Role of carnitine palmitoyltransferase I in the regulation of hepatic ketogenesis during the onset and reversal of chronic diabetes

Barbara D. GRANTHAM and Victor A. ZAMMIT
Hannah Research Institute, Ayr, Scotland KA6 5HL, U.K.

1. The kinetic properties of overt carnitine palmitoyltransferase (CPT I, EC 2.3.1.21) were studied in rat liver mitochondria isolated from untreated, diabetic and insulin-treated diabetic animals. A comparison was made of the time courses required for the changes in these properties of CPT I to occur and for the development of ketosis during the induction of chronic diabetes and its reversal by insulin treatment. 2. The development of hyperketonaemia over the first 5 days of insulin withdrawal from streptozotocin-treated rats was accompanied by parallel increases in the activity of CPT I and in the Iₜₜ (concentration required to produce 50% inhibition) of the enzyme for malonyl-CoA. 3. The rapid reversal of the ketogenic state by treatment of chronically diabetic rats with 6 units of regular insulin was not accompanied by any change in the properties of CPT I over the first 4 h. Higher doses of insulin (15 units), delivered throughout a 4 h period, resulted in an increase in the affinity of CPT I for malonyl-CoA, but the sensitivity of the enzyme to the inhibitor was still significantly lower than in mitochondria from normal animals. 4. Conversely, when insulin treatment was continued over a 24 h period, full restoration of the sensitivity of the enzyme to malonyl-CoA was achieved. However, the activity of the enzyme was only decreased marginally. 5. These results are discussed in terms of the possibility that the major regulatory sites of the rate of hepatic oxidation may vary in different phases of the induction and reversal of chronic diabetes.

INTRODUCTION

It is well established that regulation of the activity of overt carnitine palmitoyltransferase (CPT I) plays a central role in the partitioning of hepatic long-chain fatty acid metabolism between esterification and oxidation (see McGarry & Foster, 1980; Zammit, 1984). However, few studies have been performed in which the importance of the individual kinetic parameters of the enzyme in mediating changes in the oxidative capacity of the liver have been investigated. The induction of a large ketogenic capacity of the liver during the onset of chronic diabetes and the accompanying changes in the characteristics of CPT I (as well as their respective reversal by insulin treatment of diabetic rats) provide opportunities for such studies. Using such an approach, we have previously demonstrated that the major locus for the control of flux through the hepatic fatty acid oxidative pathway may differ between the induction phase of starvation ketosis and its acute reversal during the early stages of refeeding (Grantham & Zammit, 1986b). These studies also raised questions as to the mechanism(s) through which hormones, particularly insulin, may act directly on CPT I, in addition to effects on the activity of the enzyme through changes in the hepatic content of malonyl-CoA. Thus the restoration of the kinetic properties of CPT I after refeeding occurs remarkably slowly compared with the well-documented changes in circulating insulin concentration and the acute decline in ketogenic capacity of the liver (see Grantham & Zammit, 1986b).

In ketogenic states, CPT I undergoes three major changes in its properties, as measured in vitro in isolated liver mitochondria: (i) it becomes more active, (ii) it becomes less sensitive to inhibition by malonyl-CoA and (iii) it acquires a slightly lower affinity for acyl-CoA substrate (see Grantham & Zammit, 1986b). In the present study, we have investigated the progressive effects, on these properties of CPT I, induced by the withdrawal of insulin from streptozotocin-treated rats that had been maintained on insulin for several days. In this model of diabetes (developed by Blakeshear & Alberti, 1974) the capacity of the liver to oxidize non-esterified long-chain fatty acids increases only gradually over a period of days after insulin withdrawal, in spite of the rapid attainment of low serum concentrations of insulin after effective insulin withdrawal. It was therefore considered that a comparison of the time courses of changes in the kinetic properties of CPT I with that for increased ketogenic capacity of the liver would provide useful information about the importance of these changes in the characteristics of the enzyme for the development of the ketogenic state. Similarly, we were able to investigate the rapidity with which the respective CPT I parameters respond to acute or chronic insulin repletion. The data suggest that, in this model of diabetes, changes in CPT I characteristics favourable to increased fatty acid oxidation occur in parallel with the increase in hepatic ketogenic capacity during the onset of diabetic ketosis. However, the reversal of these changes occurs remarkably slowly, compared with the rapidity with which the ketogenic state is reversed after insulin treatment of chronically diabetic animals.

