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Novel Effect of C75 on Carnitine Palmitoyltransferase I Activity and Palmitate Oxidation[†]

Assia Bentebibel,[‡] David Sebastián,[‡] Laura Herrero,[‡] Eduardo López-Viñas,[§] Dolors Serra,[‡] Guillermina Asins,[‡] Paulino Gómez-Puertas,[§] and Fausto G. Hegardt*,[‡]

Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Barcelona, E-08028 Barcelona, Spain, and Centro de Biologia Molecular "Severo Ochoa", Universidad Autonoma de Madrid, Consejo Superior de Investigaciones Científicas, Cantoblanco, E-28049 Madrid, Spain

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ABSTRACT: C75 is a potential drug for the treatment of obesity. It was first identified as a competitive, irreversible inhibitor of fatty acid synthase (FAS). It has also been described as a malonyl-CoA analogue that antagonizes the allosteric inhibitory effect of malonyl-CoA on carnitine palmitoyltransferase I (CPT I), the main regulatory enzyme involved in fatty acid oxidation. On the basis of MALDI-TOF analysis, we now provide evidence that C75 can be transformed to its C75-CoA derivative. Unlike the activation produced by C75, the CoA derivative is a potent competitive inhibitor that binds tightly but reversibly to CPT I. IC_{50} values for yeast-overexpressed L- or M-CPT I isoforms, as well as for purified mitochondria from rat liver and muscle, were within the same range as those observed for etomoxiryl-CoA, a potent inhibitor of CPT I. When a pancreatic INS(823/13), muscle L6E9, or kidney HEK293 cell line was incubated directly with C75, fatty acid oxidation was inhibited. This suggests that C75 could be transformed in the cell to its C75-CoA derivative, inhibiting CPT I activity and consequently fatty acid oxidation. In vivo, a single intraperitoneal injection of C75 in mice produced short-term inhibition of CPT I activity in mitochondria from the liver, soleus, and pancreas, indicating that C75 could be transformed to its C75-CoA derivative in these tissues. Finally, in silico molecular docking studies showed that C75-CoA occupies the same pocket in CPT I as palmitoyl-CoA, suggesting an inhibiting mechanism based on mutual exclusion. Overall, our results describe a novel role for C75 in CPT I activity, highlighting the inhibitory effect of its C75-CoA derivative.

C75 is a chemically stable synthetic inhibitor of fatty acid synthase (FAS).[1] Structurally, it is a cell-permeable α-meth- ylene-γ-butyrolactone, designed to be less reactive and potentially safer than cerulenin, a natural product obtained from the fungus *Cephalosporium caerulens*. C75 lacks the reactive epoxide present in cerulenin, which enhances

* To whom correspondence should be addressed: Department of Biochemistry and Molecular Biology, School of Pharmacy, University of Barcelona, Diagonal 643, E-08028 Barcelona, Spain. Phone: +34 93 4024523. Fax: +34 93 4024520. E-mail: fgarciaheg@ub.edu and dserra@ub.edu.
[‡] University of Barcelona.
[§] Consejo Superior de Investigaciones Científicas.

chemical stability. In contrast to cerulenin, which only inactivates the β-ketoacyl-acyl synthase activity of FAS, C75 inhibits two additional FAS activities: enoyl reductase and thioesterase. With respect to the overall FAS reaction, C75 is a competitive, irreversible inhibitor against all three substrates: acetyl-CoA, malonyl-CoA, and NADPH (*1*, *2*).

C75 has been proposed for two therapeutic applications. The first is as an antitumor agent, since it induces cytostatic and cytotoxic effects in cultured tumor cells where the increase in malonyl-CoA levels, due to FAS inhibition, causes cancer cell-specific apoptosis (*3*). The second is as an anti-obesity agent, since it can alter the metabolism of neurons in the hypothalamus, where an increase in the level of malonyl-CoA serves as a secondary messenger of nutrient status, thereby mediating appetite suppression (*4*, *5*). Furthermore, it appears that C75 exerts both short- and long-term effects on food intake by preventing the upregulation of orexigenic neuropeptides and the downregulation of anorexigenic neuropeptides (*6*). In peripheral tissues (*7*), C75 has been postulated not only to increase the level of malonyl-CoA but also to act as a malonyl-CoA analogue that antagonizes the inhibitory effect on carnitine palmitoyltransferase (CPT I), the main regulatory step of mitochondrial β-oxidation (*8*). Both its central and the peripheral actions could reduce weight in lean and fat mice.

Mammalian tissues express three isoforms of CPT I: liver (L-CPT I), muscle (M-CPT I) (*8*), and brain (CPT I-C) (*9*). The liver and muscle isoforms are tightly regulated by their physiological inhibitor malonyl-CoA, which allows CPT I to signal the availability of lipid and carbohydrate fuels to the cell. The malonyl-CoA sensitivity of L-CPT I in the adult rat depends on the physiological state. It is increased by renewed feeding of carbohydrates to fasted rats, by obesity, or following administration of insulin to diabetic rats, whereas it is decreased by starvation and diabetes (*10*, *11*).

In addition to the physiological inhibition by malonyl-CoA, CPT I activity may also be inhibited by several synthetic epoxy-containing fatty acid compounds such as etomoxir, 2-TDGA (palmoxirate-tetradecyl-2-oxiranecarboxylate) and POCA {2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylic acid}. The CoA esters of these compounds formed in the cytosol inhibit long-chain fatty acid oxidation via their potent inhibitory effect on CPT I (*12*). Taking into account that the CoA derivatives, rather than their free acid forms, are the inhibitory forms of these compounds, we hypothesize that C75 could constitute an acyl-CoA synthetase substrate, with the resulting C75-CoA derivative acting as a potential inhibitor of CPT I activity. We found that C75-CoA is produced in vitro and inhibits CPT I with competitive inhibition kinetics. CPT I activity was also inhibited in mitochondria from pancreas-, muscle-, and kidney-derived cell lines incubated with C75 directly, as observed with etomoxir, revealing that the CoA derivatives of both compounds may be produced within the cell. These inhibitory effects were followed by a decrease in the level of fatty acid oxidation. Finally, in mice treated with a single intraperitoneal (ip) injection of C75, CPT I activity decreased but

subsequently recovered. We conclude that the inhibitory effect of C75-CoA is caused by strong, reversible binding to CPT I inside the palmitoyl-CoA pocket.

EXPERIMENTAL PROCEDURES

Animals. Six-week-old C57BL/6J male mice were purchased from Harlam Co. Animals were maintained under a 12 h dark/light cycle at 23 °C with free access to food and water. Experiments were performed following a 1 week acclimatization period. Male Sprague-Dawley rats (180−200 g) bred in our laboratory were used to obtain liver, pancreas, and soleus. All experimental protocols were approved by the Animal Ethics Committee at the University of Barcelona.

Materials. L-[*methyl*-³H]Carnitine hydrochloride and [1-¹⁴C]-palmitic acid were purchased from Amersham Biosciences. C75 was purchased from Alexis Biochemicals, and etomoxir was provided by H. P. O. Wolf (GMBH, Allensbach, Germany). Yeast culture media products were from Difco. The Bradford solution for protein assays was from Bio-Rad. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, and antibiotics were from Gibco-Invitrogen Corp. Defatted bovine serum albumin (BSA), palmitate, malonyl-CoA, and other chemicals were purchased from Sigma-Aldrich. Acyl-CoA synthetase from *Pseudomonas* sp. was obtained from Sigma.

Synthesis of C75-CoA and Etomoxiryl-CoA. Etomoxir and C75 were activated to CoA derivatives by long-chain acyl-CoA synthetase in the presence of CoA-SH (*13*). Etomoxir and C75 were dissolved in DMSO to a final concentration of 100 mM. The synthesis was performed with 1 μmol of each drug separately, in a total volume of 1 mL of a buffer containing 0.1% (w/v) Triton X-100, 5 mM CoA-SH, 10 mM ATP, 1 mM DTT, 10 mM MgCl₂, 100 mM MOPS-NaOH (pH 7.5), and 0.25 unit of long-chain acyl-CoA synthetase from *Pseudomonas* sp. The reaction was carried out at 35 °C for 2 h. The conversion of etomoxir and C75 to etomoxiryl-CoA and C75-CoA, respectively, was complete, as deduced from the spectrophotometric assay of the remaining free CoA, as described elsewhere (*14*). Stock aliquots, in which the final concentration of each CoA derivative was 1 mM, were stored at −20 °C and diluted in 100 mM MOPS-NaOH (pH 7.5) for activity assays.

