Enhancing hepatic mitochondrial fatty acid oxidation stimulates eating in food-deprived mice

Abdelhak Mansouri,1 Gustavo Pacheco-López,1 Deepthi Ramachandran,1 Myrtha Arnold,1 Claudia Leitner,1 Carina Prip-Buus,2,3,4 Wolfgang Langhans,1 and Núria Morral5

1Physiology and Behavior Laboratory, Institute of Food, Nutrition, and Health, ETH Zurich, Switzerland; 2INSERM, U1016, Institut Cochin, Paris, France; 3CNRS, UMR8104, Paris, France; 4Université Paris Descartes, Sorbonne Paris Cité, Faculté de Médecine Paris, France; and 5Department of Medical and Molecular Genetics, and Center for Diabetes Research, Indiana University School of Medicine, Indianapolis, Indiana

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Enhancing hepatic mitochondrial fatty acid oxidation stimulates eating in food-deprived mice. Am J Physiol Regul Integr Comp Physiol 308: R131–R137, 2015. First published November 26, 2014; doi:10.1152/ajpregu.00279.2014.—Hepatic fatty acid oxidation (FAO) has long been implicated in the control of eating. Nevertheless, direct evidence for a causal relationship between changes in hepatic FAO and changes in food intake is still missing. Here we tested whether increasing hepatic FAO via adenovirus-mediated expression of a mutated form of the key regulatory enzyme of mitochondrial FAO carnitine palmitoyltransferase 1A (CPT1mt), which is active but insensitive to inhibition by malonyl-CoA, affects eating and metabolism in mice. CPT1mt expression increased hepatic CPT1 protein levels. This resulted in an increase in circulating ketone body levels in fasted CPT1mt-expressing mice, suggesting an increase in hepatic FAO. These mice did not show any significant changes in cumulative food intake, energy expenditure, or respiratory quotient after 4-h food deprivation. After 24-h food deprivation, however, the CPT1mt-expressing mice displayed increased food intake. Thus expression of CPT1mt in the liver increases hepatic FAO capacity, but does not inhibit eating. Rather, it may even stimulate eating after prolonged food deprivation. These data do not support the hypothesis that an increase in hepatic FAO decreases food intake.

energy homeostasis; carnitine palmitoyltransferase 1a; food intake; metabolic control of eating; liver

Because food is the only source of metabolic fuels, it is reasonable to assume that feedback from metabolism contributes to the control of eating, allowing for the maintenance of a balance between the amount of calories spent and ingested (52, 53). The nervous system continuously monitors metabolism to assess the availability of energy-providing fuels such as fatty acids and glucose and to control energy intake and expenditure accordingly. Despite open questions concerning its physiological relevance, the eating-stimulatory effect of inhibitors of glucose utilization or fatty acid oxidation (FAO) is generally considered evidence for the existence of a metabolic control of eating (12, 27, 43). FAO inhibitors such as mercaptoacetate (MA, an inhibitor of acyl-CoA dehydrogenases) (45) and etomoxir or methyl-palmoxirate (inhibitors of the carnitine palmitoyltransferase-1, CPT1) (13, 14, 19) reliably stimulate eating in laboratory mice (9, 51), rats (5, 19), and humans (18). The eating-stimulatory effect of MA was associated with a decline in circulating ketone bodies and an increase in nonesterified fatty acids (NEFA), indicating an inhibition of FAO (5, 45). Moreover, FAO inhibitors require intact abdominal vagal afferents to stimulate eating (6, 20, 29, 44), suggesting that they act in the abdominal cavity to do so. Given the major role of the liver in FAO and ketogenesis (38), the eating-stimulatory effect of FAO inhibitors was hypothesized to originate in the liver, with changes in hepatocyte ADP-to-ATP ratio and membrane potential connecting hepatocyte FAO to vagal afferent activity (4, 19, 27). So far, however, all these findings were correlative, and there is no direct evidence supporting the hypothesis that the liver is the organ where the brain senses changes in peripheral FAO.

