A NOVEL MECHANISM FOR INHIBITION OF β-OXIDATION BY METHYLENECYCLOPROPYLACETYL-CoA, A METABOLITE OF HYPOGLYCIN

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1. Introduction

Hypoglycin (L-2-amino-3-methylenecyclopropylpropionic acid), the toxic principle of the Jamaican ackee fruit (*Blighia sapida*) [1–4], is converted in vivo to methylenecyclopropyl acetyl-CoA (MCPA-CoA) [5]. This metabolite is thought to inhibit fatty acid oxidation [6], following administration of hypoglycin to animals, thereby indirectly causing hypoglycaemia [2–4]. Here we show that in the presence of methylenecyclopropylacetate (MCPA) fatty acids of chain-lengths greater than C_8 are oxidised in isolated mitochondria at a decreased rate and only as far as butyrate. This is due to deacylation of short-chain acyl-CoA in the matrix accumulating as a result of the powerful and specific inhibition of butyryl-CoA dehydrogenase (EC 1.3.99.2) by MCPA-CoA.

2. Materials and methods

Hypoglycin and MCPA were prepared essentially by published methods [5,7] and acyl-carnitine and acyl-CoA esters were synthesised and characterised as described previously [8,9]. Rat liver mitochondria were isolated in 0.3 mM mannitol 2.5 mM 2(*N*-hydroxyethylpiperazin-*N'*-yl) ethanesulphonate, 0.1 mM ethyleneglycol-2-(aminoethyl)-tetraacetate, pH 7.2 [8]. Oxygen uptake by mitochondria in state 3 [9] was recorded polarographically in 3.0 ml of medium at 30°C containing 100 mM KCl, 20 mM 3-(*N*-morpholino) propanesulphonate, 2.5 mM phosphate, 5 mM $MgCl_2$, 1.0 mM EDTA, 10 mM malonate, 2.0 mM ADP and 9 mg defatted bovine serum albumin, pH 7.2. With these conditions acyl groups are quantitatively converted to acetoacetate and the oxygen uptake is a direct measure of the flux through β-oxidation [8]. Extracts of acetone-dried rat liver mitochondria (2 g) were made by stirring with 10 ml of 10 mM potassium phosphate, pH 7.2 at 0°C, followed by centrifugation (4.5×10^6 *g* min) and passage through a column of Sephadex G_{25} pre-equilibrated with phosphate buffer. Protein was measured by a biuret method [8]. Acyl-CoA dehydrogenase activities (EC 1.3.99.–) were assayed at 20°C and pH 7.2 with 60 μM acyl-CoA as substrate [10]. Acyl-CoA hydrolase (EC 3.1.2.–) activity was assayed spectrophotometrically at 324 nm and 20°C in 20 mM phosphate, pH 7.2, using 0.5 mM aldrithiol-4 to follow CoA.SH released.

3. Results and discussion

The rate and extent of oxidation of most even-chain acyl-carnitines were decreased, and that of hexanoyl-carnitine and butyryl-carnitine was completely blocked, following pre-incubation of liver mitochondria with MCPA (fig. 1). The degree of impairment of oxidation of each substrate was independent of the concentration of MCPA in the range 10 μM–0 mM, although at lower concentrations longer pre-incubation (up to 15 min) was necessary to get maximun inhibition.

Inhibition of oxidation of butyryl-carnitine is explained by the lack of detectable butyryl-CoA dehydrogenase activity after sonication of liver mitochondria that had been incubated with MCPA [11] or isolated from livers of hypoglycaemic rats 4 h after administration of hypoglycin [11, 12]. By contrast, palmitoyl-CoA (octanoyl-CoA) dehydrogenase (EC 1. 3. 99. 3)

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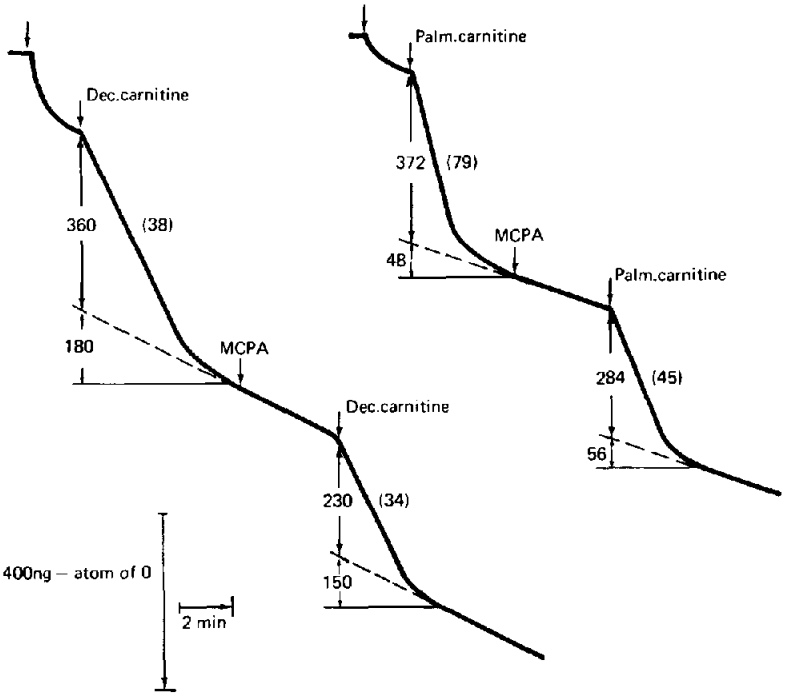

