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C75 ACTIVATES MALONYL-CoA SENSITIVE AND INSENSITIVE COMPONENTS OF THE CPT SYSTEM

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ABSTRACT

Carnitine palmitoyltransferase I (CPT-I) and II (CPT-II) enzymes are components of the carnitine palmitoyltransferase shuttle system which allows entry of long-chain fatty acids into the mitochondrial matrix for subsequent oxidation. This system is tightly regulated by malonyl-CoA levels since this metabolite is a strong reversible inhibitor of the CPT-I enzyme. There are two distinct CPT-I isotypes (CPT-Iα and CPT-Iβ), that exhibit different sensitivity to malonyl-CoA inhibition. Because of its ability to inhibit fatty acid synthase (FAS), C75 is able to increase malonyl-CoA intracellular levels. Paradoxically it also activates long-chain fatty acid oxidation. To identify the exact target of C75 within the CPT system, we expressed individually the different components of the system in the yeast *Pichia pastoris*. We show here that C75 acts on recombinant CPT-Iα, but also on the other CPT-I isotype (CPT-Iβ) and the malonyl-CoA insensitive component of the CPT system, CPT-II.

INTRODUCTION

C75 was initially synthesized as a chemically stable inhibitor of mammalian fatty acid synthase (FAS) [1]. FAS catalyses long-chain fatty acid synthesis through the condensation of acetyl-CoA and malonyl-CoA. Malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase I (CPT-I), a component of the CPT system, which controls the entry of long-chain fatty acids into the mitochondria [2]. Thus, it could be expected that FAS inhibition would increase malonyl-CoA levels that in turn would down regulate fatty acid β-oxidation through CPT-I inhibition. However, it has been shown that C75 increases fatty acid oxidation in diet induced obesity [3].

C75 exerts its actions through several mechanisms. In the hypothalamus, C75 causes suppression of food intake by preventing fasting-induced up-regulation of the orexigenic neuropeptides NPY and AgRP and down-regulation of the anorexigenic neuropeptides POMC and CART [4-6]. In the peripherical tissues C75 acts on two key steps of lipid metabolism: FAS and CPT. As a result of FAS inhibition, lipid synthesis is down regulated and malonyl-CoA level increase. However, *In vitro* studies using primary rat hepatocytes and cell line models showed that C75-mediated increase of malonyl-CoA concentration does not cause inhibition of fatty acid oxidation as predicted but on the contrary revealed that C75 is able to activate the CPT system in the presence of inhibitory concentrations of malonyl-CoA [3]. Altogether these mechanisms of action are responsible for the loose of appetite, the reduction of adipose mass [4] and fatty liver [5] observed after C75 treatment.

The CPT system is involved in the translocation of long chain acyl-CoA across the mitochondria membrane, and is composed of three enzymes: CPT-I, carnitine/acylcarnitine translocase, and CPT-II. The CPT-I activity catalyzes the initial

step of the process by converting the acyl-CoA into acylcarnitine esters in the presence of L-carnitine. The carnitine/acylcarnitine translocase promotes the transit of acylcarnitine esters across the mitochondrial inner membrane and, finally, the CPT-II enzyme regenerates the long-chain acyl-CoA from the acylcarnitine esters, hence providing substrates for β-oxidation. Because CPT-I catalyzes the first rate-limiting step in the carnitine shuttle system and because of its tightly regulation by its physiological inhibitor malonyl-CoA, it is considered the most critical step in controlling fatty acid flux through the β-oxidation pathway [2]. However, at the transcriptional level CPT-I is not the sole enzyme regulated since it has been showed the CPT-II is regulated by starvation [7] and PPAR (Peroxisome Proliferator Activated Receptor) [8].

