Effects of Palmitoyl CoA on Citrate and Malate Transport by Rat Liver Mitochondria

(tricarboxylate transport system/fatty acid synthesis/surface molecules/metabolic regulation)

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ABSTRACT Palmitoyl CoA inhibited citrate transport from isolated rat liver mitochondria. Under conditions described, 50% inhibition was observed at about 6-8 nmol/mg of mitochondrial protein per ml. The percentage inhibition was inversely proportional to the concentration of the counter-transporting anion in the medium. Although comparable levels of palmitoyl CoA had little effect on malate exit on the dicarboxylate carrier, higher concentrations inhibited both citrate and malate transport.

The specificity of the inhibition by palmitoyl CoA was investigated by examination of the effects on the tricarboxylate transport system of fatty acids, CoASH, acetyl CoA, palmitoylcarnitine, deoxycholate, and other molecules with surface active properties. None of the above compounds, at sublytic concentrations, inhibited citrate transport appreciably. The inhibition of citrate transport by low concentrations of palmitoyl CoA was rapid and could be prevented or partially reversed by addition of albumin.

In animals who are starved, fed a high fat diet, or rendered insulin-deficient, the rate of synthesis of hepatic fatty acid is greatly diminished (1, 2). The possible control mechanisms responsible for this, and for the related hyperlipogenesis that follows feeding carbohydrate to starved rats, have been discussed in recent reviews (3, 4). One of the potential control points that has received relatively little attention is the transport system in mitochondrial membranes responsible for the exit of citrate from mitochondria into the cytosol. Citrate in the cytosol of the cell serves as substrate in the citrate lyase (EC 4.1.3.6) reaction (5), thereby generating acetyl CoA. Acetyl CoA so formed provides all carbons from which fatty acids are synthesized in the cytosol. Accordingly, considerable attention has been devoted to an evaluation of the regulation of the citrate lyase reaction in fatty acid synthesis (4, 6). Even though concentrations of citrate lyase tend to be elevated when rates of fatty acid synthesis are high, the altered activities of citrate lyase cannot be directly correlated with fluxes in fatty acid synthesis during all physiological states (7). It appears that changes in the activity of this enzyme follow the changes in rates of fatty acid biosynthesis and do not initiate them (7). The possibility remains open, however, that the rate of exit of citrate from mitochondria could functionally regulate the rate of acetyl CoA formation, independent of variations in the amount of citrate lyase present.

Results to be presented in this report demonstrate that low concentrations of palmitoyl CoA inhibit citrate transport, while higher concentrations inhibit both citrate and malate transport. We shall discuss the importance of these observa-

tions in relation to the possible mechanisms controlling rates of fatty acid synthesis.

METHODS

Rats (150 g Wistar, obtained from High Oak Ranch, Goodwood, Ontario) were allowed free access to Purina rat chow before they were killed. Liver mitochondria were isolated by conventional methods in a medium containing 0.25 M sucrose, 5.0 mM Tris·HCl, and 1 mM ethylene glycol-bis (β -aminoethyl ether-*N,N'*-tetraacetic acid (EGTA), pH 7.4 (8). The preparation was resuspended to give a final protein concentration of 30 mg/ml. Mitochondrial protein was determined by the method of Lowry *et al.* (9). Concentrations of agents used in all studies appear in figures or text. Graphs shown in individual figures are representative of three or more separate experiments in each case.

Methods used for determination of [14 C]malate and [14 C]citrate exchanges, as well as for tricarboxylate anion loading, were the same as those used by Robinson *et al.* (10). The 14 C label in the tricarboxylate compounds is present in citrate and isocitrate. For convenience, throughout this report we will use [14 C]citrate to represent both these anions. The method for L-[14 C]malate loading was that of Robinson and Williams (11). Exchange experiments were monitored at 8° for 2 min as described (10, 12).

