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BRAIN-SPECIFIC CARNITINE PALMITOYLTRANSFERASE-1C: ROLE IN CNS FATTY ACID METABOLISM, FOOD INTAKE AND BODY WEIGHT

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Abstract

While the brain does not utilize fatty acids as a primary energy source, recent evidence shows that intermediates of fatty acid metabolism serve as hypothalamic sensors of energy status. Increased hypothalamic malonyl-CoA, an intermediate in fatty acid synthesis, is indicative of energy surplus and leads to the suppression of food intake and increased energy expenditure. Malonyl-CoA functions as an inhibitor of CPT1, a mitochondrial outer membrane enzyme that initiates translocation of fatty acids into mitochondria for oxidation. The mammalian brain expresses a unique homologous CPT1, CPT1c, that binds malonyl-CoA tightly but does not support fatty acid oxidation *in vivo*, in hypothalamic explants or in heterologous cell culture systems. CPT1c KO mice under fasted or refed conditions do not exhibit an altered CNS transcriptome of genes known to be involved in fatty acid metabolism. CPT1c KO mice exhibit normal levels of metabolites and of hypothalamic malonyl-CoA and fatty acyl-CoA levels either in the fasted or refed states. However, CPT1c KO mice exhibit decreased food intake and lower body weight than WT littermates. In contrast, CPT1c KO mice gain excessive body weight and body fat when fed a high-fat diet while maintaining lower or equivalent food intake. Heterozygous mice display an intermediate phenotype. These findings provide further evidence that CPT1c plays a role in maintaining energy homeostasis, but not through altered fatty acid oxidation.

Keywords

fatty acid synthase; AMPK; carnitine palmitoyl-transferase; CPT1c; acetyl-CoA carboxylase; malonyl-CoA

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INTRODUCTION

Cytokines and endocrine hormones control food intake and energy expenditure and are critical for maintaining homeostasis and averting serious health problems such as those associated with obesity. In addition, a recently-identified system for maintaining energy balance utilizes an ancient nutrient-sensing pathway with which the CNS monitors energy needs by assessing current energy surplus/deficit and responding by modulating appetite and peripheral energy expenditure (Wolfgang and Lane 2006b). It was recently found that key regulatory enzymes and intermediates in the fatty acid biosynthetic pathway act as hypothalamic sensors that monitor energy status and adjust food intake and increase energy expenditure accordingly. Specifically, changes in the level of malonyl-CoA, a well-characterized intermediate in fatty acid synthesis, was found to modulate energy balance in the hypothalamus (Wolfgang and Lane 2006a). Thus, increasing hypothalamic malonyl-CoA provokes anorexia and increased peripheral energy expenditure leading to a leaner phenotype (Loftus et al. 2000; Hu et al. 2003; Cha et al. 2005). Conversely, lowering malonyl-CoA by over-expressing malonyl-CoA decarboxylase in the hypothalamus increases food intake and produces obesity in rodent models showing that this system is both sufficient and required for the suppression of food intake (He et al. 2006). The physiological relevance of this mode of regulation is supported by the demonstration that fluctuation of hypothalamic malonyl-CoA level caused by fasting (0.3μM) and refeeding (1.2μM) produce concomitant changes in food intake, energy expenditure and neuropeptide expression in the hypothalamus (Shimokawa et al. 2002; Gao and Lane 2003; Hu et al. 2003).

The molecular mechanism by which hypothalamic malonyl-CoA transmits satiety signals has not been elucidated. The brain-specific carnitine palmitoyltransferase-1c (CPT1c) is a candidate as a downstream target of malonyl-CoA because it possesses appropriate characteristics (Price et al. 2002; Wolfgang et al. 2006; Dai et al. 2007). Like CPT1a (liver) and CPT1b (muscle), CPT1c is localized in the outer membrane of mitochondria(Dai et al. 2007). Malonyl-CoA, which is known to inhibit both CPT1a and CPT1b, binds tightly to CPT1c (K_D = ~0.3μM) within its dynamic range in the hypothalamus (Wolfgang et al. 2006). Furthermore, CPT1c (Dai et al. 2007) and its mRNA (Lein et al. 2007) is enriched in areas of the brain that are known to regulate feeding behavior. Finally, we have shown that a mouse knockout of CPT1c results in a phenotype consistent with a malonyl-CoA target protein. CPT1c KO mice exhibit reduced food intake and body weight on a standard low-fat diet (Wolfgang et al. 2006). Thus, CPT1c possesses biochemical and physiological characteristics to place it downstream of malonyl-CoA in the hypothalamic signaling pathway that regulates food intake and energy expenditure.

It is firmly established that CPT1a and CPT1b, homologues of CPT1c, catalyze the initiating step of fatty acid oxidation by which long-chain acyl-CoAs are translocated from the cytoplasm into the mitochondrial matrix, the site containing the enzymes of the β-oxidation pathway (McGarry et al. 1977; McGarry et al. 1978; McGarry and Foster 1980; McGarry 1995a, b). The enzymatic reaction catalyzed by CPT1a and CPT1b involves the transfer of the fatty acyl group from acyl-CoA to carnitine. The fatty acyl-carnitine then traverses the inner mitochondrial membrane via a cation transporter into the matrix. Despite extensive investigations in our (Wolfgang et al. 2006) and another (Price et al. 2002) laboratory and the high degree of amino acid sequence identity/similarity (>50%/>66%) of CPT1c to CPT1a, it has not been possible to demonstrate an enzymatic activity for CPT1c (Price et al. 2002) (Wolfgang et al. 2006). It is surprising that this unique ‘enzyme’ is expressed in the CNS, since neural tissue does not normally use fatty acids as a major physiological fuel. Rather, the CNS relies on glucose as the primary fuel when carbohydrate is available, or ketones during fasting and high-fat feeding. These facts suggest a unique, perhaps regulatory, function for CPT1c.

Here we explore the biochemical properties of CPT1c *ex vivo* and *in vivo* and verify that CPT1c does not facilitate the oxidation of long-chain fatty acids under a variety of conditions. Moreover, we show that disruption of the CPT1c gene produces a phenotype that is consistent with a regulatory role.

