C75 is converted to C75-CoA in the hypothalamus, where it inhibits carnitine palmitoyltransferase 1 and decreases food intake and body weight

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ARTICLE INFO

ABSTRACT

Central nervous system administration of C75 produces hypophagia and weight loss in rodents identifying C75 as a potential drug against obesity and type 2 diabetes. However, the mechanism underlying this effect is unknown. Here we show that C75-CoA is generated chemically, *in vitro* and *in vivo* from C75 and that it is a potent inhibitor of carnitine palmitoyltranferase 1 (CPT1), the rate-limiting step of fatty-acid oxidation. Three-D docking and kinetic analysis support the inhibitory effect of C75-CoA on CPT1. Central nervous system administration of C75 in rats led to C75-CoA production, inhibition of CPT1 and lower body weight and food intake. Our results suggest that inhibition of CPT1, and thus increased availability of fatty acids in the hypothalamus, contribute to the pharmacological mechanism of C75 to decrease food intake.

1. Introduction

The brain plays an important role in the evaluation and control of energy homeostasis. Blood concentrations of glucose and fatty acids are sensed by neurons of the hypothalamus, which adjusts feeding behaviour and monitors fatty-acid metabolism. Several laboratories have attempted to design anti-obesity drugs and modulate fatty-acid metabolism to inhibit food intake. C75 is a synthetic inhibitor of fatty-acid synthase (FAS) [1] and has been proposed as an anti-obesity agent since its administration decreases food intake and body weight in rodents [2–5]. C75 can alter the metabolism of neurons in the hypothalamus, where an increase in the level of malonyl-CoA due to FAS inhibition serves as a secondary

messenger of nutrient status, thereby mediating the suppression of food intake [6,7]. Hypothalamic levels of long-chain fatty acyl-CoA (LCFA-CoA) also signal nutrient availability and control food intake [8].

Malonyl-CoA signals the availability of lipid and carbohydrate fuels [9] and acts as a physiological inhibitor of the enzyme carnitine palmitoyltransferase 1 (CPT1). CPT1 catalyzes the first step in the transport of LCFA from the cytoplasm to the mitochondria, and is the rate-limiting step in β-oxidation. Mammalian tissues express three CPT1 isoforms: CPT1A, CPT1B and CPT1C which differ in their sensitivity to malonyl-CoA and tissue distribution [10,11]. CPT1A and CPT1C are expressed in the brain. CPT1A is located in the mitochondrial membrane and CPT1C is expressed in the endoplasmic reticulum of neurons where although it has CPT1 activity, it does not participate in mitochondrial fatty-acid oxidation [12]. Interestingly, our group has generated a mutant form of CPT1A insensitive to malonyl-CoA (CPT1A M593S), and a mutant form of carnitine acetyltransferase (CrAT) that swaps its preference from short to LCFA-CoA (CrAT D356A/M564G). These mutants have allowed us to examine the structural requirements of substrates and inhibitors [13–15].

The inhibition of FAS by C75 produces an accumulation of malonyl-CoA which is difficult to reconcile with the activation of CPT1 reported by others [2,16–18]. To unravel this paradox the mechanism of action of C75 needs to be examined. We recently demonstrated that C75 is converted *in vitro* to C75-CoA, a potent inhibitor of CPT1 [19]. CPT1 activity was also inhibited in mitochondria from pancreas-, muscle-, and kidney-derived cell lines incubated with C75, which indicates that C75-CoA is produced in these cells. This inhibition was followed by a decrease in fatty-acid oxidation. The role of CPT1 in heart, liver and pancreatic β-cells makes it a potential target in the treatment of diabetes, obesity, and other human diseases.

Here we show that C75-CoA, and not C75, directly inhibits CPT1 activity. We also show that C75-CoA is formed in the hypothalamus in a dose-dependent way, where it inhibits CPT1 activity and decreases food intake and body weight. These results indicate that direct inhibition of CPT1 by C75-CoA in the hypothalamus could control body weight and feeding behaviour.

2. Materials and methods

2.1. Materials

L-[Methyl-^{3}H]carnitine hydrochloride was purchased from Amersham Biosciences (GE Healthcare, Europe, Barcelona, Spain). Yeast culture media products were from Difco™ Laboratories (Detroit, MI). Bradford solution for protein assays was from Bio-Rad Laboratories (Barcelona, Spain). RPMI 1640 was from Gibco-Invitrogen Corporation (Barcelona, Spain). C75, C_{17}-CoA, defatted bovine serum albumin (BSA), palmitoyl-CoA, malonyl-CoA, and other chemicals were from Sigma–Aldrich (Madrid, Spain). Acyl-CoA synthetase from *Pseudomonas* sp. was from Sigma (Madrid, Spain). Etomoxir was provided by H.P.O. Wolf (GMBH, Allensbach, Germany).

2.2. Animals

Sprague-Dawley female rats (210–230 g) were bred in our laboratory. Animals were maintained under a 12 h dark/light cycle with free access to food (2014, Harlan) and water. All experimental protocols were approved by the Animal Ethics Committee at the University of Barcelona, in accordance with current legislation.

2.3. Cannulation surgery

Chronic (i.c.v.) cannulae were stereotaxically implanted into lateral ventricle under ketamine (Imalgene, 90 mg/kg) and xylazine (Rompun, 11 mg/kg) anesthesia. The coordinates were 1.0 mm posterior to bregma, 1.4 mm lateral of the sagittal sinus and 6.2 mm ventral to the dura mater. Rats received subcutaneous injection of buprenorphine (0.05 mg/kg) for analgesia.

2.4. Stereotaxic microinjection

Rats were anesthetized as previously described and immobilized in a stereotaxic apparatus. Injections were given bilaterally into the ARC nucleus of the hypothalamus (2.7 mm posterior to bregma, 0.2 mm lateral of the sagittal sinus and 9.8 mm ventral to the dura mater). 30 min after the injection rats were sacrificed and hypothalamus was dissected.

2.5. Treatments

After 1 week of postsurgical recovery, cannula placements were verified by assessing a feeding response to ghrelin [20]. I.c.v. injection (5 μL) of etomoxir (190 μg), C75 (40 μg) or vehicle (RMPI 1640) were performed with a microliter syringe (Hamilton). For feeding experiments, rats received single injections of vehicle or compound dissolved in vehicle 30 min before the light was turned off. We measured intakes of chow, corrected for spillage, at 1, 2, 4 and 22 h. Body weight was measured after 22 h. For the activity experiments three rats were microinjected (ARC) with 2 μL of RPMI 1640 medium as a control or with 2 μL of C75 dissolved in the same medium at 33 mM final concentration. For the LC–MS/MS analysis, two rats were microinjected (ARC) with 2 μL of RPMI 1640 medium as control or with 2 μL of C75 dissolved in the same medium at 15, 30 or 60 mM final concentration. 30 min after injection rats were killed and hypothalamus was excised and stored at −80 °C for acyl-CoA extraction.