MATERIALS AND METHODS

Animals

These were female Wistar rats (A. Tuck and Sons, Rayleigh, Essex, U.K.) weighing 180–230 g. Insulin
deficiency was induced by a single intraperitoneal injection of streptozotocin (100 mg/kg body wt.). After 2 days (during which the animals were given a 5% glucose solution to drink), induction of diabetes was ascertained by measurement of the glucose concentration in blood samples obtained from the tail of lightly ether-anæsthetized animals. Only animals which, at this stage, had a blood glucose concentration higher than 18 mM were used. During subsequent chronic diabetes these animals developed higher blood glucose concentrations.) The animals were then maintained on a daily injection of protamine–zinc insulin (4–6 units) for 5 days as described by Blackshear & Alberti (1974). Rats were used for the preparation of liver mitochondria after the indicated intervals from the last injection of insulin. Aortic blood samples were obtained (at the time of liver sampling) for measurements of blood glucose and ketone bodies as well as serum insulin concentrations. Acute insulin treatment of diabetic rats was achieved by intraperitoneal injection of crystalline insulin as indicated. Longer-term insulin treatment was achieved by injection of protamine–zinc insulin as described above.

Preparation of mitochondria and assay of CPT I activity

Animals were anaesthetized with pentobarbitone (60 mg/kg body wt.). After 20 min, the liver was excised and mitochondria were prepared as described previously (Granath & Zammit, 1986b). The final mitochondrial pellet was resuspended (in medium containing 150 mM-KCl, 5 mM-Tris/HCl and 1 mM-EGTA, pH 7.4 at 0 °C) at a concentration of about 50 mg of protein/ml. CPT I activity was measured radiochemically at 37 °C as described previously (Granath & Zammit, 1986b). The assay medium contained 150 mM-KCl, 5 mM-Tris, 1 mM-EGTA, 1 mM-dithiothreitol, 3 mM-L-[methyl-3H]carnitine (0.08 Ci/mmol), the indicated concentrations of palmitoyl-CoA and malonyl-CoA, 10 mg of defatted albumin/ml, 1 μg of Antimycin A/ml and 1 μg of rotenone/ml. The final volume was 2.0 ml and the pH was 7.4 at 37 °C. Mitochondria (about 2 mg of protein) were incubated in the assay medium at 37 °C for 2 min before initiation of the reactions by addition of L-carnitine. Reactions were terminated after a further 2 min by addition of 0.3 ml of 6 M-HCl. Incorporation of 3H into acylcarnitine was measured as described previously (Robinson & Zammit, 1982).

Materials

The sources of these were as described previously (Granath & Zammit, 1986b). In addition, streptozotocin was a gift from the Upjohn Chemical Co. (Kalamazoo, MI, U.S.A.), and protamine–zinc insulin (bovine; 100 units/ml) was from Weddel Pharmaceuticals (Wrexham, Clwyd, Wales, U.K.). Crystalline insulin was obtained from Sigma (Poole, Dorset, U.K.).

RESULTS

Treatment of rats with streptozotocin resulted in acute elevation of blood glucose concentrations after 2 days, accompanied by a 15–20% decrease in body weight (results not shown). Maintenance of these animals on protamine–zinc insulin, for the subsequent 5 days, stabilized their blood glucose concentrations at values approximating to normal. Normal growth rates were also restored. At 24 h after the last insulin injection, blood glucose and ketone-body concentrations were still within the normal range (Fig. 1 and Table 1). This was presumed to be due to residual insulin in the circulation, owing to the slow-release preparation of insulin used (see Blackshear & Alberti, 1974). These animals (24 h after last insulin treatment) are referred to as ‘controls’, to distinguish them from ‘normal’ animals, which were not treated with streptozotocin. After a further 24 h of insulin withdrawal, blood glucose concentrations increased markedly (Table 1); there was little further increase during subsequent days of insulin withdrawal. These observations are similar to those of Blackshear & Alberti (1974), who also reported that, 48 h after the last insulin treatment, the plasma concentration of non-esterified fatty acids is also fully elevated to maximal values, whereas serum insulin concentrations decrease to values below normal (cf. Table 1). By contrast, the concentration of ketone bodies in the blood increased only very gradually over the first 5 days of insulin withdrawal (Fig. 1). Acute (4 h) treatment of these animals with insulin resulted in a rapid decline in blood glucose and ketone-body concentrations to normal values. Longer-term (24 h) treatment also resulted in the normalization of these parameters.