MATERIAL AND METHODS

Animals

Mice with a targeted knockout of exons 1 and 2 of the *cpt1c* gene were propagated and genotyped as previously described (Wolfgang et al. 2006). Mice were fed a standard lab chow (Harlan 2018) after weaning. For longitudinal diet studies, age and sex matched littermates where produced *en mass* via *in vitro* fertilization. The diet studies were conducted with 10% kcal from fat (Research Diets D12450B) or 60% kcal from fat (Research Diets D12492) diets for the time indicated. All procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and under the approval of the Johns Hopkins Medical School Animal Care and Use Committee.

Quantitative RT-PCR

Total RNA was isolated from tissue homogenized using Trizol (Invitrogen) and further purified by an RNeasy column (Qiagen, Valencia, CA). All qPCR experiments were conducted with a Mx3000P Quantitative PCR System (Stratagene, La Jolla, CA). Primers were made to amplify a 100 base product.

Immunoblotting

Immunoblotting for human CPT1c was done using affinity purified antibodies produced in rabbits against a human-specific peptide. Anti-GFP was purchased from Santa Cruz. A horseradish peroxidase-conjugated secondary anti-rabbit or anti-mouse antibody was used and visualized using SuperSignal chemiilluminescent substrate (Pierce).

Analysis of metabolic parameters

Blood samples were taken from cut tailtips of conscious fasted (14h) mice. Plasma was collected for measurement of glucose (glucose oxidase method; QuantiChrom, Bioassay Systems, Hayward, CA), triglycerides (enzymatic method, Sigma Diagnostics, St. Louis, MO), non-esterified fatty acids (enzymatic method, NEFA-C, Wako Pure Chemicals Industries, Osaka, Japan) and total Ketones (β-hydroxybutyrate and acetoacetate) (Wako). Carnitine and acylcarnitine were measured in fasting mice as previously described (Cox et al. 2001). Fatty acyl-CoAs were measured as previously described (Hammond et al. 2005).

Measurement of fatty acid oxidation

Cos-1 cells were grown in DMEM with 10 % FBS under 10% CO2. Retroviral expression vectors (pLNCX2, Clontech) were engineered to express CPT1a-eCFP and CPT1c-eCFP fusion genes. These retroviruses or one expressing GFP alone were added to Cos-1 cells and stable lines were selected with G418. Fatty acid oxidation was measured in sealed tubes equipped with center wells. Cells were treated as described, trypsinized and placed in tubes containing radiolabeled fatty acid. A stopper containing a centerwell was added and the cells were shaken at 37 degrees for 2 hours. The reaction was stopped by adding H_2SO_4 (1N final concentration) to the cells and the radiolabeled $^{14}CO_2$ was trapped after acidification in 1N NaOH soaked Whatman paper in the center well. (50mCi/mmol) [1-^{14}C] oleic acid or palmitic acid (Perkin Elmer) were used as substrate. Tissue explants from fasted animals were rapidly dissected and assayed identically as above.

RESULTS

Capacity of CPT1c and CPT1a to support long-chain fatty acid oxidation

It has been established that CPT1a and CPT1b facilitate the transfer of long-chain fatty acyl-CoAs from the cytoplasm to the matrix of mitochondria, the site of fatty acid oxidation. These enzymes catalyze the transfer of the fatty acyl group from CoA to carnitine allowing the acyl-carnitine to traverse the inner mitochondrial membrane via a transport channel. Previously, we and others showed that CPT1c does not catalyze this acyl transfer reaction under heterologous *in vitro* conditions (Price et al. 2002; Wolfgang et al. 2006). This is surprising since the amino acid sequence of CPT1c is highly homologous to those of CPT1a and CPT1b and possesses all of the amino acid residues known to be essential for efficient catalysis. We reasoned, therefore, that the *in vitro* enzymatic assay might lack required interacting components including allosteric activators. Therefore, we directly tested the ability of CPT1c to facilitate long chain fatty acid oxidation in a cellular context.

COS-1 cells were infected with retrovirus encoding either GFP, CPT1c-cyan fluorescent protein or CPT1a-cyan fluorescent protein fusions and selected with neomycin to obtain stable clones. The cells stably expressed the fusion proteins and exhibited appropriate punctate cytoplasmic localization consistent with mitochondrial localization (Fig. 1A,B). First, we tested the ability of the cells to oxidize oleic acid (C18:1) and palmitic acid (C16:0), by incubation with the ^{14}C-fatty acid and quantifying the released $^{14}CO_2$. COS-1-GFP cells exhibited little or no oxidation of the ^{14}C-fatty acids, whereas cells expressing CPT1a showed robust oxidation. However, no enhancement of fatty acid oxidation was detected in cells expressing CPT1c (Fig. 1C,D). Similar results were obtained with cells transiently expressing a C-terminal FLAG tagged CPT1a and CPT1c, as well as untagged CPT1a and CPT1c using both mouse and human cDNAs (results not shown).

Next, we tested the possibility that CPT1c requires an activator or a covalent modifying kinase to activate fatty acid oxidation. Therefore, we treated each cell line with AICAR, 8-Bromo CAMP, phorbol ester (PMA), ionomycin or KCl, and measured the oxidation of ^{14}C-oleic acid. Both AICAR and cAMP increased the oxidation of oleic acid in cells expressing CPT1a, but not in cells expressing CPT1c where no significant enhancement of oxidation occurred under any condition (Fig. 1E).

Finally, we determined whether properties unique to neurons might be required to support the activation of fatty acid oxidation by CPT1c. Thus, hypothalamic and cortical explants from WT and CPT1c KO mice were tested for their ability to oxidize ^{14}C-oleic acid. No difference in fatty acid oxidation by explants from WT and KO mice was observed (Fig. 2A). Of note, the explants exhibited weak fatty acid oxidation activity consistent with previous observations that the brain does not efficiently utilize fatty acids as a physiological fuel. There is no difference in fasting or fed hypothalamic malonyl-CoA in WT and CPT1c KO mice (Fig. 2B). We also examined plasma free carnitine and acyl-carnitine levels by mass spectrometric analysis in fasted WT and CPT1c KO animals. No differences were observed in either free carnitine or acyl-carnitine levels indicating the KO mice do not exhibit any overt systemic oxidation defects or spill-over of these derivatives into the circulation because of disruption of the CPT1c gene (Fig. 2C). Furthermore, there were no changes in the mRNA for CPT1a, CPT1b, CPT2, Crot, or Crat in CPT1c KO hypothalamus showing that the lack of differences was not do to compensation by another CPT (data not shown).

