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Effects of C-methylated carnitine analogs on rates of mitochondrial fatty acid oxidation

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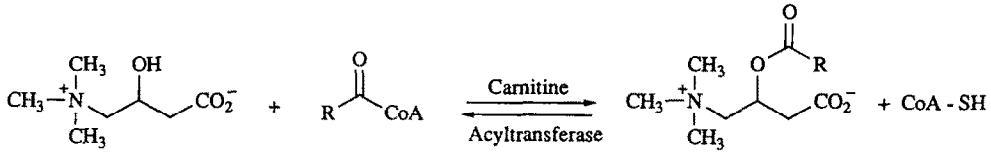
Abstract—Carnitine analogs containing one and/or two methyl substituents on the α-, β-, or γ-carbon were evaluated in isolated rat liver mitochondria for their effects on fatty acid oxidation. Their abilities to either support, in the absence of carnitine, or inhibit, in the presence of carnitine, carnitine-dependent fatty acid oxidation were determined by the conversion of radiolabeled [1-^{14}C]palmitic acid to acid-soluble radiolabeled products. None of the methylcarnitine analogs were observed to be significant inhibitors of palmitate oxidation at concentrations (1.0 mM) up to ten times that for L-carnitine. The two diastereomers of D,L-4-methylcarnitine, however, were able to support palmitate oxidation in the absence of carnitine, and rates were roughly 40% of that obtained with equimolar (0.1 mM) L-carnitine.

L-Carnitine (Table 1) serves as a substrate for several carnitine acyltransferases that, as illustrated below, reversibly catalyze the esterification of carnitine to acyl groups [1–3]. The carnitine acyltransferases are classified according to chain length specificity for the acyl group. Carnitine acetyltransferase (CAT)* is selective for short chain acyl-CoAs, carnitine octanoyltransferase (COT) utilizes medium chain length acyl-CoAs, and carnitine palmitoyltransferase (CPT) utilizes long chain acyl-CoAs. Considerable overlap of acyl chain length selectivity occurs for COT and CPT.



The physiological function for CPT is the best understood among these enzymes. The inner mitochondrial membrane is impermeable to long chain acyl-CoAs, and at least two different forms of mitochondrial CPT are essential for the transport of long chain fatty acids into mitochondria. CPT-1 is a tightly membrane-bound protein exposed to the outside of the mitochondrial matrix, which esterifies carnitine to a long chain fatty acyl-CoA. Carnitine-acylcarnitine translocase is then responsible for shuttling acylcarnitines across the mitochondrial inner membrane into the mitochondrial matrix. CPT-2 is a membrane-bound protein exposed to the inside of the mitochondrial matrix, which releases the fatty acyl group from the acylcarnitine as the fatty acyl-CoA.

CPT-1 is a key regulatory site for the control of fatty acid oxidation and ketogenesis, and is strongly inhibited by endogenous malonyl-CoA, an intermediate in the biosynthesis of fatty acids [1]. Synthetic inhibitors of CPT-1 have been described which slow fatty acid oxidation rates, inhibit ketogenesis, and lower blood glucose, suggesting applications for the treatment of diabetes [4]. Most of the known, effective antiketogenic CPT-1 inhibitors are analogs of long chain fatty acids or fatty acylcarnitines, including tetradecyloxiranecarboxylic acid [5], 2-[6-(4-chlorophenoxy)hexyl]oxirane-2-carboxylic acid [6], and hemipalmitoylcarnitinium [7]. However, with the exception of aminocarnitine [8], few nonacylated carnitine analogs have proven to be effective antiketogenic CPT-1 inhibitors. We proposed that the incorporation of extra steric bulk around the hydroxyl group of carnitine might interfere with acyl group transfer and thus inhibit fatty acid oxidation to provide antiketogenic effects. Here we report on the evaluation of α-, β-, and γ-methylated carnitines (structures **1–5**, Table 1) as supporters or inhibitors of carnitine-dependent fatty acid oxidation in isolated rat liver mitochondria.

* Abbreviations: CAT, carnitine acetyltransferase; COT, carnitine octanoyltransferase; and CPT, carnitine palmitoyltransferase.

