Doctoral Thesis

Fatty acid metabolism and control of food intake

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Fatty Acid Metabolism and Control of Food Intake

A Dissertation submitted to the
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1. Summary

The major hypothesis for the metabolic control of eating posits that eating is inversely related to the rate of fuel oxidation. In line with this idea, blockade of fatty acid oxidation (FAO) by peripheral administration of inhibitors of FAO stimulates feeding in many species, including humans. Even though it is not proven yet that the liver is involved in this feeding-stimulatory effect, several findings suggest that this is the case. Yet, whether an increased hepatic FAO inhibits feeding is unknown. Therefore, the central aim of this thesis was to determine whether enhanced hepatic FAO reduces food intake.

In the first study, we infused the long chain fatty acid (LCFA) oleic acid (OA) and the medium chain fatty acid (MCFA) caprylic acid (CA) into the hepatic portal vein (HPV) of male rats to assess whether HPV infusion of these two fatty acids reduces food intake and whether their relative efficacy differs. MCFA are oxidized faster in the liver because they are absorbed directly into the hepatic portal vein and because they can enter the mitochondria independent of the enzyme carnitine-palmitoyl-transferase 1 (CPT 1). Hence, we hypothesized that HPV CA will have a greater feeding-inhibitory potency than HPV OA. This hypothesis was not supported. Rather, 6-h HPV infusion of 14 μg/min OA produced a robust inhibition of feeding, whereas a dose of CA that was 14 times larger (200 μg/min) than that of OA failed to have any effect on feeding. OA (14 μg/min) and an 80-fold greater dose of CA (1100 μg/min) inhibited feeding similarly. These findings indicate that increased hepatic FAO is unlikely to be responsible for the feeding-inhibitory effect of HPV OA. The plasma concentrations of the liver enzymes γ-glutamyltransferase (γ-GT) and alanine aminotransferase (ALT), the pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), and the stress hormone corticosterone (Cort) all remained in the physiological range after HPV OA, indicating that liver toxicity was also not the cause of HPV OA’s potent effect on feeding.
Summary

In the second study, 90-min, dark onset HPV infusion of CA (2.3 mg/min) after 18-h food-deprivation reduced the size of the first meal about 40% and reduced 24-h food intake by about 13%. The feeding-inhibitory effect of CA originated in the liver because identical infusions of CA into the vena cava did not affect food intake. The postprandial decreases in plasma free fatty acids (FFA) and β-hydroxybutyrate (BHB) were attenuated in HPV CA-treated rats, indicating that hepatic FAO was increased in HPV CA infused rats relative to controls. This is consistent with the hypothesis that an increased hepatic FAO reduces food intake, but does not prove that there is a causal link between increased hepatic FAO and reduced food intake. Further the finding that animals started eating after food deprivation although their hepatic FAO was enhanced clearly demonstrates that beside hepatic FAO other mechanisms must be involved in the regulation of food intake.

A conditioned taste aversion test was done in separate rats. Some, but not all, rats displayed aversions to saccharine paired with HPV CA infusions, but there was no significant association between the feeding-inhibitory effect of CA on the conditioning day and saccharine intake on the test day. Plasma concentrations of IL-6, TNF-α, γ-GT, ALT and Cort stayed at basal levels. Taken together, these data suggest that the feeding-inhibitory effect of CA was not due only to aversion or toxicity.

In the third study we wanted to develop an alternative approach to test the hypothesis that increased hepatic FAO can inhibit feeding behavior. We used transgenic methods to increase the expression of CPT 1α, an enzyme which catalyzes the rate limiting step of hepatic FAO, in the 293T human embryonic kidney cell line. Activation of the CPT 1α transgene through an inducible tet-on gene expression system increased mitochondrial long-chain fatty acid oxidation about 6-fold. Increasing palmitic acid concentration, however, decreased viability of CPT 1α over-expressing cells, and CPT 1α over-expression increased cell death. These data encourage the use of this transgenic system for future in-vivo studies of the roles of fatty acid oxidation in the control of metabolism and energy balance.
Zusammenfassung

2. Zusammenfassung


In der ersten Studie infundierten wir die langkettige Fettsäure (LCFA) Ölsäure (OA) und die mittelkettige Fettsäure (MCFA) Caprylsäure (CA) in die hepatische Pfortader (HPV) von männlichen Ratten, um zu untersuchen, ob die HPV Infusion dieser beiden Fettsäuren die Nahrungsaufnahme reduziert und ob diese sich in ihrer relativen Wirksamkeit unterscheiden. MCFA werden in der Leber schneller oxidiert, da sie direkt in die HPV resorbiert werden und da sie unabhängig von Carnitin-Palmitoyl-Transferase 1 (CPT 1) in die Mitochondrien eintreten können. Wir vermuteten deshalb, dass CA nach HPV Infusion den Verzehr stärker reduziert als OA.