2.6. C75-CoA and acyl-CoAs extraction and quantification

Acyl-CoAs were extracted as described elsewhere [21], with some modifications. All procedures were performed at 0–4 °C. Hypothalamus from control- and C75-microinjected rats was thawed and 1 mL of 100 mM KH_2PO_4 pH 4.9 was added. 16 nmol of heptadecanoyl-CoA (C_{17}-CoA), as an internal standard, was added to all samples, which were then sonicated for 20 s. One millilitre of n-propanol was added and the emulsions were sonicated for a further 20 s, left on ice for 20 s, and sonicated again for 20 s. To this solution, 0.125 mL

of saturated $(NH_4)_2SO_4$ was added, followed by 2 mL of 100% acetonitrile, and the mixture was then vortexed for 5 min at 4 °C. The tubes were then centrifuged for 5 min at 2100 × g at 4 °C. The supernatant was removed and passed through a sterile 0.2 μm filter (Millipore). The filtrate was lyophilized and re-dissolved in the same mobile phase (buffer B: 10% acetonitrile in 10 mM ammonium acetate buffer (pH 5.3)) used for the LC–MS/MS analysis. C_{17}-CoA and C75-CoA were used as standards to produce the calibration curves from which we quantified production of C75-CoA *in vivo*. The straight lines obtained had a regression coefficient of 99%.

2.7. Liquid chromatography–mass spectrometry (LC–MS/MS)

LC analyses were performed using a Perkin Elmer series 200 equipped with a quaternary pump and thermostatted auto-sampler. An XBridge (Waters) C_{18} column (50 mm × 2.1 mm, 3.5 μm) was used at room temperature and the volume injected was 15 μL. The mobile phases were buffer A, 50% acetonitrile in 10 mM ammonium acetate buffer (pH 5.3); buffer B, 10% acetonitrile in 10 mM ammonium acetate buffer (pH 5.3) and C, 100% acetone. The column was equilibrated with 15% buffer A for 10 min, and then the eluting gradient was as follows: 15% buffer A to 90% buffer A in 5.5 min, then 90% buffer A to 50% buffer A, 5% C to 45% C in 15.5 min, and 50% buffer A, 45% C in 25 min. The flow rate was 0.4 mL/min.

MS and MS/MS experiments were performed on an API 3000 triple-quadrupole mass spectrometer equipped with a Turbo Ionspray source. All the analyses were performed in positive mode with the following settings: capillary voltage, +3000 V; nebulizer gas (N_2), 10 (arbitrary units); curtain gas (N_2), 12 (arbitrary units); collision gas (N_2), 4 (arbitrary units); declustering potential, +30 V; focusing potential, +200 V; entrance potential, +10 V; collision energy, +40 V; collision cell exit potential, +15 V. Drying gas (N_2) was heated to 300 °C and introduced at a flow-rate of 6000 $cm^3 min^{-1}$. Full scan data acquisition was performed scanning from m/z 500 to 1200 in profile mode and using a cycle time of 2 s with a step size of 0.1 u and a pause between each scan of 2 ms. In order to achieve maximum sensitivity, samples were injected in multiple reaction monitoring mode (MRM) by monitoring the 1022.5/515.6 and the 1020.7/513.5 transitions, obtained through product ion scan experiments with the C75-CoA and C_{17}-CoA standard, respectively. To calculate the C75-CoA concentration in the samples, 15 μL of increasing concentrations of C75-CoA and C_{17}-CoA from 10 to 5000 nM was injected. The areas of the resulting peaks were used to produce the calibration curve. The C_{17}-CoA was used as internal standard to calculate the percentage of recovery in the acyl-CoA extraction.

2.8. Enzymatic synthesis of C75-CoA and etomoxiryl-CoA

C75 was converted first, *in vitro*, to its CoA derivative by long-chain acyl-CoA synthetase in the presence of CoA, as described previously [19]. The unreacted CoA from the C75-CoA activation solution (1 mL) was removed by incubation for 30 min at 4 °C with 1.25 mL of SulfoLink Coupling Gel (Pierce Biotechnology), as carnitine acyltransferase activity is inhibited by CoA [22]. C75-CoA synthesized in this enzymatic reaction was used as a standard for LC–MS/MS analysis. The conversion of etomoxir to etomoxiryl-CoA was nearly complete, as deduced from the fluorometric assay of the remaining free CoA, as described elsewhere [23].

2.9. Non-enzymatic synthesis of C75-CoA

In the second experiment, a solution of C75 and CoA (pH 8.4) was stirred in the absence of acyl-CoA synthetase. The structure and relative stereochemistry of the C75-CoA adduct was determined by NMR. CoA sodium salt hydrate (8.6 mg), and $Na_3PO_4·12H_2O$ (7.6 mg) were added to a solution of (±)-C75 (2.5 mg) in D_2O (0.8 mL) in an NMR tube. The structure of the C75-CoA adduct was fully determined by ^1H and ^{13}C NMR, gCOSY and gHSQC experiments.

2.10. Expression of rat CrAT and CPT1 in Saccharomyces cerevisiae

Plasmids pYESCrAT^{wt} [14], pYESCrAT$^{D356A/M564G}$ [15], pYESLCPT^{wt} [24], pYESLCPT^{M593S} [13] containing CrAT wt, CrAT D356A/M564G mutant, CPT1A wt and CPT1A M593S mutant, respectively, were expressed in yeast cells, and mitochondrial cell extracts were prepared as previously described [24]. An *S. cerevisiae* strain devoid of COT and CPT1 activity and lacking the endogenous *CAT2* gene (FY23Δ*cat2* (MATa trp1 ura3 Δ*cat2*::LEU2)) was used as an expression system [25].

2.11. Determination of carnitine acyltransferase activity

Mitochondrial-enriched fractions were obtained by differential centrifugation [26], with minor modifications. All protein concentrations were determined using the Bio-Rad protein assay with bovine serum albumin as a standard.

Two methods were used for the assay of carnitine acyltransferase: a radiometric method [24] and an endpoint fluorometric method [14,23]. The radiometric assay was used in all cases, unless otherwise indicated.

