Cycloheximide blocks changes in rat liver carnitine palmitoyltransferase 1 activity in starvation

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1. Starvation (24 h) increased the maximum activity of carnitine palmitoyltransferase 1 in rat liver and increased the concentration of malonyl-CoA required to cause 50% inhibition of the enzyme (I_{50}). 2. Re-feeding (24 h) with a standard cube diet or a high-carbohydrate diet reversed both of these changes, whereas re-feeding with a high-fat diet did not. 3. Administration of cycloheximide (200 μ g/100 g body wt.) blocked the increases in carnitine palmitoyltransferase 1 activity and I_{50} on starvation. 4. It is suggested that increase in carnitine palmitoyltransferase 1 activity in starvation may involve synthesis of new enzyme.

In rat liver, CPT₁, the overt form of carnitine palmitoyltransferase, is controlled by both long- and short-term regulatory mechanisms. Starvation (Harper & Saggerson, 1975; Bremer, 1981; Saggerson *et al.*, 1982; Saggerson & Carpenter, 1982), alloxan-diabetes (Harano *et al.*, 1972) and hyperthyroidism (Stakkestad & Bremer, 1983) increase CPT₁ activity relative to mitochondrial protein, whereas the activity is decreased in hypothyroidism (Saggerson *et al.*, 1982; Stakkestad & Bremer, 1983). Culture of hepatocytes with glucagon for 24 h also increases CPT₁ activity (Harano *et al.*, 1982). The question of whether these changes reflect alterations in the amount of enzyme protein or modification of existing enzyme has not been considered in detail. Norum (1965), using an isotope-exchange assay, found that treatment of rats with ethionine, puromycin or actinomycin did not significantly prevent the increase in CPT activity in starvation. However, it is questionable whether that particular assay discriminates between CPT₁ and CPT₂ activities, since, when it is used, only approx. 40% of observed CPT activity in isolated mitochondria is suppressible by malonyl-CoA under conditions where fatty acid oxidation is totally suppressed (Assay I of McGarry *et al.*, 1978).

Abbreviations used: CPT₁ and CPT₂, the overt and latent forms respectively of carnitine palmitoyltransferase (EC 2.3.1.21); I_{50} , the concentration of malonyl-CoA required to inhibit CPT₁ activity by 50%; I_{max} , the percentage inhibition of CPT₁ at infinite malonyl-CoA concn.

In the short term CPT₁ can be potently inhibited by malonyl-CoA (McGarry *et al.*, 1978). The sensitivity of the enzyme to this inhibitor, however, is itself regulated in the long term. Starvation (Bremer, 1981; Saggerson & Carpenter, 1981*a,b*; Robinson & Zammit, 1982), hyperthyroidism (Stakkestad & Bremer, 1983) and a ketotic diabetic state (Cook *et al.*, 1984) all result in diminished sensitivity to malonyl-CoA.

In this study we have reconsidered the effects of inhibition of protein synthesis on the starvation-induced changes in hepatic CPT₁, using a direct assay of the enzyme. We report that a single dose of cycloheximide can prevent both the increase in activity and the changes in sensitivity.

Materials and methods

Animals

These were male Sprague–Dawley rats bred at University College London and fed on GR3-EK cube diet (E. Dixon and Sons, Ware, Herts., U.K.). Starvation (24 h) and re-feeding procedures were started at 10:00–10:30 h. ‘Cycloheximide-treated’ animals received a single intraperitoneal injection of cycloheximide (2 mg/ml in 0.15 M-NaCl) at a dose of 200 μ g/100 g body wt. ‘Saline-treated’ animals were similarly injected with an equivalent volume of the 0.15 M-NaCl vehicle. When re-feeding was performed after 24 h of starvation, this was for a further 24 h period. The animals were either re-fed with the standard GR3-EK cube diet or with high-carbohydrate or high-fat diets made