Fig.1. Inhibition by MCPA of the state 3 rate of oxidation of acyl-carnitines in rat liver mitochondria. Mitochondria (10 mg of protein) were added where indicated (by unlabelled arrows); 20 μM decanoyl-carnitine or 10 μM palmitoyl-carnitine and 1.0 mM MCPA were then added as shown. The rates of oxygen uptake (ng atom O/min/mg of protein) during a pulse of acyl-carnitine oxidation are given in parentheses. The amount of oxygen consumed (ng atom O) on addition of acyl-carnitine is indicated by the vertical arrows; the upper one for each pulse gives the apparent increase, and the lower one the apparent contribution of endogenous respiration. For the control pulses the sum of these values was close to that required for the complete oxidation of acyl-carnitines to acetoacetate and this indicated that endogenous respiration was suppressed.

[10] was not inhibited [11]. Palmitoyl-carnitine oxidation was unimpaired in mitochondria uncoupled by addition of 0.2 μM trifluoromethoxycarbonylcyanide phenylhydrazone, 10 mM arsenate and valinomycin (0.2 mg/ml) even after prolonged incubation with 1.0 mM MCPA, indicating that prior conversion of MCPA to MCPA–CoA by butyryl-CoA (medium chain acyl-CoA) synthetase (EC 6. 2. 1. 2) in the matrix is necessary for inhibition (see [8]). We have failed to prepare MCPA-CoA, either chemically or enzymically using purified butyryl-CoA synthetase [13], suggesting that MCPA-CoA is very unstable. However, there was a progressive and apparently irreversible inactivation of butyryl-CoA dehydrogenase (fig. 2) when purified ox liver butyryl-CoA dehydrogenase [10] and rat liver butyryl-CoA synthetase, or extracts of rat liver mitochondria, were incubated with MCPA, CoA-SH and MgATP (all of which were necessary for inactivation), indicating that MCPA-CoA is the inhibitory species derived from hypoglycin.

The decrease in the percentage actually consumed of the theoretical oxygen uptake required for complete oxidation of acyl-carnitine to acetoacetate, increased with decreasing chain-length from palmitoyl-carnitine to octanoyl-carnitine in MCPA-inhibited mitochondria. This agrees quantitatively with conversion of acyl-CoA, generated in the matrix from exogenous acyl-carnitine, to butyrate and acetoacetate (fig.3), for example.

Palmitoyl-CoA + $6O_2$ → 3 Acetoacetate + Butyrate + CoA.SH. The total CoA.SH content of rat liver mitochondria is about 3 nmol/mg of protein [8] which is much less than the amount of acyl-carnitine that can be oxidised (at least 10 nmol/mg mitochondrial protein, not shown) so that repeated recycling of CoA.SH

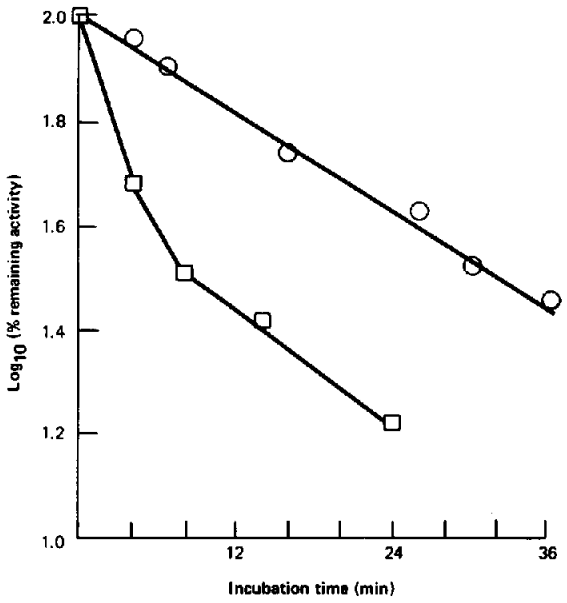

Fig.2. Inactivation of butyryl-CoA dehydrogenase by a metabolite of MCPA. Purified ox liver butyryl-CoA dehydrogenase (50 nU) and rat liver butyryl-CoA synthetase (1 nU) (○), or extracts of rat liver mitochondria (8 mg protein/ml) (□), were incubated with 0.5 mM MCPA, 0.04 mM CoA.SH and 10 mM MgATP at 30°C in 10 mM phosphate, pH 7.2. Butyryl-CoA dehydrogenase was assayed at various times during the incubation.

