Sensitivity of inhibition of rat liver mitochondrial outer-membrane carnitine palmitoyltransferase by malonyl-CoA to chemical- and temperature-induced changes in membrane fluidity

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We have tested the possibility that alterations in the fluidity of the outer membrane of rat liver mitochondria could result in changes in the sensitivity of overt carnitine palmitoyltransferase (CPT I) to malonyl-CoA [Zammit (1986) *Biochem. Soc. Trans.* 14, 676–679]. The sensitivity of CPT I to malonyl-CoA inhibition was measured by using highly purified mitochondrial outer membranes prepared from fed or 48 h-starved rats in the presence and absence of agents that increase membrane fluidity by perturbing membrane lipid order [benzyl alcohol, isoamyl alcohol (3-methylbutan-1-ol) and 2-(2-methoxyethoxy)ethyl-8-(*cis*-2-n-octylpropyl)octanoate (A_2C)]. All these agents resulted in marked decreases in the ability of malonyl-CoA to inhibit CPT I. This effect was accompanied by a modest increase in the absolute activity of CPT I in the absence of malonyl-CoA when the short-chain alcohols were used, but not when A_2C was used, suggesting that the effect of increased membrane fluidity to decrease the malonyl-CoA sensitivity of CPT I may occur independently from other actions that may affect more directly the active site of the enzyme. In confirmation of the potential importance of fluidity changes, we showed that a marked increase in sensitivity of CPT I to malonyl-CoA could be produced when assays were performed at lower temperatures than those normally employed. These observations are discussed in the context of the slowness of the changes in CPT I sensitivity to malonyl-CoA inhibition that are induced by physiological perturbations.

INTRODUCTION

The sensitivity of mitochondrial outer-membrane carnitine palmitoyltransferase (CPT I) to malonyl-CoA inhibition changes with physiological state in rat liver (see [1] for review). Under conditions of increased fatty acid oxidation, e.g. starvation, diabetes, the enzyme becomes less sensitive to malonyl-CoA inhibition. This adaptation amplifies the effect of changes in hepatic malonyl-CoA concentration, as decreases in malonyl-CoA content of the liver are accompanied by decreased sensitivity [2]. Moreover, the two parameters appear to be positively related in the intact rat [3].

The changes in malonyl-CoA sensitivity of CPT I have two components. One is inherent to the outer membrane itself in which CPT I resides [4]. This is evidenced by the fact that changes in sensitivity are retained during the preparation of mitochondria [5] or outer-membrane preparations [4] which may take several hours. Superimposed on these inherent properties of the enzyme and/or membrane, however, is a second component that can be mimicked *in vitro*. Incubation of mitochondria at 37 °C in the absence of malonyl-CoA results in a gradual decrease in sensitivity to malonyl-CoA [5,6]. Conversely, incubation in the presence of malonyl-CoA results in a time-dependent increase in sensitivity of the enzyme to the inhibitor [5,6]. Moreover, this increased sensitivity is retained if, after incubation, the mitochondria are cooled and maintained near 0 °C [7]. It is only lost gradually on re-incubation of the mitochondria at 37 °C.

The latter observations prompted us to suggest that the enzyme could adopt conformations that were more and less sensitive, respectively, to malonyl-CoA inhibition [2,5,8]. According to this hypothesis, at higher temperatures and in the absence of malonyl-CoA the favoured conformation would be the insensitive one, whereas in the presence of malonyl-CoA the enzyme would adopt a sensitive conformation and could be locked in this state

by rapid cooling of the mitochondria. To the extent that, in the intact liver, mitochondria always experience some concentration of malonyl-CoA (that is higher in the less ketogenic states), some of the difference in their sensitivity *in vitro* before incubation at 37 °C may be due to a residual influence of the hepatic malonyl-CoA they experienced before extraction of the tissue. By using short assay periods, we were able to demonstrate [5] that it takes about 2–4 min of incubation at 37 °C fully to abolish this effect. Any residual effect of malonyl-CoA concentrations *in vivo*, however, are always superimposed on the inherent component, as, after incubation at 37 °C, differences in sensitivity to malonyl-CoA between the enzyme in mitochondria isolated from fed, starved and diabetic animals are still clearly discernible, although much smaller than those observed on initial incubation [5,6].

