Elucidation of the Mechanism by Which (+)-Acylcarnitines Inhibit Mitochondrial Fatty Acid Transport*

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It is well established that medium and long chain (+)-acylcarnitines (i.e. fatty acid esters of the unnatural D-isomer of carnitine) inhibit the oxidation of long chain fatty acids in mammalian tissues by interfering with some component(s) of the mitochondrial carnitine palmitoyltransferase (CPT) system. However, whether their site of action is at the level of CPT I (outer membrane), CPT II (inner membrane), carnitine-acylcarnitine translocase (CACT, inner membrane), or some combination of these elements has never been resolved. We chose to readdress this question using rat liver mitochondria and employing a variety of assays that distinguish between the three enzyme activities. The effect on each of (+)-acylcarnitine, (+)-hexanoylcarnitine, (+)-octanoylcarnitine, (+)-decanoylcarnitine, and (+)-palmitoylcarnitine was examined. Contrary to long-standing belief, none of these agents was found to impact significantly upon the activity of CPT I or CPT II. Whereas (+)-acylcarnitine also failed to influence CACT, both (+)-octanoylcarnitine and (+)-palmitoylcarnitine strongly inhibited this enzyme with a similar IC_{50} value (~35 μM) under the assay conditions employed. Remarkably, (+)-decanoylcarnitine was even more potent (IC_{50} ~5 μM), whereas (+)-hexanoylcarnitine was far less potent (IC_{50} >200 μM). These findings resolve a 35-year-old puzzle by establishing unambiguously that medium and long chain (+)-acylcarnitines suppress mitochondrial fatty acid transport solely through the inhibition of the CACT component. They also reveal a surprising rank order of potency among the various (+)-acylcarnitines in this respect and should prove useful in the design of future experiments in which selective blockade of CACT is desired.

EXPERIMENTAL PROCEDURES

Animals and Mitochondrial Preparation—Male Harlan Sprague-Dawley rats (~300 g of body weight) were maintained on regular Purina chow and used in the fed state. Liver mitochondria were isolated as described by McGarry et al. (12) using Method A and suspended in ice-cold 150 mM KCl, 10 mM Tris-HCl, pH 7.2. The protein concentration was measured using the method of Lowry et al. (13).

CPT Assays—Mitochondrial CPT activity was determined in a variety of ways, all at 30 °C. In Assay 1, the reaction was followed in the direction palmitoyl-CoA + [1-14C]carnitine → palmitoyl[1-14C]carnitine + CoASH. The reaction mixture contained in a final volume of 0.5 ml: 30 mM KCl, 105 mM Tris-HCl, pH 7.2, 50 μM palmitoyl-CoA, 500 μM [1-14C]carnitine (~280,000 cpm), 1% fatty acid-free bovine serum albumin, 4 mM ATP, 4 mM MgCl₂, 0.25 mM GSH, 2 mM KCN, 40 μg/ml rotenone, and ~0.25 mg of mitochondrial protein. To measure CPT I activity the mitochondria were kept intact, and incubations were carried out in the absence or presence of 100 μM malonyl-CoA or the

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1 CPT, carnitine palmitoyltransferase; DC, decanoylcarnitine; OC, octanoylcarnitine; CACT, carnitine-acylcarnitine translocase; OG, octylglucoside; PC, palmitoyl carnitine; HC, hexanoylcarnitine.
\(\text{CoASH} \), CPT I cannot operate, but palmitoyl-[14C]-L-carnitine and other components listed for Assay 1. Under these conditions (absence of carnitine, and 

\[\text{[14C]}\text{L-carnitine has been given previously (3–6, 12, 14).}\]

\[\text{i.e. isi still formed because of the combined activity of CACT and internal L-carnitine can enter the matrix via CACT, and label is exchanged}\]

\[\text{m\text{tained initially 25}[14C]L-carnitine (\text{50,000 cpm}), 100 \mu M \text{CoASH, and ~0.25 mg of mitochondrial protein together with the other components used in Assay 1, and the rate of loss of label from palmitoylcarnitine was followed for over 4 min.}\]