Together, these findings argue that CPT1c does not facilitate the oxidation of fatty acids in the CNS, but may have a unique substrate not involved in fatty acid oxidation. This is rather surprising because of the high degree of amino acid sequence similarity between CPT1c and

CPT1a and CPT1b. It should be noted that we have tested approximately 50 fatty acyl-CoA donor substrates (Wolfgang et al. 2006), as well as other acceptor substrates including ethanolamine, serine, choline, and sphingosine, none of which gave rise to catalytic activity *in vitro* with cell-free preparations (results not shown).

Effect of nutritional state on metabolic parameters of CPT1c KO mice

In an effort to gain further insight into the physiological role of CPT1c in energy balance, we subjected CPT1c KO and WT mice to different nutritional states and measured metabolic, gene expression and behavioral responses. A large group of age- and sex-matched CPT1c KO and WT littermates were produced *en mass* by *in vitro* fertilization. KO and WT littermates were randomly placed into two groups: 1) fasted for 30h or 2) fasted for 24h, then refed for 6h. It was found that both male and female CPT1c KO mice weighed ~2g less than their WT littermates before and after fasting (Fig. 3A) consistent with our previous results (Wolfgang et al. 2006). It was also observed that CPT1c KO mice exhibited a tendency (though not statistically significant) to eat less after fasting (Fig. 3B). The mice were euthanized and plasma and tissue were collected immediately for analysis. The levels of plasma constituents in the WT and KO groups did not differ significantly, but in all cases did exhibit the expected increase in NEFA and total ketone levels and lowered blood glucose level caused by fasting (Fig. 3C).

We next addressed the question of whether disruption of the CPT1c gene altered the expression patterns of genes involved in fatty acid metabolism to rule out compensation by other carnitine transferase genes. Total RNA was isolated from the hippocampus of mice in each treatment group and subjected to quantitative rtPCR analysis of key genes involved in fatty acid metabolism (Table 1). We focused on the hippocampus because of the large amount of accessible tissue and the high level of expression of CPT1c as indicated by immunohistochemistry and Western blotting (Dai et al. 2007). Importantly, the level of malonyl-CoA in the hippocampus, like that in the hypothalamus, is dynamic undergoing large changes caused by fasting and refeeding (Wolfgang and Lane, unpublished results). As shown in Table 1 rtPCR confirmed the disruption of the CPT1c gene in the KO mice. Importantly, compensatory changes in the expression of the other carnitine acyltransferases were not observed due to disruption of the CPT1c gene (Table 1). Likewise, no significant differences in the expression of other genes involved in fatty acid metabolic pathways resulted from knocking out the CPT1c gene (Table 1). Nor did microarray analysis of hippocampal RNA from CPT1c WT and KO revealed significant differences in the fatty acid metabolic machinery (Wolfgang and Lane unpublished results). These findings showed that the loss of CPT1c did not affect the transcription of numerous key fatty acid metabolic genes nor was there transcriptional compensation by other known CPTs.

We also determined whether knocking out the CPT1c gene altered the level of long-chain acyl-CoAs in the CNS of WT and CPT1c KO mice under fasted or fed conditions as outlined above. Were CPT1c required for the mitochondrial translocation into or metabolism of long-chain fatty acids in the hypothalamus, we would have expected the build-up of their corresponding long-chain acyl-CoAs in the CNS tissues of CPT1c KO mice. We also determined the long-chain fatty acyl-CoA levels in the hippocampus and cortex of WT and CPT1c KO mice under fasting and refed conditions (Table 2). Again, we focused on hippocampus and cortex because of their large size and relative expression of CPT1c (hippocampus > cortex). The hippocampus showed no significant difference in the long chain acyl-CoA species analyzed and further did not show differences between fasted and refed animals. This is surprising since it has been reported that fasting and refeeding can alter the long chain acyl-CoA species in the hypothalamus of rats (He et al. 2006). However, we did not directly replicate those experiments. The CPT1c KO cortex did show a

statistically significant increase in the long chain acyl-CoA species. However, since CPT1c is not expressed as high a level in the cortex as other parts of the brain such as hippocampus, and hypothalamus it is unclear if these data are physiologically relevant.

Effect of feeding a high-fat on CPT1c KO and WT mice

Preliminary experiments indicated that feeding a high-fat diet (45% kcal from fat) caused increased body weight gain by CPT1c KO mice compared to littermate WT mice. These findings were expanded to more critically test the effect of feeding a diet with higher fat content (60% kcal from fat) for a more extended period of time, i.e. 13 weeks, to both homozygous and heterozygous CPT1c KO and WT mice. As illustrated in Fig. 4A homozygous CPT1c$^{(-/-)}$ mice gained ~100% more and heterozygous CPT1c$^{(+/-)}$ mice gained ~50% more body weight than WT mice (Fig. 4A). The fact that heterozygous mice express half the amount of CPT1c as WT mice and gain an intermediate amount more body weight, indicates that CPT1c is co-dominantly expressed. It is of interest that when fed a low fat diet, there was no difference in body weight gain between CPT1c KO and WT mice ((Wolfgang et al. 2006); and data not shown).