Two main isotypes of CPT-I exist: CPT-Iα and CPT-Iβ. CPT-Iα is highly expressed in liver and in a wide variety of tissues. CPT-Iβ is only expressed in muscle, adipose tissue, heart, and testis. Both enzymes differ markedly in their kinetics characteristics, namely the K_m for carnitine and the sensibility to malonyl-CoA inhibition. CPT-Iα shows a lower K_m for carnitine and a low sensitivity to malonyl-CoA inhibition (higher IC_{50}), while CPT-Iβ has a high K_m for carnitine and a higher sensitivity to malonyl-CoA (lower IC_{50}) [2]. These kinetic characteristics are preserved by the recombinants enzymes when expressed in *Pichia pastoris* [9-11]. The differences between both isotypes are not universally conserved since it has been recently showed that porcine CPT-I isotypes behave as natural chimera of CPT-I isotypes: pig CPT-Iα has a high affinity for carnitine but also high sensibility to malonyl-CoA inhibition [12, 13] while pig CPT-Iβ has a low affinity for carnitine and a low sensibility to malonyl-CoA inhibition [14].

Thupari *et al.* [3] concluded that C75 stimulates CPT-I activity. However, considering the experimental approach used, the action of C75 on the malonyl-CoA insensitive

component of the CPT system can not be discarded, since under the assay conditions both CPT-I and CPT-II catalyze the same reaction, the formation of palmitoylcarnitine from carnitine and palmitoyl-CoA [11, 15].

Moreover, Thupari *et al.* [3] worked with different cellular systems - primary hepatocytes, NCF-7 human breast cells cancer line and with mouse 3T3-L1 adipocytes- that all express the α isotype of CPT-I (mouse 3T3-L1 adipocytes, unlike other adipocytes express the α isotype [16]). Hence, they could not investigate the effects of C75 on CPT-Iβ, the isotype expressed in adipose tissue, one of the tissues of major interest with regard to β-oxidation.

To distinguish between CPT-I and CPT-II activities, and also to study the β isotype, we expressed individually the different CPT enzymes in the yeast *Pichia pastoris*, devoid of endogenous CPT activity, and analysed the effect of C75. This study was complemented with experimental setups in mammalian cell lines that allowed distinction between CPT-I and CPT-II activities.

MATERIALS AND METHODS

Construction of Plasmids for CPT Expression in P. pastoris – All expression plasmids used for the expression of rat CPT-Iα [11], pig CPT-Iα [12], human CPT-Iβ [9], and CPT-II [11], were constructed in a similar way in which an EcoRI site was introduced immediately upstream of the ATG start codon to enable cloning into the unique EcoRI site located 3' of the glyceraldehid-3-phosphate dehydrogenase gene promoter (GAPp) in pHWO10 plasmid [11, 17] The constructs were linearized in the GAP gene promoter by digestion with *AvrII* and integrated into the *GAPp* locus of *P. pastoris* GS115 by electroporation [18]. Histidine prototrophic transformants were selected on YND plates and grown on YND medium. Mitochondria were isolated by disrupting the yeast cells with glass beads as previously described [11, 19].

Carnitine palmitoyltransferase assay in P. pastors mitochondria – CPT activity was assayed by the forward exchange method using L-[^{3}H] carnitine as previously described [11]. In a total volume of 0.5 mL, the standard enzyme assay mixture contained 0.2 mM (for pig CPT-Iα, rat CPT-Iα, and rat CPT-II) or 1 mM (for human CPT-Iβ) of L-[^{3}H]carnitine (~5 000 dpm/nmol) 80 μM palmitoyl-CoA, 20 mM HEPES (pH 7.0), 1% fatty acid-free albumin, and 40-75 mM KCl, with or without 10 μM malonyl-CoA and/or 200 μM C75 (Alexis Biochemicals) as indicated. Reactions were initiated by addition of 0.1 mg of isolated intact yeast mitochondria. The reaction was linear up to 4 min, and all incubations were done at 30 ºC for 3 min. with or without malonyl-CoA and/or C75, as indicated. Reactions were stopped by addition of 6% perchloric acid and were then centrifuged at 2000 rpm for 7 min. The resulting pellet was suspended in water, and the product [^{3}H] palmitoylcarnitine was extracted with butanol at low pH. After centrifugation at 2000 rpm for 2 min, an aliquot of the butanol phase was transferred to a vial for radioactive counting