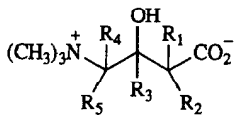
Methods

Racemic methylcarnitines **1–5** (Table 1) were synthesized as we reported previously [9]. Of these, compounds **1** and **3** contain two chiral centers and thus each exists as two diastereomers. Compound **1** was evaluated in the present study as the 1:1 diastereomeric mixture produced during synthesis. The synthesis of compound **3** provided a 3:1 diastereomeric mixture but, unlike **1**, these were separated chromatographically prior to evaluation. The most abundant diastereomer was **3a**, and the least abundant diastereomer was **3b** (see Tables 1 and 2). We previously reported the stereochemical assignments as (3*S*,4*S*;3*R*,4*R*) for racemic **3a** and (3*S*,4*R*;3*R*,4*S*) for racemic **3b** [10].

Rat liver mitochondria were isolated according to a literature procedure [11]. Briefly, male Sprague–Dawley rats weighing 100–150 g were used, having been allowed free access to food and water. Following formation of the mitochondrial pellet in 0.25 M sucrose, it was suspended in ice-cold 154 mM KCl and used in this form for all incubations.

Rates of fatty acid oxidation in the isolated rat liver mitochondria were evaluated using a modification of the

Table 1. Structures of carnitine and the methylcarnitine analogs



Compound	R_1	R_2	R_3	R_4	R_5
Carnitine	H	H	H	H	H
1	CH_3	H	H	H	H
2	H	H	CH_3	H	H
3	H	H	H	CH_3	H
4	CH_3	CH_3	H	H	H
5	H	H	H	CH_3	CH_3

procedure described by McGarry and Foster [12]. Incubations were carried out in 15-mL Corex centrifuge tubes, which initially contained in 2.2 mL of a sucrose Tris phosphate buffer, pH 7.4, the following reagents: 40 μM [1-^{14}C]palmitic acid (0.4 μCi), 0.8% fatty-acid-free albumin, 4 mM ATP, 1 mM ADP, 50 μM CoA, and 250 μM reduced glutathione. The following were added, as needed, in 100 μL buffer to give the indicated final concentration: 100 μM L-carnitine, 20 μM malonyl-CoA, 100 μM analog (**1–5**), and/or 1000 μM analog (**1–5**). To this was added 0.2 mL of the ice-cold mitochondrial suspension (1 to 1.5 mg protein; determined by the method of Lowry *et al.* [13] using bovine serum albumin as standard), and the tubes were shaken at 80 oscillations/min at 30°. The final reaction volume was 2.5 mL. The reactions were terminated after 5 min by the addition of 0.4 mL of 35% (w/v) $HClO_4$, and the precipitate was removed by centrifugation at 20,000 *g*. The relative quantity of palmitate converted to total acid-soluble products was determined by liquid scintillation counting of 0.2 mL of the $HClO_4$ supernatant. The counts produced without added carnitine (<8% of counts for controls) were subtracted. Radioactive CO_2 was not collected since previous literature studies [14], using similar procedures, have shown that labeled CO_2 accounts for less than 1% of the label present in acid-soluble products.

Results and Discussion

Inhibitors of CPT-1 slow rates of fatty acid oxidation and are antiketogenic, suggesting possible therapeutic applications in diabetes. We were interested in determining if simple methylcarnitines of the type described in Table 1 would inhibit rates of mitochondrial fatty acid oxidation. These compounds contain added methyl groups near the hydroxyl group of carnitine. It was anticipated that some of the methylcarnitines might undergo efficient binding to the carnitine binding site on CPT-1, but the extra steric bulk may inhibit acyl group transfer. Additionally, we wanted to evaluate the effects of these structural changes on the ability of methylcarnitines to substitute for carnitine in supporting fatty acid oxidation. Both studies can indirectly

Table 2. Effects of methylcarnitines on rates of palmitate oxidation in isolated rat liver mitochondria

	Palmitate oxidation* (% of control)		
Compound	0.1 mM Analog No carnitine	0.1 mM Analog 0.1 mM L-Carnitine	1.0 mM Analog 0.1 mM L-Carnitine
1†	—‡	—§	90.2 ± 3.3 (N = 4, P < 0.05)‖
2	—	—	92.0 ± 2.5 (N = 3, P < 0.05)
3a†	45.1 ± 7.8 (N = 4, P < 0.01)	—	81.8 ± 5.2 (N = 4, P < 0.05)
3b†	35.7 ± 6.1 (N = 4, P < 0.01)	—	75.7 ± 3.9 (N = 4, P < 0.01)
4	—	—	81.4 ± 5.0 (N = 4, P < 0.05)
5	—	—	84.2 ± 3.7 (N = 4, P < 0.05)

* Controls contained 0.1 mM L-carnitine but no analog. The absolute quantity (mean) of palmitate oxidized in 5 min was 6.77 nmol. Data represent the means ± SD of N runs. The sensitivity of the system to inhibition was checked by the addition of 20 μM malonyl-CoA, a known inhibitor, which reduced rates of palmitate oxidation to 16% (mean) of controls.