\[\text{In Assay 3, used only with intact mitochondria, the initial reactants were 125 [14C]l-carnitine (\text{280,000 cpm}), 125 \mu M \text{palmitoyl-l-carnitine, and ~0.75 mg of mitochondrial protein together with the other components listed for Assay 1. Under these conditions (absence of external CoASH), CPT I cannot operate, but palmitoyl-[14C]-l-carnitine is still formed because of the combined activity of CACT and internal CPT II, i.e. both the external [14C]l-carnitine and unlabeled palmitoyl-l-carnitine can enter the matrix via CACT, and label is exchanged between them through the action of CPT II using the matrix pool of CoASH. In this}&

\[\text{TABLE I}\]

\[\text{Effects of malonyl-CoA and (+)-acylcarnitines on components of the CPT system in rat liver mitochondria}\]

\[\text{Assays 1–3 were carried out as described under “Experimental Procedures.” Values are means ± S.E. for three experiments.}\]

\[\begin{array}{|c|c|c|c|}
\hline
\text{Table} & \text{Assay 1} & \text{Assay 2} & \text{Assay 3} \\
\hline
& \text{Intact, CPT I, left → right} & \text{OG-solubilized, CPT II, left → right} & \text{Intact, CPT II + CACT, isotope exchange} \\
\hline
\text{Control} & 100^b & 100^b & 100^d \\
\text{Malonyl-CoA (100 \mu M)} & 21.7 ± 3.1 & 90.6 ± 2.1 & 99.9 ± 1.1 \\
\text{(+)-AC (200 \mu M)} & 98.5 ± 2.0 & 101.3 ± 1.3 & 98.5 ± 3.6 \\
\text{(+)-HC (200 \mu M)} & 98.5 ± 2.7 & 97.2 ± 2.7 & 99.2 ± 1.5 \\
\text{(+)-OC (200 \mu M)} & 101.0 ± 3.5 & 102.0 ± 3.2 & 101.3 ± 1.5 \\
\text{(+)-DC (200 \mu M)} & 96.0 ± 4.2 & 98.3 ± 2.4 & 96.2 ± 3.0 \\
\text{(+)-PC (50 \mu M)} & 103.1 ± 3.5 & 101.2 ± 2.5 & 95.6 ± 1.9 \\
\text{(+)-PC (200 \mu M)} & 93.4 ± 3.7 & 89.6 ± 1.0 & 92.5 ± 3.2 \\
\hline
\end{array}\]

\[\text{a 2.9 nmol \cdot min}^{-1} \cdot \text{mg mitochondrial protein}^{-1}.\]

\[\text{b 5.0 nmol \cdot min}^{-1} \cdot \text{mg mitochondrial protein}^{-1}.\]

\[\text{c 4.4 nmol \cdot min}^{-1} \cdot \text{mg mitochondrial protein}^{-1}.\]

\[\text{d 2745 cpm appearing in palmitoyl-l-carnitine \cdot min}^{-1} \cdot \text{mg mitochondrial protein}^{-1}.\]

\[\text{AC, acetylcarnitine.}\]

\[\text{indicated concentration of (+)-acylcarnitine. Under these conditions, the assay monitors only the external malonyl-CoA-sensitive CPT I activity together with a small and variable contribution of internal CPT II, resulting from the unavoidable minor damage of mitochondria during the isolation procedure (14). CPT II activity was measured using the same system except that the mitochondrial suspension was first brought to 1% (w/v) with octyl glucoside (OG) and kept on ice for 30 min before assay. This treatment destroys CPT I activity while causing complete release of the detergent-stable and malonyl-CoA-insensitive CPT II isoform (14). Reactions were initiated by the addition of mitochondria and were terminated with 0.5 ml of 1.2 M HCl after 4 min. The palmitoyl-[14C]-l-carnitine formed was extracted with butanol and quantified by scintillation counting (12, 14).}\]