Given the limitations of the pharmacological approach to stimulate specifically hepatic FAO, several studies adopted transfection or transduction approaches to express FAO key enzymes in particular cell types or tissues. CPT1A is the rate-limiting enzyme of mitochondrial long-chain FAO (33). It catalyzes the transfer of an acyl group to carnitine in the outer mitochondrial membrane. The acyl-carnitine complex translocates the acyl moiety into mitochondria, where FAO occurs (3, 32). CPT1A is allosterically inhibited by malonyl-CoA, a metabolite generated during de novo lipogenesis (7, 10). Transfection of human embryonic 293T kidney cells with the CPT1A cDNA increased the capacity of these cells to oxidize long-chain fatty acids (22). Moreover, adenovirus-mediated expression of a mutated form of CPT1A, CPT1mt, which is constitutively active but insensitive to inhibition by malonyl-CoA (35), increased mitochondrial FAO in cultured rat hepatocytes (1) and C2C12 muscle cells (17). A first attempt to increase hepatic FAO in vivo was reported by Stefanovic-Racic and colleagues, who overexpressed CPT1A in the rat liver using an adenovirus. They found an increase in hepatic FAO flux and a reduction in diet-induced hepatic steatosis (46). Later, others reported that adenovirus-mediated expression of the peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1-α) in the rat liver caused an increase in FAO and a reduction in hepatic steatosis as well as reduced circulating triacylglycerol levels (37). In mice, long-term expression of CPT1mt using an adenovirus (AAV) increased hepatic FAO flux and prevented diet-induced obesity and hepatic steatosis (39). The expression of CPT1mt even reversed an already established insulin resistance (34). Moreover, the CPT1mt was exclusively expressed in the liver, documenting the reliability of the adenovirus to specifically target this tissue (34).
None of these studies, however, investigated the effect of increased hepatic FAO on body energy homeostasis and eating. The present study closed this gap. To examine the role of hepatic FAO in eating behavior, we injected an adenovirus expressing CPT1mt via the tail vein in mice fed a chow diet. Fourteen days later, when the CPT1mt protein levels in the liver were presumably at their highest level (34), cumulative food intake and energy expenditure were assessed during 48 h either after 4-h or 24-h food deprivation. Overall, our results do not support the hypothesis that an increase in hepatic FAO inhibits eating.

**MATERIALS AND METHODS**

**Animals.** Male 8- to 11-wk-old C57BL/6 mice bred at the ETH-Schwerenbach laboratory animal facility were used. Before their transfer to metabolic cages, mice were group-housed in Makroten cages (22.0 × 16.0 × 14.0 cm, L × W × H) with ad libitum access to tap water and laboratory chow (4.15 kcal/g, N 3436, Kliba, Kaiseraugst, Switzerland). Mice were adapted to a 12:12 h light/dark cycle in a climate-controlled colony room (22 ± 2°C and 60% relative humidity) for 11 days (Fig. 1). All procedures were approved by the Canton of Zurich Veterinary Office.

**Adenovirus administration.** Recombinant adenovirus vectors encoding either β-galactosidase (β-gal) as a control or the CPT1M593S mutant (CPT1mt) were produced as previously described (1). Mice were injected 1 × 10⁹ infectious particles (IP) of β-gal (n = 12) or CPT1mt (n = 12) in a final volume of 300 µl sterile phosphate-buffered saline via the tail vein, as described (36) (Fig. 1). After adenovirus injections, mice were kept in a BL2 room and allowed to recover for 6 days.

**Indirect calorimetry and food intake assessment.** The mice were placed in metabolic cages to automatically monitor food intake, CO₂ release, O₂ consumption, and physical activity using the PhenoMaster (TSE systems, Bad Homburg, Germany) open-circuit calorimetry system with controlled temperature and humidity. The mice were allowed to adapt to the single cage in the metabolic cages for 8 days. On days 14 and 16 after adenovirus administration they were food deprived either for 4 or 24 h and then offered food ad libitum while the system recorded the above-mentioned parameters during the food deprivation period and the subsequent 48 h with access to food (Fig. 1).