Diese Annahme wurde jedoch nicht bestätigt. Vielmehr erzeugte eine 6-stündige HPV Infusion vom 14 µg/min OA eine starke Hemmung der Nahrungsaufnahme, wohingegen eine 14-mal höhere CA Dosis (200 µg/min) keinen Effekt auf die Nahrungsaufnahme ausübte. OA (14 µg/min) und eine 80-fach höhere CA Dosis (1100 mg/min) hemmten die Nahrungsaufnahme ähnlich stark. Nach diesen Ergebnissen ist es unwahrscheinlich, dass eine erhöhte hepatische FAO für den verzehrsreduzierenden Effekt von OA verantwortlich ist. Die Plasmakonzentrationen der Leberenzyme γ-Glutamyl-Transferase (γ-GT) und Alanin-Aminotransferase (ALT), der proinflammatorischen Zytokine Interleukin-6 (IL-6) und Tumor-Nekrosis-Faktor-α
Zusammenfassung

(TNF-α) und des Stresshormons Corticosteron (Cort) blieben alle nach der HPV OA Infusion im physiologischen Bereich. Demzufolge war auch ein toxischer Effekt von OA auf die Leber nicht der Grund des starken verzehrsreduzierenden Effektes.

In der zweiten Studie reduzierte eine 90-minütige HPV Infusion von CA (2.3 mg/min), die nach einer 18-stündigen Fastenperiode mit Beginn Dunkelphase startete, die Größe der ersten Nachtmahlzeit um 40% und die 24-h Futteraufnahme um 13%. Der verzehrsreduzierende Effekt von CA ging von der Leber aus, da die gleiche CA Infusion in die Vena Cava die Nahrungsaufnahme nicht beeinflusste. Der postprandiale Abfall der freien Fettsäuren (FFA) und des β-Hydroxybutyrates (BHB) im Plasma war in den HPV CA behandelten Ratten schwächer, was darauf hindeutet, dass die hepatische FAO in HPV CA Infundierten im Vergleich zu den Kontrolltieren erhöht war. Dies stimmt mit der Hypothese überein, dass eine erhöhte hepatische FAO die Nahrungsaufnahme reduziert, beweist aber nicht, dass ein kausaler Zusammenhang zwischen erhöhter hepatischer FAO und reduzierter Nahrungsaufnahme besteht.

Ein Geschmacksaversionstest wurde mit separaten Tieren durchgeführt. Einige, aber nicht alle Ratten zeigten dabei Aversionen gegenüber Saccharin-Lösung, deren Verzehr mit der HPV CA Infusion gekoppelt war. Es gab aber keine signifikante Assoziation zwischen dem verzehrsreduzierenden CA Effekt am Konditionierungstag und der Aufnahme der Saccharinlösung am Testtag. Die Plasmakonzentrationen von IL-6, TNF-α, γ-GT, ALT und Cort blieben auf basalem Niveau. All diese Ergebnisse deuten darauf hin, dass der Hemmeffekt von CA auf den Verzehr nicht ausschließlich auf einer Aversion oder Toxizität basierte.

In der dritten Studie wollten wir einem alternativen Ansatz entwickeln, um die Hypothese zu testen, dass eine erhöhte hepatische FAO die Nahrungsaufnahme hemmen kann. Wir nutzten transgene Methoden, um die Expression von CPT 1α in 293 T humanen embryonalen Nierenzellen zu erhöhen. CPT 1α ist ein Enzym, das den geschwindigkeitsbestimmenden Schritt der FAO katalysiert. Die Aktivierung des CPT 1α Transgens durch ein induzierbares tet-on Gen-Expressionssystem erhöhte die
Zusammenfassung

3. Introduction

Obesity is a health problem and is threatening to become a global epidemic (Kopelman, 2000; Seidell, 2000; World Health Organisation, 1998). The prevalence of overweight has risen dramatically over the past two decades (Foreyt and Goodrick, 1995). Obesity presents a serious danger to health because it increases the risk of developing many chronic diseases, such as type 2 diabetes, cardiovascular disease and different forms of cancer (Kopelman, 2000; Office of the Surgeon General, 2001; World Health Organization (WHO), 2004). Although genetic factors play an important role in the development of obesity, we cannot blame our genes for the increasing rate of obesity especially in the timeframe of the past two decades. The culprit is an environment which promotes behaviors that cause obesity. What behaviors contribute to obesity and how does the environment foster these behaviors? Generally speaking, obesity arises only when energy intake exceeds energy expenditure. Our environment is characterized by an essentially unlimited supply of convenient, relatively inexpensive, highly palatable, energy-dense foods and big portion sizes coupled with a sedentary lifestyle (Hill and Peters, 1998). The contribution of fat, which is subject of this thesis, to overweight, is seen as following: Most high fat diets have a high caloric density which leads to a high fat hyperphagia. But on the other hand, an increased caloric intake and body weight do not occur when the density of the high fat diet is reduced (Geary et al., 1979).