In this paper we investigate whether the mechanism underlying the two components may not, in fact, be the same one, namely that changes in fluidity of the outer membrane affect the conformational state of the enzyme so as to result in changes in sensitivity to malonyl-CoA. Although this was proposed several years ago [2], and malonyl-CoA itself was shown to affect the fluidity of whole mitochondrial membranes [9], no reports have appeared in which the effects of experimentally induced changes in outer-membrane fluidity on sensitivity of CPT I to malonyl-CoA were studied. We find that the sensitivity of CPT I to malonyl-CoA inhibition in very pure outer membranes is exquisitely dependent on the fluidity of the membrane when this is manipulated by temperature or known membrane-disrupting agents.

MATERIALS AND METHODS

Animals

These were male Wistar rats (300–400 g) that were either fed *ad libitum* on a standard laboratory chow (Biosure, Poole, Dorset,

U.K.) or deprived of food for 48 h before use. All had continuous access to water.

Mitochondrial outer membranes

Preparation of rat liver mitochondrial outer membranes was exactly as described previously [4] and included Percoll-density-gradient purification of the starting mitochondria and Sepharose-cytochrome *c* affinity purification of the crude outer-membrane preparations.

Assay of CPT activity and [14 C]malonyl-CoA binding

CPT activity was measured at the temperatures indicated, as described previously [10]. In all experiments the membranes were preincubated for 2 min before initiation of the reaction by addition of L-carnitine. Membrane-fluidizing agents were included in the assay medium during this period of pre-incubation. For 2-(2-methoxyethoxy)ethyl-8-(*cis*-2-n-octylpropyl)octanoate (A_2C), the compound was first added to the membranes in assay medium and co-sonicated (2×20 s). The assay period was 2 min when performed at 37 °C and 4 min at 20 °C. The formation of acyl- 14 C-carnitine was quantified as described by Borrebaek [11]. [14 C]Malonyl-CoA binding to the outer membranes was performed at 0 °C as in [4] with additions as indicated in the Figure legends. After addition of ligand, the incubations were continued for 20 min and the amount of specific binding was determined by filtration of the membranes [4]. Appropriate controls were run concurrently.

Assay of marker enzyme activities

The activities of catalase, monoamine oxidase, NADPH-cytochrome *c* reductase and succinate dehydrogenase were assayed for each preparation of membranes, by the methods described elsewhere [10]. Routinely, purifications of outer membranes with respect to whole mitochondria were approx. 200-fold, and no detectable activity of the microsomal or peroxisomal markers was observed in the final membrane preparation (see [4]).

Materials

The sources of most materials were as described previously [4,10]. In addition, A_2C , benzyl alcohol and isoamyl alcohol (3-methylpropan-1-ol) were from Sigma Chemical Co. (Poole, Dorset, U.K.).

RESULTS AND DISCUSSION

We have investigated the effects of temperature and membrane-fluidizing agents on the sensitivity of CPT I to malonyl-CoA in outer-membrane preparations. In the course of these studies we also had the opportunity to observe any effects on absolute activities of the enzyme.

Effects of membrane-fluidizing agents

The effects of benzyl alcohol, isoamyl alcohol and A_2C on the sensitivity of CPT I in purified outer membranes of mitochondria from fed or starved rats were tested. The use of several different agents decreased the possibility that any effects observed in their presence could arise from the specific interactions of the individual chemicals with a particular membrane component. Consequently, common effects of these chemicals can be interpreted to result from their common characteristic, namely to increase membrane fluidity.

In Fig. 1, the effects of increasing concentrations of benzyl alcohol on the activity of CPT I in the absence and presence of a sub-optimal concentration of malonyl-CoA are shown. At higher concentrations of the alcohol the absolute activity of

Fig. 1. Effects of increasing concentration of benzyl alcohol on the activity of CPT I in rat liver mitochondrial outer membranes

CPT I was assayed at 37 °C in the absence (▲) or presence (■) of 10 μM-malonyl-CoA in membranes isolated from fed rats. Values are means ($\pm$ S.E.M.) for three separate determinations on different membrane preparations.

CPT I (measured at sub-optimal palmitoyl-CoA concentration) was increased by about 30%. However, this effect was maximal at relatively low concentrations of benzyl alcohol. The modest increase in CPT activity was accompanied by a much larger effect on the fraction of this activity that could be inhibited by malonyl-CoA (10 μM). The latter effect was investigated further by using several concentrations of malonyl-CoA (Fig. 2). Even at the relatively low concentration of 20 mM-benzyl alcohol there was a marked decrease in the sensitivity of CPT I to malonyl-CoA. The benzyl alcohol-induced decrease in sensitivity observed for the enzyme in membranes isolated from fed animals (Fig. 2a) was equivalent to that observed between the sensitivity in membranes isolated from fed and starved animals ([14]; compare Figs. 2a and 2b). An additional effect of benzyl alcohol was observed in membranes from starved animals to decrease the sensitivity even further (Fig. 2b). This was not surprising, as the degree of insensitivity observed for CPT I in mitochondria from 48 h-starved normal animals is only modest compared with that observed in starved pregnant rats [3], suggesting that further desensitization of CPT to malonyl-CoA can be induced by physiological conditions that result in a more severe ketogenic state than the normal 48 h-starved state. It should be noted that the effects of benzyl alcohol observed in the present study are likely to have been under-estimates of potentially larger effects, as the high concentrations of KCl used for the assay of CPT are known to counteract the effects on membrane fluidity [12].