Activity of CPT I and affinity for palmitoyl-CoA

The activity of CPT I was measured at different palmitoyl-CoA concentrations in mitochondria isolated from normal and control animals, as well as diabetic animals from which insulin was withdrawn for 48, 72 or 120 h. Both the activity and affinity of the enzyme for palmitoyl-CoA were identical in mitochondria from
Hepatic carnitine palmitoyltransferase in diabetes

Vol. 249

Table 1. Concentrations of glucose, acetoacetate and 3-hydroxybutyrate in whole blood, and of insulin in serum of normal, control and diabetic rats

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Diabetic</td>
</tr>
<tr>
<td></td>
<td>15 units of RI (4 h)</td>
<td>6 units of RI (4 h)</td>
</tr>
<tr>
<td>Glucose (μmol/ml)</td>
<td>12.2±0.6 (3)</td>
<td>10.3±0.4 (3)</td>
</tr>
<tr>
<td>Acetoacetate (μmol/ml)</td>
<td>0.6±0.1 (3)</td>
<td>0.1±0.1 (3)</td>
</tr>
<tr>
<td>3-Hydroxybutyrate (μmol/ml)</td>
<td>&lt;0.1 (3)</td>
<td>0.1±0.1 (3)</td>
</tr>
<tr>
<td>Insulin (units/ml)</td>
<td>21±3 (3)</td>
<td>19±3 (3)</td>
</tr>
</tbody>
</table>

Values are means ± S.E.M. for the number of determinations shown in parentheses. For diabetic animals, the time (h) from the last injection of insulin is given. 1. Insulin-treated diabetic animals were maintained on 120 h-diabetic rats, and the insulin preparations indicated. Abbreviations: RI, regular insulin; PZI, protamine zinc insulin.

Fig. 2. Changes in the activity of hepatic CPT I in isolated liver mitochondria during the induction and reversal of diabetic ketosis in the rat

CPT I activity was assayed in the presence of different concentrations of palmitoyl-CoA (in the presence of 10 mg of albumin/ml; see the Materials and methods section) in mitochondria isolated from normal (○) and control (●) rats, and from diabetic animals from which insulin had been withdrawn for 48 h (■), 72 h (△) and 120 h (□).

normal and control rats (Figs. 2 and 3). However, the activity of the enzyme (expressed per mg of mitochondrial protein) increased over the first several days after insulin withdrawal (Figs. 1 and 2). Continued insulin withdrawal for up to 120 h increased the activity (at 540 μM-palmitoyl-CoA, in the presence of 10 mg of albumin/ml) to a value that was 2.5-fold higher than in mitochondria from normal or control animals. The decrease in CPT I affinity for palmitoyl-CoA characteristic of ketogenic states (Brady et al., 1985; Grantham & Zammit, 1986b) was apparent 48 h after the last insulin injection (Fig. 3).

Reversal of these effects of chronic diabetes on CPT I by insulin treatment of 120 h-diabetic rats occurred relatively slowly when compared with the reversal of the ketogenic hyperglycaemic state. Thus the activity of CPT I was unchanged, after 4 h of either type of acute insulin treatment (from that shown for 120 h-diabetic rats in Fig. 2). Similarly, treatment of diabetic rats for 24 h with insulin resulted in only a relatively small (20%) decrease in the activity of CPT I, to a value that was still 2.0-fold higher than that in mitochondria from normal or control rats; the affinity of CPT I for palmitoyl-CoA appeared not to be restored to normal values even after 24 h of insulin treatment of chronically diabetic rats (results not shown).

