The role of changes in the sensitivity of hepatic mitochondrial overt carnitine palmitoyltransferase in determining the onset of the ketosis of starvation in the rat

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The relationships between the increase in blood ketone-body concentrations and several parameters that can potentially influence the rate of hepatic fatty acid oxidation were studied during progressive starvation (up to 24 h) in the rat in order to discover whether the sensitivity of mitochondrial overt carnitine palmitoyltransferase (CPT I) to malonyl-CoA plays an important part in determining the intrahepatic potential for fatty acid oxidation during the onset of ketogenic conditions. A rapid increase in blood ketone-body concentration occurred between 12 and 16 h of starvation, several hours after the marked fall in hepatic malonyl-CoA and in serum insulin concentrations and

doubling of plasma non-esterified fatty acid (NEFA) concentration. Consequently, both the changes in hepatic malonyl-CoA and serum NEFA preceded the increase in blood ketone-body concentration by several hours. The maximal activity of CPT I increased gradually throughout the 24 h period of starvation, but the increases did not become significant before 18 h of starvation. By contrast, the sensitivity of CPT I to malonyl-CoA and the increase in blood ketone-body concentration followed an identical time course, demonstrating the central importance of this parameter in determining the ketogenic response of the liver to the onset of the starved state.

INTRODUCTION

Mitochondrial overt carnitine palmitoyltransferase (CPT I) catalyses a reaction at which major control of the rate of fatty acid oxidation occurs [1]. The main regulatory property of the enzyme is its inhibition by malonyl-CoA [2]. In the liver, the effects of changes in malonyl-CoA concentration are amplified by concurrent alterations in the sensitivity of the enzyme to the inhibitor [3]. Thus low malonyl-CoA concentrations (e.g. in the liver of starved rats) are accompanied by a decreased sensitivity (increased K_i) to malonyl-CoA [4,5], as well as increased expression of CPT I protein [6]. The importance of the changes in sensitivity of CPT I to malonyl-CoA in determining the ability of acyl-carnitine synthesis to compete for cytosolic acyl-CoA has been explored previously by correlating the time courses of changes in the kinetic characteristics of hepatic CPT I with the changing relationship between plasma non-esterified fatty acid (NEFA) and ketone-body concentrations during the onset and reversal of ketosis in streptozotocin-treated diabetic rats [7]. It was concluded that, whereas the changes in CPT I sensitivity to malonyl-CoA were a key factor in determining the flux through CPT I during the onset of the ketogenic states, the reversal of the adaptation in CPT I sensitivity lagged considerably behind the reversal of the ketotic state upon insulin treatment [7]. Therefore control at the CPT I step is high during the onset of ketosis but it is much lower during the early phase of its reversal. In studies conducted *in vivo* on rats in which the hepatic fatty acids were selectively labelled, it was shown that re-feeding of starved rats or insulin-treatment of diabetic animals did not significantly alter the ability of CPT I to compete for acyl-CoA for several hours after the reversal of the ketotic state was begun [8–10]. Re-establishment of control by the CPT I-catalysed reaction took at least 8 h and coincided with the regaining of sensitivity to malonyl-CoA inhibition by the enzyme. These observations have

highlighted the important role that changes in the malonyl-CoA sensitivity of CPT I play in the control of hepatic fatty-acid oxidation during the starved-to-re-fed transition. However, to our knowledge, no studies have been performed to assess the role of changes in the sensitivity of CPT I to malonyl-CoA during the onset of starvation and specifically to determine the temporal relationships between the onset of increased ketone-body formation by the liver and the changes in CPT I expression of catalytic activity and/or kinetic characteristics. Therefore in the present study we have addressed these questions. The data indicate that, among the extra- and intra-hepatic mechanisms involved, the loss of sensitivity of CPT I to malonyl-CoA is a major factor in determining the onset of increased ketone-body production by the liver during the progression of starvation.

MATERIALS AND METHODS

Animals

Female Wistar rats (180–200 g) maintained on a chow diet provided *ad libitum* (see [8] for details) were used. Food was removed from the rats for up to 24 h, starting 1 h into the light period of a 12 h light/12 h dark cycle. Water was available throughout.

Blood and liver sampling and preparation of mitochondria

Rats were anaesthetized with pentobarbitone (60 mg/kg). After a 15 min period of stabilization the abdominal cavity was opened and either an aortic blood sample (0.5 ml) was taken for determination of blood and plasma metabolite concentrations or the left lateral lobe of the liver was freeze-clamped for malonyl-CoA determinations. The rest of the liver was removed and homogenized using a Teflon–glass homogenizer and ice-cold

Abbreviations used: CPT I, mitochondrial overt carnitine palmitoyltransferase; NEFA, non-esterified fatty acids.