All in all, it is reasonable to assume that over-consumption in calories contributes to obesity. This does not mean, however, that food intake is not regulated in obese people.

3.1 The control of food intake

Very often obese people are able to maintain their body weight constant over a long period of time, this on a higher level compared to normal weight people. This finding suggest that also in obese people, the control of food intake is guaranteed by a
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widely distributed neural network in the brain that integrates oropharyngeal food stimuli (including those resulting in hedonic perception), gastrointestinal (GI) signals, metabolic signals (including signals related to body adiposity), and environmental and experimental contingencies (Geary and Schwartz, 2005). Most animals ingestive behavior is organized as meals that are separated by intervals of noneating, therefore the meal is the “biological unit of eating”. A meal answers the basic question in nutrition: namely when and what to eat and how much (Geary and Schwartz, 2005). Ingestion of food during meals stimulates positive feedback signals from the mouth that contributes to the maintenance of eating and negative feedback signals from the mouth, stomach, small intestine, and perhaps post-absorptive sites that ultimately terminate eating (Geary and Schwartz, 2005). After meal termination a feeling of satiation (the subjective experiences and behaviors produced during a meal by eating that cause the termination of the meal) appears (Geary, 2004). Satiation has to be differentiated from postprandial satiation (the subjective experiences and behaviors associated with the lack of eating during the intermeal interval; the physiological mechanisms of postprandial satiety are thought to be partially independent from those of satiation) (Geary, 2004).

Finally, on eating short-term and long-term influences are exerted: Short term controls are supposed to act on individual meals and to be independent of energy balance (Geary and Schwartz, 2005), whereas long-term influences are connected to the energy balance (the matching of energy intake in the form of food to energy output; energy output is the energy expenditure in the form of physical or metabolic work or heat) (Geary and Schwartz, 2005). Long-term influences are supposed to be independent from those influences affecting individual meals. Furthermore the amount eaten over any period of time is completely determined by the number and size of meals (Geary and Schwartz, 2005).
3.1.1. High fat diet and control of food intake

Several findings indicate that dietary fat influences the development of hyperphagia and obesity. In experimental rats or mice maintained on normal, low-fat chow, obesity develops only slowly with age, even when they are housed in small cages that limit physical activity. But if sedentary animals are provided with ad libitum high-fat diets (≥ 35% of energy from fat), they reliably increase energy intake, increase the efficiency of body fat gain, and rapidly become obese (Graham et al., 1990; Sclafani, 1989). It appears that several factors contribute to this (Rolls and Hammer, 1995). First, high fat diets, like very sweet diets, are highly palatable, which may lead to hyperphagia and obesity. Second, this effect may be due to an increase in energy density. This is because increased energy intake and body weight often do not occur when the density of the high fat diet is reduced (Geary et al., 1979). In contrast, when the energy density of the fat is not controlled, there is usually a positive relationship between the level of fat intake and adiposity or body weight which has been reported both in animals (Salmon and Flatt, 1985) and humans (Astrup et al., 2000; Bray and Popkin, 1998; Huot et al., 2004; Mosca et al., 2004; Saris et al., 2000; Satia-Abouta et al., 2002). Third, relative increases in the fat content of the diet may increase energy intake because at least under some conditions fat has a smaller satiety effect than carbohydrate or protein (Rolls and Hammer, 1995).

Ingested fat also has a variety of actions that contribute to the inhibition of eating. Many of these derive from pre-absorptive actions of fat and are thought to be mediated mainly through gut peptide hormones. In general their potency is shown by comparison of intragastrical (IG) versus intravenous (IV) fat infusions. IG infusions of lipids are more potent in inhibiting daily food intake than IV lipid infusions, which suggests an important role for gut signals in the control of food intake (Burggraf et al., 1997; Welch et al., 1985). Other actions of fat that inhibit eating are mediated by post-absorptive mechanisms, which are in particular the subject of this thesis, namely the fatty acid oxidation (FAO). The following sections review the current status of both pre-
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and post-absorptive actions of fat on eating in order to provide a context for the research performed in this thesis.

3.2 Pre-absorptive actions of fat controlling food intake

Gut peptides that serve as satiety signals are released from the upper gastrointestinal tract during the course of a meal. Their actions are mostly in the periphery, and very often the satiety messages induced by gastro-intestinal peptides are transmitted to the brain by vagal afferent neurons and received in visceral sensory fields of the dorsal hindbrain. How the central neural processing transforms satiety messages into an appropriate behavior (the cessation of eating) and into an appropriate sensation (satisfaction) is almost unknown (Beglinger and Degen, 2004). That the small intestine is a crucial source of satiety signals - mainly short term signals - has been shown by several studies revealing that infusion of lipids and carbohydrates into the small intestine inhibits food intake to a much greater extent than when the same nutrients are given intravenously (Drewe et al., 1992; Smith and Gibbs, 1984). The following paragraphs briefly review the most important gut peptide hormones that are thought to contribute to the control of eating by fat.