Adenosine triphosphate, 2-oxoglutarate, citric acid, antimycin A, rotenone, and palmitoyl CoA were obtained from Sigma Chemical Co. (St. Louis, Mo.). 1,2,3-Benzenetricarboxylate and 2-pentylmalonate were obtained from K & K Laboratories Inc. (Plainsview, N.Y.), and L-malate from Eastman Organic Chemicals (Rochester, N.Y.). L-[U- 14 C]-malate and [14 C]sodium bicarbonate were obtained from Amersham-Searle (Don Mills, Ontario). (-)-Palmitoylcarnitine and (+)-palmitoylcarnitine were synthesized by the procedures of Bremer (13), with palmitoyl chloride and stereoisomers of carnitine kindly provided by Otsuka Pharmaceuticals (Naruto, Tokushima, Japan).

RESULTS

In confirmation of earlier observations (10), [14 C]citrate preloaded into mitochondria was retained in the mitochondrial fraction unless unlabeled L-malate or citrate was added to the extramitochondrial space. With the "inhibitor stop" technique, with 1,2,3-benzenetricarboxylate (12), we observed that palmitoyl CoA (75 μ M, 22 nmol/mg of protein per ml)

FIG. 1. Effects of palmitoyl CoA on the transport of citrate across mitochondrial membranes of rat liver at various time intervals. Rat-liver mitochondria (3.4 mg of protein), loaded with [^{14}C]citrate, were added to 1 ml of incubation medium containing 125 mM KCl and 20 mM Tris·HCl at pH 7.4 and 8°. Citrate (1.0 mM), with 75 μM palmitoyl CoA (open circles) or without palmitoyl CoA (solid circles), was added to this basic medium. 1,2,3-Benzenetricarboxylate (50 mM) was added at each time interval in order to stop any further exchange from taking place. The mitochondria were separated by centrifugation and the supernatant fractions were deproteinized, evacuated, and counted for [^{14}C]citrate (10). An unseparated sample was also treated and counted to allow calculations of the total [^{14}C]citrate in the system. The extent of the exchange during each time period was expressed as percentage exchange, where the percentage exchange equals the

$$\frac{\text{Supernatant dpm in incubation} - \text{supernatant dpm at zero time}}{\text{Mitochondrial dpm at zero time}} \times 100$$

rapidly inhibited the transport of citrate across the mitochondrial membrane (Fig. 1). The concentration of palmitoyl CoA required for 50% inhibition of citrate transport was 32 μM when 1 mM citrate was the exchanging anion and 42 μM

TABLE 1. Effects of metabolic intermediates on the exit of [^{14}C]citrate from rat liver mitochondria

Addition to incubation medium	Concentration (μM)	cpm In supernatant	% Exchange
None	—	656	—
Citrate	1000	3326	89
+ CoASH	25	3386	90
	100	3243	87
+ Acetyl CoA	25	3301	88
	100	3619	97
+ Palmitoyl-d-carnitine	50	2948	79
	100	3341	89
+ Palmitoyl-l-carnitine	50	3368	90
	100	3380	90
+ Palmitoyl CoA	40	1112	30
	80	453	0

FIG. 2. Effects of palmitoyl CoA on citrate/[^{14}C]citrate and on malate/[^{14}C]citrate exchanges. Rat liver mitochondria (6 mg protein/ml) loaded with [^{14}C]citrate were incubated as described in Fig. 1 in media containing 1 mM citrate (●—●) or 1 mM L-malate (○—○) at 8°. After 2 min the mitochondria were separated and analyzed as described in Fig. 1. The percentage inhibition of the tricarboxylate carrier is calculated by taking the percentage exchange after 2 min in the absence of palmitoyl CoA as the baseline value. These values were 44 and 21%, respectively, for the citrate- and malate-mediated exchanges.

in the presence of 1 mM L-malate (Fig. 2). The use of higher external citrate concentrations (2.5 or 10 mM) decreased the inhibition by palmitoyl CoA (Fig. 3), suggesting the existence of a competition. Also, the inhibition at a given concentration of palmitoyl CoA was decreased at higher mitochondrial protein concentrations (Fig. 4). The inhibitory effect of palmitoyl CoA could be prevented by prior addition of albumin, and it could be partially reversed by the subsequent addition of albumin (Fig. 5). From data presented in Figs. 4 and 5, it follows that the molar ratio of palmitoyl CoA to protein is a more important determinant in the consideration of inhibitory effects than is the absolute concentration of palmitoyl CoA. An inhibition of citrate transport of 50% was usually observed when about 6 nmol of added palmitoyl CoA/mg of mitochondrial protein was present under experimental conditions used (Figs. 4 and 6).