Mass Spectrometry. The MALDI-TOF mass spectra of C75 and etomoxir, as well as their CoA derivatives C75-CoA and etomoxiryl-CoA, were obtained on a Voyager DE-RP (Applied Biosystems) mass spectrometer equipped with a nitrogen laser (337 nm, 3 ns pulse). The acceleration voltage was set to 20 kV. Data were acquired in the reflector mode with delay times of 320 ns for both positive and negative polarities. Spectra were calibrated externally using a calibration mixture (Calibration Mixture 1, Applied Biosystems): CHCA, des-Arg¹-bradykinin, angiotensin I, Glu¹-fibrinopeptide B, and neurotensin *m/z* 300−1700. Samples were prepared by diluting 1 μL of each drug in the activation buffer to 100 μL with H₂O, and mixing 1 μL of this diluted solution with 1 μL of matrix solution [10 mg/mL 2,5-dihydroxybenzoic acid (2,5-DHB, Aldrich) in a 1:1 methanol/water mixture]. One microliter of the sample/matrix mixture was spotted onto the stainless steel sample plate, allowed to evaporate to dryness in air, and introduced into the mass spectrometer. Spectra were acquired in the positive and

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[1] Abbreviations: FAS, fatty acid synthase; L-CPT I, carnitine palmitoyltransferase I, liver isoform; M-CPT I, carnitine palmitoyltransferase I, muscle isoform; KRBH buffer, Krebs-Ringer bicarbonate Hepes buffer.

negative ion mode. MALDI-TOF spectra were recorded by the Mass Spectrometry Service (SCT, University of Barcelona).

Expression of CPT I in Saccharomyces cerevisiae. S. cerevisiae was chosen as a heterologous expression system because it does not express endogenous CPT I activity. The plasmid pYES2-L-CPT I, which encodes the liver isoform of CPT I, was obtained as previously described (*15*). The plasmid pYES2-M-CPT I was obtained from the plasmid DS112-36 (*16*) containing the coding cDNA of the rat muscle CPT I isoform. The fragment that encompassed nucleotides 27−2432, including the coding region of M-CPT I, was subcloned into the *S. cerevisiae* expression plasmid pYES2 (Invitrogen). A *Hin*dIII site (underlined) was introduced by PCR immediately 5′ of the ATG start codon of M-CPT I to enable cloning into the unique *Hin*dIII site of plasmid pYES2. A consensus sequence (in boldface type), optimized for efficient translation in yeast, was also introduced in the same PCR, using the forward primer CPT I *Hin*dIII.for (5′-TCG ATA AGC **TT**A TAA AAT GGC GGA AGC ACA CCA GGC AG-3′) and the reverse primer CPT I *Hin*dIII.rev (5′-GGA AGC TTG GGC AGT GAT GT-3′). The resulting 550 bp fragment, obtained after the digestion of PCR products with *Hin*dIII, was ligated on pYES2 plasmid digested with the same restriction enzyme, thereby yielding the plasmid pYES2-M-CPT I-ATG. This plasmid was digested with *Sal*I (in cDNA of M-CPT I) and *Sph*I (in plasmid pYES2) and ligated with the CPT I *Sal*I−*Sph*I fragment (purified band of 2351 bp), producing pYES2-M-CPT Ipre. The TTTTTTA sequence (nucleotides 387−393) present in the M-CPT I cDNA, which resembles a known yeast polyadenylation signal (*17*), was subsequently changed by PCR to increase expression levels in yeast without changing the amino acid sequence, producing pYES2-M-CPT I. The appropriate substitutions and the absence of unwanted mutations were confirmed by sequencing the inserts in both directions with an Applied Biosystems 373 automated DNA sequencer. The expression of the plasmids pYES2-L-CPT I and pYES2-M-CPT I in yeast was performed as previously described (*15*).

Cell Cultures. The clonal β cell line INS(832/13), derived and selected from the parental rat insulinoma INS-1 (*18*), was cultured (passages 48−60) in a humidified atmosphere of 5% CO_2 in complete medium composed of RPMI 1640, containing 11 mM glucose and supplemented with 10% heat-inactivated FBS (Wisent Inc.), 10 mM HEPES, 2 mM glutamine, 1 mM sodium pyruvate, 50 mM 2-mercaptoethanol, 100 units/mL penicillin, and 100 μg/mL streptomycin. The maintenance culture was passaged once a week by gentle trypsinization, and cells were grown to confluence in Falcon dishes.

The L6E9 rat skeletal muscle cells were cultured in a humidified atmosphere containing 5% CO_2 in complete medium composed of DMEM containing 10% FBS (Gibco-Invitrogen Corp.), 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 mM HEPES (pH 7.4) (growth medium). Preconfluent myoblasts (80−90%) were induced to differentiate by lowering the level of FBS to a final concentration of 2% (differentiation medium). All experiments were performed with completely differentiated myotubes (after 4 days in differentiation medium).

Human embryonic kidney (HEK) 293 cells obtained from ECACC (European Collection of Cell Cultures) were cultured in a humidified atmosphere containing 5% CO_2 in complete medium composed of DMEM containing 10% FCS (Biological Industries), 100 units/mL penicillin, and 100 μg/mL streptomycin. Cells were grown to 80% confluence.

Preparation of Mitochondrial Fractions. Mitochondria-enriched fractions from yeast overexpressing L- and M-CPT I were obtained as previously described (*15*). Mitochondria-enriched cell fractions from INS(832/13), L6E9, and HEK293 cells cultured in 15 cm dishes were obtained with a glass homogenizer as previously described (*19*). The pellet, in which the mitochondria remain largely intact, was used directly for CPT I activity assays. Mitochondria-enriched fractions were obtained from rat and mouse muscle as described elsewhere (*20*), with minor modifications. Two soleus muscle samples of each animal were homogenized separately in 250 mM sucrose buffer using an omni mixer and then centrifuged at 1000*g* for 15 min. The pellet was homogenized and centrifuged at 600*g* for 10 min. The resulting supernatant was centrifuged at 15000*g* for 15 min, and the pellet was resuspended in 100 μL of a buffer containing 250 mM sucrose and 150 mM KCl. Mitochondria-enriched fractions from rat and mouse liver were obtained by homogenization in a buffer containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl (pH 7.4) (*21*). The liver suspension was centrifuged at 560*g* for 15 min, and the supernatant was further centrifuged at 12000*g* for 20 min. The pellet was resuspended in 2 mL of homogenization buffer, centrifuged for 10 min at 7000*g*, washed, and resuspended in 1 mL of the homogenization buffer. To obtain mitochondria-enriched fractions from mice pancreas, tissue was homogenized in a buffer containing 250 mM sucrose, 20 mM Tris-HCl (pH 7.4), 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 10 μg/mL leupeptin, 4 μg/mL aprotinin, 2 μg/mL pepstatin, and 100 μM PMSF. The homogenate was subjected to differential centrifugation at 900*g* for 10 min and at 5500*g* for 10 min. The pellet was resuspended with a Dounce homogenizer and centrifuged at 2000*g* for 2 min and at 4000*g* for 8 min. Finally, the pellet was resuspended in 250 μL of 250 mM sucrose (*22*). All the processes were performed at 4 °C, and fractions were assayed immediately for determination of CPT I activity.