Energy expenditure (EE) was normalized to body weight and was calculated using the following equation: EE (kcal) = (3.941 VO₂ + 1.106 VCO₂)/1,000.

**Terminal experiment.** Mice were euthanized by decapitation in the middle of the light phase on day 21 after adenovirus injection under fed or 7-h food-deprived conditions (Fig. 1). Blood was collected in 1.5-ml Eppendorf tubes, allowed to clot for 5 min, and kept on ice. After centrifugation (5 min, 2,400 g) the serum was stored at −20°C until further analysis. The liver was quickly removed, immediately deep frozen in liquid nitrogen, and stored at −80°C until further analysis.

**Metabolite analysis.** Serum levels of NEFA and β-hydroxybutyrate (BHB) were determined using an enzyme-based reaction, measuring the absorbance of the resulting metabolite using Cobas Mira analyzer (Cobas Mira; LaRoche, Basel, Switzerland) (26).

**Immunoblotting.** About 30 mg of liver tissue was lysed in RIPA buffer (radioimmuno precipitation assay buffer, 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Nonidet-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (complete EDTA-free, 04693132001, diluted 1:25 in RIPA buffer, Roche) for about 15 min and then centrifuged (4 min, 9,600 g, 4°C). The supernatant containing the proteins was transferred into new tubes, and protein content was estimated using a specific kit (DC Protein Assay, Bio-Rad). Pure water (Milli-Q) and 3 µl Laemmli buffer (1% SDS, 20% β-mercaptoethanol, 1.92 M glycine, 0.25 M Tris, 1% bromphenol blue, at pH 8.45) were used to prepare protein sample dilutions (25 µg in 30 µl), which were then subjected to heating at 95°C for 5 min. Protein separation was done by electrophoresis using a stacking gel [0.67 ml 30% acrylamide, 3.6 ml Milli-Q, 0.625 ml upper gel buffer (1 M Tris-HCl at pH 6.8), 0.05 ml 10% SDS, 50 µl 10% ammonium persulfate (APS), 5 µl tetramethylthiylenediamine (TEMED)] and a separating gel [4 ml 30% acrylamide, 3.3 ml MilliQ, 2.5 ml lower gel buffer (1.5 M Tris-HCl at pH 8.8), 0.1 ml 10% SDS, 100 µl 10% APS, 6 µl TEMED]. The proteins were transferred to a 0.2-µm nitrocellulose membrane using electrophoresis at 90 V in transfer buffer (50 mM Tris base, 380 mM glycine, 0.1% SDS, 20% methanol). Then the membrane was washed with distilled water and blocked with 5% fat-free milk in TBST [0.1% Tween 20 in TBS (8.0 g NaCl, 0.2 g KCl, 1.15 g NaHPO₄, 0.2 g KH₂PO₄ in 1 liter of distilled water at pH 7.4)]. The membrane was incubated with the primary antibody [anti-CPT1A (42) that recognizes both endogenous CPT1A and the mutated form of CPT1, CPTmt; anti-carnitine palmitoyltransferase 2 (CPT2) (1); anti-AMPKα (Cell Signaling Technology); anti-p-AMPKαThr172 (Cell Signaling Technology); or anti-β-actin (Sigma)] of the desired protein in milk solution (5% milk in TBST) overnight at 4°C. After being washed with TBST three times for 10 min each, the membrane was incubated with the appropriate horseradish peroxidase-linked secondary antibody (anti-mouse or anti-rabbit) against the primary

![Fig. 1. Experimental design.](http://ajpregu.physiology.org/)
antibody in the milk solution for 1 h at room temperature. The membrane was washed again with TBST and the band revealed using detection reagents (chemiluminescence-based detection using luminol and H₂O₂) and analyzed using ImageQuant LAS 4000 mini (ImageQuant, GE Health Care).

Statistical analysis. The data are presented as means ± SE. Depending on the type of experiments, the significant differences (P < 0.05) were tested either using Student’s t-test or one-way analysis of variance (ANOVA) followed by the Newman-Keuls test. The statistical analysis and the graph design were performed using GraphPad Prism software (Version 5).