Sensitivity of CPT I to malonyl-CoA inhibition

Withdrawal of insulin from diabetic animals for 120 h produced the expected decrease in sensitivity of CPT I to
malonyl-CoA inhibition (compared with control or normal animals; cf. Cook et al., 1984; Brady et al., 1985). Intermediate periods (48 h, 72 h) of withdrawal of insulin from diabetic animals resulted in intermediate effects on the sensitivity of CPT I to malonyl-CoA inhibition (Fig. 4).

The ability of insulin treatment to reverse the effects of diabetes on the sensitivity of CPT I to malonyl-CoA inhibition depended on the dose of the hormone administered. However, it did not appear to be related to the degree of normalization of blood glucose or ketone-body concentrations (Table 1). Two series of acute experiments were performed, in which animals received either a single dose of regular insulin (6 units, intra-peritoneally) 4 h before being sampled (cf. Gamble & Cook, 1985; Cook & Gamble, 1987) or a total of 15 units of insulin administered in equal doses over the 4 h period at hourly intervals (cf. Easom & Zammit, 1986). In the first series, blood glucose and ketone-body concentrations were decreased to normal values. However, an increase in sensitivity of CPT I to malonyl-CoA inhibition, over that observed in 120 h-diabetic rats, was not observed (Fig. 5). By using the hourly doses of insulin (3 units/h for 4 h), it was possible to obtain higher serum insulin concentrations (Table 1). Under these conditions, a distinct increase in sensitivity of CPT I to malonyl-CoA inhibition was observed, which nevertheless still left the enzyme in a considerably desensitized state compared with CPT I in mitochondria from normal or control animals (Fig. 5). In contrast, treatment of diabetic rats with protamine-zinc insulin for 24 h (three injections each of 10 units at 8 h intervals) resulted in complete restoration of the sensitivity of CPT I to malonyl-CoA inhibition to that characteristic of the normal state (Fig. 5), even though the serum insulin concentration at the time of liver sampling was much lower than after acute (hourly) insulin treatment. However, it has to be emphasized that, as expected, both treatments resulted in extremely high (non-physiological) concentrations of insulin (Table 1).

**DISCUSSION**

The assay conditions used in the present study were chosen to give a low sensitivity of CPT I to malonyl-CoA (i.e. high $I_{50}$ values) in order to minimize interference from the destruction of malonyl-CoA by deacylase activity of mitochondria with the accurate quantitative assessment of this parameter. Therefore the high concentrations of malonyl-CoA required to effect complete inhibition of CPT I (compared with those that occur in vivo; Zammit, 1981) should not be interpreted as indicating that malonyl-CoA effects on CPT I are not relevant to the control of CPT I activity in vivo. Similarly, because of the high albumin concentrations used in the assay medium, the maximal activity of CPT I was observed at total concentrations of palmitoyl-CoA in excess of 500 $\mu$M. Preliminary experiments confirmed that, at this concentration of palmitoyl-CoA, CPT activity was due almost exclusively to CPT I (overt) activity. Thus treatment of mitochondria with bromo-
Hepatic carnitine palmitoyltransferase in diabetes

CPT I activity (see legend to Fig. 4) was assayed in liver mitochondria isolated from ‘control’ rats (○) and from rats that were chronically (120 h) diabetic (□), or diabetic and treated with a single intraperitoneal injection of 6 units of regular insulin (▲) 4 h before liver sampling, or hourly injections each of 3 units of regular insulin for 4 h before liver sampling (▼), or injections each of 10 units of protamine–zinc insulin at 8 h intervals for 24 h (☆). Values are means ± S.E.M. for three or four determinations on separate mitochondrial preparations.

palmitate, CoA, ATP and carnitine (which results in total inhibition of palmitoyl-CoA plus carnitine oxidation, but not of palmitoylcarnitine oxidation, by rat liver mitochondria; see Grantham & Zammit, 1986a) resulted in 95% inhibition of CPT activity measured at 540 μM-palmitoyl-CoA. This degree of inhibition was similar to that observed at 130 μM-palmitoyl-CoA, indicating that no deterrent effects were apparent even at the higher concentration of palmitoyl-CoA.