Determination of CPT I Activity. CPT I activity was measured in mitochondria-enriched fractions obtained from yeast, cultured cells, or tissues as described above. CPT I activity in 3−4 μg of yeast protein extracts, 10−15 μg of mitochondria-enriched cell fractions, or 20 μg of mitochondria fractions from tissues was determined by the radiometric method as previously described (*15*). Extracts were preincubated at 30 °C for different times in the presence or absence of drugs. Enzyme activity was assayed for 4 min at 30 °C in a total volume of 200 μL. The substrates were 50 μM palmitoyl-CoA and 400 or 1000 μM L-[*methyl*-^{3}H]-carnitine for L- and M-CPT I isoforms, respectively. In yeast extracts, only the overexpressed L-CPT I or M-CPT I activity was present. In tissues and cell culture extracts, both CPT I (malonyl-CoA-sensitive) and CPT II (insensitive to malonyl-CoA) were present. Thus, in these fractions, CPT I activity was determined as the malonyl-CoA/etomoxiryl-CoA-sensitive CPT activity. CPT II activity, which is also present in mammalian mitochondrial extracts, was always subtracted

from the total activity to calculate specific CPT I activity. The presence of CPT activity insensitive to malonyl-CoA (CPT II activity) in mitochondria obtained from cell cultures was less than 5% and thus was not taken into consideration. Drugs or their CoA derivatives were preincubated with the enzyme between 1 and 5 min depending on the assay. Drug concentrations ranging from 0.01 to 50 μM were used to estimate the IC_{50} value. IC_{50} corresponds to the inhibitor concentration that inhibits 50% of the enzyme activity. Malonyl-CoA (50 μM) was used for malonyl-CoA inhibition assays. C75-CoA concentrations were varied from 1 to 5 μM to examine the dependence of CPT I activity on increasing palmitoyl-CoA concentration. In all cases, the molar ratio of palmitoyl-CoA to albumin was kept at 5:1 to avoid the presence of free acyl-CoA and its deleterious detergent effects and to prevent the formation of micelles. Kinetic constants (K_m and V_{max}) were determined by Lineweaver–Burk analysis. Inhibition constants (K_I and k_{inact}) were determined at 20 μM palmitoyl-CoA by nonlinear parameter estimation (*23*, *24*), using SigmaPlot version 8.0. All protein concentrations were determined using the Bio-Rad protein assay with bovine albumin as a standard.

Washing and Dialysis Assays. The binding of CoA derivatives to CPT I was assessed as described previously (*25*), with some modifications. Yeast mitochondria-enriched fractions overexpressing L-CPT I were preincubated for 5 min at 30 °C with each CoA derivative at 50 μM. One aliquot was used directly for the CPT I activity assay (unwashed), and the other aliquot was centrifuged at 13000g for 5 min at 4 °C and resuspended (washed) in 5 mM Tris-HCl (pH 7.2), 150 mM KCl, 2 μg/mL leupeptine, 0.5 μM benzamidine, 1 μg/mL pepstatin, and 1 mM PMSF before the assay. The CPT I activity assay was conducted for 4 min at 30 °C as described above.

To verify the reversibility of the interaction of C75-CoA with CPT I, dialysis assays were performed. Mitochondria-enriched fractions (160 μg) obtained from yeast cells expressing L-CPT I were preincubated at 30 °C for 5 min (without the drug) or with a final concentration of 50 μM C75-CoA or 50 μM etomoxiryl-CoA and then dialyzed in buffer containing 10 mM Hepes (pH 7.4), 1 mM EDTA, and 10% glycerol at 4 °C. Aliquots were taken before dialysis (0 h), and 24 and 36 h thereafter, and assayed for CPT I activity.

C75 Treatment of Cell Cultures and Administration to Mice. Cells were incubated with either C75 at 10, 20, 30, or 40 μg/mL or etomoxir at 30 or 40 μg/mL in culture medium. Stock solutions of C75 and etomoxir were prepared at 100 mM in DMSO. Control cells were incubated with the same amount of DMSO. L6E9 myotubes were incubated for 2 h at 37 °C, and INS(832/13) and HEK293 cells were incubated for 1 h at 37 °C. Subsequently, the cells were washed in PBS, and either the CPT I activity, the level of palmitate oxidation, or cell viability was measured.

Mice were given a single ip injection of either C75 or etomoxir, dissolved in RPMI 1640 medium, at 20 mg/kg of body weight or medium alone for control. Animals were killed at different times postinjection, and mitochondria-enriched fractions from liver, soleus, and pancreas were obtained as described above. Fractions were assayed immediately to measure CPT I activity.

Assessment of Fatty Acid Oxidation. Palmitate oxidation to CO_2 was assessed in culture cells grown in 12-well plates. On the day of the assay, cells were washed in KRBH with 0.1% defatted BSA, preincubated at 37 °C for 30 min in KRBH with 1% BSA, and washed in KRBH with 0.1% BSA. Cells were then incubated for 2 h at 37 °C with fresh KRBH containing 2.5 mM glucose in the presence of 0.8 mM carnitine with 0.25 mM palmitate and 1 μCi/mL [1-^{14}C]-palmitic acid bound to 1% (w/v) BSA. Oxidation measurements were performed by a CO_2-capture system assay as previously described (*26*).

Viability Cell Culture Assays. To evaluate the cytotoxic effect of the drugs, an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was performed (*27*). Cells were seeded in 12-well plates and incubated with drugs as described above. Subsequently, 200 μL of 0.25% (w/v) MTT was added to each well, and cells were further incubated for 2 h. The resulting formazan crystals were then solubilized by adding 1 mL of MTT lysis solution [10% (w/v) SDS and 1 mM acetic acid in DMSO], and the absorbance at 570 nm was measured. The results are expressed as the percentage of absorbance related to control cells.

In Silico Molecular Docking. A new three-dimensional model for CPT I was constructed using homology modeling procedures based on structural alignments of CPT I sequence and utilizing the coordinates of the recently described protein structure of the mouse carnitine octanoyltransferase (*28*). The three-dimensional model of the free C75-CoA molecule was prepared using molecular-orbital calculation methods implemented in Mopac (*29*). The in silico docking programs Autogrid and Autodock (*30*, *31*) were used to generate and evaluate low-energy conformational models for the putative ligand position, thus providing a model for the interaction of C75-CoA with the active center of CPT I.

Statistical Analysis. Data are expressed as the mean ± the standard error of at least three independent experiments. The significance of differences was assessed using the unpaired Student's *t* test.

RESULTS

Synthesis and Analysis of C75-CoA. To determine whether C75 is converted to C75-CoA as described for etomoxir, each drug was incubated independently in the presence of CoA and acyl-CoA synthetase, as described in Experimental Procedures. The production of stable CoA derivatives was then analyzed by MALDI-TOF. Figure 1A shows a peak of 1020.4 Da corresponding to the molecular mass of the C75-CoA formed in the reaction. According to this molecular mass, CoA-SH binds to C75 by opening the furan group (Figure 6B), without the loss of a water molecule. A similar analysis was performed for etomoxir (Figure 1B). This figure shows a peak of 1064.0 Da for etomoxiryl-CoA. The chemical structure of the product formed is shown in Figure 6B, in which the CoA-SH binds to etomoxir by opening the epoxid group, without the loss of a water molecule. Other peaks correspond to products derived from CoA, C75, or etomoxir.