\[\text{Assay 2 measured the activity of CPT II in OG-treated mitochondria in the “physiological” direction, i.e. palmitoyl-[14C]-l-carnitine + CoASH → palmitoyl-CoA + [14C]-l-carnitine. Here the reaction mixture contained initially 25 \mu M palmitoyl-[14C]-l-carnitine (\text{50,000 cpm}), 100 \mu M CoASH, and ~0.25 mg of mitochondrial protein together with the other components used in Assay 1, and the rate of loss of label from palmitoylcarnitine was followed for over 4 min.}\]

\[\text{In Assay 3, used only with intact mitochondria, the initial reactants were 125 [14C]l-carnitine (\text{280,000 cpm}), 125 \mu M \text{palmitoyl-l-carnitine, and ~0.75 mg of mitochondrial protein together with the other components listed for Assay 1. Under these conditions (absence of external CoASH), CPT I cannot operate, but palmitoyl-[14C]-l-carnitine is still formed because of the combined activity of CACT and internal CPT II, i.e. both the external [14C]l-carnitine and unlabeled palmitoyl-l-carnitine can enter the matrix via CACT, and label is exchanged between them through the action of CPT II using the matrix pool of CoASH. In this isotope exchange assay, the appearance of label in palmitoylcarnitine was monitored for over 4 min.}\]

\[\text{In all three assays, the reaction rate was essentially linear over the 4-min period.}\]

\[\text{Materials—The sources of all reaction components and (+)-acylcarnitines have been given previously (3–6, 12, 14).}\]

\[\text{RESULTS}\]

\[\text{CPT I and CPT II catalyze the reversible reaction: palmitoyl-CoA + carnitine ⇔ palmitoylcarnitine + CoASH. We shall refer to the reaction generating palmitoylcarnitine as “left → right” and the opposite reaction as “right → left.” As seen from Table I, 100 \mu M malonyl-CoA had the expected inhibitory effect on CPT I (Assay 1, intact mitochondria), the suppression being ~80%. The residual activity stemmed from (a) partial damage to the mitochondria during their preparation, allowing limited exposure of the usually latent malonyl-CoA-insensitive CPT II, and (b) some desensitization of CPT I to the inhibitor, a phenomenon that always occurs when dealing with mitochondria from fed animals if extreme precautions are not taken to maintain the mitochondria at 0–4 °C at every step before assay (15, 16). The key point to be emphasized, however, is that none of the (+)-acylcarnitines tested impacted significantly on CPT I activity or suppressed the activity of CPT II, regardless of the direction in which the reaction was measured (Assays 1 and 2 with OG-solubilized mitochondria). In other experiments, raising the concentration of (+)-OC to 1 mM still had no impact on Assays 1 and 2.}\]

\[\text{The small effect of malonyl-CoA seen in the assay of CPT II from “left → right” was not because of enzyme inhibition but rather because of low-grade release of free CoASH resulting from nonspecific breakdown of malonyl-CoA by the detergent-solubilized mitochondria. This has the effect of displacing the equilibrium position of the reaction to the left and is not observed when the assay is run in the presence of thiol reagents, such as N-ethylmaleimide or sodium tetrathionate, to trap any CoASH generated in the system (data not shown). Also, the apparent 10% suppression of CPT II activity caused by 200 \mu M (+)-PC in Assay 1 with OG-solubilized mitochondria was probably a detergent effect resulting from the simultaneous presence of OG and the amphiphatic long chain acylcarnitine at high concentration. A similar phenomenon was observed previously using other detergent mixtures (17).}\]

\[\text{In sharp contrast to their lack of effect in Assays 1 and 2, both medium and long chain (+)-acylcarnitines (but not (+)-acyetylcarnitine) markedly suppressed the exchange of [14C] between t-carnitine and palmitoyl-t-carnitine in Assay 3, the degree of inhibition being 97, 83, 78, and 31% by (+)-DC,}\]
(+)-Acylcarnitines and Carnitine-Acylcarnitine Translocase