The changes in the kinetic parameters of CPT I induced by chronic diabetes were very similar to those induced by 48 h-starvation (Grantham & Zammit, 1986b). Similar changes in malonyl-CoA-sensitivity of the enzyme were reported by Gamble & Cook (1985) for mitochondria isolated from animals killed only 48 h after streptozotocin treatment. Therefore it can be assumed that the subsequent period of maintenance of acutely diabetic animals on insulin (‘control rats’) resulted in complete reversal of these changes in the present study, as there were no significant differences between the properties of the enzyme in mitochondria from normal and control rats. Subsequent withdrawal of insulin from these animals, however, resulted in a much slower development of ketosis than in the acutely diabetic rats, in spite of a much more rapid development of hyper-glycaemia and increased plasma concentrations of non-esterified fatty acids (Table 1, and Blackshear & Alberti, 1974). These observations suggest that the slow increase in ketosis reflects a genuine increase in the intrinsic ketogenic capacity of the liver after 48 h of insulin withdrawal, under conditions of constantly high rates of delivery of fatty acids to the liver, although it is possible that the developing ketosis is partly due to decreased utilization of ketone bodies by peripheral tissues in chronically diabetic rats (Blackshear & Alberti, 1974).

The present observations suggest that this apparent increase in the ketogenic capacity of the liver is accompanied by a parallel increase, over the same time scale, in the maximal activity of CPT I and in its I₅₀ for malonyl-CoA. Consequently, it is suggested that the observed changes in these parameters may be involved in mediating the gradual increase in hepatic ketogenic capacity at a time when fatty acid release from adipose tissue is maximal. The metabolic result of these changes in the properties of CPT I would, of course, amplify the effects of decreased hepatic malonyl-CoA concentrations (Foerster et al., 1979; Lynen, 1979). The small decrease in affinity of CPT I for palmitoyl-CoA was established after only 48 h of insulin withdrawal. Such a change was observed previously after 48 h starvation of rats and, although tentative suggestions have been made for its significance in vivo (Grantham & Zammit, 1986h), its possible role remains unknown.

The reversal of the changes in CPT I characteristics (induced by chronic diabetes) after insulin treatment occurred much more gradually than the restoration of normoglycaemia and normoketonaemia. Acute treatment with 6 units of regular insulin resulted in normalization of blood glucose and ketone-body concentrations, with no apparent change in the sensitivity of CPT I to malonyl-CoA inhibition. Higher doses of insulin, delivered regularly over a 4 h period, only partially restored this parameter towards normal values. However, full restoration of normal sensitivity was observed after 24 h treatment with insulin, even though the rate of administration of insulin (maximum rate = 1.25 units/h) was lower than that in the acute experiments (3.75 units/h), and the ultimate resulting plasma insulin concentration was similar, at the time of liver sampling, to that obtained in the acute experiments. Consequently, the length of time over which elevated plasma insulin concentrations are maintained appears to be important in determining the degree of reversal of the desensitization of CPT I to malonyl-CoA inhibition. Very high insulin concentrations can also achieve partial reversal of desensitization, but this is still evident after 4 h of elevated insulin concentrations.

These observations suggest that CPT I does not respond to insulin with the same rapidity as the antilipolytic effect of the hormone on adipose tissue and its effects on glucose turnover. The time scale of these effects of insulin on CPT I also contrasts with that observed in previous studies on the effects of insulin treatment of diabetic rats on other hepatic metabolic processes (e.g. phosphorylation state of 3-hydroxy-3-methylglutaryl-CoA reductase), which are very rapid (Easom & Zammit, 1986). In this context, however, it is essential to stress that the protocols used in studies involving the preparation of isolated mitochondria are not designed to observe or preserve rapid changes in post-translational modification of enzymes (such as reversible covalent phosphorylation; see Grantham & Zammit, 1986b).

Nevertheless, since the same conditions were used
throughout the present study, it is valid to emphasize that, whereas parallel changes occurred in the apparent increase in hepatic ketogenic rate and in the properties of CPT I during the onset of the ketotic state, there was no such temporal correlation during the acute treatment of diabetic rats with insulin. It is suggested that, whereas CPT I is an important site at which regulation is exerted in order to mediate the gradual increase in hepatic ketogenesis during the onset of the diabetic state, it does not appear to play such a role during the acute reversal of the ketosis. It is likely that the rapid decline in blood ketone-body concentration after insulin treatment of chronically diabetic rats is due primarily to the anti-lipolytic effect of insulin on adipose tissue, although it is also possible that acetyl-CoA generated from continued (although much diminished) oxidation of fatty acids may be diverted towards citrate synthesis.