Carnitine palmitoyltransferase assay in mammalian cell lines - Cells were grown to confluence and collected in PBS (Phosphate buffer saline). Cells were collected by centrifugation and resuspended in buffer A (KCl 150mM, Tris HCl 5mM pH 7.2) and broken with 10 cycles of glass homogenizer (tight fitting pestle). Mitochondria were collected by centrifugation (16000g, 5 min. 4ºC) and resuspended in buffer A. At this step mitochondria could either be used for total CPT activity assay or processed for CPT-II activity determination by frozen and thawed, and then incubated for 30 minutes at 4°C in presence of 1% octylglucoside. This treatment inhibits CPT-I activity [20] and hence, once the membrane debris are removed by centrifugation (16000g, 2 min at 4ºC), allow direct measurement of CPT-II activity.

For both total CPT and CPT-II assay, 100µg of protein were used for the enzymatic assay. The CPT assay was performed using the same protocol as for *P. pastoris* mitochondria. To ensure that the CPT-II assay worked correctly an alternative protocol was developed: before harvest, cells were treated for 2 hours with etomoxir or vehicle (DMSO) in order to irreversibly inhibit CPT-I activity. Cells were then collected and processed as for total CPT or CPTII assay. Etomoxir inhibition of CPT-I activity gave the same results than measured by octylglucoside treatment of mitochondrial preparations, which validated the assay (results not shown).

RESULTS AND DISCUSSION.

To study the effect of C75 on the CPT system, recombinant rat CPT-Iα and human CPT-Iβ were expressed in *Pichia pastoris*. As shown in Figure 1A and 1B, C75 activates rat CPT-Iα (23.18%) and human CPT-Iβ (33.02%) recombinant enzymes. The stimulatory effect of C75 is maintained when the CPT-I activity is measured in presence of 10 μM malonyl-CoA: rat CPT-Iα is activated 23.35% and human CPT-Iβ 44.44%. We also expressed pig CPT-Iα, since this enzyme is a natural chimera which a k_m for carnitine characteristic of the α isotype but a sensitivity to malonyl-CoA inhibition characteristic of the β isotype [12]. Figure 1C shows that C75 activates pig CPT-Iα in the absence (23.88%) or in the presence of malonyl-CoA 10 μM (28.88%).

The increase in the activity of CPT-Iα by C75, even in the presence of inhibitory concentration of malonyl-CoA, has been used as an argument to explain the C75 paradox, in which this compound is able to simultaneously stimulate fatty acid β-oxidation and malonyl-CoA accumulation [3]. However, the total activity of CPT-I, in the presence of C75 and malonyl-CoA, still remains lower than the activity in the absence of malonyl-CoA (Figure 1), questioning such explanation in physiologic conditions.

C75 stimulation of CPT-I activity was lower than reported in primary culture cells [3], and appears unrelated with the different sensibility to malonyl-CoA of the recombinant enzymes. Therefore, we studied the effect the C75 on CPT-II activity. Figure 2A shows that C75 stimulates recombinant CPT-II activity (30.73%) in a similar way than the CPT-I activity. To confirm this result we determined C75 effect on endogenous CPT-II activity from CHO and Cos-7 cell lines. Since CPT-II or CPT-I *in vitro* determinations are based in the same assay [11, 15], we took advantage of octylglucoside, a detergent that promotes specific inactivation of the CPT-I activity [20], to exclusively assay CPT-

II activity. Figure 2B shows that C75 stimulates the activity of CPT-II enzyme in CHO (90.29%) and Cos-7 (123.91%) cell lines.

All together these results indicate that C75 acts directly on the CPT shuttle system, since C75 activation extends to different isotypes of CPT-I and to the CPT-II enzyme. Therefore, this result could explain the activation of the fatty acid oxidation by C75 in different tissues, independently of the CPT-I isotype expressed.