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medium containing 250 mM sucrose, 1 mM EGTA and 10 mM Tris (pH 7.4 at 4 °C). Mitochondrial fractions were obtained by differential centrifugation. Briefly, after low speed centrifugation (500 *g* for 10 min at 4 °C) to remove cell debris, the supernatant was centrifuged at 6500 *g* for 10 min at 4 °C. The pellet was resuspended in buffer and centrifuged at 6000 *g* for 10 min at 4 °C to obtain the final mitochondrial pellet.

Enzyme assays and metabolite analyses

CPT I activity was measured by following the rate of [³H]-palmitoylcarnitine formation from [³H]carnitine and palmitoyl-CoA. The assay medium (1 ml) contained 150 mM KCl, 1 mM EGTA, 5 mM Tris/HCl (pH 7.4), 5 mM ATP, 1 % (w/v) defatted BSA, 1.6 mM dithiothreitol, 4 µg rotenone/ml, 2 µg antimycin A/ml and 0.52 mM L-[³H]carnitine (specific activity 770 d.p.m./nmol). When maximal activity of CPT I was measured the palmitoyl-CoA concentration was 110 µM. To obtain sensitivity curves, the palmitoyl-CoA concentration was 35 µM and malonyl-CoA was added at concentrations ranging from 0.2–100 µM. The assays were performed at 37 °C and 0.5 mg of mitochondrial protein was used. The reactions were started by addition of [³H]carnitine after a 2 min equilibration period and were terminated, after a further 2 min, by the addition of 0.3 ml of 6 M HCl. Separation of [³H]palmitoylcarnitine was performed by butanol extraction [12].

Analyses of blood ketone bodies (in HClO₄ extracts of whole

blood) and plasma NEFA were performed as described previously [9]. Malonyl-CoA was measured in HClO₄ extracts of frozen liver tissue, as described in [12], using [¹⁴C]butyryl-CoA as primer. Insulin was measured in serum prepared from aortic blood samples by competitive ELISA.

Materials

Palmitoyl-CoA was from Pharmacia (St Albans, Herts, U.K.). Malonyl-CoA and L-carnitine were from Sigma (Poole, Dorset, U.K.). The NEFA-C kit was from Alpha Laboratories (Eastleigh, Hants, U.K.). [³H]carnitine and [¹⁴C]butyryl-CoA were from Amersham International (Amersham, Bucks, U.K.).

RESULTS

Relationship between serum insulin, plasma NEFA and blood ketone-body concentrations

The serum insulin concentration decreased steadily to reach a minimum value after 12 h of starvation. The relatively modest decrease in serum insulin that occurred during the first 4 h was accompanied by a doubling of the plasma NEFA concentration, shown in Figures 1 and 2. There was no further increase in

Figure 1 Responses of (a) plasma NEFA concentration and (b) blood ketone-body concentration to progressive starvation in the rat

Food was withdrawn from rats previously fed *ad libitum* at zero time, which was equivalent to 1 h into the light phase of a 12 h light/12 h dark cycle. At the times indicated animals were anaesthetized and, after a period of stabilization (15 min), the abdominal cavity was opened and a sample of aortic blood was obtained. Values are the means $\pm$ S.E.M. for four to nine animals for each time point.

Figure 2 Changes in (a) the concentration of serum insulin and (b) the concentration of hepatic malonyl-CoA during the progression of starvation for a 24 h period

Rats were starved as described in the legend to Figure 1. At the times indicated the rats were anaesthetized, the abdominal cavity was opened and either a sample of aortic blood was obtained or the left lateral lobe of the liver was freeze-clamped between tongs cooled in liquid N₂. Mitochondria were prepared from the remaining liver (see the Materials and methods section). Values are the means $\pm$ S.E.M. for determinations on four to nine animals for each time point.

Figure 3 Changes in (a) the IC_{50} of mitochondrial CPT I for malonyl-CoA and (b) the expression of the maximal activity of CPT I measured in mitochondria isolated from the liver of rats starved for periods up to 24 h

Values statistically significantly different from those obtained at zero time are indicated by an asterisk. Values are the means $\pm$ S.E.M. for four separate determinations.