These conclusions are similar to those drawn from previous studies on the acute effects of refeeding of starved rats (Grantham & Zammit, 1986b). Acute depression of the ketogenic capacity of the liver on refeeding is not accompanied by any reversal of the changes in CPT I induced by starvation during the first several hours of refeeding. Even after 6 h of refeeding, only partial reversal of the desensitization of CPT I to malonyl-CoA inhibition is observed (Grantham & Zammit, 1986b). The present observations appear to be at variance, however, with those of Gamble & Cook (1985) and Cook & Gamble (1987), who reported much more rapid effects of insulin on the properties of CPT I in rats subjected to streptozotocin treatment 48 h before being killed. The reasons for such a discrepancy between the two sets of data are not known. It is possible that the diabetic models used in the respective studies are sufficiently different to result in different responses to insulin treatment. However, it is noteworthy that, even during the induction of diabetic ketosis, the properties of CPT I change only gradually, over a number of days, after insulin withdrawal, in spite of the relatively rapid attainment of high plasma concentrations of non-esterified fatty acids, hyperglycaemia and hypoinsulin- aemia (Table 1, and Blackshear & Alberti, 1974).

Moreover, it is possible that different modes of expressing the malonyl-CoA-sensitivity data (K₅ or l₄₈₅) may result in different emphasis being placed on different aspects of the behaviour of this parameter in vivo.

It is of interest to compare the relatively slow response of CPT to insulin withdrawal and replenishment with the time scale of the responses of the ketogenic capacity of isolated perfused liver, reported by Woodside & Heimberg (1976). These authors showed that, during the first 6 h after commencement of anti-insulin treatment of rats, the concentration of plasma non-esterified fatty acids increases to a maximum, whereas blood ketone-body concentrations are unaffected. During this period, the intrinsic capacity of perfused liver to oxidize a fixed load of fatty acid delivery increased only very gradually. Similarly, insulin treatment of diabetic rats results in the rapid (<2 h) depression of plasma non-esterified fatty acid and blood ketone-body concentrations, without any decrease, during the first 4 h, in the intrinsic capacity of the perfused liver to oxidize a fixed load of fatty acids to ketones (Woodside & Heimberg, 1976).

In conclusion, we suggest that CPT I is an important locus for the control of hepatic fatty oxidation during the onset of diabetic ketosis. By contrast, the major determinant of the rate of hepatic fatty acid oxidation after acute insulin treatment of diabetic rats is likely to be the rate of fatty acid delivery to the liver (cf. Woodside & Heimberg, 1976). The central role of malonyl-CoA in the control of the oxidation pathway appears to be partially restored after several hours, and fully restored after 24 h, of insulin treatment, although the maximal activity of CPT I remains elevated.

The physiological significance of the time courses of these changes can be tentatively suggested. A gradual increase in ketogenic capacity of the liver after insulin withdrawal would enable a greater proportion of the high rate of delivery of fatty acids to the liver to be diverted towards acylglycerol synthesis, thus increasing hepatic lipoprotein secretion for utilization by peripheral tissues in which the activity of lipoprotein lipase is not insulin-dependent (see Brindley, 1981). Conversely, a gradual decline in the fatty acid oxidative capacity of the liver after acute insulin treatment or refeeding of starved rats may serve to maintain the minimal rates of oxidation of fatty acids necessary to keep the intramitochondrial acetyl-CoA concentration elevated. This would enable the continued activity of pyruvate carboxylase for a short period, during which active gluconeogenesis may continue (Katz et al., 1986).

We thank Dr. D. J. Flint for performing the determinations of serum insulin concentrations and Mr. C. G. Corstorphine for expert assistance. This work was supported by the British Diabetic Association.

REFERENCES


Received 9 April 1987/7 August 1987; accepted 21 September 1987