Effects of C75 and C75-CoA on Yeast-Expressed L- and M-CPT I Activity. Increasing concentrations of synthesized C75-CoA or etomoxiryl-CoA were independently incubated with yeast-overexpressed mitochondrial L- or M-CPT I. Both
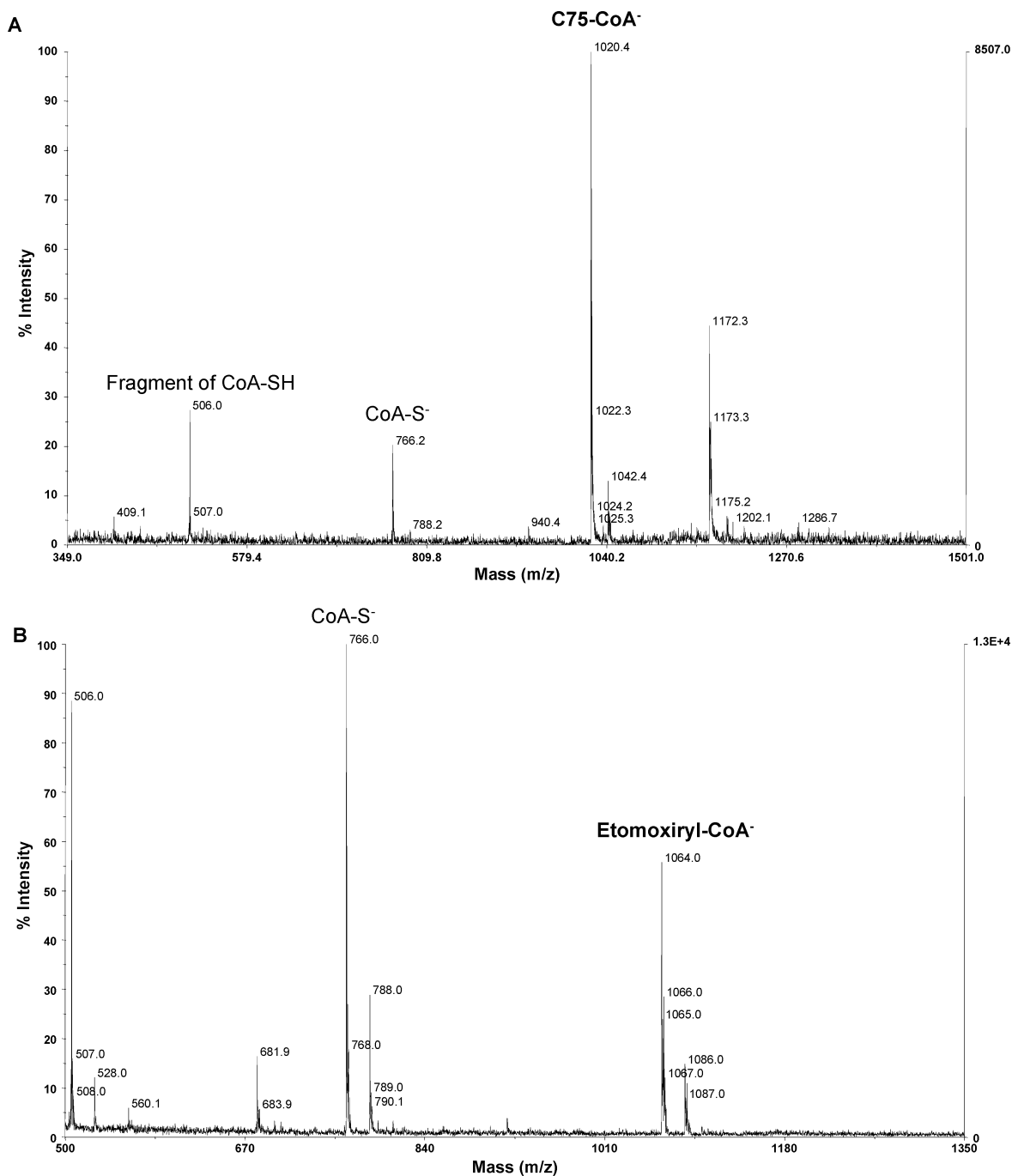
FIGURE 1: MALDI-TOF spectra of C75-CoA and etomoxiryl-CoA. Spectra were directly obtained from the reaction product of C75 and CoA-SH, and etomoxir and CoA-SH using a Voyager-DE-RP from Applied Biosystems with DHB (10 mg/mL in a 1:1 water/methanol mixture). Detection was accomplished with a reflector and in the negative mode. The product C75-CoA (A) is confirmed by the peak at 1020.4 Da and etomoxiryl-CoA (B) by the peak at 1064.0 Da.

CoA derivatives strongly inhibited L- and M-CPT I isoforms with similar kinetics (Figure 2). Almost complete inhibition of CPT I was observed at 50 μM C75-CoA. IC_{50} values for C75-CoA were 0.24 and 0.36 μM for L- and M-CPT I isoforms, respectively (Table 1). IC_{50} values for etomoxiryl-CoA were 4.06 and 3.10 μM for L- and M-CPT I isoforms, respectively (Table 1). Only the CoA derivatives inhibited CPT I activity. When mitochondrial yeast extracts were incubated with 40 or 200 μM C75, a 20−30% increase in CPT I activity was observed. However, etomoxir did not produce CPT I activation.

Analysis of Binding of C75-CoA to CPT I. To assess whether binding of C75-CoA to CPT I is stable, yeast mitochondria-enriched fractions overexpressing L-CPT I were incubated with 50 μM C75-CoA, etomoxiryl-CoA, or malonyl-CoA, or with buffer alone as a control. Following preincubation for 5 min, the effects of washing were tested. Extracts were assayed directly (unwashed samples) or centrifuged and resuspended in buffer (washed samples) (see Experimental Procedures) and assayed for CPT I activity. As shown in Figure 3A, the inhibition by malonyl-CoA was lost when extracts were washed, with CPT I activity recovering by 91% with respect to the washed control. However, both C75-CoA and etomoxiryl-CoA produced persistent inhibition, at levels of 75 and 79%, respectively, with respect to washed control fractions. The experiments
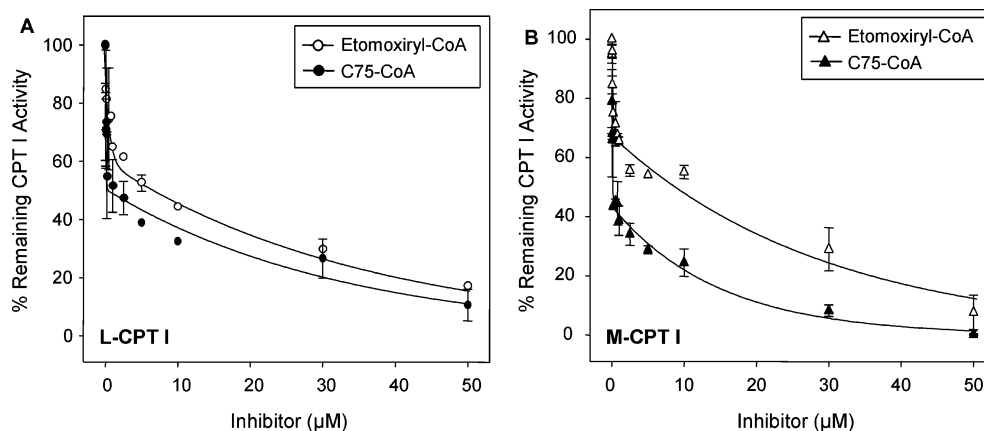
FIGURE 2: Effects of C75-CoA and etomoxiryl-CoA on the activity of liver (L) and muscle (M) isoforms of CPT I overexpressed in yeast *S. cerevisiae*. L-CPT I (A) and M-CPT I (B) were overexpressed in yeast and mitochondrial fractions were preincubated for 5 min with increasing concentrations of etomoxiryl-CoA (○ and △) and C75-CoA (● and ▲). CPT I activity was measured, and data are expressed relative to control values in the absence of drugs (100%) as the mean of three independent experiments.

Table 1: IC_{50} Values of CPT I for C75-CoA and Etomoxiryl-CoA[a]

	IC_{50} (μM)	
	C75-CoA	etomoxiryl-CoA
yeast overexpressing L-CPT I	0.24 ± 0.01	4.06 ± 0.78
yeast overexpressing M-CPT I	0.36 ± 0.18	3.10 ± 0.06
rat liver	0.25 ± 0.13	0.70 ± 0.10
rat muscle	0.015 ± 0.005	0.04 ± 0.01
INS(832/13) cells	0.25 ± 0.16	1.21 ± 0.35
L6E9 myotubes	0.46 ± 0.21	2.87 ± 0.8

[a] Mitochondrial fractions obtained from rat liver, rat muscle, cultured cells, and yeast overexpressing CPT I were assayed for CPT I activity in the presence of C75-CoA and etomoxiryl-CoA. IC_{50} values were calculated as described in Experimental Procedures.

with C75-CoA and etomoxiryl-CoA demonstrate that they were tightly bound to CPT I.

Fifty and one hundred percent of the CPT I activity was recovered after dialysis for 24 and 36 h, respectively, in C75-CoA-treated fractions, though not in those treated with etomoxiryl-CoA (Figure 3B). The C75-CoA−protein complex was undone during dialysis, showing tight but reversible binding. In contrast, there was no CPT I activity recovery when etomoxiryl-CoA was used, which is consistent with data demonstrating that protein and etomoxiryl-CoA formed covalent adducts.