CPTII deficiency is among the most common inborn errors of mitochondrial fatty acid β-oxidation. Recently, in CPT-II-deficient cultured human skin fibroblasts, it has been observed that up-regulation of CPTII expression mediated by bezafibrate improves fatty acid oxidation rate [21]. This data is consistent with the presence of a PPRE in the 5'-flanking region of the human CPTII gene [8] and therefore validates the CPTII as a potential pharmacological target. We show now that CPTII gene can also be regulated at the post-transcriptional level by C75.

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REFERENCES

[1] F.P. Kuhajda, E.S. Pizer, J.N. Li, N.S. Mani, G.L. Frehywot, and C.A. Townsend, Synthesis and antitumor activity of an inhibitor of fatty acid synthase, Proc. Natl. Acad. Sci. 97 (2000) 3450-3454.

[2] J.D. McGarry, and N.F. Brown. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis, Eur. J. Biochem. 244 (1997) 1-14.

[3] J.N. Thupari, L.E. Landree, G.V. Ronnett, and F.P. Kuhajda, C75 increases peripheral energy utilization and fatty acid oxidation in diet-induced obesity, Proc. Natl. Acad. Sci. 99 (2002) 9498-9502.

[4] M.V. Kumar, T. Shimokawa, T.R. Nagy, and M.D. Lane. Differential effects of a centrally acting fatty acid synthase inhibitor in lean and obese mice, Proc. Natl. Acad. Sci. 99 (2002) 1921-1925

[5] T.M. Loftus, D.E. Jaworsky, G.L. Frehywot, C.A. Townsend, G.V. Ronnett, M.D. Lane, and F.P. Kuhajda, Reduced food intake and body weight in mice treated with fatty acid synthase inhibitors, Science 288 (2000) 2379-2381.

[6] T. Shimokawa, M.V. Kumar, and M.D. Lane, Effect of a fatty acid synthase inhibitor on food intake and expression of hypothalamic neuropeptides, Proc. Natl. Acad. Sci. 99 (2002) 66-71.

[7] E. Sekoguchi, N. Sato, A. Yasui, S. Fukada, Y. Nimura, H. Aburatani, K. Ikeda, and A. Matsuura, A novel mitochondrial carnitine-acylcarnitine translocase induced by partial hepatectomy and fasting, J. Biol. Chem. 278 (2003) 38796-38802.

[8] M.J. Barrero, N. Camarero, P.F. Marrero, and D. Haro, Control of human carnitine palmitoyltransferase II gene transcription by peroxisome proliferator-

activated receptor through a partially conserved peroxisome proliferator-responsive element, Biochem. J. 369 (2003) 721-729.

[9] H. Zhu, J. Shi, Y. de Vries, D,N, Arvidson, J.M. Cregg, and G. Woldegiorgis, Functional studies of yeast-expressed human heart muscle carnitine palmitoyltransferase I, Arch. Biochem. Biophys. 347 (1997) 53–61

[10] H. Zhu, J. Shi, J.M. Cregg, and G. Woldegiorgis, Reconstitution of highly expressed human heart muscle carnitine palmitoyltransferase I, Biochem. Biophys. Res. Commun. 239 (1997) 498-502.

[11] Y. de Vries, D.N. Arvidson, H.R. Waterham, J.M. Cregg, G. Woldegiorgis, Functional characterization of mitochondrial carnitine palmitoyltransferases I and II expressed in the yeast Pichia pastoris, Biochemistry 36 (1997) 5285-5292.

[12] C. Nicot, F.G. Hegardt, G. Woldegiorgis, D. Haro, P.F. Marrero, Pig liver carnitine palmitoyltransferase I, with low Km for carnitine and high sensitivity to malonyl-CoA inhibition, is a natural chimera of rat liver and muscle enzymes, Biochemistry 40 (2001) 2260-2266.