(+)-OC, (+)-PC, and (+)-HC, respectively, when used at a fixed concentration of 200 μM (Table I). When each compound was tested over a range of concentrations, the IC50 values for (+)-DC, (+)-OC, (+)-PC, and (+)-HC were found to be ~5, 33, 38, and >200 μM, respectively, under the experimental conditions employed here (Fig. 1). As CPT I cannot function during Assay 3 because of the absence of external CoASH, malonyl-CoA, not surprisingly, was totally without effect (Table I). The unique feature of this assay is that it monitors the combined activity of CACT and internal CPT II in intact mitochondria. Because neither CPT I nor CPT II is influenced by any of the (+)-acylcarnitines examined here (see above), it follows that the C6-C16 derivatives act only on the CACT component of the mitochondrial CPT system.

DISCUSSION

In the present study, we have revisited an issue that surprisingly has lain dormant and unresolved for more than 30 years, namely the question of how (+)-acylcarnitines suppress the mitochondrial oxidation of long chain fatty acids. It was originally proposed that they do so by inhibiting one or both of the CPT enzymes (7, 11). Indeed based on this assumption, this laboratory and others used (+)-DC and (+)-OC to block hepatic fatty acid oxidation and ketone body production in the perfused rat liver (1–3, 6) and to effect the rapid reversal of diabetic ketoacidosis in intact alloxanized rats (4, 5). Subsequently, however, Pande (8) and Pande and Parvin (10) demonstrated that the sequential operation of CPT I and CPT II in mitochondrial long chain fatty acid oxidation is coupled by a third component, i.e. CACT, which allows the one-for-one exchange of L-carnitine and acyl-L-carnitine across the mitochondrial inner membrane. Furthermore, they demonstrated that CACT can be inhibited by certain (+)-acylcarnitines, although the assay employed showed a rather high background activity, and a detailed comparison of the relative effectiveness of different chain length (+)-acylcarnitines was not carried out (8, 10). This observation left open the question of whether such compounds act not only on CACT but also at the level of CPT I and/or CPT II. Armed with our current and more sophisticated understanding of the CPT system (9) together with specific assays for its various components, we considered it important to readdress this matter, albeit after a 25-year hiatus. What emerges is an unambiguous answer to the issue at hand, namely that over a broad range of chain lengths (from C2 to C16) none of the (+)-acylcarnitines that we tested affect CPT I or CPT II in mitochondria from rat liver (and presumably from other tissues). By contrast, whereas (+)-acylcarnitines also had no impact on CACT activity, the medium and long chain (+)-acylcarnitines did inhibit this enzyme. Interestingly, under the defined conditions of our assay, (+)-DC (IC50 ~5 μM) proved to be far more potent than either (+)-OC or (+)-PC, both of which displayed a similar IC50 value (~35 μM), whereas (+)-HC was the least effective (IC50 >200 μM). Thus, it is solely through the inhibition of CACT that these agents block hepatic ketogenesis from long chain fatty acids (1–6). Because the entire CPT system is bypassed in the oxidation of medium chain fatty acids, the failure of (+)-acylcarnitines to suppress ketogenesis from octanote (1, 6) is readily explained.

The current findings also provide explanations for two additional longstanding and puzzling observations. First, why using increasing concentrations of (+)-PC did Fritz and Marquis (7) see an initial apparent inhibition followed by a subsequent paradoxical stimulation of CPT activity in rat heart mitochondria? Almost certainly this is because they employed an isotope exchange assay in which the initial reactants were palmitoyl-L-carnitine, [3H]carnitine, and CoASH, and the rate of appearance of labeled palmitoylcarnitine was measured. It is now clear that this assay monitors the combined activity of CPT I, CPT II, and CACT and that the suppression of isotope exchange observed at the lower concentrations of (+)-PC (7) resulted not from the inhibition of CPT I or CPT II as the authors concluded but from the suppression of CACT activity. The enhancement of isotope exchange seen with much higher concentrations of (+)-PC (7) undoubtedly stemmed from the detergent effect of this agent (which the authors actually documented) to cause the inactivation of CPT I, loss of translocase function, and simultaneous enhancement of the detergent-stable CPT II.