up in the laboratory (Saggerson & Tomassi, 1971). The approximate composition of the high-carbohydrate diet (w/w) was 45% sucrose, 10% casein, 3% purified wood cellulose, 2.5% dried yeast, 2.5% salt mixture (Hawk & Oser, 1965), 6% sodium alginate and 31% water. The high-fat diet was: 20% corn oil, 20% margarine, 10% casein, 1% sucrose, 2.5% dried yeast, 2.5% salt mixture, 6.5% wood cellulose, 6% sodium alginate, 31% water. 'Cycloheximide-treated fed' animals received cycloheximide 24 h before death. 'Cycloheximide-treated starved' animals also received cycloheximide 24 h before death at the start of the starvation period. 'Cycloheximide-treated re-fed' animals received the drug after a 24 h period of starvation immediately before the 24 h re-feeding period.

Chemicals

Cycloheximide was obtained from Sigma Chemical Co. (Kingston-upon-Thames, Surrey, U.K.). Sources of other chemicals have been described previously (Saggerson *et al.*, 1982).

Experimental methods

Liver mitochondria were obtained as described by Saggerson (1982) and finally suspended in 0.3 M-sucrose medium containing 10 mM-Tris/HCl buffer (pH 7.4), 1 mM-EGTA and 1 mM-dithiothreitol to give a protein concentration of approx. 6 mg/ml.

CPT₁ assays were performed within 30 min of the isolation of mitochondria. Intact mitochondria (50 µl) were preincubated at 25°C for 4 min in 1.0 ml containing 150 mM-sucrose, 60 mM-KCl, 25 mM-Tris/HCl (pH 7.4), 1 mM-EGTA, 1 mM-dithiothreitol, fatty acid-poor albumin (1.3 mg/ml) and the indicated concentrations of palmitoyl-CoA and malonyl-CoA. The reaction was initiated by addition of 25 µl containing 0.4 µmol of L-carnitine and 0.5 µCi of L-[Me-³H]carnitine. After 4 min the reaction was terminated and treated as described by Saggerson *et al.* (1982). The maximum activity of CPT₁ was measured with 100 µM-palmitoyl-CoA (Saggerson & Carpenter, 1981b, 1982; Saggerson *et al.*, 1982). Inhibition of CPT₁ by malonyl-CoA was measured with 40 µM-palmitoyl-CoA and the following malonyl-CoA concentrations: 0, 2, 3, 5, 10, 20, 50, 100 µM. In every case plots of (percentage inhibition of CPT₁)⁻¹ versus [malonyl-CoA]⁻¹ were linear ($r = 0.98-1.0$; mean of 40 experiments = 0.992), permitting the percentage of overt CPT that is suppressible by malonyl-CoA ($I_{\max}$) to be derived from the intercept on the ordinate (Saggerson & Carpenter, 1981a). The concentration of malonyl-CoA (I_{50}) that inhibits 50% of the malonyl-CoA-suppressible activity can therefore be derived from this plot. All CPT assays under these conditions were linear over the whole of the

4 min assay period in the presence and absence of malonyl-CoA. We did not observe the slow, malonyl-CoA-induced, time-dependent, increase in sensitivity to malonyl-CoA reported by Zammit (1984). Either this phenomenon does not occur under our assay conditions or it is completed during the 4 min preincubation period.

Portions of mitochondria were also sonicated (MSE sonicator at full power, 8 µm peak-to-peak) for 2 min at 2°C; 25 µl portions were then taken for assay of 'total CPT' with 40 µM-palmitoyl-CoA. CPT₂ activity is maximal at 40 µM-palmitoyl-CoA (Saggerson & Carpenter, 1983) and this was therefore calculated from:

$$\begin{aligned} \text{CPT}_2 = & \text{'total CPT' (at 40 } \mu\text{M-palmitoyl-CoA)} \\ & - \text{'malonyl-CoA-suppressible' CPT}_1 \\ & \quad \text{(at 40 } \mu\text{M-palmitoyl-CoA)}. \end{aligned}$$

Glutamate dehydrogenase (EC 1.4.1.2) was assayed in portions of mitochondria treated with Triton X-100 (0.1%, w/v) by the method of Martin & Denton (1970).