To assess the effect of a shorter period of feeding a high-fat (60% cal from fat) diet on body weight and food intake, we placed male and female WT and CPT1c KO mice on either a low-fat or high-fat diet for two weeks. At the beginning of the study, the CPT1c KO mice weighed ~2g less than WT littermates (Fig. 4B) consistent with our previous results. After just two weeks of feeding the high-fat diet CPT1c KO mice gained much more body weight gain (~2.5-fold for males and ~2.0-fold for females) than WT littermates (Fig. 4C). Despite the fact that initial body weights were lower for the CPT1c KO mice, the differences in total body weight were substantial by the end of the experiment (Fig. 4D). Despite greater body weight gain, the KO mice ate significantly less (females) or about the same (males) amount of diet (Fig. 4E). This finding suggests a lower energy expenditure/unit of body weight gain and is consistent with our previous report (Wolfgang et al. 2006) indicating that peripheral energy expenditure of CPT1c KO mice is suppressed in comparison to WT mice. These findings suggest that CPT1c in the CNS has a regulatory role in fatty acid metabolism mice.

It should also be noted that at the end of the study all mice fed the ketogenic high-fat diet exhibited the expected changes in blood metabolites, i.e. higher free fatty acid and ketone levels (Fig. 4F). There were no, however, significant differences between the CPT1c KO and WT mice.

DISCUSSION

CPT1c, a recently discovered mitochondrial protein, which is expressed in neurons of the CNS and possesses enigmatic characteristics (Price et al. 2002). It is not required for life nor is it essential for neuronal survival. CPT1c KO mice are viable, fertile and do not suffer dire consequences upon fasting or feeding a high fat diet, conditions that lead to a shift in energy source from glucose to ketones (Wolfgang et al. 2006). CPT1c KO mice have no obvious histological abnormalities even in later life. Unlike its counterparts in liver (CPT1a) and muscle (CPT1b), CPT1c does not appear to participate directly in fatty acid oxidation in so far as we have determined to date. However, disruption of the CPT1c gene reveals a complex metabolic phenotype (e.g., the response to feeding a high fat diet) suggesting that CPT1c plays a regulatory, rather than a direct metabolic role. These and other observations are consistent with the hypothesis that CPT1c is a target of the metabolic intermediate, malonyl-CoA, to control body weight.

The expression of CPT1c is restricted to the CNS and is enriched in neural feeding centers within the hypothalamus. Therefore, CPT1c is located appropriately to play a role in energy

homeostasis. However, CPT1c is ubiquitously expressed in neurons throughout the brain with highest expression in hippocampal neurons suggesting it also serves a broader role (Price et al. 2002) (Lein et al. 2007).

The biochemical reaction that CPT1c catalyzes has been difficult to elucidate, if indeed it functions as an enzyme. CPT1c has a striking amino acid similarity to other CPTs and it is unclear from structural considerations why CPT1c does not behave as a putative member of this family. We have performed 3-dimensional modeling based on the crystal structure coordinates of the carnitine octanoyl transferase, CROT (Jogl and Tong 2003), which suggests that CPT1c possesses a binding pocket large enough to accommodate a long chain fatty acyl group. However, CPT1c does not catalyze acyl transfer to carnitine when assayed with a large array (~50) of potential acyl-CoA substrate donors (Wolfgang et al. 2006) and here we report no evidence, either *in vitro* or *in vivo*, for a role in fatty acid oxidation. In addition, we have tested several other potential acceptor substrates including serine, ethanolamine, choline and sphingosine derivatives none of which served as acceptors. It is still possible, of course, that a unique substrate exists for a CPT1c-catalyzed enzymatic reaction. However, we believe it unlikely that we have missed a CPT1c substrate involved in fatty acid oxidation given the extensive *in vitro* and *ex vivo* analyses reported in the present paper. More likely explanations are that CPT1c functions in a non-enzymatic process or that the reaction carried out has unique or restrictive activation requirements (e.g. allosteric or covalent modification) that were not satisfied.

Malonyl-CoA is an allosteric inhibitor of CPT1a and CPT1b, which catalyze the initiating step of fatty acid oxidation (McGarry et al. 1977; McGarry et al. 1978; McGarry and Foster 1980; McGarry 1995a, b). Malonyl-CoA is the product of the ACC catalyzed carboxylation of acetyl-CoA and serves as the basic chain elongation unit for fatty acid synthase. Inhibition of CPT1 by malonyl-CoA ensures that fatty acid oxidation and fatty acid synthesis does not occur concomitantly in cell types where both processes coexist. Therefore, we reasoned that CPT1c might be inhibited by malonyl-CoA in an analogous manner. Indeed, CPT1c does bind to malonyl-CoA tightly within the dynamic concentration range that occurs in the hypothalamus (Wolfgang et al. 2006), but since an enzymatic reaction has not been discovered, it is possible that malonyl-CoA binding has another role. Nevertheless, it is of great interest that the CPT1c KO produces a phenotype expected of a target protein.

The fatty acid biosynthetic pathway in the hypothalamus has clearly been shown to be involved in body weight regulation from both a pharmacological and genetic perspective (Wolfgang and Lane 2006b). The nexus to these systems appears to be the regulation of malonyl-CoA level. Malonyl-CoA concentration is regulated in large part by the rate of synthesis by ACC, which is in turn regulated/inhibited by phosphorylation/inhibition by 5’-AMPK, a cellular sensor of changes in the [AMP]/[ATP] ratio which inhibits several biosynthetic enzymes in times of energy deficit (Hardie 2004; Kahn et al. 2005). Consistent with malonyl-CoA as a mediator of energy homeostasis (Wolfgang and Lane 2006a) several laboratories have now shown that modulation of AMPK leads to coordinated changes in food intake (Andersson et al. 2004; Minokoshi et al. 2004).

Elevation of hypothalamic malonyl-CoA by the inhibition of FAS rapidly inhibits food intake and increases energy expenditure (Wolfgang and Lane 2006a). Another non-lipogenic tissue that utilizes malonyl-CoA as a signaling intermediate is skeletal muscle. Muscle utilizes malonyl-CoA not for the elongation of fatty acids, since muscle has little to no FAS, but rather to regulate CPT1b and thereby, fatty acid oxidation (Cha et al. 2006). This constitutes a precedent for malonyl-CoA as a signaling intermediate.

Another signaling protein that requires anchorage to the mitochondrial outer membrane to be active is MAVS. MAVS functions to enable IFN signaling via Nfκb activation after viral infection (Seth et al. 2005). Several viruses produce peptidases to evade this response by clipping MAVS from the mitochondria (Li et al. 2005). It is not known why this signaling protein requires association with the mitochondria, but it is clear that anchorage specifically to mitochondria is required.