2.11.1. Radiometric method
The forward reaction of carnitine acyltransferase activity was assayed in mitochondrial-enriched fractions obtained from yeast (5 μg protein for CrAT D356A/M564G and 3–4 μg protein for CPT1A) and from rat hypothalamus (100 μg protein for CPT1). Enzyme activity was assayed for 4 min at 30 °C in a total volume of 200 μL. The substrates were 400 μM L-[methyl-^3H]-carnitine, and 50 μM myristoyl-CoA (for CrAT D356A/M564G) or palmitoyl-CoA (for CPT1A). Drugs were preincubated with the enzyme for 1–5 min depending on the assay. Drug concentrations ranging from 0.01 to 60 μM were used to calculate the IC_{50}. Enzyme assays were carried out to deduce the effects of the free acid (C75) versus the CoA ester (C75-CoA), that is, the potency of C75-CoA as inhibitor relative to C75 as an activator. In this case, CPT1A wt overexpressed in yeast (8 μg protein) was preincubated for 5 min with increasing concentrations of C75 (5–100 μM), C75-CoA (5–50 μM) with or without 100 μM C75 at each C75-CoA concentration. Enzyme activity was compared to the control (DMSO). In all cases, the molar ratio of acyl-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.

2.11.2. Fluorometric method

The forward reaction of carnitine acyltransferase activity was assayed for 8 min at 30 °C in a solution containing 0.1 mM acyl-CoA, 1.5 mM EDTA, 1.5 mM L-carnitine, and 40 mM Hepes buffer, pH 7.8, in a total volume of 600 μL. Acetyl-CoA was used as substrate for CrAT wt. Reactions were started by the addition of 5 μg of yeast-expressed protein. Increasing concentrations of C75-CoA, etomoxiryl-CoA or malonyl-CoA were independently incubated with yeast-overexpressed mitochondrial CrAT wt. Parallel (blank) assays were run in the absence of L-carnitine.

2.12. Construction of rat CrAT and CPT1A models

A model of rat CrAT wt enzyme was constructed by homology modelling using as templates the structures deposited in the Protein Data Bank (PDB) corresponding to human (1NM8) [27], and mouse CrAT (1NDB, 1NDF and 1NDI) [28], essentially as described elsewhere [14]. CrAT D356A/M564G was modelled by the same procedures using rat CrAT wt as template. CPT1A was also modelled using as templates the structures of mouse carnitine octanoyltransferase (1XL7, 1XL8) [29] and carnitine palmitoyltransferase 2 (PDB entry 2H4T) [30], essentially as described elsewhere [31,32]. The quality of the models was checked using the WHAT-CHECK routines [33] from the WHAT-IF program [34] and the PROCHECK validation program from the SWISS-MODEL server facilities [35]. To optimize geometries and release local constraints or inappropriate contacts, the modelled structures were energy minimized with the implementation of the GROMOS 43B1 force field in the program DeepView [36], using 500 steps of steepest descent minimization followed by 500 steps of conjugate-gradient minimization.

2.13. In silico molecular docking

Structural models of the molecular docking of the inhibitors C75-CoA and malonyl-CoA to the active site of the receptor proteins CPT1A, and CrAT mutant D356A/M564G were performed using the suite of programs included in the Autodock 3.0 package [35], as described elsewhere [14].

2.14. Statistical analysis

Data are represented as mean ± S.E.M. Student t-test was used for statistical analysis. Different experimental groups were compared with a one-way ANOVA followed by Turkey's test for comparisons post hoc. A probability level of $P < 0.05$ was considered significant.

3. Results

3.1. Synthesis and chemical characterization of C75-CoA by NMR

We have shown that C75-CoA can be synthesized from C75 and CoA in the presence of acyl-CoA synthetase from

Pseudomonas sp. [19]. Here we show that the MS spectrum of C75-CoA gave an $[M + H]^+$ ion at m/z 1022.5, corresponding to the protonated molecule of C75-CoA. This ion corresponded to the formation of the C75-CoA molecule by conjugation of the CoA group without the loss of a water molecule. The product showed the following: a fragmentation ion at m/z 515.6, which was assigned to the C75-pantethenoic group, and an ion at m/z 428.1, which corresponds to adenosine 3′-diphosphate (Supplemental Fig. S1).

We hypothesised that the –SH group in CoA would be sufficiently nucleophilic to add to the electrophilic methylene group in C75 without any external agent (acyl-CoA synthetase) in an alkaline medium in which the thiol group is partially deprotonated. We mixed both reagents, C75 and HSCoA in a deuterated water solution at pH 8.4 (Fig. 1A) and the mixture was analyzed by ^{1}H NMR, comparing the results with the unreacted C75 and CoA. The peaks assigned to the methylene group (black triangle) progressively decreased and a new signal at ~2.9 ppm appeared due to the formation of the new saturated methylene group (white triangle) (Fig. 1B). These changes were fast (30 min). In a parallel experiment at neutral pH the same transformation was observed, albeit much more slowly (data not shown).

The structure and relative stereochemistry of the C75-CoA adduct shown in Fig. 1C were determined by one- and two-dimensional ^{1}H and ^{13}C NMR techniques in order to assign every signal (gCOSY and gHSQC experiments) [37]. In particular, the *trans–trans* relative configuration on the lactone ring shown in Fig. 1C is well established on the basis of the observed coupling constants (J), especially $J_{d,e}$. These coupling constants depend on the dihedral angle (H–C–C–H) of the protons involved and give information about their relative position. When we compared the coupling constants of the lactone ring moiety of our adduct with those reported [38,39] for the related compounds I and II (Fig. 1C) high correlation with II was observed, suggesting the same relative configuration.

As described above, we used two methods to obtain C75-CoA. In one, a solution of C75 and CoA was incubated in the presence of acyl-CoA synthetase. In the other, the adduct was synthesised in the absence of the enzyme. We aimed to determine whether the structural and inhibitory properties of the C75-CoA adducts depended on the presence of acyl-CoA synthetase in their synthesis. Two analytical tests were applied. First, a comparative mass/mass spectroscopy analysis showed that the C75-CoA synthesised in the presence of the enzyme (Supplemental Fig. S1) had the same ion fragmentation profile as that synthesised in the absence of the enzyme (data not shown). Second, we assayed their capacity to inhibit CPT1, obtaining identical results for both adducts (Fig. 1D). We conclude that the two adducts synthesized in the presence or absence of the enzyme have identical structure and properties.