High-speed supernatants were obtained by centrifuging post-mitochondrial supernatants for 1 h at 105 000 g_{av} . Portions of these were used for the assay of fatty acid synthetase and glucose-6-phosphate dehydrogenase (EC 1.1.1.49) as described, respectively, by Saggerson & Greenbaum (1970) and Martin & Denton (1970).

Mitochondrial protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Expression of results

CPT activities were expressed relative to mitochondrial protein. Glutamate dehydrogenase activity was measured in the initial whole homogenates and in the mitochondrial fractions. Glutamate dehydrogenase was therefore used as a reference enzyme to assess the recovery of CPT and mitochondrial protein and to express these values per total liver. All values are given as means $\pm$ S.E.M. The numbers of individual experiments are indicated in parentheses in the Tables. Statistical significance was determined by Student's *t* test for unpaired samples.

Results and discussion

Table 1 shows general data for the animals used in the study. It was noted that cycloheximide treatment slightly decreased the loss in body weight on starvation (19.5 ± 0.6 g for 29 saline-treated animals versus 15.9 ± 1.2 g for 10 cycloheximide-treated animals; $P < 0.02$). This was in part due to the smaller decrease in liver weight. Cycloheximide increases the content of triacylglycerol in the livers of fed animals and suppresses

Table 1. General data
Significance of changes in fatty acid synthetase and glucose-6-phosphate dehydrogenase activities is indicated as follows: for differences from the fed state, ^a, ^b indicate respectively $P < 0.02$, $P < 0.01$; for effects of cycloheximide, ^c, ^a, ^e indicate respectively $P < 0.02$, $P < 0.01$, $P < 0.001$.

Treatment of animals	Saline-treated (S) or cycloheximide-treated (C)	Body wt. (g)			Liver wt.		Enzyme activities ($\mu\text{mol/min}$ per total liver)				Mitochondrial protein (mg per total liver)
		Day 0	Day 1	Day 2	(g)	(% of body wt.)	Fatty acid synthetase	Glucose-6-phosphate dehydrogenase	Glutamate dehydrogenase		
Fed (6)	S	175 $\pm$ 3	180 $\pm$ 3	—	7.8 $\pm$ 0.4	4.4 $\pm$ 0.2	4.0 $\pm$ 0.5 (6)	14.5 $\pm$ 1.1 (6)	1309 $\pm$ 39 (4)	643 $\pm$ 33 (4)	
Fed (9)	C	171 $\pm$ 3	166 $\pm$ 2	—	7.1 $\pm$ 0.1	4.3 $\pm$ 0.1	2.9 $\pm$ 0.2 (9)	12.5 $\pm$ 0.9 (9)	1341 $\pm$ 62 (6)	567 $\pm$ 24 (6)	
Starved (6)	S	177 $\pm$ 3	156 $\pm$ 3	—	5.6 $\pm$ 0.1	3.6 $\pm$ 0.1	1.7 $\pm$ 0.1 ^b (9)	10.6 $\pm$ 0.7 ^a (9)	1085 $\pm$ 70 (7)	484 $\pm$ 10 (7)	
Starved (10)	C	177 $\pm$ 4	161 $\pm$ 3	—	6.9 $\pm$ 0.1	4.3 $\pm$ 0.1	2.6 $\pm$ 0.3 ^c (10)	13.9 $\pm$ 1.4 (6)	1220 $\pm$ 90 (4)	548 $\pm$ 22 (4)	
Starved, re-fed with cube diet (7)	S	180 $\pm$ 4	162 $\pm$ 3	182 $\pm$ 3	9.4 $\pm$ 0.2	5.1 $\pm$ 0.1	7.8 $\pm$ 1.2 ^a (6)	28.6 $\pm$ 3.2 ^b (6)	1204 $\pm$ 100 (4)	647 $\pm$ 32 (4)	
Starved, re-fed with cube diet (8)	C	172 $\pm$ 4	157 $\pm$ 3	160 $\pm$ 3	5.9 $\pm$ 0.2	3.7 $\pm$ 0.1	1.8 $\pm$ 0.2 ^{b,d} (6)	10.0 $\pm$ 0.7 ^e (6)	1554 $\pm$ 48 (4)	643 $\pm$ 7 (4)	
Starved, re-fed with high-carbohydrate diet (4)	S	178 $\pm$ 3	159 $\pm$ 4	173 $\pm$ 4	9.4 $\pm$ 0.7	5.4 $\pm$ 0.3	—	—	—	—	
Starved, re-fed with high-fat diet (4)	S	183 $\pm$ 1	165 $\pm$ 1	171 $\pm$ 2	6.1 $\pm$ 0.2	3.6 $\pm$ 0.2	—	—	—	—	