We suggest that CPT1c is anchored to the mitochondria because it plays a role in fatty acid metabolism, however, to date we have been unable to identify biochemical alterations in fatty acid metabolism in CPT1c KO animals. CPT1c KO animals do not appear to accumulate long chain fatty acyl-CoAs in the hypothalamus or the CNS (Table 2) or free fatty acids (data not shown). Elucidation of the biochemical reaction(s) in which CPT1c participates will be necessary to understanding how nutrient signaling is mediated.

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Abbreviations

FAS	fatty acid synthase
CPT	carnitine palmitoyl-transferase
ACC	acetyl-CoA carboxylase
CNS	central nervous system
SCD	stearoyl-CoA desaturase
AICAR	5-Aminoimidazole-4-carboxamide-1-b-D-ribofuranoside
PMA	Phorbol 12-myristate 13-acetate
8-Br cAMP	8-Bromo camp

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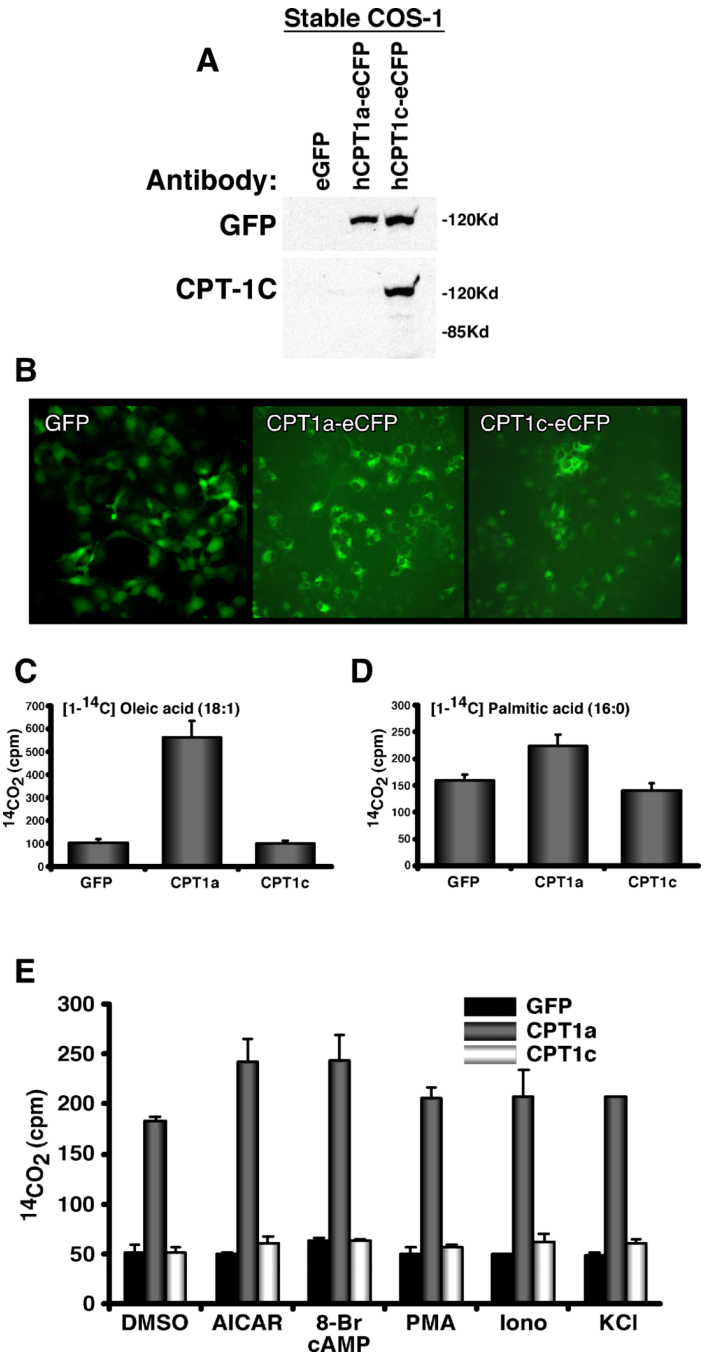


Figure 1.

Oxidation of fatty acids by CPT1a and CPT1c in stably transfected Cos-1 cells. (A) Western blot using anti-GFP or anti-human CPT1c antibodies. Expression of CPT1a and CPT1c carboxy-terminal eCFP fusion proteins are detected in lysate of stably expressing Cos-1 cells. (B) Epifluorescent images of cells expressing fusion proteins (20× objective). (C, D) Two hour oxidation of [1-^{14}C] fatty acids (oleic or palmitic) to $^{14}CO_2$ by the stably transfected cos-1 cells (51mCi/mmol; total CPM added: 310,000). N=4. (E) Stably expressing Cos-1 cells were pretreated for 1 hour by AICAR (2.5mM), camp (2.5mM) PMA (10μM), Ionomycin (1μM), or KCL (60mM), and oxidation of [1-^{14}C] oleic acid to $^{14}CO_2$

was measured for 2 hours (51mCi/mmol; total CPM added: 310,000). Experiments performed in triplicate.