C75-CoA Inhibits CPT I Activity. To examine whether the CPT I enzyme source could modify the response to C75-CoA, we carried out additional experiments with freshly isolated mitochondria from rat liver (L-CPT I) and rat muscle (M-CPT I). Both C75-CoA and etomoxiryl-CoA inhibited CPT I activity with similar kinetics (Figure 4A,B). CPT I from fresh mitochondria proved to be more sensitive than that from yeast extract; at 10 μM CoA derivative, CPT I was almost completely inhibited. IC_{50} values for C75-CoA were 0.25 and 0.015 μM for CPT I from rat liver and rat muscle, respectively (Table 1). IC_{50} values for etomoxiryl-CoA were 0.70 and 0.04 μM for CPT I from rat liver and rat muscle, respectively (Table 1). C75-CoA appears to be a stronger inhibitor for M- than for L-CPT I.

The inhibitory effects of C75-CoA and etomoxiryl-CoA were also tested on purified mitochondria from pancreas [INS(832/13)] and muscle (L6E9) cultured cell lines. In all cases, CPT I activity was strongly inhibited by increasing concentrations of both CoA derivatives (Figure 4C,D). The
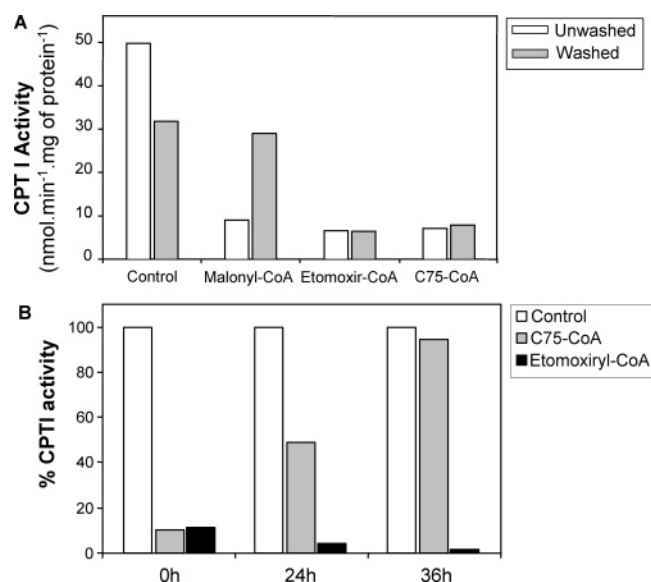

FIGURE 3: Analysis of binding of C75-CoA to L-CPT I activity overexpressed in yeast *S. cerevisiae*. (A) Three micrograms of mitochondria from yeast overexpressing L-CPT I was preincubated for 5 min with 50 μM C75-CoA, etomoxiryl-CoA, and malonyl-CoA and washed with buffer or left unwashed, as described in Experimental Procedures. Specific activity is represented as the mean of three independent experiments. (B) The resulting 160 μg of mitochondria-enriched fractions from yeast cells expressing L-CPT I was preincubated at 30 °C for 5 min without the drug or with a final concentration of 50 μM C75-CoA or 50 μM etomoxiryl-CoA and then dialyzed. Aliquots were taken prior to dialysis (0 h) and 24 and 36 h after dialysis and assayed for CPT I activity.

IC_{50} values for C75-CoA were 0.25 and 0.46 μM for INS-(812/13) and L6E9 cells, respectively. The IC_{50} values for etomoxiryl-CoA were 1.21 and 2.87 μM for INS(823/13) and L6E9 cells, respectively (Table 1). C75-CoA was a more potent CPT I inhibitor than etomoxiryl-CoA in all cases.

To define the type of inhibition of C75-CoA on CPT I activity, we performed experiments with varying C75-CoA and palmitoyl-CoA concentrations. Lineweaver−Burk plots for CPT I activity at different palmitoyl-CoA concentrations for the enzyme were linear (Figure 5). The palmitoyl-CoA concentrations ranged from 1 to 100 μM. The observed K_m values for palmitoyl-CoA were 4.5, 33.6, 61.6, and 126.0 μM at C75-CoA concentrations of 0, 1, 2, and 5 μM, respectively, but no change was observed in the intrinsic

MITOCHONDRIA OBTAINED FROM TISSUES
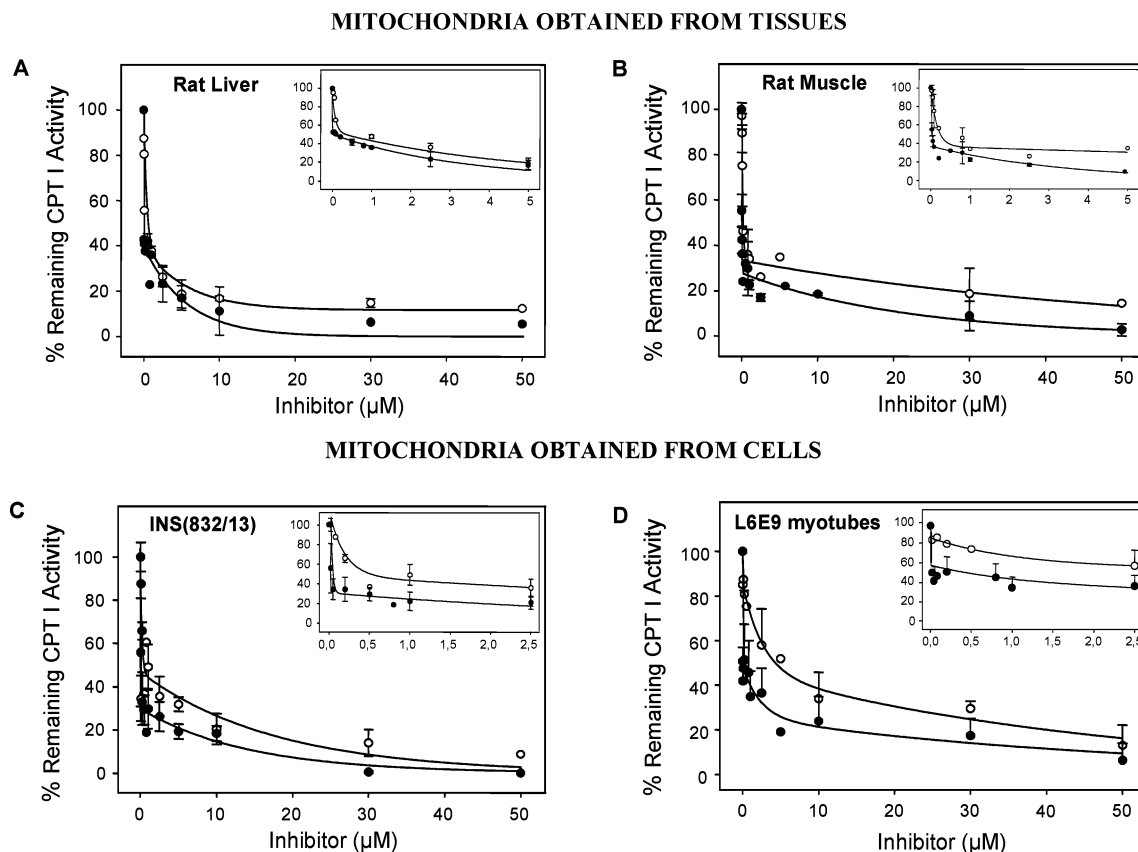

MITOCHONDRIA OBTAINED FROM CELLS

FIGURE 4: Effects of C75-CoA and etomoxiryl-CoA on CPT I activity. Mitochondria isolated from rat liver (A), rat muscle (B), INS(823/13) cells (C), or L6E9 myotubes (D) were preincubated for 1 min with increasing concentrations of C75-CoA (●) or etomoxiryl-CoA (○), and CPT I activity was assayed as described in Experimental Procedures. Data represent the mean of at least three independent experiments and are expressed relative to control values in the absence of the inhibitor (100%). The inset shows an expanded dose–response curve for the two inhibitors.
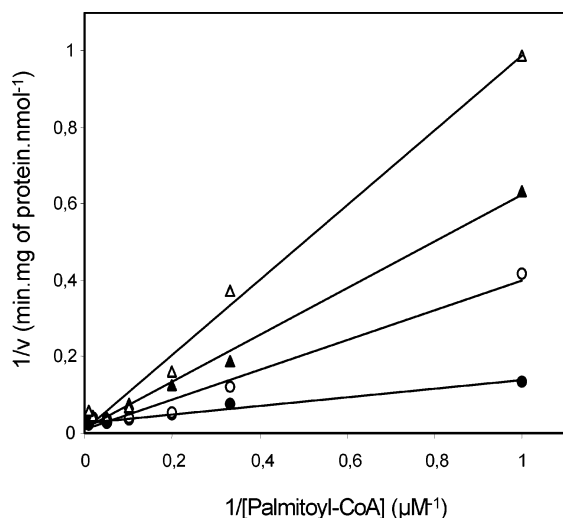