3.2. Effect of C75 and C75-CoA on CPT1 activity

To examine the controversial effect of C75 on CPT1 activity we incubated yeast mitochondria with increased concentrations of C75 (Fig. 2A). The results reported indicate that free C75 alone is neither an activator nor an inhibitor of CPT1. We also
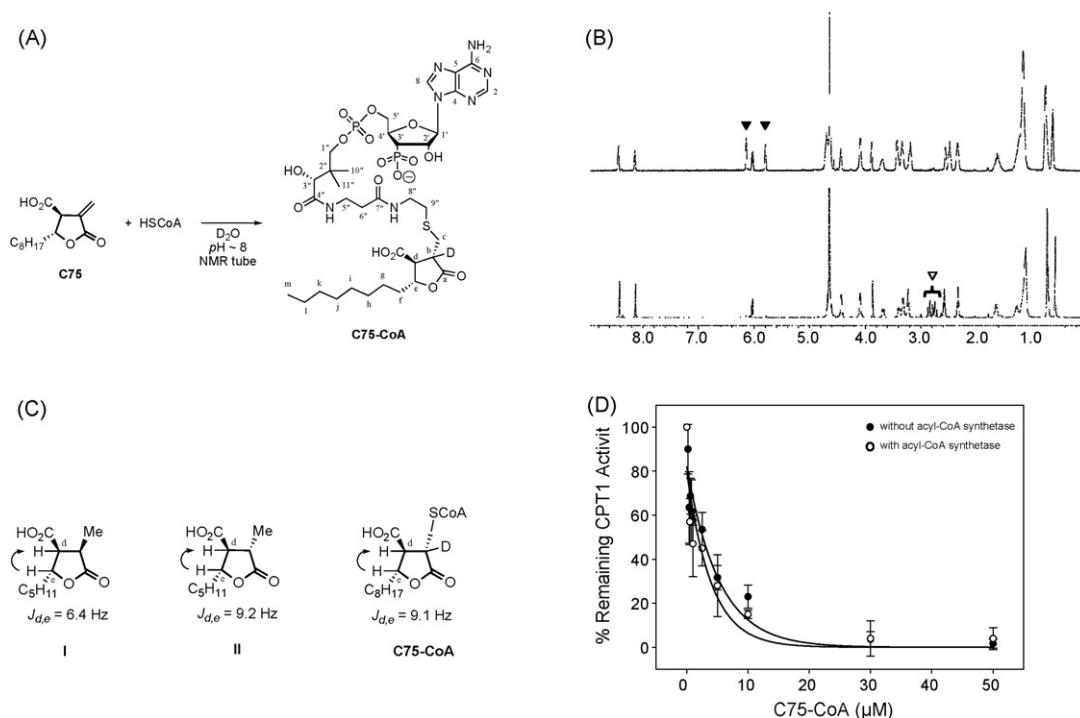
Fig. 1 – NMR structural studies. (A) Incubation of a ~1:1 mixture of C75 and CoA in D_2O (with 10% of DMSO-d6 in order to increase the solubility of C75). Peaks assigned to the methylene group (black triangles), peaks assigned to the saturated methylene groups (white triangles). (B) ^1H NMR (300 MHz) of a ~1:1 mixture of C75 and CoA in D_2O at pH ~8 at time 0 min (upper panel) and ^1H NMR (400 MHz) of a ~1:1 mixture of C75 and CoA in D_2O at pH ~8 after 30 min (lower panel). (C) Comparison of coupling constants (J) of C75-CoA adduct lactone ring with those reported early for related compounds I and II. (D) Comparison between the CPT1 inhibitory properties of C75-CoA synthesized in absence (●) and in presence (○) of acyl-CoA synthetase.
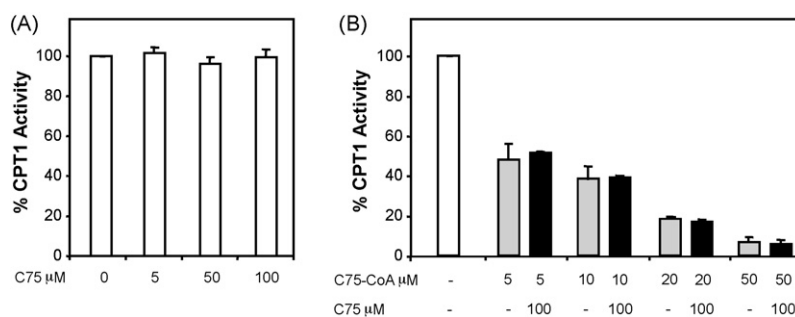

Fig. 2 – CPT1A wt overexpressed in yeast is inhibited by C75-CoA in presence of C75. (A) Mitochondrial fractions from yeast overexpression CPT1A wt were preincubated for 5 min with increasing concentrations of C75 and activity was measured. (B) Comparative CPT1A wt activity of mitochondrial fractions from yeast, incubated with increasing concentrations of C75-CoA and in presence or absence of a fixed concentration of C75 (100 μM). Data are represented as a percentage of the control value in the presence of DMSO and the results are the average of three independent experiments.

performed kinetic inhibitory experiments in which, in addition to increasing concentrations of C75-CoA, a fixed concentration of C75 (100 μM) was added to the mixture. CPT1 inhibition was not counteracted by the fixed amount of free C75 at any concentration of C75-CoA. In other words, even at the highest C75 concentrations, the free product did not overcome the inhibition by C75-CoA (Fig. 2B).

3.3. Inhibitory effects of C75-CoA on yeast-expressed mutated CPT1A and CrAT

To examine the structural requirements of the interaction between C75-CoA and CPT1 we carried out C75-CoA inhibitory experiments where we used specific acyltransferase mutants. We used CrAT D356A/M564G, which has a deep hydrophobic
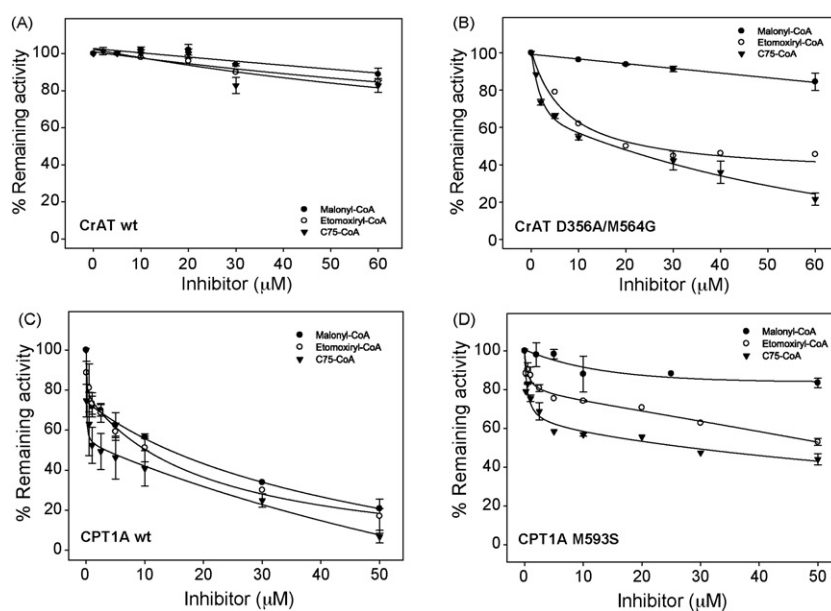
Fig. 3 – Effects of malonyl-CoA, etomoxiryl-CoA and C75-CoA on the activity of yeast-expressed wt CPT1A and CrAT, and the point mutants CPT1A M593S and CrAT D356A/M564G. Mitochondrial extracts from yeast expressing (A) CrAT wt, (C) CPT1A wt, and point mutants (B) CrAT D356A/M564G and (D) CPT1A M593S, were preincubated with increasing concentrations of malonyl-CoA (●), etomoxiryl-CoA (○) and C75-CoA (▼). Carnitine acyltransferase activity was measured, and data are expressed relative to control values in the absence of drugs (100%) as the mean of at least two independent experiments.

pocket for the binding of LCFA-CoAs instead of short acyl-CoAs and behaves like CPT1A in terms of acyl-CoA specificity [15]. We also used CPT1A M593S, which is active but insensitive to malonyl-CoA inhibition [13]. Experiments with etomoxiryl-CoA, a pharmacological inhibitor of CPT1, and malonyl-CoA, its physiological inhibitor, were carried out as controls.