The tricarboxylic acid carrier could not be inhibited by free CoASH (100 μM), acetyl CoA (100 μM), either stereoisomer of palmitoylcarnitine (100 μM) (Table 1), or by oleic acid (2.8 mM) (Table 2). The tricarboxylate carrier could be inhibited by certain detergents (e.g., Tween 80), but this was not a prominent characteristic of all surface active compounds (e.g., deoxycholate and Tween 20) (Table 2). Further, the concentrations required for inhibition by nonspecific detergents was very close to those associated with release of the intramitochondrial [^{14}C]citrate in the absence of exchanging anion (1 mM citrate) (Table 2). At these concentrations, mitochondrial lysis probably occurred.

The possible effects of palmitoyl CoA on the dicarboxylate transport system were also examined. Using rat liver mitochondria preloaded with [^{14}C]malate, we measured the exchange of malate with inorganic phosphate at 8° for two min. We compared the effects of palmitoyl CoA on the dicarboxylate carrier and on the tricarboxylate carrier in aliquots of

FIG. 3. Effects of citrate concentration on inhibition of citrate/[^{14}C]citrate exchange by palmitoyl CoA. Experiments were conducted as described in the legend to Fig. 1, with a time interval of 2 min. The protein concentration was 3.8 mg/ml, and the citrate concentration in the incubation medium was 1 mM (●—●), 2.5 mM (*—*), or 10 mM (○—○).

mitochondria prepared from the same rat livers. At low concentrations of palmitoyl CoA, citrate/[^{14}C]citrate exchange was inhibited to a greater extent than was P_i /[^{14}C]malate exchange (Fig. 6). To achieve an inhibition of the dicarboxylate carrier of 50%, about 21 nmol of palmitoyl CoA per mg of mitochondrial protein was required. Note that this ratio is about three times larger than that required to obtain a comparable inhibition of the tricarboxylate carrier (Fig. 6).

The inhibition by palmitoyl CoA of the exchange of P_i /[^{14}C]malate was considerably greater than the inhibition of the citrate/[^{14}C]malate exchange (Fig. 7).

FIG. 4. Effects of mitochondrial protein concentration on the inhibition of citrate/[^{14}C]citrate exchange by palmitoyl CoA. The mitochondrial protein concentrations were 6 mg/ml (●—●), 3 mg/ml (○—○), or 1.5 mg/ml (*—*). Experimental conditions were otherwise the same as those described in the legend to Fig. 3, with 1 mM citrate.

FIG. 5. Reversal by albumin of inhibition of citrate/[^{14}C]citrate exchange induced by palmitoyl CoA. For details of incubations, see legend to Fig. 1. Mitochondria (2.65 mg of protein/ml) were incubated for either 60 or 120 sec before the addition of 1,2,3-benzenetricarboxylate (50 mM). Palmitoyl CoA was present at the concentrations (μM) to the right of each curve. Albumin (1.25 mg/ml) was present in those incubations noted by the dashed parts of curves connected to stars. Albumin was added at time zero in the absence of palmitoyl CoA, or after 60 sec when palmitoyl CoA was present.

FIG. 6. Effects of palmitoyl CoA on the dicarboxylate and tricarboxylate carriers. For details of experimental procedures, see the legend to Fig. 2. Mitochondrial protein concentrations in graphs depicted were 2.58 and 3.2 mg/ml for the tricarboxylate and dicarboxylate experiments, respectively. Mitochondria obtained from the livers of four rats were divided into two portions, one of which was loaded with [^{14}C]citrate, and the other of which was loaded with [^{14}C]malate by procedures described in *Methods* (10, 11). The exchanging anion was 1 mM citrate for the tricarboxylate carrier (curve T) and 5 mM inorganic phosphate for the dicarboxylate carrier (curve D). The percentage inhibition was calculated as described in the legend to Fig. 2.