Table 2. *Carnitine palmitoyltransferase measurements*
Calculation of values and expression of results is described in the Materials and methods section. Unless stated otherwise, the number of observations was as given in the first column. Significance of changes is indicated as follows: for differences from the fed state, ^a, ^b, ^c indicate respectively $P < 0.02$, $P < 0.01$, $P < 0.001$; for effects of cycloheximide, ^d, ^e, ^f indicate respectively $P < 0.02$, $P < 0.01$, $P < 0.001$.

Treatment of animals	Saline-treated (S) or cycloheximide-treated (C)	CPT ₁ measured at 40 μM-palmitoyl-CoA				CPT ₁ maximum activity measured at 100 μM-palmitoyl-CoA			CPT ₂ maximum activity (nmol/min per mg of mitochondrial protein)
		Malonyl-CoA-suppressible activity (nmol/min per mg of mitochondrial protein)	I _{max} (%)	I ₅₀ (μM)	(nmol/min per mg of mitochondrial protein)	(nmol/min per total liver)	(nmol/min per 100 g body wt.)		
Fed (4)	S	3.16 ± 0.20	87 ± 5	4.3 ± 0.4	4.96 ± 0.37	3155 ± 100	1749 ± 51	5.60 ± 0.16	
Fed (6)	C	2.52 ± 0.16	74 ± 2	4.6 ± 0.6	4.42 ± 0.20	2509 ± 160 ^e	1488 ± 95	6.64 ± 0.22	
Starved (7)	S	4.22 ± 0.25 ^a	81 ± 4	21.5 ± 3.4 ^b	8.10 ± 0.32 ^c	3856 ± 127 ^b	2419 ± 90 ^c	6.83 ± 0.30 ^b	
Starved (7)	C	3.02 ± 0.06 ^{a,e}	75 ± 2	6.8 ± 0.6 ^e	5.45 ± 0.21 ^{b,f}	2979 ± 126 ^f	1773 ± 74 ^f	6.97 ± 0.22	
Starved, re-fed with cube diet (4)	S	3.98 ± 0.26	95 ± 1	5.8 ± 0.8	5.49 ± 0.10	3541 ± 164	1949 ± 86	5.86 ± 0.64	
Starved, re-fed with cube diet (4)	C	3.77 ± 0.15 ^c	84 ± 6	6.5 ± 0.9	6.35 ± 0.19 ^{c,e}	4079 ± 128 ^c	2471 ± 134 ^{c,d}	7.92 ± 0.45	
Starved, re-fed with high-carbohydrate diet (4)	S	3.11 ± 0.24	86 ± 3	8.7 ± 1.2 ^a	5.78 ± 0.44	—	—	6.17 ± 0.46	
Starved, re-fed with high-fat diet (4)	S	4.11 ± 0.22 ^a	80 ± 6	36.1 ± 6.0 ^b	9.00 ± 0.40 ^c	—	—	7.11 ± 0.25 ^b	