RESULTS

Effect of adenovirus-mediated expression of CPT1mt in the liver on eating and energy homeostasis. Based on a previous study using the same adenoviral vector and dose, we assumed that the highest level of CPT1mt expression was achieved after 16 days of adenovirus administration and used this time point for our calorimetry studies (34). Mice were food-deprived for 4 h before the beginning of the dark cycle. The recording of cumulative food intake during the subsequent 48 h did not reveal any significant difference between groups (Fig. 2). To examine the impact of a longer fast, food was withdrawn for 24 h. Remarkably, this longer period of food deprivation induced a significant increase of cumulative food intake in CPT1mt-expressing mice compared with β-gal-expressing (control) mice (Fig. 2).

Energy expenditure and respiratory exchange ratio (RER) after 4 h of food deprivation did not reveal any significant difference between CPT1mt and β-gal-treated mice (Fig. 3, A and B). Both groups displayed RER peak values close to 1.0 during the dark cycle, when mice are more active and eat more (and carbohydrate is the predominant fuel source), and a decrease to ~0.85 during the light cycle, when activity and food intake decrease and fatty acids are also used as fuel. Twenty-four hours of food deprivation reduced the RER during the fasting period in both groups, with a tendency of a greater decrease in CPT1mt-treated mice, which may reflect a higher breakdown and fatty acid release from the adipose tissue. Instead, in the β-gal-expressing animals, the RER gradually decreased to ~0.85 during the same period, similar to what was observed after 4 h food deprivation (Fig. 3A).

Adenovirus-mediated expression of CPT1mt in the liver increased the expression of CPT1A protein. At the end of the experiment, immunoblotting analysis of liver tissue was performed to confirm CPT1A overexpression. In mice treated with adeno-CPT1mt, expression of CPT1A (but not CPT2) increased, both in the fed state and after 7 h of food deprivation, indicating that the overall amount of CPT1A was indeed higher than in β-gal-expressing control animals in both states. Interestingly, the upregulation of CPT1A protein expression was associated with an increase in the phosphorylation of the key regulatory enzyme of cellular energy homeostasis AMPK (p-AMPK) only in the fed condition, suggesting a change in fuel flux during a period when CPT1A is normally not active (Fig. 4).

Adenovirus-mediated expression of CPT1mt in the liver increased circulating ketone bodies and NEFA. The analysis of circulating levels of BHB as an indicator of FAO and ketogenesis, or NEFA as an indicator of lipolysis, did not reveal any difference between the CPT1mt and β-gal-treated (control) mice in the fed state (Fig. 5, A and B). Seven hours of food deprivation did, however, increase the plasma levels of NEFA in both groups, suggesting a similar degree of triglyceride breakdown and fatty acid release from the adipose tissue. Serum BHB increased significantly in both groups, as expected. However, the CPT1mt-transfected animals displayed higher levels of BHB, as anticipated from increased FAO levels.