3.2.1. Cholecystokinin (CCK)

CCK is the most studied satiety signal and is secreted in several molecular forms, primarily CCK-8, CCK-33, CCK-58 in rats, within the duodenal and jejunal mucosa. CCK is also synthesized within the central nervous system, primarily in the form of CCK-4. The two receptors that mediate the effects of CCK are termed CCK-1 (formerly CCK-A, for alimentary) receptor, which is found primarily in the gastrointestinal system, and CCK-2 (former CCK-B, for brain) receptor, which is found primarily within the CNS (Strader and Woods, 2005).

CCK release in the intestine occurs in response to nutrient stimulation. CCK is released by hydrolysis products of fat and protein in the chyme in the small intestine (Douglas et al., 1990; Lewis and Williams, 1990). Its physiological actions appear to
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include inhibition of gastric emptying, gastric acid secretion, stimulates the gallbladder contraction and exocrine pancreatic secretion and inhibition of appetite (Beglinger and Degen, 2004).

About 30 years ago Gibbs et al. first demonstrated that exogenous administration of either purified CCK or CCK-8 into the peritoneal cavity of rats reduced meal size (Gibbs et al., 1973). By increasing doses of CCK a greater reduction in food intake is obtained. Furthermore CCK must be administered near the start of a meal to be effective, i.e. if CCK is administered more than 15 min before animals begin to eat, it has no effect on meal size (Gibbs et al., 1973). Subdiaphragmatic vagotomy or selective damage to vagal efferents (Lorenz and Goldman, 1982; Moran et al., 1997) eliminates the anorectic effect of CCK demonstrating that vagal sensory fibers are necessary to convey the satiety signal to the brainstem. However, in this context, it should be mentioned that CCK also causes animals to consume smaller meals when centrally administered (Zhang et al., 1986).

Gibbs et al. observed that IP injection of CCK during sham feeding elicited the complete sequence of satiety, whereas quinine adulteration (the bitter taste of quinine induces anorexia) of the liquid diet stopped sham feeding, but did not elicit the complete sequence (Antin et al., 1975). Further, endogenous CCK can be blocked by CCK-1 antagonists and this blockage increases meal size in animals and humans. All these findings shows that CCK is a natural satiety factor (Beglinger et al., 2001; Hewson et al., 1988; Reidelberger and O'Rourke, 1989).

Behavioral experiments indicate that CCK is truly a short-term, meal-reducing signal. This is illustrated by the fact that repeated or long-term chronic (Crawley and Beinfeld, 1983) or intermittent (West et al., 1984) administration of CCK to rats has no effect on weight loss. When the size of every meal is reduced by CCK, animals compensate by increasing meal frequency (West et al., 1984).

3.2.2. Enterostatin

Dietary triglycerides are intraluminally hydrolyzed by pancreatic lipase and its protein cofactor colipase. Colipase is secreted as pro-colipase, activated by trypsin, to
yield colipase and a pentapeptide namely enterostatin. Unlike colipase, enterostatin is not important for the digestion of fat and acts as a negative feedback regulator during fat intake which in turn prevents the over-consumption of fat (Berger et al., 2004).

Enterostatin specifically inhibits fat intake after peripheral (intra-duodenal, intra-gastric or intra-peritoneal) or central administration in rats that are adapted to a high fat diet (Lin and York, 1998). When enterostatin was given IV it inhibited high-fat food intake in a narrow concentration range, a lower dose was ineffective and a higher dose even increased food intake, demonstrating a U-shaped dose-response curve for enterostatin.

The mechanism of action for enterostatin is not completely understood. Based on studies with OLEFTA rats, lacking the CCK-1 receptor, it was concluded that the enterostatin signal is mediated through peripheral or central CCK-1 receptors (Lin et al., 2003). Other studies suggest that enterostatin interferes with μ-opioid receptors or μ-opioid pathways and thereby causing a decreased reward during fat intake (Berger et al., 1998; Lin et al., 1998; White et al., 2000). Finally, also β-subunit of F1F0-ATP synthase localized in plasma membranes is a potential target protein for enterostatin. Interaction with F1F0-ATP synthase might influence ATP production (Berger et al., 2004). Therefore, the exact mechanism how enterostatin reduces food intake and the physiological relevance of enterostatin in the regulation of food intake is still unclear.

