more similar to those of CPT I in mitochondria of the animals fed *ad libitum* than of the 24 h-starved rat. It is suggested that these

Figure 2 Absence of change in the sensitivity of CPT I to malonyl-CoA inhibition before and after a meal

Livers were obtained from rats before (○) and after (●) a meal (3 h) in meal-fed rats. The activity of CPT I was measured in isolated liver mitochondria in the presence of the indicated concentrations of malonyl-CoA. The positions of the curves for the enzyme in mitochondria from rats fed *ad libitum* (—○—) and 24 h-starved rats (---●---) are shown (re-drawn after ref. [8]). Values are means ($\pm$ S.E.M.) of 4 determinations on separate mitochondrial preparations. The maximal activity of CPT I was 20.2 ± 1.2 and 22.2 ± 3.8 nmol/mg per mg of mitochondrial protein (at 37 °C) before and after the 3 h meal, respectively.

Figure 3 Time course of increase in hepatic malonyl-CoA content in the liver during a meal

Values are means ($\pm$ S.E.M.) for 4 determinations. Concentrations obtained for livers of normal 24 h-starved rats (S) and rats fed *ad libitum* (F) are shown for comparison (data from [8]).

characteristics explain the milder degree of ketonaemia observed in meal-fed rats [14]. It is likely that the milder ketonaemia is due to a lower overall hepatic utilization of fatty acids, as the $^{14}\text{CO}_2/[^{14}\text{C}]\text{glycerolipid}$ ratio is high in the 21 h-starved meal-fed rat (Figure 1).

(ii) Hepatic malonyl-CoA concentration

The above observations raise the questions as to whether (a) the malonyl-CoA concentration in pre-meal rat livers has to be decreased below the value obtained for 24 h-starved rats in order

to attain the high $^{14}\text{CO}_2/[^{14}\text{C}]\text{glycerolipid}$ ratios shown in Figure 1(c) and (b) whether the relatively high degree of sensitivity of CPT I in meal-fed rats is sufficient, by itself, to ensure that a rapid switch away from oxidation occurs during the first 2 h of the meal. The answer to both these questions appears to be negative [but see (iii) below]. First, the hepatic malonyl-CoA concentration is not significantly different between 24 h-starved and pre-meal rats (Figure 3). Second, it increases to values that are 2.5- and 4-fold higher than those attained in refed 24 h-starved animals after only 1 and 2 h after commencement of the meal, respectively (Figure 3). This confirms the findings in [22] and explains the rapid resumption of fatty acid synthesis upon feeding of meal-fed rats [13].

These observations in meal-fed rats emphasize the point that the absolute concentration of malonyl-CoA can be used, by itself, to control the rate of fatty acid oxidation directly under certain physiological conditions and that changes in CPT I sensitivity do not always necessarily play a central role (see, e.g., suggestion in [19]). In this instance, the overshoot in hepatic malonyl-CoA concentration to values well above those obtained in rats fed *ad libitum* ensures that the inhibition of CPT I is prompt and near-total, and complements the relatively high sensitivity and low activity of the enzyme already present in the pre-meal rat (Figure 2) in bringing about the rapid curtailment of acylcarnitine formation. Consequently the oxidation/esterification ratio (Figure 1c) is decreased very rapidly to values even lower than those observed in the animals fed *ad libitum*.

(iii) Kinetic characteristics of acetyl-CoA carboxylase

In 24 h-starved-refed rats we observed a biphasic change in the properties of acetyl-CoA carboxylase upon refeeding [2]. Thus the K_m for citrate declined from 8 mM to 5.2 mM within 1 h, followed by a further fall to 3 mM over the following 11 h [2]. Similarly, the expressed/total (E/T) activity ratio of the enzyme showed a rapid increase equivalent to about 10% of total enzyme activity, followed over the next 21 h by further increases equivalent to 20% of total activity [2]. Both these parameters reflect the phosphorylation status of acetyl-CoA carboxylase, and these time courses are indicative of a two-phase dephosphorylation of the sites on the enzyme that affect catalytic activity and/or affinity for citrate (see [23] for review). Figure 4 illustrates that the changes in K_m and E/T ratio are much more rapid and of a greater extent for acetyl-CoA carboxylase in the cytosol of livers of meal-fed rats during a meal. They are also monophasic. The K_m value for citrate fell from 7.9 ± 0.8 mM to 2.9 ± 0.3 mM within 2 h. Similarly, the increase in E/T ratio was maximal within this period, indicating that dephosphorylation of acetyl-CoA carboxylase was completed very rapidly, in sharp contrast with the situation in 24 h-starved-refed rats [2]. The absolute activity of carboxylase was not significantly different between pre-meal (0.23 ± 0.02 $\mu\text{mol/min per g wet wt.}$) and 24 h-starved rats (0.20 ± 0.02 $\mu\text{mol/min per g wet wt.}$).