FIG. 7. Effects of palmitoyl CoA on inorganic phosphate/[^{14}C]malate and citrate/[^{14}C]malate exchanges. Mitochondria (4.5 mg of protein) loaded with [^{14}C]malate (11) were incubated in a basal medium containing either 1 mM citrate (●—●) or 5 mM inorganic phosphate (☆--☆) for 2 min in the presence of concentrations of palmitoyl CoA indicated. Experimental procedures were otherwise the same as those described in the legends to Figs. 1 and 2.

DISCUSSION

The major finding presented demonstrates that palmitoyl CoA, at concentrations of about 6–8 nmol/mg mitochondrial protein, reduces the rate of exit of citrate from mitochondria by 50% under experimental conditions described.

Although some of the effects of long-chain fatty acyl CoA derivatives on various enzymes result from nonspecific alteration of protein structure caused by the surface active properties of these compounds (14), palmitoyl CoA reversibly inhibits certain systems in a specific manner. For example, the percentage inhibition by palmitoyl CoA of the exit of citrate from mitochondria was diminished when the citrate concentration in the medium was elevated (Fig. 3). This concentration dependence, with greatest inhibition at lower citrate levels, supports the specificity of palmitoyl CoA action on the tricarboxylate transport system. In addition, the differential effects of palmitoyl CoA on the dicarboxylate and the tricarboxylate transport systems suggest specificity of action. As shown in Fig. 6, when P_i /[^{14}C]malate exchange was measured, the concentration of palmitoyl CoA required to achieve 50% inhibition was about three times the amount observed for inhibition of the tricarboxylate carrier by 50%. As shown in Fig. 7, palmitoyl CoA at relatively high concentrations (225 μM) inhibited the P_i /[^{14}C]malate exchange almost completely, whereas the citrate/[^{14}C]malate exchange was inhibited by less than 25% at comparable palmitoyl CoA levels. Other data depicted in the tables and in Figs. 1 and 5 provide supplementary evidence that the effects of palmitoyl CoA on transport processes are specific.

Reports on the effects of long-chain fatty acyl CoA derivatives on other mitochondrial transport systems have recently appeared (15–17). Palmitoyl CoA and oleoyl CoA inhibited transport of ADP into rat liver mitochondria, whereas the free fatty acids had no effect. The rapid inhibition by fatty

TABLE 2. Effect of surface active agents on the exit of [^{14}C]citrate from rat liver mitochondria

Exp. no.	Additions to the incubation medium	Concentration ($\mu\text{g/ml}$)	Total dpm in the supernatant	
			0 Citrate	1.0 mM Citrate
1	Sodium dodecyl sulfate (SDS)	0	611	2028
		40	661	1857
		100	593	1903
		200	942	1194
2	Tween 20	0	816	2887
		4	807	2781
		10	703	2432
		20	1176	2448
3	Deoxycholate	0	462	1486
		5	477	1525
		25	523	1211
		100	1239	1586
4	Oleic acid	0	457	1471
		20	435	1333
		200	474	1311
		800	483	1222
5	Tween 80	0	338	1351
		80	419	1385
		400	519	1086
		800	486	930
6	Palmitoyl CoA	2000	829	862
		0	979	5001
		37.5	1501	2091
		75	1072	1432

acyl CoA derivatives was abolished by addition of carnitine, indicating that the acyl CoA derivatives and not the acyl-carnitine derivatives were the inhibitors of the ADP translocase system (15).

The fatty acid biosynthetic pathway appears to be highly integrated, both with respect to the coordinate nature of the control of synthesis of various enzymes associated with the pathway and with respect to the multiple sites of inhibition of fatty acid synthesis by elevated concentrations of long-chain fatty acyl CoA derivatives (3, 18). In addition, cellular mechanisms exist that coordinate the rates of other pathways in relation to that of fatty acid synthesis. There tends to be a direct correlation between fluxes of hepatic fatty acid oxidation and gluconeogenesis (19, 20), and these rates are inversely related to that of fatty acid synthesis (3, 21). It is tempting to postulate that these processes are in part integrated by the control of anion transport from mitochondria to the cytosol. Although it is not yet possible to evaluate quantitatively the relative importance of the inhibitory effects of palmitoyl CoA on each of the reactions that is influenced (14), we offer the hypothesis that a major site of control of fatty acid synthesis exists at the level of product inhibition on the first reaction of the sequence required for generating cytosolic acetyl CoA, namely an inhibition of citrate transport by palmitoyl CoA.