3.2.3. Peptide tyrosine-tyrosine (PYY)

The most likely peptide to mediate the "ileal brake" phenomenon (Ileal nutrient inhibits food intake and this physiological phenomenon has been called the "ileal brake") is the peptide tyrosine-tyrosine (PYY) which is a member of the pancreatic polypeptide family that also includes pancreatic polypeptide (PP) and neuropeptide Y (NPY). PYY is synthesized and secreted by L cells in the distal ileum and colon. In fact, most L cells that secrete glucagon-like peptide-1 (GLP-1) also secrete PYY. PYY is secreted as PYY (1-36) and is degraded to PYY (3-36) by dipeptidyl peptidase-IV (DPP-IV). Receptors that mediate the effects of PYY belong to the NPY receptor family and include Y1, Y2,
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Y4 and Y5. PYY (1-36) is an agonist for the Y1 and Y2 receptor and is a potent orexigen. PYY (3-36) displays a highly selective agonist activity for the Y2 receptor and it has been reported to reduce food intake (Strader and Woods, 2005).

3.2.4. Apolipoprotein A-IV (apo A-IV)

Apolipoprotein A-IV is a 46-kDa protein in humans that is synthesized mainly by intestinal villus epithelial cells (Doi et al., 2001) with a higher synthesis in the jejunum than in the ileum (Abumrad et al., 1999). In rodents, apo A-IV is a smaller protein of 43-kDa that is synthesized by both the small intestine and the liver, with the small intestine producing the higher amount (Fukagawa et al., 1994; Wu and Windmueller, 1979).

Stimulation of apo A-IV secretion by the enterocytes is stimulated by active lipid absorption, i.e. the assembly and transport of chylomicrons is necessary to stimulate intestinal apo A-IV synthesis (Hayashi et al., 1990). This lymphatic apo A-IV output follows a circadian rhythm with increased secretion just before feeding at dark onset and peaked midway through the dark period (Fukagawa et al., 1994).

For apo A-IV a physiological role in the short-term control of food intake was suggested, since IV administration of apo A-IV produced significant dose-dependent inhibition of food intake in the first 30 min after administration. Thereafter it had no anorectic effect anymore (Fujimoto et al., 1992). Apo A-IV is also present in the hypothalamus and the potency to reduce feeding is about 50 times higher when apo A-IV is directly administered into the third cerebroventricular of rats. Blocking of endogenous apo A-IV by third ventricle injection of antirat apo A-IV serum induced feeding during the light phase when rats usually do not eat. These finding indicates that Apo A-IV exerts its satiety effect centrally. In ad libitum fed rats, hypothalamic apo A-IV mRNA and protein levels also changed across the diurnal cycle. However, in contrast to intestinal apo A-IV secretion which was stimulated by fat intake, hypothalamic apo A-IV mRNA levels and food intake were inversely related with a nadir in the middle of the dark phase (Tso and Liu, 2004). The fluctuation of the hypothalamic apo A-IV mRNA and protein levels could be modified by food restriction.
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When rats had only access to food during the light phase, the lowest hypothalamic apo A-IV mRNA levels were measured during this feeding period in the light phase. So far it is not clear what the relationship between circulating apo A-IV and brain apo A-IV is and whether peripheral apo A-IV can cross the blood-brain barrier to access the central nervous system (Tso and Liu, 2004).

As already mentioned apo A-IV is a short term satiety factor; however, the finding that increased levels of intestinal apo A-IV induced by active fat absorption were attenuated by IV leptin infusion (Doi et al., 2001) indicates that it interacts with long term satiety signals. Leptin is an important component of lipid homeostasis as its level in the blood circulation is directly correlated with the amount of fat in the body (Frederich et al., 1995). Leptin is a peptide that is synthesized and secreted by adipocytes (Maffei et al., 1995). Its acute administration decreases food intake and increases energy expenditure. Circulating leptin increases as an individual becomes obese. One hypothesis of obesity is that the intestinal apo A-IV response to lipids is attenuated by increasing leptin levels (Tso and Liu, 2004). Presently the molecular mechanism is not known how leptin modifies intestinal apo A-IV levels.

3.2.5. Glucagon-like peptide-1 (GLP-1)

GLP-1 has been shown to potently inhibit eating in man and animals (Flint et al., 1998; Turton et al., 1996). GLP-1 is cleaved from proglucagon in mucosal L cells of the distal portion of the ileum and in the A cells of the pancreas. GLP-1 secretion starts within minutes after meal onset although the L cells are located primarily in the lower gastrointestinal tract, suggesting it is controlled by neural signaling from the upper to the lower gastrointestinal tract. GLP-1 stimulates insulin and inhibits pancreatic glucagon secretion and thus prepares the organism for the arrival of glucose (Nauck, 1998). So far it was assumed that GLP-1 secretion is mainly stimulated by carbohydrate ingestion. However, recently, it also has been demonstrated that free fatty acids induce secretion of GLP-1 by binding at a G-protein-coupled receptor (GRP120) that is expressed in the intestine (Hirasawa et al., 2005).
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3.2.6. Ghrelin

Most gastro-intestinal signals suppress food intake by reducing meal size, however, the recently discovered gut peptide Ghrelin is an exception. Ghrelin is a peptide hormone synthesized and secreted from the fundic region of the stomach; its administration causes an increase in food intake by increasing meal size. Ghrelin is a ligand for the growth hormone secretagogue receptor (Strader and Woods, 2005). Fasting increases plasma levels of ghrelin (Cummings et al., 2001), and exogenous ghrelin exhibits potent orexigenic properties when administered peripherally (Tschop et al., 2000) or centrally (Tschop et al., 2000; Wren et al., 2001). Ghrelin has also been linked to the anticipatory aspects of meal ingestion because levels peak shortly before scheduled meals in humans and rats and fall shortly after the meals end (Cummings et al., 2001).