NEFA concentration thereafter. The maximal NEFA concentrations observed in this study were relatively modest (0.25 mEq/l) and were related to the age and adiposity of animals used, as plasma NEFA concentrations were increased to twice this value in heavier (250 g) animals (results not shown), presumably due to the greater adiposity of older animals. By contrast to the early response of plasma NEFA to starvation, ketone-body concentrations began to increase only after 12 h starvation (Figure 1b), i.e. several hours after the increase in NEFA. This asynchrony between the increases in the two parameters suggested that intrahepatic mechanisms could be restricting the rate of fatty acid oxidation in the liver. Therefore time courses were obtained for other parameters that are thought to affect CPT I activity *in vivo* in order to identify the changes that most closely approximated the increase in blood ketone-body concentration.

Hepatic malonyl-CoA concentration, CPT I activity and sensitivity

The hepatic malonyl-CoA concentration decreased in parallel with the decrease in serum insulin (Figure 2), when results were expressed per unit wet-tissue weight, and the lowest value was attained at the 12 h time point. Thereafter values remained low up to 18 h but were significantly increased by 24 h (Figure 2a). By contrast, the sensitivity of CPT I to malonyl-CoA inhibition was similar to the value obtained for fed animals, even after 12 h starvation but increased rapidly between 12 and 16 h of starvation (Figure 3a) in parallel with the increase in blood ketone-body

concentration (Figure 1b). No significant increase was observed in CPT I maximal activity in isolated liver mitochondria even after 18 h of starvation, i.e. beyond the period of starvation at which a significant increase in blood ketone-body concentration was observed. A significant increase in CPT I activity was observed only after 24 h starvation (Figure 3b).

DISCUSSION

The above data indicate that, of the time courses for any the parameters studied only that for the decrease in sensitivity (increase in IC_{50}) of CPT I for malonyl-CoA coincided with the time course for the increase in blood ketones. Thus substantial changes in both plasma NEFA concentration (an index of the rate of delivery of NEFA to the liver) and in hepatic malonyl-CoA concentration occurred before an increase in blood ketone bodies was observed. These parameters appeared to respond very rapidly to the decrease in plasma serum insulin concentration, as would be expected from the anticipated loss of the anti-lipolytic action of insulin on adipose tissue and the activatory effect of the hormone on hepatic acetyl-CoA carboxylase [1]. In spite of these changes, there was no immediate increase in blood ketone-body concentration, suggesting that flux through CPT I continued to be largely inhibited even when the concentration of malonyl-CoA was decreased by over 70 % (Figure 2b). Similarly, although a tendency for a gradual increase in the maximal activity of CPT I was apparent throughout the 24 h period, a significant increase occurred too late in the 24 h period studied to account for the abrupt rise in blood ketone-body concentration which occurred between 12 and 14 h after food withdrawal. It is concluded, therefore, that the loss of sensitivity of CPT I to malonyl-CoA is the most likely kinetic characteristic of CPT I to be involved in determining the timing of the onset of increased ketone production by the liver. When the decreased sensitivity occurs the other prerequisites (increased substrate supply and lowered inhibitor concentration) are already established, and this combination enables acylcarnitine formation to proceed at an enhanced rate resulting in the rapid development of ketonaemia observed between 12 and 14 h after food removal.

The timing of the increase in IC_{50} is of interest with respect to the possible mechanisms involved in mediating it. It is evident that, contrary to the relationship between serum insulin and hepatic malonyl-CoA concentration, there was a substantial lag between the attainment of low insulin concentration and the onset of the rise in IC_{50} for malonyl-CoA. This is similar to the lag for the reversal of the effect observed either on refeeding of starved rats [8] or insulin treatment of streptozotocin-diabetic animals [9]. It reinforces the suggestion that, if insulin is involved in changes in the sensitivity of CPT I for malonyl-CoA, the changes are mediated probably not through direct action on CPT I but through changes in the lipid composition of the mitochondrial outer membrane, of which CPT I is an integral protein [14]. The time required to bring about the changes in malonyl-CoA sensitivity is comparable with that required for the alteration in phospholipid and/or cholesterol content of the membrane and for the possible remodelling of the acyl-chain composition of the phospholipids, which may affect the molecular order of the membrane lipids with which CPT I interacts. Experiments *in vitro* have suggested that malonyl-CoA inhibition of CPT I is extremely sensitive to the molecular order of the membrane lipids, and that the changes observed *in vivo* can be mimicked by chemical- or temperature-induced alterations in membrane fluidity [15]. In addition, incorporation of specific phospholipids into the mitochondrial outer membrane *in vitro* induces large changes in the IC_{50} value for malonyl-CoA in-

hibition [16]. It is also of interest that recent studies have shown that when isolated rat hepatocytes are cultured in the presence of insulin the sensitivity of CPT I to malonyl-CoA is increased, but that this effect takes several hours to be expressed [17].

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