FIGURE 5: Lineweaver–Burk plot for the inhibition of L-CPT I by C75-CoA. Mitochondria-enriched fractions from yeast-overexpressed L-CPT I (3–4 μg) were preincubated with C75-CoA for 5 min and then incubated for 4 min at 30 °C with various palmitoyl-CoA concentrations in the presence of 400 μM carnitine. The concentrations of C75-CoA were (●) 0, (○) 1, (▲) 2, and (△) 5 μM. Each point is the mean of two different experiments.

catalytic activity of the enzyme (3.98 ± 0.83 nmol min^{-1} mg^{-1}). Inhibition constants were determined by nonlinear regression analysis and were 0.23 ± 0.08 μM for the apparent inhibition constant (K_I) and 0.09 ± 0.004 min^{-1} for the inactivation constant (k_{inact}). The results of the inhibition

kinetics revealed that C75-CoA is a competitive inhibitor with respect to palmitoyl-CoA.

Molecular Model of Docking of C75-CoA into L-CPT I. The recent crystallization of carnitine octanoyltransferase (COT) (*28*), a member of the carnitine acyltransferase family, allowed us to improve the previous three-dimensional model of CPT I based on the carnitine acetyltransferase crystal (*32*). Using docking analysis, an in silico model was constructed for the interaction between the active center of CPT I and C75-CoA (Figure 5A). Refined in silico docking techniques were used, allowing free rotation of the ligand acyl chain bonds. This model suggests that the inhibitor C75-CoA fits into the enzyme in a manner similar to that of the physiological substrate palmitoyl-CoA. While the CoA segment in both molecules is positioned in almost the same orientation, the aliphatic tail of C75 fits into the hydrophobic cavity of CPT I defined by α-helix 12 and β-strands 1, 13, and 14 of the protein, just where the acyl group of palmitoyl-CoA is most likely positioned during normal enzymatic processes. The head of C75, bound to the sulfur atom of CoA, is located in the proximity of the catalytic residue His473.

Effects of C75 on CPT I Activity and Fatty Acid Oxidation in Cultured Cells. To assess whether CPT I inhibition is followed by a similar decrease in the extent of fatty acid oxidation, the three cultured cell lines [INS(812/13), L6E9, and HEK293] were incubated with C75 or etomoxir. It was not necessary in this case to transform the drugs to their CoA derivatives, since this conversion is assumed to occur inside the cells via endogenous acyl-CoA synthetase. CPT I activity
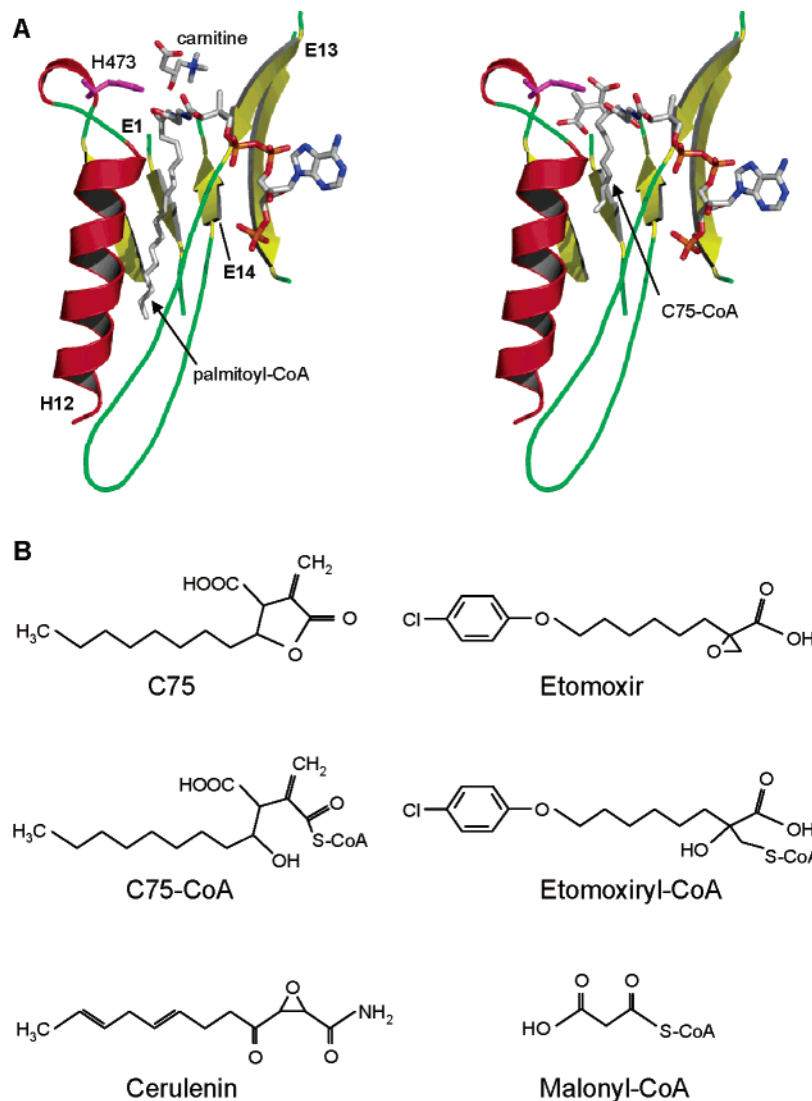
FIGURE 6: Proposed model for the location of C75-CoA in the CPT I active center. (A) Comparison of the location of a molecule of palmitoyl-CoA in the active center of CPT I (left) and the proposed model for the location of C75-CoA in the same enzyme locus (right). The positions of carnitine and catalytic His473 (magenta), as well as secondary structure elements α-helix 12 and β-strands 1, 13, and 14, surrounding the acyl cavity, are indicated. The aliphatic tail of the inhibitor molecule is located in the hydrophobic pocket present in the CPT I active center, a position similar to that proposed for the substrate. The "head" of C75 occupies the central cavity of the enzyme. (B) Structures of known compounds affecting CPT I activity are included at the bottom for comparison.

decreased with increasing C75 concentrations (Figure 7). CPT I activity was reduced by 49, 62, and 62% in pancreatic INS(832/13), muscle L6E9, and kidney HEK293 cells, respectively, at maximal C75 concentrations of 30, 40, and 30 μg/mL, respectively. In parallel, the level of [1-^{14}C]-palmitate oxidation was reduced by 62, 84, and 68% in pancreatic INS(832/13), muscle L6E9, and kidney HEK293 cells, respectively, at maximal C75 concentrations of 30, 40, and 30 μg/mL, respectively. When etomoxir was used, CPT I activity was reduced by 80, 52, and 71% in pancreatic INS-(832/13), muscle L6E9, and kidney HEK293 cells, respectively, while the level of palmitate oxidation was consequently reduced by 71, 78, and 77%, respectively. To rule out the possibility that the inhibition of palmitate oxidation reflected an increase in the level of cell death caused by C75 or etomoxir, we performed viability experiments using the MTT assay. In all cases, cell viability at the drug concentrations that were used was higher than 98% of that of control samples.