3.3. Post-absorptive actions of fat controlling food intake

The importance of pre-absorptive satiety signals in the regulation of intake of fat meals is unquestionable, yet several lines of evidence (see reviews (Friedman, 1998; Langhans, 1996; Leonhardt and Langhans, 2004; Scharrer, 1999)) suggest that post-absorptive signals are also involved. For example, in tests of rats fed a liquid diet after food deprivation, Horn et al. (Horn et al., 1996) demonstrated that oral preloads of 1.5 ml corn oil had no effect on subsequent food intake until 90 min later when food was offered immediately, whereas when food was offered 4 h after the preload, food intake was reduced within 30 min. Furthermore, by 4 h after preloads, most of the ingested fat had been absorbed. These findings suggest that ingested fat can act at post-absorptive sites to decrease food intake.
Inhibition of fatty acid oxidation

In 1986 Scharrer and Langhans (Scharrer and Langhans, 1986) showed for the first time that blocking of the fatty acid oxidation increases eating. The fatty acid oxidation inhibitor mercaptoacetate (MA) inhibits the acyl-CoA-dehydrogenases which are located in the mitochondrial matrix (Bauche et al., 1981).

MA increased food intake in rats fed a high fat diet (18% fat, 46% starch; w/w), but not in rats fed a low fat diet (3.3% fat, 77% starch) suggesting that the potency of MA to induce eating is positively correlated with the fat content of the diet. MA increased food intake primarily through an acute effect on the duration of the intermeal interval (Langhans and Scharrer, 1987b), indicating that fatty acid oxidation contributes to the maintenance of postprandial satiety.

A role for fatty acid oxidation in the control of eating was also demonstrated by Friedman et al. (Friedman et al., 1986) showing that the treatment with the fatty acid oxidation inhibitor methyl palmoxirate increased food intake in rats fed a high fat, but not in rats fed a low fat diet.

Further, in a macronutrient preference study (Singer et al., 1997) MA increased intake of carbohydrate and protein and decreased intake of fat, i.e. rats appear to avoid the nutrient they can not oxidize in response to MA, and over consume nutrients which they can utilize.

A potent hyperphagic effect of fatty acid oxidation inhibitors has also been established in mice (Del Prete et al., 1998) and suckling rats (Swithers, 1997).

Also in humans the CPT 1 inhibitor etomoxir clearly decreased plasma β-hydroxybutyrate (BHB), i.e. fatty acid oxidation, and increased meal size (26%) and tended to shorten the subsequent intermeal interval (13%) of 10 normal weight males with habitual dietary fat intakes between 38 and 50% of their daily energy intake (Kahler et al., 1999).
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3.3.1.1. Role of the liver

To this date it is not clear whether the inhibition of fatty acid oxidation in the liver or in any other single organ is sufficient to increase food intake. The liver plays a key role in whole body fat metabolism (Seifter and England, 1994). The principal aspects of fat metabolism in which the liver plays a unique or key role are the conversion of carbohydrates into fatty acids (de novo lipogenesis), the uptake, storage, conversion and release of fatty acids and, hence, the control of blood levels and the oxidation of fatty acids, including the production of ketone bodies which serve as an energy source for other organs in a fasting state. The liver has a very high metabolic rate, accounting for only 4% of body weight, but for 20% of normal basal total energy expenditure. Normally, the liver covers most of its own energy (ATP) needs by oxidizing fatty acids (Seifter and England, 1994). Therefore it is reasonable to assume that the liver is sensitive to changes in fatty acid availability and/or metabolism.

As already mentioned, hepatocytes rely in particular on fatty acid oxidation (Seifter and England, 1994) for ATP generation, and changes in energy status of hepatocytes may therefore be involved in the transduction of a reduced fatty acid oxidation into an afferent neural signal. In line with this assumption hyperphagia appears to be triggered by a decrease in energy status (ATP) of hepatocytes (measured by assaying for adenine nucleotides) (Friedman et al., 1999). Different metabolic inhibitors in fact synergistically decreased the hepatic ATP/ADP ratio and the phosphorylation potential, and increased food intake (Horn et al., 2004; Ji et al., 2000).