[13] C. Nicot, J. Relat, G. Woldegiorgis, D. Haro, and P.F. Marrero, Pig liver carnitine palmitoyltransferase. Chimera studies show that both the N- and C-terminal regions of the enzyme are important for the unusual high malonyl-CoA sensitivity, J. Biol. Chem. 277 (2002)10044-10049.

[14] J. Relat J, C. Nicot, M. Gacias, G. Woldegiorgis, P.F. Marrero, and D. Haro, Pig muscle carnitine palmitoyltransferase I (CPTIbeta), with low K(m) for carnitine and low sensitivity to malonyl-CoA inhibition, has kinetic characteristics similar to those of the rat liver (CPTIalpha) enzyme, Biochemistry 43 (2004) 12686-12691.

[15] K.F. Woeltje, V. Esser, B.C. Weis, A. Sen, W.F. Cox, M.J. McPhaul, C.A. Slaughter, D.W. Foster, J.D. McGarry, Cloning, sequencing, and expression of a cDNA encoding rat liver mitochondrial carnitine palmitoyltransferase II, J. Biol. Chem. 265 (1990) 10720-107255.

[16] N.F. Brown, J.K. Hill, V. Esser, J.L. Kirkland, B.E Corkey, D.W. Foster, J.D. McGarry, Mouse white adipocytes and 3T3-L1 cells display an anomalous pattern of carnitine palmitoyltransferase (CPT) I isoform expression during differentiation. Inter-tissue and inter-species expression of CPT I and CPT II enzymes, Biochem. J. 327 (1997) 225-231

[17] H.R. Waterham, M.E. Digan, P.J. Koutz, S.V. Lair, and J.M. Cregg, Isolation of the Pichia pastoris glyceraldehyde-3-phosphate dehydrogenase gene and regulation and use of its promoter, Gene 186 (1997) 37-44.

[18] J. Shi, H. Zhu, D.N. Arvidson, and G. Woldegiorgis, The first 28 N-terminal aminoacid residues of human heart muscle carnitine palmitoyltransferase I are essential for malonyl CoA sensitivity and high-affinity binding, Biochemistry. 39 (1999) 712-717.

[19] J. Dai J, H. Zhu, J. Shi, and G. Woldegiorgis, Identification by mutagenesis of conserved arginine and tryptophan residues in rat liver carnitine palmitoyltransferase I important for catalytic activity, J. Biol. Chem. 275 (2000) 22020-22024.

[20] K.F. Woeltje, M. Kuwajima, D.W. Foster, and J.D. McGarry, Characterization of the mitochondrial carnitine palmitoyltransferase enzyme system. II. Use of detergents and antibodies, J. Biol. Chem. 262 (1987) 9822-9827.

[21] F. Djouadi, J.P. Bonnefont, L. Thuillier, V. Droin, N. Khadom, A. Munnich, and J. Bastin, Correction of fatty acid oxidation in carnitine palmitoyl transferase 2-deficient cultured skin fibroblasts by bezafibrate, Pediatr. Res. 54 (2003) 446-451

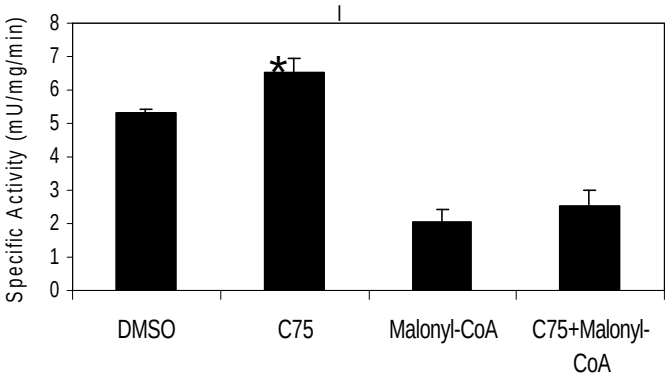
FIGURE LEGENDS

Figure 1. C75 activates recombinant CPT-Iα, and CPT-Iβ. *Pichia pastoris* were transformed with the plasmids encoding for the rat CPT-Iα (A), human CPT-Iβ (B), or pig CPT-Iα (C). Mitochondria isolated from these strains were assayed for CPT activity in the presence of DMSO (vehicle), 200 μM of C75, or 10 μM of malonyl-CoA, as described in the Material and Methods section. Results are an average of at least three separate experiments with at least two independent mitochondrial preparations. * ($p<0.005$). ** ($p<0.05$)