The rapid and full response of acetyl-CoA carboxylase to the ingestion of a meal in the rats used in the present study is likely to be attributable to the size of the meal itself and the hormonal response to it. Meal-fed rats ingested 4–6 g of food within the first 1 h, and about 11 g within 3 h (see the Materials and methods section), whereas 24 h-starved rats only consume 3–4 g of the diet within the first 3 h (see [8]). Other adaptations may also increase the efficiency of absorption from the gut in meal-fed animals [24,25], resulting in increased glucose absorption [26]. These differences will be accompanied by a greater distension of the stomach. Such features of meal feeding may result in a more acute hormone-release pattern in these animals. Thus the surge

Figure 4 Diurnal changes in (a) K_a for citrate and (b) expressed/total activity ratio for cytosolic acetyl-CoA carboxylase in the liver of meal-fed rats

The duration of the meal (3 h) is indicated by the thick axis. Values are means ($\pm$ S.E.M.) for 3–5 separate determinations. Values for animals fed *ad libitum* (F) are given for comparison (from [8]).

in plasma insulin concentration induced by the meal (5-fold) occurs earlier and reaches a higher peak than in starved-refed animals [26]. Even when intubated with a standard quantity of food (2 g), rats trained to meal-feed show a higher insulin response than 22 h-starved rats previously fed *ad libitum* [24]. It is noteworthy that the very sharp insulin peak observed during the first 1 h of a meal is also accompanied by a peak in glucagon secretion [26]. However, the insulin/glucagon molar ratio increases 3-fold within 2 h and 5-fold after 4 h from the start of a meal [26]. These observations suggest that the liver of the meal-fed rat is likely to be exposed to higher insulin concentrations much more rapidly after the commencement of a meal and that this may be responsible for the sharper transitions observed in the present study. It is not known whether the increased insulin sensitivity displayed by some tissues of meal-fed rats (see e.g. [27,28]) extends also to the liver.

Changes in glycerolipid synthesis and secretion

In 24 h-starved rats, refeeding results in acute changes in the partitioning of the fatty acid label into triacylglycerols and phospholipids as well as in the fraction of newly synthesized triacylglycerol that is secreted by the liver into the plasma [8]. We found that the values for each of these parameters in the livers of pre-meal rats were very similar to those observed for 24 h-starved rats. Also, both the increase in [14 C]phospholipid/[14 C]total glycerolipid ratio (Figure 5a) and the decrease in the fraction of triacylglycerol secreted during the 60 min experimental period (Figure 5b) were similar to those observed previously in 24 h-starved-refed rats [8], although they tended to be more acute.

Figure 5 Effect of ingestion of the meal on (a) incorporation of labelled fatty acid into phospholipid relative to total acylglycerol synthesis, and (b) fractional secretion rate of labelled triacylglycerol into the plasma

Values are means ($\pm$ S.E.M.) for 4 separate determinations. Data for animals fed *ad libitum*, before (F) and after (S) a 24 h fast (from [8]), are shown for comparison.

The time courses for the two effects closely mirrored each other, again suggesting a common causative factor [8].

The fact that the time courses for changes in the dynamics of glycerolipid synthesis and secretion were similar to those observed in starved-refed rats suggests that adaptations of fatty acid metabolism in meal-fed rats are pathway-specific, affecting fatty acid oxidation and synthesis but neither the partitioning of glycerolipids between triacylglycerol and phospholipid synthesis nor the fractional secretion rate of triacylglycerols.

General conclusion

The concept of a malonyl-CoA/acetyl-CoA regulatory cascade, the effects of which are transmitted across the mitochondrial inner membrane via acylcarnitine synthesis [8], has now been shown to be valid also in the case of the much more rapid transition between the 'starved' and 'fed' states experienced during diurnal food consumption in awake, unrestrained, meal-fed rats. In addition, it is shown that the roles played by changes in the kinetic characteristics of acetyl-CoA carboxylase and CPT I are different from those observed for 24 h-starved-refed rats. Consequently, the roles of changes in the absolute concentration of malonyl-CoA and of the sensitivity of CPT I to the inhibitor in mediating the altered partitioning of hepatic fatty acids upon

refeeding are reversed in meal-fed rats with respect to the situation in 24 h-starved-refed animals.

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