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1. Masoro, E. J. (1962) *J. Lipid Res.* 3, 149–164.

2. Fritz, I. B. (1961) *Physiol. Rev.* **41**, 52-129.
3. Tepperman, J. & Tepperman, H. M. (1970) *Fed. Proc.* **29**, 1284-1293.
4. Lowenstein, J. M. (1968) *Biochem. Soc. Symp.* **27**, 61-86.
5. Srere, P. A. (1962) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. 5, pp. 641-644.
6. Srere, P. A. (1965) *Nature* **205**, 766-770.
7. Foster, D. W. & Srere, P. A. (1968) *J. Biol. Chem.* **243**, 1926-1930.
8. Robinson, B. H. & Chappell, J. B. (1967) *Biochem. Biophys. Res. Commun.* **28**, 249-255.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, A. J. (1951) *J. Biol. Chem.* **193**, 265-275.
10. Robinson, B. H., Williams, G. R., Halperin, M. L. & Leznoff, C. C. (1970) *Eur. J. Biochem.* **15**, 263-272.
11. Robinson, B. H. & Williams, G. R. (1970) *Biochim. Biophys. Acta* **216**, 63-70.
12. Robinson, B. H., Williams, G. R., Halperin, M. L. & Leznoff, C. C. (1971) *Eur. J. Biochem.* **20**, 65-71.
13. Bremer, J. (1968) in *Biochemical Preparations*, ed. Lands, W. E. M. (John Wiley & Sons, Inc., New York), Vol. 12, pp. 69-73.
14. Taketa, K. & Pogell, B. M. (1966) *J. Biol. Chem.* **241**, 720-726.
15. Shug, A., Lerner, E., Elson, C. & Shrago, E. (1971) *Biochem. Biophys. Res. Commun.* **43**, 557-563.
16. Pande, S. V. & Blanchaer, M. C. (1971) *J. Biol. Chem.* **246**, 402-411.
17. McLean, P., Gumaa, K. A. & Greenbaum, A. L. (1971) *FEBS Lett.* **17**, 345-350.
18. Halperin, M. & Robinson, B. H. (1972) in *Proceedings of the Symposium on Insulin Action*, ed. Fritz, I. B. (Academic Press, New York), in press.
19. Lardy, H. A., Foster, D. O., Young, J. W., Shrago, E. & Ray, P. D. (1965) *J. Cell. Comp. Physiol.* **66** (Suppl. 1), 39-53.
20. Krebs, H. A. (1966) *Advan. Enzyme Regul.* **4**, 339-353.
21. Fritz, I. B. (1967) *Perspect. Biol. Med.* **10**, 643-677.

Correction. In the article "RNA in Human Leukemic Cells Related to the RNA of a Mouse Leukemia Virus", by Hehlmann, R., Kufe, D. & Spiegelman, S., which appeared in the February 1972 issue of *Proc. Nat. Acad. Sci. USA* **69**, 435-439, Figs. 2 and 3 (pp. 436 and 437) were transposed during printing and should be reversed (see below).

FIG. 2. (A-D) Cs_2SO_4 density profiles of RLV-[^3H]DNA hybridized to polysomal RNAs of four leukemic samples. Polysomal RNA was isolated from buffy coats of patients showing clinical manifestation of acute lymphocytic leukemia (A, C, and D) and of acute myelogenous leukemia (B). The cells were disrupted with a Dounce homogenizer, and cytoplasmic pellets were prepared as described under Fig. 1B. 300 μg of polysomal RNA were hybridized to RLV-[^3H]DNA in 60- μl volumes, and the reactions were analyzed by Cs_2SO_4 density centrifugation.

FIG. 3. (A-D) Cs_2SO_4 density centrifugation of RLV-[^3H]DNA after annealing to polysomal RNA isolated from (A) normal white buffy coat, (B) phytohemagglutinin-stimulated lymphocytes, (C) buffy coat of a leukemic patient in clinical remission, and (D) fetal lung. The polysomal RNA input was 300 μg , except in (C) where 1000 μg were used per 60- μl hybridization reaction. Subsequent analysis on Cs_2SO_4 was described in Fig. 1.