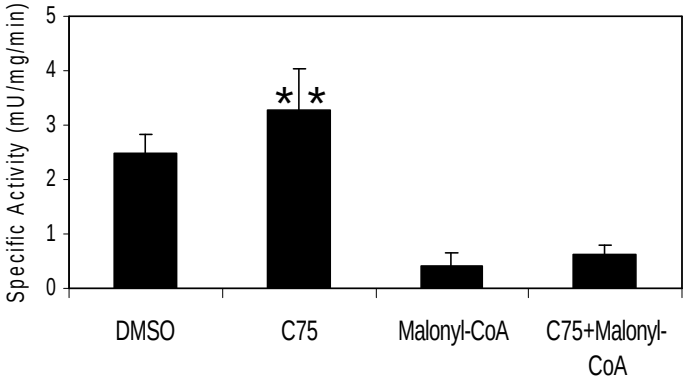
Figure 2. C75 activates CPT-II. (A) *Pichia pastoris* were transformed with the plasmid encoding for the rat CPT-II. Mitochondria isolated from these strains were assayed for CPT activity in the presence of DMSO (vehicle) or 200 μM of C75, as described in the Material and Methods section. Results are an average of three separate experiments with at least two independent mitochondrial preparations. (B) Cos-7 or CHO mitochondria were treated with octylglucoside to inactivated CPT-I, as described in the Material and Methods section. Mitochondria were subsequently assayed for CPTII activity in the presence of DMSO (vehicle) or 200 μM of C75. Results are an average of at least three separate experiments with at least two independent mitochondrial preparations. * ($p<0.005$).

Figure

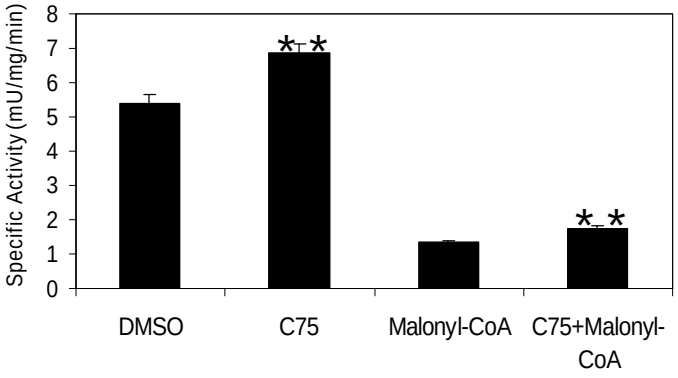
(A) Rat CPTIα



(B) Human CPTIβ

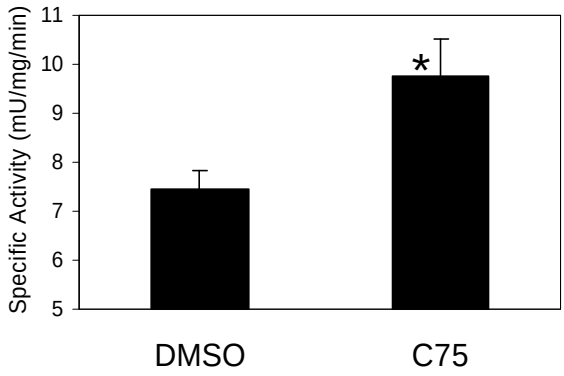


(C) Pig CPTIα



Figure

(A) Rat CPTII activity



(B) CPTII activity