DISCUSSION

The hypothesis of a relationship between peripheral FAO and eating is mainly based on the capacity of peripherally administered FAO inhibitors, such as MA (27) and methyl palmitoxirate (13, 20) or etomoxir (19), to stimulate eating. Given the high FAO capacity of the liver and the dependence of the behavioral effect on intact abdominal vagal afferents, it was hypothesized that an eating-modulatory signal is derived from hepatic FAO and relayed to the brain via vagal afferents (14, 27). Attempts to investigate the effects of an increase in peripheral FAO on eating are sparse and mainly based on the use of pharmacological agonists of the peroxisome proliferator-activated receptor-α (PPAR-α) (15, 24). Peripheral PPAR-α ac-
Activation did indeed inhibit eating in several studies in rats (15, 23, 24, 40, 41). Moreover, Ji et al. (23) showed that the reduction in food intake, weight gain, and adiposity produced by fenofibrate in obesity-prone rats was associated with an increase in the expression of CPT-1A mRNA in the liver, suggesting a relationship between increased hepatic FAO and the inhibition of eating. This finding does not, however, exclude another or an additional site of action because CPT-1 mRNA levels were measured only in the liver. Furthermore, an increased expression at the mRNA level does not always translate into an increase at the protein level, and other studies that attempted to localize the effect of PPAR-α appeared to indicate that the observed inhibition of eating was not due to a stimulation of hepatic FAO (5, 13, 31). Friedman and colleagues (13) reported that both a high and a low dose of methyl palmitate inhibited hepatic FAO and induced a decline in circulating BHB levels, but only the high dose stimulated eating, indicating that a decrease in hepatic FAO per se is not sufficient to stimulate eating. Instead, the authors interpreted their findings as evidence for a relationship between changes in hepatic energy metabolism and changes in eating (13). Our results extend these findings by indicating that also a stimulation of hepatic FAO is not sufficient to inhibit eating. Furthermore, we used a different approach based on the use of adenovirus to stimulate hepatic FAO by expressing a mutated form of CPT1A that is no longer controlled by malonyl-CoA (1, 17, 34, 35) in the liver. We chose this particular mutated form of CPT1 to also avoid the usual reciprocal interaction between FAO and glucose metabolism. Indeed, the adenovirus-mediated expression of CPT1mt in the liver of mice effectively increased hepatic FAO also in mice fed chow diet (rich in carbohydrate and low in fat) (34). The adenovirus was used as gene transfer vehicle because it specifically targets the liver and does not transduce brain, heart, skeletal muscle, or white adipose tissue after intravenous administration (11, 34). With the use of this approach an increase in CPT1A protein levels in the liver was achieved. This is likely to have only moderately raised hepatic FAO, given that serum ketone bodies did not increase.

Fig. 3. Adenovirus-mediated expression of CPT1mt in the liver did not affect body energy homeostasis. Mice were treated as described in Fig. 2. Oxygen consumption (VO₂) and CO₂ (VCO₂) release were assessed using an automatic system for 48 h, 14 days after transfection and after 4 or 24 h food deprivation (A, B), as well as during the 24-h food deprivation period (C). Data are reported as means ± SE. *P < 0.05.
increase in the fed state, although they were significantly higher in the CPT1mt group after food deprivation. These data are in agreement with a previous study that used the same adenovirus vector and dose to treat hepatic steatosis (34). Indeed, in this previous study, the adenovirus-mediated expression of CPT1mt in the liver did increase the expression of CPT1A at the mRNA and protein levels as well CPT1 activity and FAO flux (34).

The assessment of cumulative food intake and energy homeostasis after a short (4 h) period of food deprivation did not reveal any differences in eating or energy homeostasis between CPT1mt and β-gal-expressing mice, despite the significant increase in the hepatic expression of CPT1A in the CPT1mt-treated animals. Twenty-four hours of food deprivation did, however, increase food intake as well as the RER in CPT1mt-expressing mice, without significantly affecting energy expen-
diture. This raises the question of why the enhancement of hepatic FAO caused an increase rather than a decrease in food intake in CPT1mt-treated mice? At present we can only speculate about the underlying mechanisms. Perhaps the specific increase in hepatic FAO caused a decrease in FAO in another organ with the critical sensors. This assumption is indirectly supported by the fact that RER was increased during the light phase, indicating that despite an increase in hepatic FAO whole body FAO was decreased. Another possible explanation is, of course, that changes in peripheral FAO, independent of the organ or location where these changes occur, have no effect on food intake, and that the expression of CPT1mt in the liver stimulated eating after 24 h food deprivation through the production of a metabolite (perhaps a ketone body) or some other unknown mechanism.