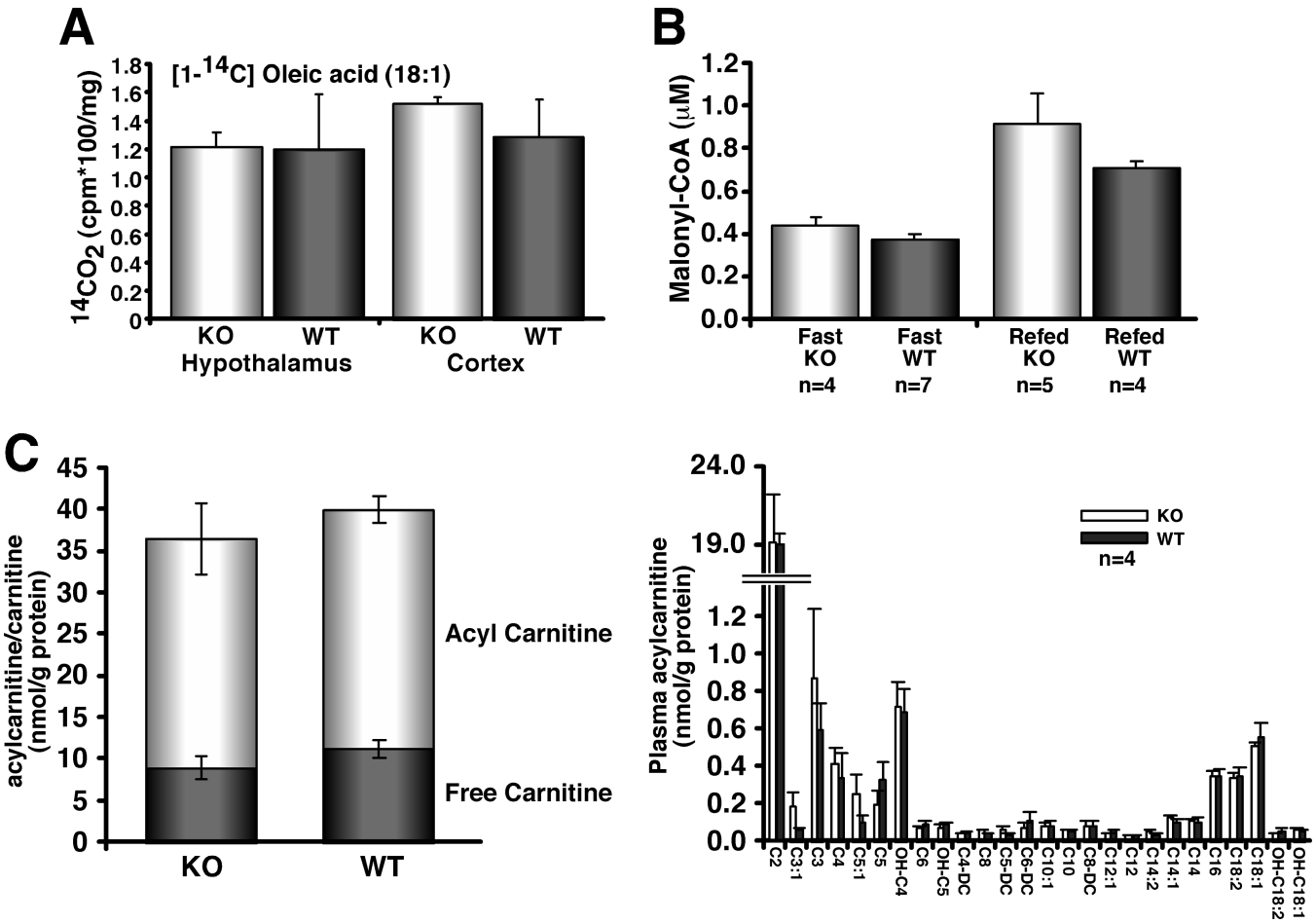
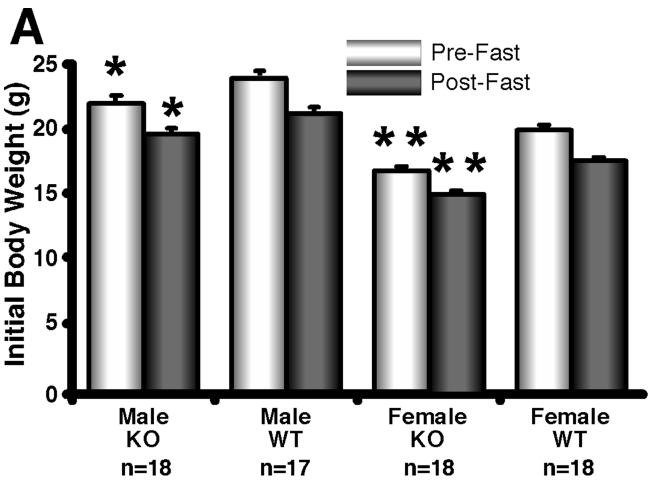
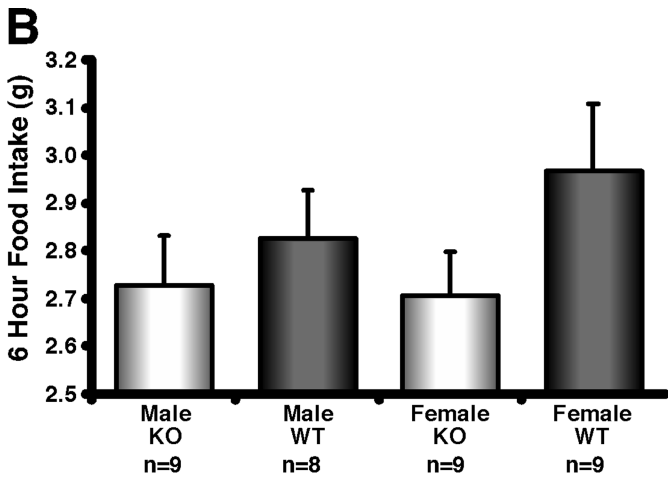


Figure 2.

Effect of CPT1c deletion on the metabolism of fatty acids *in vivo*. (A) CPT1c KO or WT littermates were fasted overnight for 24 hours, euthanized and hypothalamus and cortex were rapidly dissected and oxidation of [1-^{14}C] oleic acid (51mCi/mmol; total CPM added: 310,000) to $^{14}CO_2$ was measured for 2 hours (n=4). (B) CPT1c KO or WT littermates were fasted overnight for 24 hours or fasted for 24 hours and refed standard chow for 2 hours. The hypothalamus was collected and malonyl-CoA concentration was measured by the malonyl-CoA recycling assay. (C) CPT1c KO or WT littermates were fasted for 24 hours, euthanized and plasma was collected and analyzed by mass spectrometry for carnitne and acylcarnitine species (n=4).