Second, why in the studies of McGarry and Foster (6) with the perfused rat liver was the inhibitory effect of (+)-OC on ketogenesis from oleate so readily overcome by the addition of (+)-octanoyl-L-carnitine ((+)-OC) or L-carnitine itself to the perfusate? Knowing now that the anti-ketogenic effect of (+)-OC results from the inhibition of CACT rather than the inhibition of CPT I or CPT II, a simple answer is evident. As shown by Pande (8), (+)-acylcarnitines act as competitive inhibitors against L-carnitine in the CACT reaction. Clearly, under our experimental conditions where (+)-OC underwent β-oxidation, it was the accumulation of intracellular L-carnitine (which we measured at the time (6)) that overcame the attenuation of oleate oxidation by (+)-OC. The provision of exogenous carnitine obviously had the same effect (6).

We consider the current findings to be important for several reasons. First, they resolve a number of hitherto confusing observations surrounding the mechanism of action of (+)-acylcarnitines on the mitochondrial CPT system. Second, Assay 3 as described here provides a simple method for measuring the combined activities of mitochondrial CACT and CPT II. This could have practical utility in that it affords a selective screening tool for potential modulators of CACT activity provided that the test compound is first shown not to alter the activity of CPT II. Third, the data reveal a striking and surprising difference in potency between the various (+)-acylcarnitines with regard to the inhibition of CACT and establish the C10 compound as a particularly effective agent for this purpose (Unfortunately, the C12 and C14 homologues were not available for study). Finally, given the recent explosion of interest in the role of the CPT system in fuel homeostasis, the knowledge gained from this study will allow more accurate interpretation of future experiments in which (+)-acylcarnitines might be used as inhibitors of mitochondrial fatty acid oxidation.

REFERENCES

1. Williamson, J. R., Browning, E. T., Scholz, R., Kreisberg, R. A., and Fritz, I. (1968) Diabetes 17, 194–208
2. Williamson, J. R., Browning, E. T., Thurman, R. G., and Scholz, R. (1969) J. Biol. Chem. 244, 5055–5064
3. McGarry, J. D., Meier, J. M., and Foster, D. W. (1973) J. Biol. Chem. 248, 270–278
4. McGarry, J. D., and Foster, D. W. (1973) J. Clin. Invest. 52, 877–884
5. McGarry, J. D., and Foster, D. W. (1974) Diabetes 23, 485–493
6. McGarry, J. D., and Foster, D. W. (1974) J. Biol. Chem. 249, 7984–7990
7. Fritz, I. B., and Marquis, N. R. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 1226–1233
8. Pande, S. V. (1975) Proc. Natl. Acad. Sci. U. S. A. 278, 883–887
9. McGarry, J. D., Brown, N. F. (1997) Eur. J. Biochem. 244, 1–14
10. Pande, S. V., and Parvin, R. (1976) J. Biol. Chem. 251, 6683–6691
11. Delisle, G., and Fritz, I. B. (1987) Proc. Natl. Acad. Sci. U. S. A. 58, 790–797
12. McGarry, J. D., Mills, S. E., Long, C. S., and Foster, D. W. (1983) Biochem. J. 214, 21–28
13. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
14. Esser, V., Brown, N. F., Cowan, A. T., Foster, D. W., and McGarry, J. D. (1996) J. Biol. Chem. 271, 6972–6977
15. Zanetti, V. A., Clark, G., Corstorphine, C. G., and Gray, S. R. (1984) Biochem. J. 222, 335–342
16. McGarry, J. D., Brown, N. F., Inthapassou, P. P., Park, D. L., Cook, B. A., and Foster, D. W. (1992) in New Developments in Fatty Acid Oxidation (Coates, P., and Tanaka, K., eds) Vol. 375, pp. 47–61, John Wiley & Sons, New York
17. Woeltje, K. F., Kuwajima, M., Foster, D. W., and McGarry, J. D. (1987) J. Biol. Chem. 262, 9822–9827