Similar effects to those of benzyl alcohol were observed with isoamyl alcohol, although much higher concentrations of this less hydrophobic compound were needed to achieve the same effects (results not shown). A_2C , which has a totally different molecular structure, but is known to result in increased fluidity, owing to membrane perturbation [13,14], also gave a decrease in

Fig. 2. Fluidization of rat liver mitochondrial outer membranes with benzyl alcohol decreases the sensitivity of CPT I to malonyl-CoA

CPT I was assayed at 37 °C in the absence (●) or presence (◆) of 20 mM-benzyl alcohol at different concentrations of malonyl-CoA. Outer membranes were prepared from (a) fed ($n = 7$) or (b) starved ($n = 6$) rats. Values are means ($\pm$ S.E.M.) for separate determinations on different membrane preparations. The absolute activities of CPT I in the absence of malonyl-CoA were (a) 52.3 ± 4.7 and 68.1 ± 7.2 nmol/min per mg of protein and (b) 113.8 ± 19.8 and 151.2 ± 28.1 nmol/min per mg of protein in the absence and presence of 20 mM-benzyl alcohol respectively.

sensitivity of CPT to malonyl-CoA (Fig. 3), suggesting that these changes were genuinely due to increases in membrane fluidity. However, the effects of A_2C were smaller than we expected. This appeared to be due to difficulty in achieving the incorporation of A_2C into the outer membrane, as it was found that only co-sonication of membranes with A_2C was successful in obtaining a sufficiently high effective concentration to partition into the membranes. The presence of a high KCl concentration may also have contributed to this effect (see above).

Effects of temperature on CPT I sensitivity to malonyl-CoA

In all our previous studies (see, e.g., [3,4,15,16]), we have used 37 °C as the temperature for assays of CPT activity, so as to reproduce, as much as possible, the physiological conditions of the enzyme. However, in most other laboratories, assays are performed at lower temperatures [17,18]. In view of the above

Fig. 3. Effects of A_2C to decrease the sensitivity of CPT I to malonyl-CoA

Outer membranes from 48 h-starved rats were sonicated in assay buffer either in the absence (■) or in the presence (□) of 10 μ M- A_2C (in dimethyl sulphoxide) for two periods of 10 s each. They were then used to measure CPT I activity at 37 °C in the presence of different concentrations of malonyl-CoA. The effect was demonstrated reproducibly at a single concentration (20 μ M) of malonyl-CoA on three different outer-membrane preparations before the experiment shown was performed. Sonication of membranes in the presence of A_2C resulted in 8–10 % decrease in CPT activity assayed in the absence of malonyl-CoA, relative to controls which were sonicated with dimethyl sulphoxide only (0.1 % of final volume).

Fig. 4. The sensitivity of CPT I to malonyl-CoA is temperature-dependent

CPT I was assayed in outer membranes isolated and purified from 48 h-starved rats at either 37 °C (▲) or 20 °C (●) in the presence of different concentrations of malonyl-CoA. Values are means ($\pm$ S.E.M.) for three determinations on separate membrane preparations.

observations, it was desirable to ascertain whether a decrease in fluidity induced by a lower temperature resulted in an increase in sensitivity of CPT I to malonyl-CoA. Fig. 4 shows that this did occur. The effect of lowering the assay temperature from 37 °C to 20 °C was extremely large, in keeping with previous estimates that a 6 °C increase in temperature is equivalent to the addition of 40 mM-benzyl alcohol to membranes [19]. When the sensitivity

of CPT I to malonyl-CoA at 20 °C was tested in membranes isolated from fed rats (i.e. in which, at 37 °C, CPT I is already more sensitive), the increased inhibition induced by the lower temperature resulted in > 90 % of the enzyme being inhibited even at 10 μ M-malonyl-CoA (result not shown). Such an effect of temperature could account for the discrepancies between the IC_{50} values for malonyl-CoA obtained by different laboratories even under otherwise comparable conditions.