C75-CoA and etomoxiryl-CoA inhibited CrAT D356A/M564G but had little effect on CrAT wt (Fig. 3A and B). At 60 μM of C75-CoA, the remaining CrAT activity was 83% for CrAT wt, and only 21% of the original level for CrAT D356A/M564G. For etomoxiryl-CoA, at a concentration of 60 μM, the remaining activity was 86% for CrAT wt, and 46% for the CrAT mutant. These results indicate that CrAT wt is insensitive to these drugs because the long aliphatic chain of C75-CoA and etomoxiryl-CoA does not fit in the shallow cavity of the wt enzyme, where only the acyl group of short-chain acyl-CoAs such as acetyl- and butiryl-CoA can enter. However, when this cavity was open and accessible to longer acyl-CoAs, as in the CrAT D356A/M564G mutant, the enzyme became sensitive to these inhibitors. In contrast, malonyl-CoA had little effect on mutant or wt CrAT (Fig. 3A and B). These results show that CrAT D356A/M564G, like the wt enzyme, does not contain the structural determinants needed for the initial interaction with malonyl-CoA and enzyme inhibition.

Next we compared the inhibitory effect of C75-CoA on CPT1A wt and CPT1A M593S. At 50 μM of C75-CoA, the remaining activity of the M593S mutant was about 45% of the original level (Fig. 3D), whereas the activity of the CPT1A wt was almost abolished (Fig. 3C). A similar effect was observed with etomoxiryl-CoA. Malonyl-CoA, as expected, inhibited CPT1A wt while the activity of the malonyl-CoA-insensitive enzyme (CPT1A M593S) was unaffected. The IC_{50} for C75-CoA

and CPT1A M593S was 108-fold higher than that for CPT1A wt (25.9 μM vs. 0.24 μM). In the case of etomoxiryl-CoA the IC_{50} increased 31-fold compared with the wt enzyme (168 μM vs. 4.1 μM). This indicates that CPT1A inhibitors in addition to fitting in the hydrophobic pocked also need to interact with the malonyl-CoA site of CPT1A to produce potent inhibition.

3.4. Molecular model of docking of C75-CoA into CPT1A wt, and CrAT D356A/M564G

Since CPT1 has not yet been crystallized, we do not know the location of C75-CoA in the CPT1 crystal. Therefore, we used computational docking methods to identify a putative location of C75-CoA molecule in the 3D models of the catalytic core of CPT1A wt and of CrAT D356A/M564G. The model locates the C75-CoA molecule (Fig. 4A) in CTP1A wt in a position that coincides with the site of palmitoyl-CoA substrate [32], thus introducing the aliphatic chain into the hydrophobic cavity in the protein active centre. The carboxyl group in the head of the C75-CoA molecule is positioned at the equivalent locus for the carnitine substrate in the CPT1A wt models, partially filling it. This indicates that this inhibitor could hamper the correct positioning of the carnitine substrate. Moreover, the head of C75-CoA is also located near the catalytic His residue (His[473]), which would impair the catalytic activity of CPT1A. The position of the carboxyl group on C75-CoA (Fig. 4A) is similar to that proposed for the dicarbonyl moiety of malonyl-CoA on CPT1A [32], which thus interferes with the correct positioning of carnitine substrate (Fig. 4C). This shared mode of action could be explained in part by the presence of a carboxyl group in carnitine, C75-CoA and malonyl-CoA molecules (Fig. 4D), putatively located in the active centre of the enzyme.
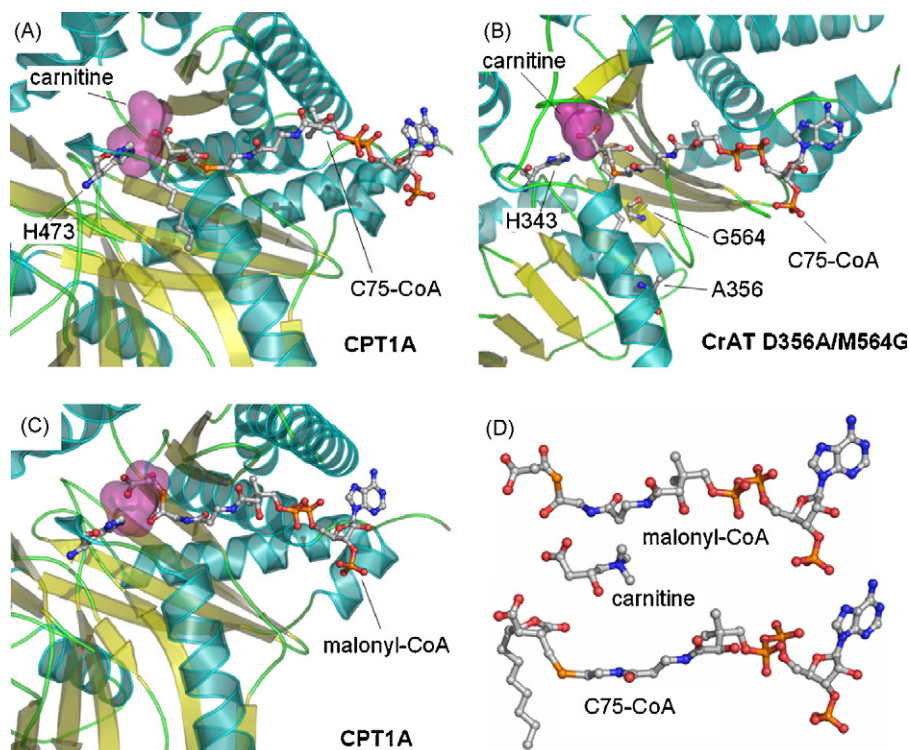
Fig. 4 – *In silico* docking of inhibitors to 3D models for the active centres of wt CPT1A and CrAT D356A/M564G mutant. (A) Predicted docked solution for C75-CoA molecule in the active centre of wt CPT1A. Location of catalytic residue His[473] is indicated. Carboxylate group of C75-CoA partially occupies the volume corresponding to the carnitine substrate molecule (magenta). (B) Putative position for C75-CoA docking solution for C75-CoA in the active centre of CrAT D356A/M564G. The aliphatic chain of the inhibitor is located in the tunnel opened by mutated residues A356 and G564. The carboxylate group of C75-CoA is placed in the carnitine locus (magenta). Location of catalytic His[343] is also indicated. (C) Docking of malonyl-CoA on CPT1A wt showing a similar positioning to C75-CoA, locating the dicarbonyl moiety in the space usually occupied by carnitine. (D) Molecular structures of malonyl-CoA, carnitine and C75-CoA, showing a similar arrangement of their corresponding carboxyl groups. Molecular representations were performed using PyMOL (DeLano Scientific, San Carlos CA).