† Compounds **1** and **3** each contain two chiral centers, and each exist as two diastereomers. Compound **1** was evaluated as the 1:1 diastereomeric mixture produced during synthesis. The diastereomers of **3** were separated chromatographically; **3a** represents the most abundant pure diastereomer resulting from synthesis, while **3b** represents the least abundant pure diastereomer.

‡ No statistically significant rates of palmitate oxidation were observed as compared with runs with no carnitine and no analog, which produced background counts of less than 8% of controls.

§ No statistically significant change in rates of palmitate oxidation was observed as compared with controls.

‖ Significance compared with controls was determined by Student's *t*-test.

provide information regarding binding requirements for CPT-1.

The compounds in Table 1 were thus evaluated in isolated, whole rat liver mitochondria for (a) their ability to support the oxidation of [1-^{14}C]palmitic acid in the absence of added carnitine, and (b) their ability to inhibit the oxidation of [1-^{14}C]palmitic acid in the presence of 0.1 mM L-carnitine. The results are given in Table 2.

None of the D,L-methylcarnitines (**1–5**) were inhibitors of carnitine-supported palmitate oxidation when present in an equimolar concentration (0.1 mM) with L-carnitine. When present at ten times (1.0 mM) the L-carnitine concentration, relatively modest reductions in palmitate oxidation were observed. These results revealed that modest increases in steric bulk on the α-, β-, and γ-positions of carnitine, using the methylcarnitines described here, do not provide a viable approach to new antiketogenic agents. Furthermore, since good interactions with the carnitine binding site on CPT-1 would result in either inhibition of carnitine-dependent fatty acid oxidation, or support of such oxidation in the absence of carnitine, methylcarnitines that cause neither effect (**1**, **2**, **4**, and **5**; see below) must not interact favorably with CPT-1.

Similarly, most of the D,L-methylcarnitines (**1**, **2**, **4**, and **5**) were unable to support mitochondrial fatty acid oxidation in the absence of carnitine. However, both diastereomers of D,L-4-methylcarnitine (**3a** and **3b**) were roughly 40% as effective as L-carnitine in supporting palmitate oxidation in the absence of added L-carnitine. The lowered effectiveness for **3a** and **3b** may result from either poorer interactions with the mitochondrial transport enzymes (CPT-1, the translocase, and CPT-2) and/or from substrate activity which resides in only the L-isomer (as with carnitine). Thus, it is clear that the modest increase in steric bulk provided by a single methyl group at the 4-position of carnitine, in either of the two stereochemistries provided by **3a** and **3b**, is well tolerated by the binding site on CPT-1. However, D,L-4,4-dimethylcarnitine (**5**) was not able to similarly support mitochondrial palmitate oxidation. When coupled with the observation that **5** was also not an effective inhibitor of carnitine-dependent fatty acid oxidation, it can be concluded that additional steric bulk at the 4-position of carnitine, as in **5**, is not tolerated by CPT-1.

In summary, these studies revealed that carnitine-dependent mitochondrial fatty acid transport is extremely sensitive to modest increases in steric bulk on the carnitine backbone. Carnitine analogs containing one methyl substituent at the α- or β-position (**1** and **2**) or dimethyl substituents at the α- or γ-position (**4** and **5**) were unable to support (in the absence of carnitine) or inhibit (in the presence of carnitine) mitochondrial palmitate oxidation. Such effects likely result from inefficient interactions with the carnitine binding site on CPT-1. In contrast, a single γ-methyl substituent (but *not* a γ,γ-dimethyl substituent) on carnitine provided an agent (**3a** and **3b**) capable of supporting mitochondrial palmitate oxidation in the absence of carnitine. This substitution must therefore be well tolerated by the carnitine binding site on CPT-1. Such information should prove useful in the future design of carnitine-based antiketogenic agents.

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