Our results also raise the question of whether the liver is truly a site of control of eating. Several studies using metabolic inhibitors failed to find a clear relationship between changes in hepatic FAO and eating (25, 28). Friedman and colleagues (13) proposed that hepatic energy status rather than FAO per se provides an eating-modulatory signal. Recent reports using pharmacological PPAR-α activation found an increase in FAO and ketogenesis in the intestine, specifically in the jejunum, but not in the liver (24). Other studies also have shown that peripheral administration of PPAR-α agonists stimulated intestinal rather than hepatic FAO (49, 50). In addition, the FAO inhibitors with an eating-stimulatory effect do require intact abdominal vagal afferents to increase food intake (6, 30). Hepatic parenchyma does, however, scarcely contain vagal afferent fibers (2). It is therefore unclear how changes in hepatocyte energy state or FAO should affect vagal afferent signaling. On the other hand, the small intestine is the primary site exposed to all dietary nutrients, including fats. Enterocytes need energy for nutrient absorption, and the intestine is densely innervated by the vagus and the splanchnic nerves (16). Therefore, it seems reasonable to speculate that intestinal rather than hepatic FAO may influence eating.

Our findings do not argue against the possibility that metabolites including fatty acids may influence eating by acting in
the hepatic portal vein. In fact, almost 30 years ago Friedman and colleagues convincingly demonstrated that low doses of glucose reduced food intake after infusion into the hepatic portal vein, but not after infusion into the jugular vein (47, 48). This effect is presumably mediated by glucose-sensitive vagal afferents terminating in the wall of the hepatic portal vein. Several years ago some of us observed that hepatic portal vein infusion of caprylic acid reduced food intake more potently than infusion of the same dose of caprylic acid into the vena cava (21). Although this effect was accompanied by an increase in circulating ketone bodies reflecting an increase in hepatic FAO, these findings were only correlative, and the effect might well have been due to a direct action of the caprylic acid on G protein-coupled receptors expressed on hepatic portal vein vagal afferents (8).

In short, our present findings do not support the hypothesis that enhancing hepatic FAO reduces food intake. We used an approach that targets specifically the liver to increase mitochondrial FAO. Nevertheless, and despite the clear evidence for a stimulation of hepatic FAO, we did not see any decrease in food intake. Rather, food intake was increased. The results of the present study do not exclude, however, a possible role of hepatic FAO in the control of eating, at least during prolonged food deprivation, but not in line with the original hypothesis (45), and they seem to exclude hepatic FAO as a key player in the control of eating in the fed state.

Perspectives and Significance

Given the controversial discussion about the role of dietary fat in a healthy diet and the evidence for a contribution of high dietary fat levels to the development of obesity, it is important to ask how ingested fat can influence eating. Hepatic FAO has been implicated in the control of eating for almost 30 years. The present study shows that specifically enhancing hepatic FAO does not reduce food intake, as was expected based on previous interpretations of the eating-stimulatory effect of FAO inhibitors after peripheral administration. Although recent findings suggest that the FAO inhibitor MA can affect eating through a direct effect on vagal afferents (8), substantial evidence still indicates that an inhibition of peripheral FAO stimulates (14, 19), whereas a pharmacological stimulation of peripheral FAO inhibits (24) eating. The present findings strongly indicate that the pertinent sensors are not located in the liver. Recently, the small intestine has been proposed as a possible location, but this hypothesis still awaits experimental proof. The here-observed stimulation of eating in mice with enhanced hepatic FAO capacity after a long period of food deprivation also raises the question about the mechanism involved in this surprising result. Thus further studies are required to understand the effects of peripheral FAO on eating under different experimental conditions.

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Current address for G. Pacheco-López: Health Sciences Department, Lerma Campus, Metropolitan University (UAM), Lerma, Mexico; Current address for C. Leitner: London School of Medicine and Dentistry, Queen Mary University of London, London, UK.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: A.M., G.P.-L., C.L., C.P.-B., W.L., and N.M. conceived and designed the research; A.M., G.P.-L., D.R., C.L., and N.M. analyzed data; A.M., C.P.-B., W.L., and N.M. interpreted results of experiments; A.M. prepared figures; A.M. drafted manuscript; A.M. edited and revised manuscript; G.P.-L., D.R., M.A., C.L., and N.M. performed experiments; C.P.-B., W.L., and N.M. approved final version of manuscript.

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