An analogous model was constructed for the location of C75 in CrAT D356A/M564G mutant structure. The position of the C75 aliphatic chain moiety is similar to that proposed for stearoyl-CoA in the same enzyme derivative [15], in which the hydrocarbon chain is located in the cavity that opens up when Asp[356] and Met[564] are replaced by Ala and Gly, respectively (Fig. 4B). The carboxyl group at the head of C75 is located in the carnitine locus, close to the catalytic His[343] residue as mentioned above for CPT1A.

### 3.5. C75 is converted to C75-CoA in rat hypothalamus

Since C75 has been proposed as a regulator of food intake through its action on the hypothalamus, we examined whether C75-CoA is produced in the hypothalamus following direct injection of C75. We injected 7.6, 15.2 and 30.4 µg of C75 in the arcuate (ARC) nucleus by stereotaxic surgery. Thirty minutes after injection the rats were killed and acyl-CoAs, including C75-CoA, were extracted from the hypothalamus. Analysis of the samples by LC–MS/MS showed a peak corresponding to C75-CoA (Fig. 5). The production of C75-CoA increased with the amount of C75 injected. Considering that in our conditions the average weight of the rat

hypothalamus was 50 mg, the concentrations of C75-CoA produced in the hypothalamus after an injection of 7.6, 15.2 and 30.4 µg of C75 were 3.7, 15.9 and 25.1 nmol/g tissue, respectively.

3.6. Central nervous system administration of C75 inhibits CPT1 activity and decreases food intake and body weight

Having demonstrated the conversion of C75 into C75-CoA in the rat hypothalamus, we then studied the effect of a stereotaxic microinjection of C75 into the rat ARC nucleus on CPT1 activity. Hypothalamic mitochondrial fractions from rats injected with 16.8 µg of C75 were assayed for CPT1 activity and the value was compared to hypothalamic mitochondrial fractions from rats injected with RPMI medium. Activity was decreased by 46% in C75-treated rats (Fig. 6A).

Next we administered C75 in the lateral cerebral ventricle of the rat and measured food intake and body weight. Consistent with previous results [2–5], C75 produced a decrease in food intake and body weight (Fig. 6B and C). The anorectic action of etomoxir was seen at a later point (22 h).
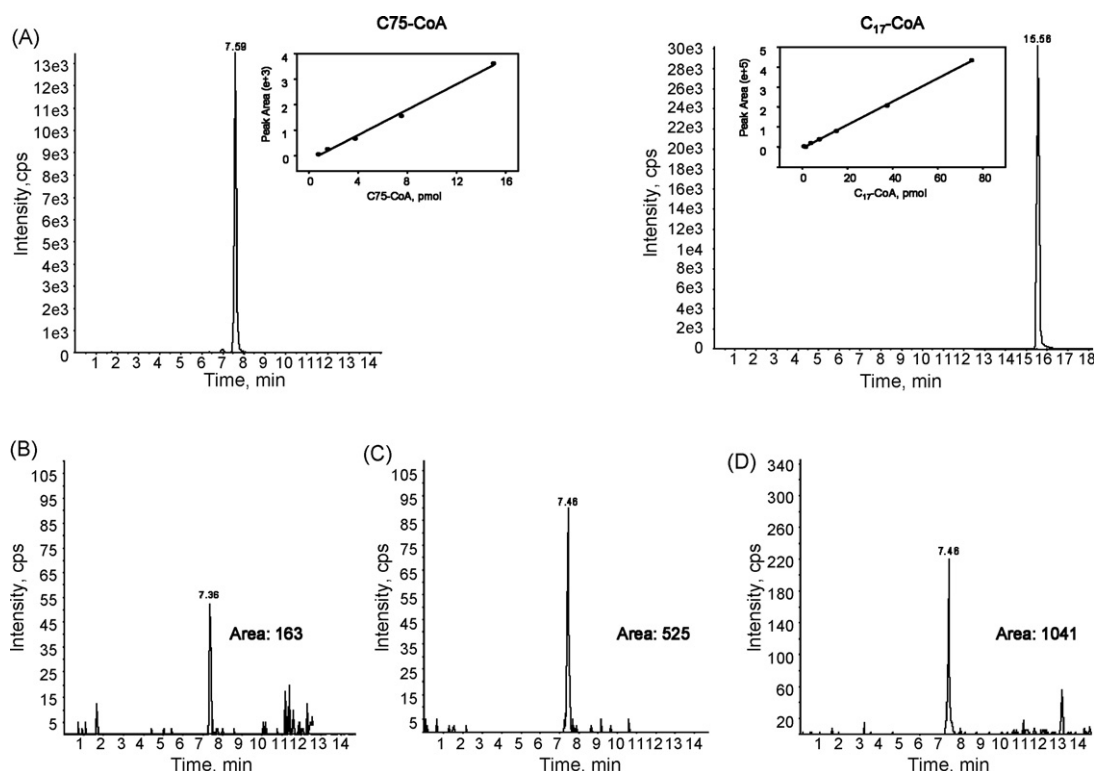
Fig. 5 – Analysis of the C75-CoA levels after hypothalamic C75 injections. (A) LC–MS/MS representative chromatograms of C75-CoA and C_{17}-CoA. Five concentrations of C75-CoA: 50, 100, 250, 500 and 1000 nM and seven concentrations of C_{17}-CoA: 50, 100, 250, 500, 1000, 2500 and 5000 nM were injected and the areas below the resulting peaks were calculated. The calibration curves are shown in insets. Representative LC chromatograms for C75-CoA obtained from rat hypothalamus after the injection in the hypothalamic ARC nucleus of 2 μL of C75 at various concentrations: (B) 15 mM, (C) 30 mM and (D) 60 mM. 30 min after injection rats were decapitated, and the hypothalamus was excised, immediately frozen and stored at −80 °C. Acyl-CoAs were extracted as described in Experimental Procedures, and C_{17}-CoA was used as internal standard. Peak areas are shown in arbitrary units.