Effects of C75 Treatment on Whole Animals. We examined the effect of C75 on CPT I activity in vivo. Mice were injected (ip) with a single dose of either C75 or etomoxir (20 mg/kg of body weight) and killed at different time points thereafter. Tissue samples (liver, soleus, and pancreas) were taken to isolate mitochondria, as described in Experimental Procedures. CPT I activity decreased rapidly in all tissues that were assayed (Figure 8) but subsequently recovered, with tissue-dependent kinetics. The level of inhibition of liver CPT I decreased by 56% at 1 h and by 73% after treatment for 3 h, while at 5 h, CPT I values were similar to those of the control. Muscle CPT I was inhibited by 80% after being treated for 30 min, recovering more rapidly than liver CPT I. In the pancreas, CPT I activity decreased by 36% after C75 treatment for 30 min, compared with the control, recovering thereafter. In no case did the level of CPT I activation exceed the control. Etomoxir also provoked an inhibition of CPT I activity in these tissues (Figure 8). After treatment for 3 h, the levels of CPT I inhibition were 97,
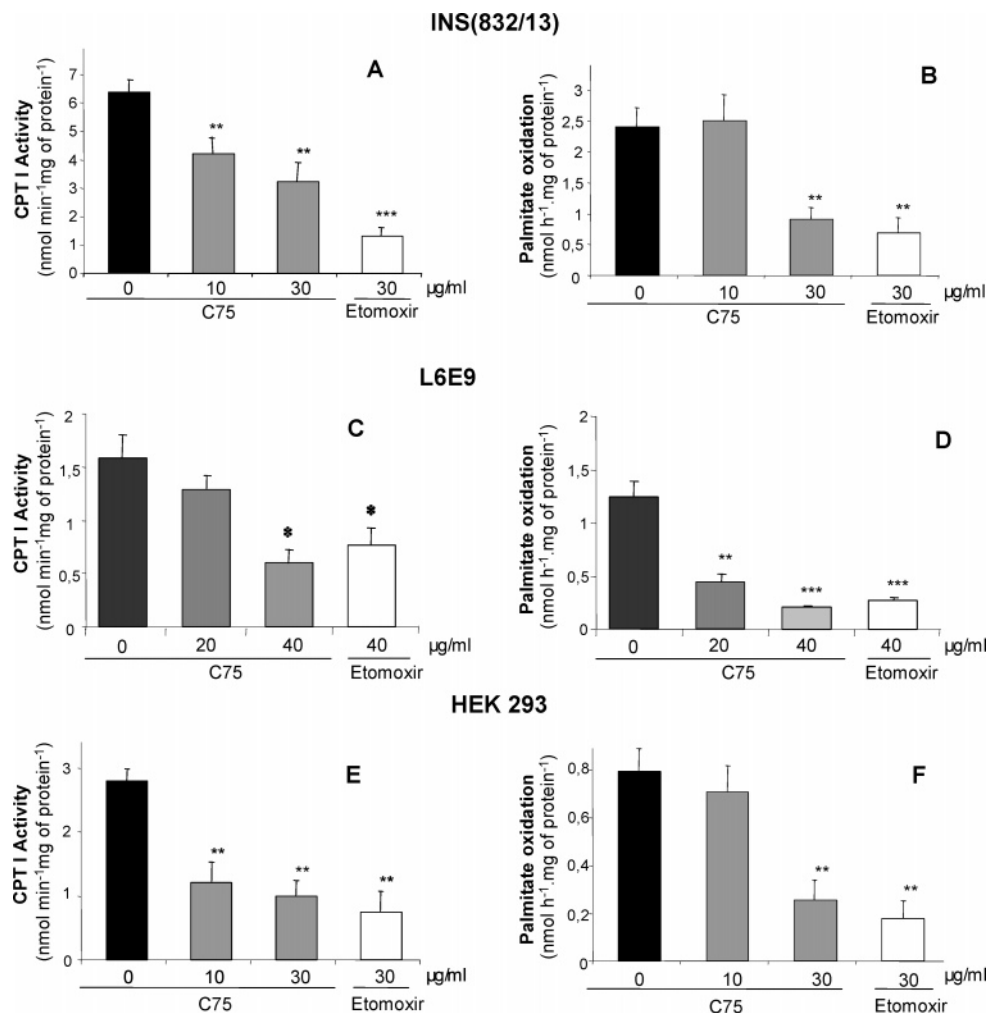
FIGURE 7: CPT I activity and palmitate oxidation in cell cultures. Cells were incubated for 2 h (L6E9) or 1 h [INS(832/13) and HEK293] with complete medium containing either 0, 10, 20, 30, and 40 μg/mL C75 or 30 and 40 μg/mL etomoxir. Mitochondria-enriched cell fractions were obtained, and 10 μg of protein was used for the CPT I activity assay. In palmitate oxidation assays, cells were preincubated for 30 min at 37 °C with KRBH with 1% BSA and then incubated for 2 h at 2.5 mM glucose in the presence of 0.8 mM carnitine, 0.25 mM palmitate, and 1 μCi/mL [1-^{14}C]palmitate. Palmitate oxidation to CO_2 was assessed as described in Experimental Procedures. Data are presented as the mean $\pm$ the standard error of three independent experiments. *P < 0.05, **P < 0.01, and ***P < 0.001 compared with the control without inhibitors.

71, and 60% in liver, muscle, and pancreas, respectively, and they were always lower than those observed following C75 treatment. These inhibitory effects of etomoxir on CPT I activity, unlike those observed following C75 treatment, were maintained for up to 5 h, producing 96, 82, and 72% CPT I inhibition in liver, muscle, and pancreas, respectively.

DISCUSSION

In this study, we show that C75 and etomoxir were transformed to their CoA derivatives by the action of an acyl-CoA synthetase. Within the cell, acyl-CoAs are formed as part of the metabolism of a variety of endogenous fatty acids, as well as some xenobiotic carboxylic acids. The synthesis of CoA derivatives is the rate-limiting step for both conjugation and inactivation of most xenobiotics. CoA conjugates increase the chemical reactivity of these compounds and may function as alternative substrates in intermediate metabolism pathways of short-, medium-, and long-chain fatty acids (*33*). Distinct mammalian long-chain and xenobiotic/medium-chain fatty acid:CoA ligases [termed acyl-CoA synthetase in revised nomenclature (*34*)] can activate long-chain,

xenobiotic, and medium-chain fatty acids. The substrate specificity and intracellular location of the acyl-CoA synthetases may explain the rate differences in the synthesis of the xenobiotic-CoA derivatives and their possible toxicity. Although C75 is a potential substrate for acyl-CoA synthetase, due to its aliphatic C8 chain and its esterifiable carboxylate groups, no studies have addressed this fact. The MALDI-TOF analysis performed in this study showed that C75-CoA was synthesized when C75 was incubated in vitro in the presence of CoA, long-chain acyl-CoA synthetase, and ATP.

Compounds such as etomoxir, TDGA, and POCA must be converted to their CoA derivative before acting as inhibitors of CPT I activity (*12*). In the same way, when C75 is transformed into its CoA derivative, it also becomes a potent inhibitor of CPT I. Under all the in vitro conditions that were tested, CPT I was clearly inhibited in a dose-dependent manner by C75-CoA. IC_{50} values observed for C75-CoA ranged from the micromolar level to the nanomolar level and were lower than those obtained for etomoxiryl-CoA, showing that C75-CoA is a stronger CPT I inhibitor
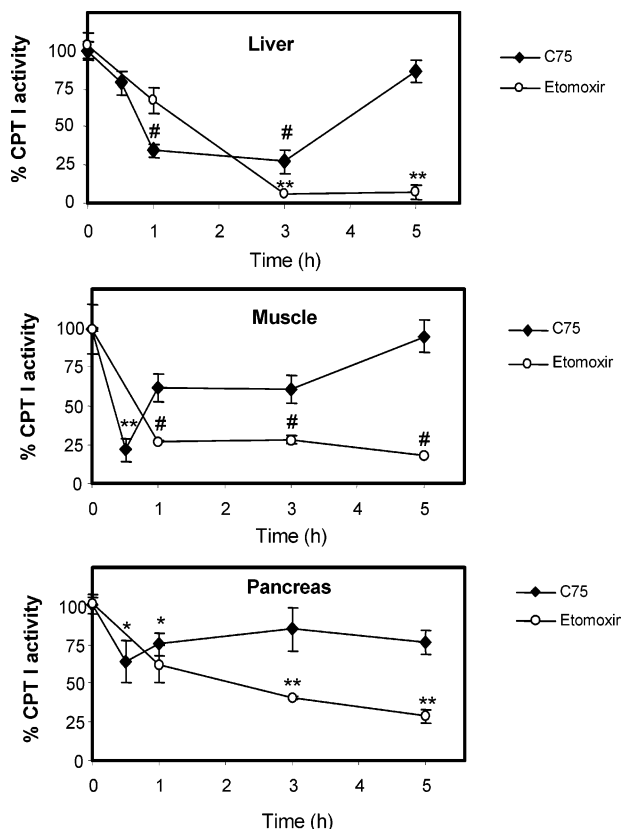
FIGURE 8: C75 effects on the whole animal. C75 or etomoxir was injected in an ip manner in mice, and animals were killed 0, 0.5, 1, 3, and 5 h after injection. Mitochondria-enriched fractions from liver, soleus, and pancreas were obtained, and CPT I activity was assayed. Results represent the mean ± the standard error of data obtained from six mice. $*P < 0.05$, $**P < 0.01$, $\#P < 0.001$ vs control (0 h).