The results of experiments, in which peripheral nerve signaling was manipulated, are also consistent with the hypothesis that MA acts in the liver to elicit an eating-stimulatory signal that is transferred to the brain via the vagal afferents. The hyperphagia of rats in response to MA is markedly attenuated by hepatic branch vagotomy (Beverly et al., 1994; Langhans and Scharrer, 1987a). Further, pretreatment with capsaicin, a toxin that destroys fine-diameter unmyelinated primary sensory neurons, including many visceral sensory neurons; transsection of the subdiaphragmatic
vagal trunk or aspiration lesion of the vagal sensory terminal fields in the area postrema
and the nucleus of the solitary tract (Ritter and Taylor, 1989b; Ritter and Taylor, 1990b)
and lesions of the lateral parabrachial nucleus (Calingasan and Ritter, 1992) all
abolished hyperphagia. In contrast, hypothalamic paraventricular lesion failed to affect
hyperphagia (Calingasan and Ritter, 1992) and eating could not be elicited by
intracerebroventricular injection of MA (Ritter and Taylor, 1989b). In addition, induction
of fos-like immunoreactivity in the brain by peripheral administration of MA was
abolished by subdiaphragmatic vagotomy (Ritter and Dinh, 1994). MA has also been
shown to influence hepatic vagal afferent activity in electrophysiological studies by
enhancing the firing rate of these afferents (Lutz et al., 1997; Lutz et al., 1996).

All these findings are in line with the hypothesis that MA acts in a peripheral
organ - possibly the liver - to elicit an eating-stimulatory signal. However, the exact
coding mechanism of portal-hepatic sensors for fatty acid oxidation is unknown.
Whereas afferent nerves readily utilize glucose, it is unlikely that they oxidize fatty acids,
but hepatocytes are probably involved in the monitoring of fatty acid oxidation by
hepatic vagal afferents. How the signal is relayed from hepatocytes to sensory fibers
must still be clarified. Langhans and Scharrer supported the idea that hepatic sodium
pump activity and membrane potential link the hepatic oxidative metabolism to hunger
and satiety (Langhans and Scharrer, 1987c).

Also, it is not exactly elucidated how this afferent signal is processed in the brain.
MA increased melanin-concentrating hormone (MCH) mRNA levels in the lateral
hypothalamic area (LHA), i.e. affecting MCH neurons in the LHA (Sergeyev et al., 2000)
and decreased galanin gene expression (galanin is a biologically active neuropeptide,
widely distributed in the central and peripheral nervous systems and the endocrine
system) and peptide immunoreactivity in the paraventricular nucleus (aPVN) of the
hypothalamus, which has a dense concentration of galanin containing neurons and
terminals (Wang et al., 1998). The orexigenic effect of MCH might well contribute to
MA-induced feeding, and the suppression of galanin activity is consistent with the fat-
sparing stimulation of feeding by MA (Singer et al., 1997). It is also known that the
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final central feeding pathway activated by inhibitors of fatty acid oxidation can be blocked by serotonergic drugs (Garosi et al., 1995).

3.3.2. Increased hepatic fatty acid oxidation and food intake

In contrast to the strong case for a feeding stimulatory effect of an inhibition of fatty acid oxidation, the evidence for a feeding suppressive effect of a stimulation of fatty acid oxidation, especially in the liver, is inconsistent and comparatively weak.

3.3.2.1. Medium chain fatty acids

Indirect evidence for a role of an increased hepatic fatty acid oxidation in control of eating is derived from studies showing that ingestion or intragastric administration of medium chain triglycerides (MCT) can inhibit eating in animals (Bray et al., 1980; Denbow et al., 1992; Furuse et al., 1992) and human (Stubbs and Harbron, 1996; Van Wymelbeke et al., 1998) more potently than long chain triglycerides (LCT). Animal and human studies have shown that the fast rate of oxidation of medium chain fatty acids (MCFA) leads to greater energy expenditure (EE), in part through activation of the sympathetic nervous system (Dulloo et al., 1996). Most animal studies have also demonstrated that the greater EE with MCFA relative to long-chain fatty acids (LCFA) results in less body weight gain and decreased size of fat depots after several months of consumption. The main difference between MCFA and LCFA is that MCFA are directly absorbed into the hepatic portal vein, whereas fatty acids from LCT are packed into chylomicrons and bypass the liver via the lymphatic system, favoring the uptake of fatty acids into adipose tissue and muscle (St Onge and Jones, 2002). MCFA do not depend on FA-binding proteins and CPT 1 to enter β-oxidation. Therefore CPT 1 is not the rate limiting step in MCFA oxidation, as it is for the oxidation of LCFA (Williamson et al., 1968). Ingestion or administration of MCT increases fat oxidation more than LCT (DeLany et al., 2000; Van Wymelbeke et al., 2001), and the increase in plasma ketone bodies, i.e. β-hydroxybutyrate, after MCT administration indicates that hepatic fatty acid
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oxidation is increased (Krotkiewski, 2001; Nakamura et al., 1994; Van Wymelbeke et al., 2001). Therefore it is tempting to speculate that the increased hepatic fatty acid oxidation after ingestion of medium chain triglycerides is responsible for the greater satiating effect of medium-chain triglycerides (MCT) compared with that of long-chain triglycerides (LCT).