Changes in fluidity and malonyl-CoA binding

Altered sensitivity of CPT I to malonyl-CoA could be due, in theory, to a change in the transmission of the conformational change induced by malonyl-CoA to the active site of the enzyme (see [4]) or to a change in the parameters of malonyl-CoA binding. We have therefore tested the effects of benzyl alcohol on the maximal binding (B_{max}) and affinity (K_D) for malonyl-CoA at the high-affinity site of outer membranes. There was no effect of benzyl alcohol on the K_D for malonyl-CoA binding ($K_D = 0.03 \pm 0.01 \mu$ M in the presence or absence of benzyl alcohol; $n = 3$). Similarly, total binding (B_{max}) was unaffected (see Fig. 5). These data showed that the marked decrease in sensitivity of CPT I to malonyl-CoA induced by an increase in fluidity was not accompanied by any change in the malonyl-CoA binding parameters. In a previous study [5] we have shown that binding of malonyl-CoA at the high-affinity site of intact mitochondria is also largely unaffected by temperature (between 10° and 37 °C). Similarly McGarry *et al.* [20] showed that maximal high-affinity binding was similar whether measured at 0 °C or 20 °C.

General considerations

The above observations strongly suggest that changes in mitochondrial outer-membrane fluidity alter the ability of malonyl-CoA binding to effect the conformational change required in CPT I for the expression of decreased affinity for acyl-CoA substrate. In this respect they argue against a mechanism of inhibition of CPT I by malonyl-CoA that involves simple steric hindrance, but in favour of the induction by malonyl-CoA of a specific conformational form of CPT I that is unfavourable for long-chain acyl-CoA binding at the active site. The data also suggest that protein-lipid interactions are involved in determining malonyl-CoA sensitivity. This would explain why

Fig. 5. Scatchard plot of [14 C]malonyl-CoA binding data obtained with liver mitochondrial outer membranes from fed rats

Binding of [14 C]malonyl-CoA was performed at 0 °C as described in the Materials and methods section, in either the absence (○) or the presence (■) of 20 mM-benzyl alcohol. The experiment illustrated is representative of three similar experiments which gave very similar results.

solubilized CPT I is relatively insensitive [21,22] to malonyl-CoA and why a partial recovery of sensitivity can be achieved by reconstitution of the solubilized material into proteoliposomes [21,23,24]. In addition, the possibility cannot be excluded that protein-protein interactions are altered by changes in fluidity. Previous observations have raised the possibility that malonyl-CoA interacts with a separate regulatory protein that is smaller than CPT I itself [10]. Other observations have indicated that CPT catalytic activity can be separated from malonyl-CoA binding [24,25] and that reconstitution of the two fractions into liposomes results in an enzyme that is malonyl-CoA sensitive [23,24].

The present findings provide an explanation for the changes in CPT I activity and malonyl-CoA sensitivity that can be achieved *in vitro* on warming mitochondria up from 0° to 37 °C (or intermediate temperatures) (see the Introduction). Interestingly, mitochondria isolated from animals in different physiological states show differences in their responses *in vitro* [8] to such changes in temperature in KCl-based media. The question inevitably arises as to whether the inherent component of the changes in sensitivity of CPT I to malonyl-CoA in mitochondria from rats in different physiological conditions are due to inherent changes in the fluidity of the membrane in the micro-environment of CPT I, such as could be produced by changes in the lipid composition of the membrane (e.g. cholesterol content, unsaturation index of the fatty acids in component phospholipids). It is evident from this study that the relatively modest changes in fluidity achieved with benzyl alcohol are of sufficient magnitude to effect changes in IC_{50} for malonyl-CoA of the same order as those observed in transitions between physiological states. Such a mechanism would explain why changes in malonyl-CoA sensitivity *in vivo* [15,16] or in cultured hepatocytes [26] only occur over the long term (12–24 h), as, presumably, they would depend on changes in the rates of synthesis and degradation of specific membrane lipids. Experimental verification of this proposal should emerge from studies on changes in the lipid composition of pure outer membranes with physiological state, and of the effects of any such changes on the fluidity of the membranes as measured with the use of mobility probes. However, we would point out that, although changes in fluidity produced by different agents may result in similar qualitative changes in the properties of CPT I, quantitative differences may persist and may depend on specific changes in membrane structure in the immediate vicinity of the enzyme. This is most clearly demonstrated in Fig. 4, where, although a decrease in temperature clearly increases the sensitivity of CPT I to malonyl-CoA in membranes from starved rats, the resulting inhibition curve is still appreciably different from that obtained with fed-rat membranes.

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