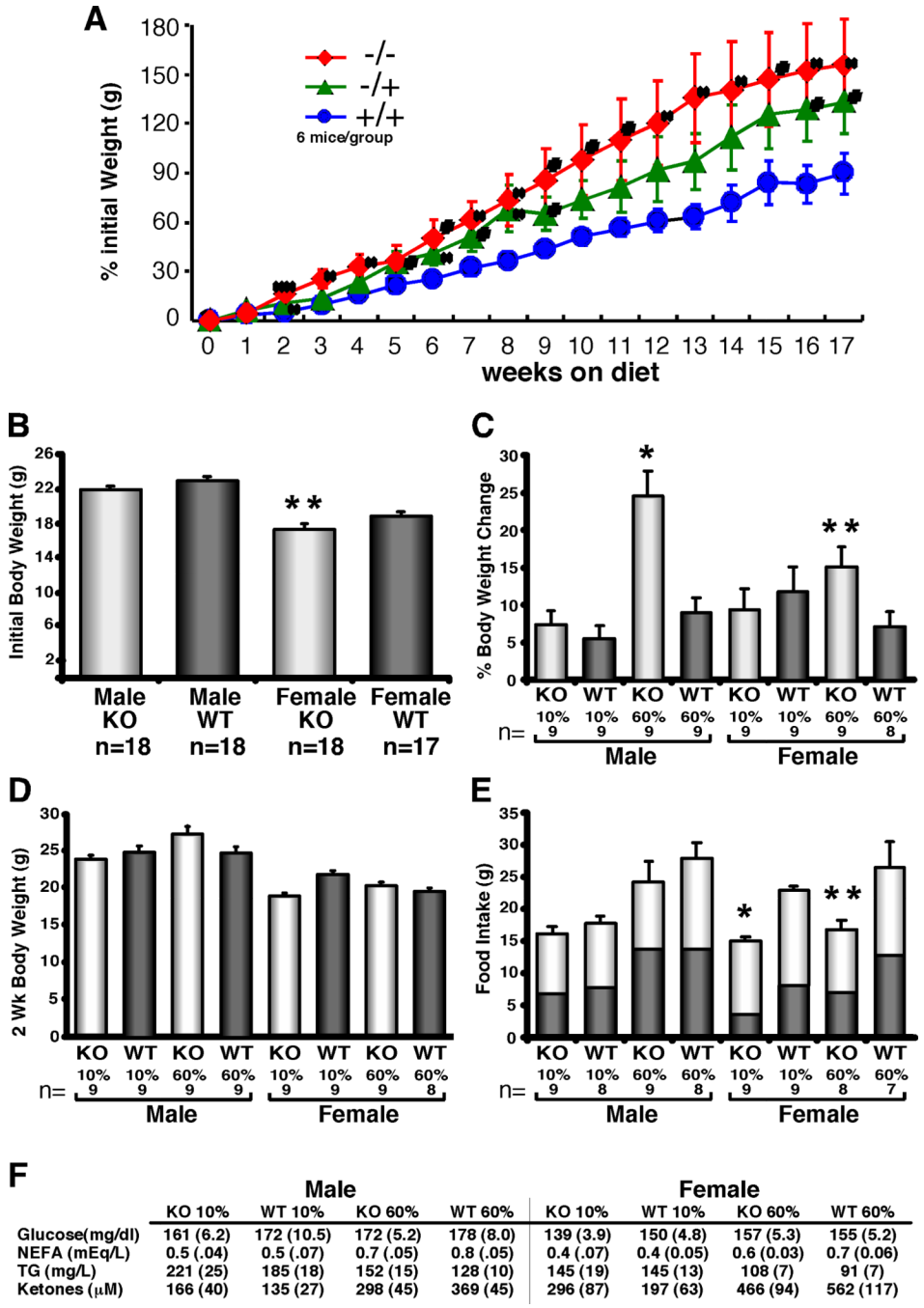


C

	Male				Female			
	KO Fasted	WT Fasted	KO Refed	WT Refed	KO Fasted	WT Fasted	KO Refed	WT Refed
Glucose(mg/dl)	118 (5.7)	119 (4.2)	156 (7.4)	157 (8.6)	99 (7.2)	115 (3.7)	143 (4.5)	158 (7.3)
NEFA (mEq/L)	0.6 (.1)	0.7 (.1)	0.3 (.02)	0.3 (.03)	1.2 (.1)	0.9 (.1)	0.3 (.02)	0.3 (.02)
TG (mg/L)	59 (5.8)	56 (6.8)	150 (9.4)	164 (10)	74 (5.9)	75 (4.8)	168 (17)	222 (19)
Ketones (μM)	643 (81)	520 (113)	209 (22)	214 (14)	1316 (163)	784 (64)	214 (12)	231 (9)

Figure 3.

Effect of fasting and refeeding on CPT1c KO mice. (A) CPT1c KO and WT mice were weighed and fasted for 24 hrs and weighed again. (B) Food intake of CPT1c KO and WT mice over a 6 hour period after a 24 hour fast. (C) Physiological plasma parameters of CPT1c KO and WT mice were determined under fasted (30 h) and refed conditions. (* $p < 0.01$; ** $p < 0.001$)



F

	Male				Female			
	KO 10%	WT 10%	KO 60%	WT 60%	KO 10%	WT 10%	KO 60%	WT 60%
Glucose(mg/dl)	161 (6.2)	172 (10.5)	172 (5.2)	178 (8.0)	139 (3.9)	150 (4.8)	157 (5.3)	155 (5.2)
NEFA (mEq/L)	0.5 (.04)	0.5 (.07)	0.7 (.05)	0.8 (.05)	0.4 (.07)	0.4 (0.05)	0.6 (0.03)	0.7 (0.06)
TG (mg/L)	221 (25)	185 (18)	152 (15)	128 (10)	145 (19)	145 (13)	108 (7)	91 (7)
Ketones (μM)	166 (40)	135 (27)	298 (45)	369 (45)	296 (87)	197 (63)	466 (94)	562 (117)

Figure 4.