4. Discussion

Energy balance is monitored by the hypothalamus, where inhibitors of FAS have been described to suppress food intake [40,41]. C75 acts on fatty-acid metabolism by inhibiting FAS activity. Inhibition of FAS produces accumulation of malonyl-CoA, which, as a physiological inhibitor of CPT1, prevents the oxidation of newly synthesized fatty acids. Moreover, C75 may activate CPT1 [2,18]. The effect of C75 on FAS is difficult to reconcile with the C75-activation of CPT1 as they have opposite effects: inhibition by malonyl-CoA and direct activation of CPT1. The simultaneous activation and inhibition of CPT1 by C75 appears paradoxical, and it has not been addressed satisfactorily. Kuhajda et al. suggested that C75 could modulate the inhibition of AMP-activated protein kinase (AMPK) which would lead to an increase in acetyl-CoA carboxylase (ACC) activity and a subsequent increase in malonyl-CoA levels [42]. This does not solve the paradox, since there would be an increase in malonyl-CoA (putatively inhibiting CPT1 activity) and a simultaneous activation of CPT1 produced by C75 itself (Fig. 7).

Here we attempt to explain the anorectic effects of C75 in terms of its inhibitory action on CPT1. Inhibition of CPT1 could prevent the oxidation of fatty acids of the ARC nucleus in the

hypothalamus leading to a local accumulation of LCFA-CoAs. The increase in LCFA-CoA is a central signal of 'nutrient abundance' which in turn activates a chain of neuronal events, via up-regulation of anorexigenic genes and down regulation of orexigenic genes, that would promote a switch in fuel sources from carbohydrates to lipids and limit food intake [8]. The detailed mechanism of this up- or down-regulation has not been addressed yet. Likewise, central administration of fatty acid suppresses food intake. Here we demonstrated that C75 is transformed into C75-CoA in the hypothalamus where it inhibits CPT1 activity. We propose that this direct effect *in vivo* of C75-CoA on CPT1 would explain by itself the inhibition of CPT1 activity. However, FAS could be inhibited by C75 in the hypothalamus. Therefore, malonyl-CoA could be in excess, inhibiting CPT1, together with C75-CoA. The anorectic effects of C75 are similar to those produced by other CPT1 inhibitors such as ST1326 and tetradecylglycidic acid, and to those of a riboprobe that specifically cleaves CPT1A mRNA [8]. The CPT1 inhibitor etomoxir also decreased feeding and reduced body weight in rats supporting those previous results [8].

It was important to discern whether C75 was an inhibitor or an activator of CPT1. Our results indicate that C75 is neither an activator nor an inhibitor of CPT1 when incubated with
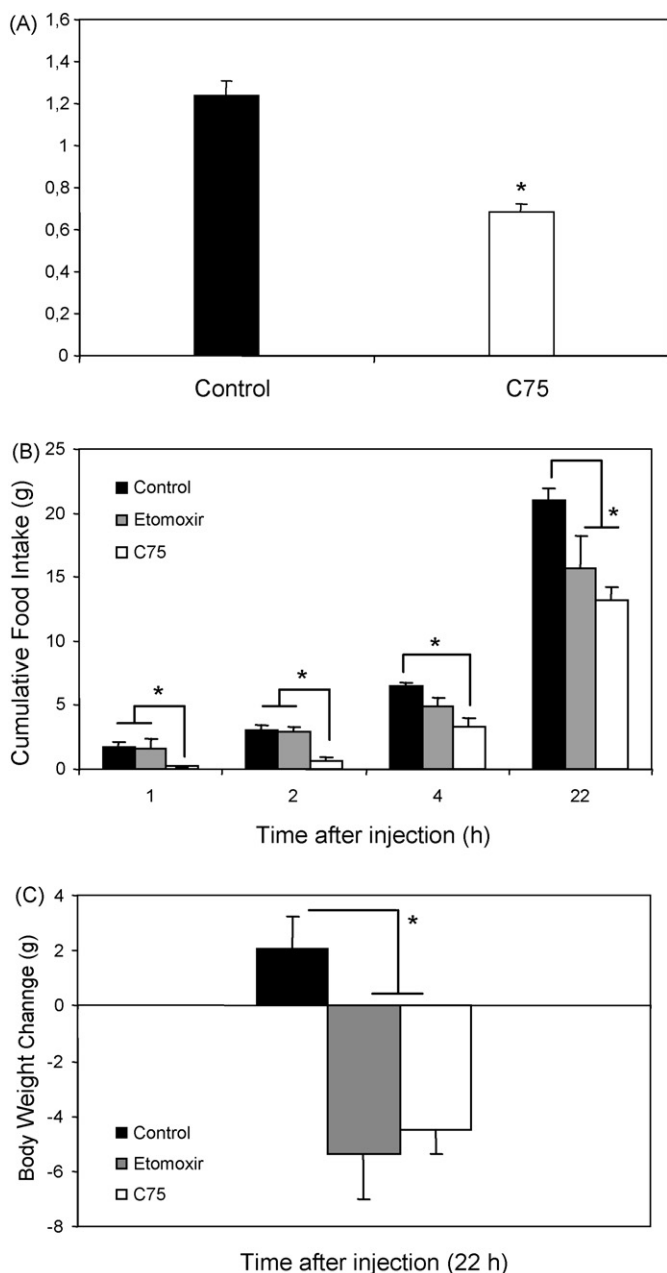
Fig. 6 – Central nervous system administration of C75 inhibits CPT1 activity and decreases food intake and body weight. (A) Determination of CPT1 activity after the injection of C75 in ARC nucleus. Control injections (n = 3; 2 μL of RPMI 1640 medium as a control, black bar). C75 injections (n = 3; 2 μL of C75 33 mM in RPMI 1640 medium, white bar). (B) Food intake measured in rats at 1, 2, 4 and 22 h after i.c.v. injection of C75 (white bars), etomoxir (grey bars) and control (RPMI medium, black bars). n = 5. (C) Body weight measured in rats at 22 h after i.c.v. injection of C75 (white bar), etomoxir (grey bar) and control (RPMI medium, black bar). n = 5. *P < 0.05.

yeast-expressed CPT1A. C75 did not overcome the inhibition caused by C75-CoA. This agrees with the observed net effect of inhibition of CPT1 activity. Results shown here and elsewhere [19] demonstrate that C75-CoA inhibits CPT1A in

vitro more strongly than etomoxiryl-CoA (IC_{50} values are 0.24 μM and 4.1 μM, respectively).