must occur. As predicted for these considerations, extracts of rat liver mitochondria rapidly deacylated butyryl-CoA and hexanoyl-CoA (apparent K_M values of 2 mM and 0.5 hexanoyl-CoA (apparent K_M values of 2 mM and 0.5 mM, and V_m values of 10 and 15 nmol/mg of protein respectively). This activity is latent in intact mitochondria and is released by sonication. Approximately the predicted amounts of free butyrate and only traces of hexanoate were detected by g.l.c. [14] as products of the oxidation of palmitoyl-carnitine in MCPA-inhibited mitochondria, while very little of these acids was formed in the absence of MCPA. Further. butyrate occurs in the blood of animals poisoned with hypoglycin [15]. The presence of short-chain acyl-CoA hydrolase with a high K_M in the matrix would oppose complete acylation of intramitochondrial CoA.SH by some unusual short-chain fatty acids, although normally intermediates of β-oxidation do not accumulate sufficiently to be deacylated [16, 17]. Similar deacylase activities have also been reported recently in mitochondria from several tissues [18–20].

The pattern of inhibition of oxidation of different acyl-carnitines by MCPA (fig. 3) contrasts with the earlier report that formation of $^{14}CO_2$ from free long-chain I-[^{14}C]-labelled fatty acids in rat liver mitochondria is impaired more strongly than from those of shorter-chain length [6]. However, formation of in-

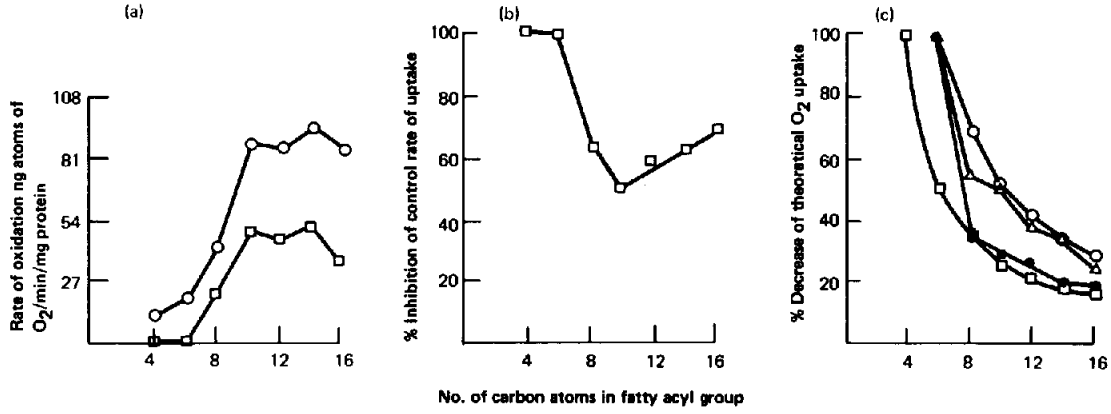

Fig.3. Characteristics of the inhibition by MCPA of the oxidation of acyl-carnitines of different chain-lengths. The rate and amount of oxygen uptake on adding 10 μM acyl-carnitines were recorded as described in the legend to fig.1. (a) Rate of oxygen uptake; control (○), inhibited (□); (b) percentage inhibition of control rates of acyl-carnitine oxidations (□); (c) theoretical and oberved percentage decreases in the size of the MCPA-inhibited pulses for the partial oxidation of acyl-carnitines compared with uninhibited pulses (fig.1); theoretical values for oxidation, as for as butyryl-CoA (□), as far as hexanoyl-CoA (○), observed values assuming that endogenous respiration, continues (△), or is suppressed (●).

hibitory MCPA-CoA in the matrix may be prevented by competition with high concentraions of those shorter medium-chain fatty acids which are also substrates for butyryl-CoA synthetase, but not by long-chain fatty acids which are activated outside (see [8]).

The inability of MCPA-inhibited mitochondria to oxidise hexanoylcarnitine can be explained if it is assumed that there is some structural organisation of the enzymes of β-oxidation (see [17]). Hexanoyl-CoA generated from long-chain acyl-CoAs by β-oxidation may have privileged access to palmitoyl-CoA dehydrogenase, compared with that formed in the matrix from exogenous hexanoyl-carnitine which may only be a substrate for butyryl-CoA dehydrogenase. This explanation also assumes that butyryl-CoA formed either by β-oxidation or from exogenous butyryl-carnitine is only a substrate for butyryl-CoA dehydrogenase. Slowing of β-oxidation by MCPA is presumably caused by short-chain acyl-CoA accumulating up to a critical concentration set by the relatively high K_M of acyl-CoA hydrolase, following inhibition of butyryl-CoA dehydrogenase. Impaired β-oxidation, together with inhibition of isovaleryl-CoA dehydrogenase [11, 12, 15] and the probable inhibition of glutaryl-CoA dehydrogenase by MCPA-CoA [15], can explain most of the pharmacological effects of hypoglycin.

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