Effect of long- and short-term high fat feeding on CPT1c KO mice. (A) Effect of a high fat diet (60% kCal from fat) on long term weight gain of CPT1c KO, Heterozygous KO and WT female mice n=6/group. (B) Initial body weight of mice on normal chow. (C) % body weight change after two weeks on a high fat diet. (D) Mean body weight after two weeks on a high fat diet. (E) food intake for the first (black bar) and second (gray bar) weeks of diet. (F) Physiological plasma parameters in CPT1c KO and WT mice fed various diets for two weeks.

Table 1

Real-time RT-PCR analysis of WT and CPT1c KO mRNA in the hippocampus of mice fasted for 24h or fasted then refed for 6h.

	WT		Cpt1c KO	
Gene	**Fasted**	**Refed**	**Fasted**	**Refed**
carnitine transferase				
Crat	1.0 [.06]	1.1 [.1]	−1.1 [.05]	1.0 [.02]
Crot	1.0 [.1]	−1.8 [.08]	−1.4 [.1]	−2.1 [.04]
Cpt1a	1.0 [.1]	−1.1 [.1]	−1.3 [.08]	−1.6 [.03]
Cpt1c	1.0 [.08]	1.0 [.08]	−4.2 [.01]	−4.4 [.02]
Cpt2	1.0 [.2]	−1.3 [.1]	1.2 [.1]	−1.5 [.06]
cytosolic thioesterase				
Acot1	1.0 [.1]	1.1 [.2]	−1.1 [.05]	−1.0 [.08]
Acot7	1.0 [.07]	1.4 [.07]	−1.2 [.03]	−1.0 [.07]
fatty acid biosynthesis				
Acaca	1.0 [.2]	1.0 [.2]	−1.2 [.1]	−1.1 [.2]
Acacb	1.0 [.2]	−1.7 [.1]	1.1 [.1]	−3.6 [.02]
Fasn	1.0 [.09]	−1.1 [.1]	−1.0 [.07]	−1.1 [.08]
Scd1	1.0 [.1]	1.1 [.07]	−1.1 [.09]	−1.0 [.1]
Scd2	1.0 [.1]	1.1 [.1]	−1.7 [.07]	1.0 [.1]
Scd3	1.0 [.2]	−1.2 [.1]	−1.7 [.04]	−1.3 [.2]
fatty acid oxidation				
Acadm	1.0 [.05]	1.1 [.09]	1.0 [.1]	−1.0 [.04]
Acadl	1.0 [.07]	−1.2 [.02]	−1.4 [.1]	−1.5 [.01]
Acadvl	1.0 [.07]	1.1 [.07]	−1.2 [.08]	−1.1 [.05]
fatty acid regulation				
Mlycd	1.0 [.1]	1.2 [.2]	−1.1 [.1]	1.1 [.2]
acyltransferase				
Agpat	1.0 [.01]	1.1 [.08]	−1.2 [.05]	−1.0 [.07]
carnitine transport				
Slc25a20	1.0 [.1]	−1.2 [.08]	1.7 [.03]	−1.1 [.1]
Slc25a29	1.0 [.07]	1.2 [.1]	−1.2 [.03]	−1.0 [.09]
Slc22a5	1.0 [.09]	1.1 [.06]	−1.1 [.1]	−1.0 [.08]
ketone utilization				
Bdh1	1.0 [.05]	−1.1 [.08]	−1.1 [.05]	1.2 [.05]
Bdh2	1.0 [.2]	1.2 [.2]	1.3 [.2]	1.2 [.2]
Oxct1	1.0 [.05]	−1.0 [.05]	−1.2 [.07]	−1.1 [.09]
Oxct2	1.0 [.1]	−1.2 [.1]	1.7 [.1]	−1.1 [.09]
Oxct2b	1.0 [.2]	−1.1 [.1]	1.9 [.2]	−1.1 [.09]
Acaa1a	1.0 [.2]	−1.1 [.1]	1.8 [.3]	−1.1 [.2]
Acaa1b	1.0 [.09]	−1.0 [.05]	−1.4 [.1]	−1.3 [.02]
Acaa2	1.0 [.2]	−1.2 [.1]	−1.3 [.1]	−1.8 [.09]

	WT		Cpt1c KO	
Gene	**Fasted**	**Refed**	**Fasted**	**Refed**
misc.				
CD36	1.0 [.1]	−1.0 [.05]	1.4 [.05]	−1.1 [.08]
Fabp7	1.0 [.1]	1.9 [.2]	1.1 [.05]	1.9 [.07]
Ucp2	1.0 [.1]	−1.0 [.1]	−1.3 [.09]	−1.4 [.04]

Fold-induction compared to WT fasted [standard deviation] n=4/group

TABLE 2

Long Chain Acyl-CoA concentration nmol/g tissue [standard error mean] n=4

Sample	C16:0	C16:1	C18:0	C18:1	C18:2	C18:3
Hippocampus						
WT fast	1.88 [0.15]	0.21 [0.02]	3.36 [0.28]	3.19 [0.19]	0.87 [0.10]	0.70 [0.08]
KO fast	2.08 [0.16]	0.23 [0.02]	3.42 [0.25]	3.65 [0.37]	0.99 [0.07]	0.77 [0.07]
WT refed	1.83 [0.07]	0.16 [0.01]	3.44 [0.12]	3.23 [0.18]	0.60 [0.04]	0.71 [0.02]
KO refed	1.87 [0.09]	0.16 [0.01]	3.26 [0.24]	3.34 [0.17]	0.60 [0.03]	0.60 [0.03]
Cortex						
WT fast	1.68 [0.05]	0.12 [0.01]	3.07 [0.09]	2.72 [0.10]	0.55 [0.06]	0.41 [0.04]
KO fast	*2.36 [0.20]	**0.18 [0.02]	**3.66 [0.11]	**3.70 [0.31]	**0.85 [0.09]	**0.59 [0.06]
WT refed	1.65 [0.16]	0.11 [0.02]	3.00 [0.23]	2.67 [0.23]	**0.38 [0.03]	0.34 [0.02]
KO refed	2.25 [0.21]	0.12 [0.02]	3.45 [0.27]	**3.43 [0.23]	0.47 [0.02]	*0.53 [0.05]

*=p<0.01;

**p=<0.05