The finding that C75 forms a CoA adduct before it can inhibit CPT1 is reminiscent of the case of etomoxir. McGarry and co-workers [43,44] showed that the CPT1 inhibitory molecules were neither tetradecylglycidic acid nor etomoxir themselves, but rather their CoA esters produced in the presence of acyl-CoA synthetase. The CoA group may direct and fix the drug molecule in the cavities of CPT1, as it does with the natural physiological inhibitor malonyl-CoA and with the substrate palmitoyl-CoA. The synthesis of C75-CoA is produced stereo specifically through the electrophilic methylene group of C75. Moreover, our finding that C75-CoA is produced in absence of acyl-CoA synthetase indicated that it could be synthesised in the hypothalamus, irrespective of whether it is expressed in hypothalamic neurons.

Our results indicate that C75-CoA is formed in the hypothalamus following stereotaxic injection of C75. Inhibition of hypothalamic CPT1 by C75-CoA *in vivo*, as seen in our experiments, is independent of the putative inhibition by malonyl-CoA, which may be formed after the action of C75 either on FAS or on AMPK. Hypothalamic CPT1 activity was determined in twice-washed mitochondria. Therefore, malonyl-CoA was unlikely to remain within CPT1, as this metabolite leaves CPT1 freely when mitochondria are washed. In contrast, C75-CoA, as it is a tight-binding inhibitor, remains bound to the enzyme after washing, which means that its inhibition is persistent [19]. Hence, C75-CoA not only inhibits CPT1 *in vitro*, but it also inhibits CPT1 activity in the hypothalamus after C75 has been converted to its CoA derivative.

That C75-CoA is an inhibitor of CPT1A is also supported by the docking analysis of the CPT1A model. Comparison of computer-calculated docking models shows that CoA is bound at the same site, whether it belongs to C75-CoA or malonyl-CoA, or palmitoyl-CoA. The carboxylic acid bound to the lactone of C75 protrudes into the carnitine site. The inhibitory mechanism of C75-CoA resembles that observed for malonyl-CoA (Fig. 4C) [32]. Several authors reported the competition between malonyl-CoA and carnitine [45,46]. The carboxylate group of malonyl-CoA and C75-CoA may partially mimic the interaction between the enzyme and the carboxylate group of carnitine, thus preventing the positioning of this substrate and inhibiting the catalytic activity of the enzyme. Several authors [47,48] suggest that the presence of two carbonyl groups in close juxtaposition in the malonyl-CoA molecule might be responsible for the interaction and the inhibitory effect on CPT1A. Because C75-CoA also has these two carbonyl groups, it may behave like malonyl-CoA. Indeed the docking models show that C75-CoA could bind to the malonyl-CoA site of the enzyme [32].

Moreover, the hydrocarbon chain of C75 is located at the same site as the hydrocarbon long-chain of palmitic acid. The kinetic experiments of inhibition of C75-CoA against palmitoyl-CoA [19] are confirmed by the docking studies, and both support the notion that C75-CoA is a strong inhibitor of CPT1A.

To assess the involvement of the hydrocarbon chain of C75-CoA positioning in the acyl group binding pocket, we carried
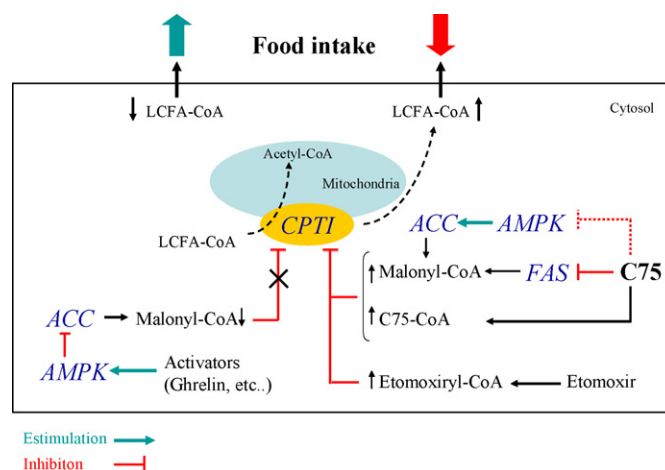
Fig. 7 – Model of action of C75 on hypothalamic fatty-acid metabolism. Energy balance is monitored by the hypothalamus. The accumulation of malonyl-CoA due to FAS-inhibition by C75 leads to a reduction in CPT1 activity. This would increase the cytosolic pool of LCFA-CoA and thus a decrease of food intake. In addition, C75 has been suggested to inhibit AMPK resulting in an activation of ACC and consequent increase of malonyl-CoA levels [42]. Here we have shown that C75 is converted into C75-CoA in the hypothalamus contributing to the direct inhibition of CPT1. Our experiments also demonstrate that other CPT1 inhibitors such as etomoxir produce similar effects decreasing food intake. On the other hand, AMPK-activators such as ghrelin [49] lead to ACC inactivation reducing malonyl-CoA levels and CPT1 inhibition. Thus, increased β-oxidation produces a reduction on LCFA-CoAs promoting food intake.

out C75-CoA inhibitory experiments with the new mutated protein, CrAT D356A/M564G. This protein has a deep hydrophobic pocket for the binding of long-chain instead of short-chain acyl-CoAs and shows CPT1-like behaviour in terms of acyl-CoA specificity, although unlike CPT1A, it is not inhibited by malonyl-CoA [15]. The inhibition by C75-CoA of the mutant CrAT D356A/M564G suggests that C75-CoA fits in the large hydrophobic pocket of this enzyme, as in CPT1A wt, and that the presence of this pocket is necessary for C75-CoA inhibition. However, CrAT D356A/M564G is not as sensitive as CPT1A wt to C75-CoA, since the IC_{50} for C75-CoA acting on CPT1A wt is 50-fold lower than that observed for the CrAT double mutant. These results indicate that factors other than the presence of a hydrophobic pocket contribute to the inhibitory potency of C75-CoA toward CPT1. CPT1A M593S, which is insensitive to malonyl-CoA inhibition, shows limited sensitivity towards C75-CoA, but its IC_{50} for C75-CoA is similar to that of CrAT double mutant (25.9 μM $vs.$ 12.8 μM, respectively). Therefore, the lack of a ''malonyl-CoA-like'' interaction between CrAT double mutant or CPT1A M593S and the carbonyl groups in the polar head of C75-CoA might explain their limited sensitivity to the inhibitor.

We conclude that C75 is converted into C75-CoA and that it strongly inhibits CPT1 in $vitro$ and in $vivo$. Docking and kinetic analysis revealed the molecular basis by which C75-CoA interacts with the enzyme and its substrates. We also show that C75-CoA is formed in $vivo$ in the hypothalamus, where it inhibits CPT1. Here the inhibition of CPT1 could alter fatty-acid oxidation, thus putatively promoting down-regulation of orexigenic genes and up-regulation of anorexigenic genes, which induces restriction in food intake. These results point to the potential use of drugs to inhibit CPT1 activity, and control food intake in the treatment of obesity and diabetes.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2008.11.020.

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