very-low-density-lipoprotein triacylglycerol secretion (Bar-On *et al.*, 1972; Roberts *et al.*, 1982). In the starved animals, cycloheximide treatment resulted in very obvious signs of 'fatty liver'. Animals re-fed with the high-carbohydrate diet after starvation regained less body weight than did those fed with the cube diet; however, the regain of liver weight was comparable in these two groups. Both the group re-fed with the high-fat diet and the group treated with cycloheximide and re-fed with the cube diet showed only small increases in body weight, with no increase in liver weight on re-feeding. We have not attempted to match energy intakes in any of the re-fed groups, but the average quantities of high-carbohydrate and high-fat diets consumed were comparable (27 and 24 g/day per rat respectively). Fatty acid synthetase and glucose-6-phosphate dehydrogenase are two cytosolic enzymes whose activities are profoundly affected by nutritional factors (Rudack *et al.*, 1971; Craig *et al.*, 1972). We therefore made measurements of these as a simple check that the administered dose of cycloheximide was adequate for inhibition of protein synthesis. This is likely to be so, since cycloheximide totally abolished the rapid rise in these activities when starved rats were re-fed.

Table 2 shows data derived from measurements of CPT activity. The maximum activity of CPT₁ is expressed per total liver and per 100 g body wt. as well as relative to mitochondrial protein, since the liver content of the latter was changed by some of the treatments. In the absence of cycloheximide, starvation increased both the CPT₁ activity and the I_{50} for malonyl-CoA, as found previously (Saggerson *et al.*, 1982; Saggerson & Carpenter, 1982), although differences in I_{max} values were slightly less. Re-feeding with the cube diet or the high-carbohydrate diet reversed these changes, whereas re-feeding with the high-fat diet did not. Qualitatively similar, but much smaller, changes in CPT₂ activity were observed with these treatments.

In the presence of cycloheximide, starvation resulted in an increase in CPT₁ maximum activity (1.03 nmol/min per mg of protein) which was only one-third of that without cycloheximide (3.14 nmol/min per mg). The CPT₁ activity measured in cycloheximide-treated starved rats was not significantly different from that in saline-treated fed animals. Cycloheximide treatment also virtually abolished the increase in the I_{50} for malonyl-CoA in starvation. Cycloheximide stimulates hepatic glycolytic flux and lipogenesis (Roberts *et al.*, 1982), a situation likely to be associated with elevation of the malonyl-CoA content. It has been suggested (Zammit, 1984) that liver mitochondria have a 'memory' of the

malonyl-CoA concentration *in vivo*, which is reflected in the I_{50} for malonyl-CoA. This could provide an explanation for our finding. Surprisingly, administration of cycloheximide to starved animals immediately before re-feeding with the cube diet partially prevented the decrease in CPT₁ activity on re-feeding, but did not prevent the return of the I_{50} for malonyl-CoA to the fed value. Cycloheximide administration had little effect on CPT₂ activity and did not block the modest rise in this activity on starvation. A high proportion of the CPT₁ activity measured at 40 μ M-palmitoyl-CoA was suppressible by malonyl-CoA. The various treatments resulted in similar but smaller changes to those seen at 100 μ M-palmitoyl-CoA. We have observed previously (Saggerson *et al.*, 1982; Saggerson & Carpenter, 1982) that long-term changes in CPT₁ activity are more pronounced if the activity is measured under V_{max} conditions (100 μ M-palmitoyl-CoA).

In conclusion, these findings suggest that the well-documented increase in CPT₁ activity on starvation may be due to synthesis of new enzyme. The observation that CPT₂ activity was unaffected by cycloheximide could explain why our conclusions are at variance with those of Norum (1965). It is probable that his assay measured both CPT₁ and CPT₂.

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