than etomoxiryl-CoA. It is well-known that the M-CPT I isoform is more sensitive than L-CPT I to its physiological inhibitor malonyl-CoA. This effect was also observed for C75-CoA. The IC_{50} value for C75-CoA acting on L-CPT I from liver mitochondrial fractions was 16-fold higher than that observed for the M-CPT I isoform from muscle mitochondria. However, these differences were not observed in overexpressed yeast extracts, which most likely reflects the expression of these proteins in a foreign system. In L6E9 myotubes, the IC_{50} value was similar to that observed in rat liver and INS(832/13) cells, consistent with the fact that L6E9 myotubes express the L-CPT I isoform rather than M-CPT I (35). Consistent with the results obtained by Yang et al. (36) and Nicot et al. (37), we also observed an increase in CPT I activity when incubating C75 alone with yeast extracts. Nevertheless, it is important to stress that the concentration of C75 alone (200 μM) used to produce maximal CPT I activation was at least 100-fold higher than that of C75-CoA (0.25 and 0.015 μM) needed to produce 50% CPT I inhibition. This suggests that C75-CoA, rather than C75 alone, is the agent responsible for these effects in vivo.

The two known CPT I inhibitors, malonyl-CoA and etomoxiryl-CoA, bind to the enzyme in different ways. Malonyl-CoA binding is reversible and not tight, consistent with its physiological role as a regulator of CPT I activity and β-oxidation, adapting its binding to each metabolic situation. Etomoxiryl-CoA is a synthetic drug designed to inhibit CPT I strongly and permanently (38). C75-CoA

exhibited certain different CPT I binding characteristics with respect to these two inhibitors. On one hand, C75-CoA bound more tightly than malonyl-CoA, as it was not released by washing. On the other hand, it also bound noncovalently, in contrast to etomoxiryl-CoA, as C75-CoA was removed by dialysis. However, the presence of carboxylic groups and the hydrocarbon chain, both in C75 and in etomoxir (Figure 5B), suggests a common mode of interaction for these two inhibitors with the active center of CPT I. Our three-dimensional model illustrating the interaction of C75-CoA with the active center of CPT I indicated a common location for the substrate palmitoyl-CoA and the inhibitor. The main differences between the two molecules, apart from the length of the hydrocarbon chain introduced into the hydrophobic cavity, are the size and chemical characteristics of the C75 "head", which is absent in the case of the natural substrate. These three-dimensional data and those from the kinetic experiments suggest a competitive mechanism between the substrate and C75-CoA. Three-dimensional data intriguingly fail to explain the action of C75 alone as a CPT I activator. Although Yang et al. (36) deduced from competitive binding experiments that C75 alone binds to different malonyl-CoA sites, the exact site at which it interacts with CPT I has yet to be determined. The lack of a CPT I crystal and the lack of a computer-generated partial three-dimensional model containing the N-terminal domain of the enzyme essential to the regulation of CPT I activity are the main handicaps to performing new docking studies which would explain C75 binding and CPT I activation.

The results obtained in cultured pancreatic, muscle, and kidney cells suggest that C75 is transformed to its CoA derivative by endogenous acyl-CoA synthetase and intracellular ATP, since the decrease in the level of palmitate oxidation correlated with the inhibition of CPT I activity. In all cases, etomoxir was used as a control of transformation, inhibition, and oxidation. To rule out the possibility that the decrease in the level of palmitate oxidation was produced by C75 cytotoxicity, viability studies were performed. Under our conditions, the viability of the three cellular models proved to be higher than 98%. Therefore, the cytotoxicity experiments provided evidence that the decrease in CPT I activity was not generated by cellular death. Instead, C75-CoA appears to be the true inhibitory molecule of CPT I activity. Depending on lipid composition and metabolism, each cell line may become more sensitive to the cytotoxic effects of C75. For instance, pancreatic INS(832/13) β-cells and kidney HEK293 cells were incubated for shorter time periods and at lower C75 concentrations than L6E9 myotubes. Other studies performed in SKBR3 breast carcinoma cells, which have a higher lipid content, exhibited increased cytotoxicity resistance. This cell line required C75 preincubation for 6 h to achieve the same cytotoxic effects (39).

Discrepancies with the findings of others, who observed CPT I activation in primary hepatocytes, in adipocytes, and in MCF-7 cells following C75 treatment, could be attributable to methodological differences (7, 36). Therefore, while Thupari et al. (7) studied permeabilized cells, we isolated purified mitochondria from tissues and from cultured cells. We believe that digitonin permeabilization, in tandem with C75 treatment, may affect the interactions between mitochondria and the cytoskeleton (40), thereby either altering CPT I activity or disturbing the assay. In this context, it has

been suggested that changes in the lipid composition of CPT I's membrane microenvironment may be important for alterations in CPT I activity (*41*, *42*).

Our in vitro experiments were also corroborated by in vivo experiments. A single intraperitoneal C75 injection produced a short-term inhibition of CPT I in mouse liver, muscle (soleus), and pancreas. In all tissues that were analyzed, CPT I activity returned to control levels after treatment for 5 h. The extent of CPT I inhibition observed in various tissues may reflect differences in the pharmacokinetics of each tissue or, alternatively, the rate of endogenous C75-CoA transformation. In contrast, the inhibition by etomoxir was maximal at 5 h. This can be explained by the fact that etomoxir covalently binds to CPT I, but C75-CoA appears to be a strong and a reversible inhibitor.

Cerulenin, the natural FAS inhibitor which formed the basis of C75's design, has been shown to inhibit CPT I and fatty acid oxidation. Our results on the effects of C75 on CPT I activity and fatty acid oxidation are similar to those reported by other groups working with cerulenin. In pancreatic islets, the level of palmitate oxidation was reduced following cerulenin incubation (*43*), consistent with our results in pancreatic cultured cells incubated with C75. In mice, cerulenin inhibits liver and muscle CPT I activity after ip injection, subsequently recovering and becoming slightly activated after a few hours (*44*). These authors concluded that the late stimulating effects of cerulenin on CPT I activity occurred via the sympathetic nervous system. Indeed, C75 may behave like cerulenin in mice, producing a biphasic effect on CPT I, i.e., an initial inhibition followed by recovery. The short-term CPT I inhibition witnessed in our experiments following ip C75 injection in mice is consistent with results obtained by Clegg et al. (*45*), who observed a reduction in the peripheral energy expenditure of rats 12 h after an ip dose of C75. Furthermore, it has recently been demonstrated that intra-cerebroventricular C75 administration increased the level of fatty acid oxidation in muscle, which was mediated by the sympathetic nervous system (*46*). Thus, the recovery of CPT I activity reported in this study might be explained if we weigh the possibility that the central C75 effect overcomes the short-term C75 peripheral action.

Finally, the fact that C75 could be transformed into its C75-CoA derivative in vivo may have implications not only for CPT I activity but also for FAS and other enzymes in different cellular pathways. This observation may explain certain contradictory findings recently published by Rohrbach et al. (*47*). These authors observed that the maximal C75 concentration in plasma was 2.6 μM after ip administration of C75 at 30 mg/kg (a concentration that suppresses appetite within 4 h), which was substantially lower than that needed to inhibit FAS in vitro (IC$_{50}$ $\sim$ 200 μM). They postulated that these results could only be explained if C75's effect on appetite suppression was independent of its inhibition of FAS in the hypothalamus. An alternative explanation would be that a metabolic product more potent than C75 (i.e., C75-CoA) might act on FAS.

In conclusion, the results presented here provide compelling evidence that C75-CoA is a potent and direct inhibitor of the two isoforms L- and M-CPT I in vitro and in vivo. Moreover, our findings suggest that both in cellular models and in lean mice C75 is expected to be transformed into its CoA derivative, thereby directly inhibiting CPT I activity.

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