3.3.2.2. Inducible-liver-specific carnitine-palmitoyl-transferase 1α (CPT 1α) over-expression

Because it is difficult to quantify the contribution of a change in fatty acid oxidation to changes in food intake, body weight and body composition caused by various substances, other approaches are needed to critically examine the contribution of fatty acid oxidation to the control of food intake and energy balance. A good target to manipulate fatty acid oxidation is the enzyme CPT 1, which catalyzes the rate limiting step in the mitochondrial oxidation of LCFA (see review (Eaton, 2002)). Long chain fatty acids are activated on the mitochondrial outer membrane by the long-chain acyl-CoA synthetase (LCAS) but the mitochondrial inner membrane is not permeable to these acyl-CoAs. The carnitine dependent transport of these activated fatty acids precedes their β-oxidative chain shortening. This transport system consists of three enzymes, carnitine palmitoyltransferase 1 (CPT 1), acyl-carnitine translocase (CACT) and carnitine palmitoyltransferase 2 (CPT-2), each with different submitochondrial localization. As a first step, acyl-CoAs formed by the catalytic action of LCAS in the mitochondrial outer membrane (MOM) are converted to acylcarnitines. This transesterification is catalyzed by CPT 1, also localized in the MOM. The reaction products, long-chain acylcarnitines, are then translocated into the mitochondrial matrix in an exchange reaction catalyzed by CACT, an integral inner membrane protein. Within the matrix the acylcarnitines are then reconverted to the respective acyl-CoAs by CPT-2, an enzyme associated with the inner leaflet of the mitochondrial inner membrane. The β-oxidation of activated fatty acids within the mitochondrial matrix is then catalyzed by the sequential action of four enzyme families (acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA...
dehydrogenase and 3-ketoacyl-CaA thiolase), each with different substrate specificity for short-, medium- and long-chain acyl-CoAs (Eaton, 2002; Kunau et al., 1995).

Three isoforms of CPT 1 have been identified: the liver isoform (L-CPT 1 or CPT 1α), which is expressed in most tissues including liver, kidney, lung and heart, but not in skeletal muscle, the muscle isoform (M-CPT or CPT 1β), which is expressed in skeletal muscle, heart and adipose tissue and the brain isoform (CPT 1γ). The finding that adenovirus-mediated over-expression of CPT 1α in rat insulinoma INS1E cells increased fatty acid oxidation (Rubi et al., 2002) confirms that transgenic over-expression of CPT 1 can be physiologically active. Therefore, transgenic animals with an increased or suppressed expression of CPT 1 should permit a critical examination of the contribution of fatty acid oxidation to the control of food intake and body weight. Yet, it is not possible to create homozygous CPT 1 knock-out animals because this mutation is lethal (Wood PA et al., 2003). In addition, given the complex interactions and redundancies in the physiological control of food intake and energy balance, a constitutive over-expression or suppression of CPT 1 in all organs might not allow identifying this enzyme’s role in different organs and in different metabolic situations because of developmental compensation.

An inducible over-expression of CPT 1α should avoid adaptive responses caused by permanent and constitutive genetic changes in classic “knock-out” or “knock-in” preparations. A tetra- or doxycycline (Dox)-regulated gene expression allows for such an inducible over-expression of a gene (Corbel and Rossi, 2002; Zhu et al., 2002). For these transgenic animals it is necessary to place a Dox-dependent transcriptional activator (tTA) under the control of an organ-specific promoter and the target gene (CPT 1α) under the control of the tet operator. Both constructs have to be inserted into mice or rat genome. The resulting animal lines carrying both transgenes should show a Dox-inducible, organ-specific CPT 1α over-expression. For example this method allows to generate a rat with an inducible, liver-specific over-expression of CPT 1α, and such a novel animal model can be used to examine the role of an increase in hepatic fatty acid oxidation in the control of food intake, body weight and metabolism under different
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conditions and without the limitation of a life-long adaptation to the change in gene expression.

3.4. Goals of current research

The preceding review indicates that two important gaps exist in the support for the hypothesis that hepatic FAO oxidation controls food intake: 1) the effects of increased hepatic FAO on food intake and 2) the site of origin of the effect. In this thesis I performed three experiments to address these gaps. The specific research aims were 1) to determine if hepatic portal infusion of the MCFA CA and/or the LCFA OA would selectively inhibit feeding; 2) to determine if the effect of hepatic portal infusion of the MCFA CA was to increase satiation, increase postprandial satiety, or both; 3) to determine the effect of hepatic portal infusions of MCFA in the liver; 4) to determine if the fatty acid oxidation is increasable by an over-expression of CPT 1α in-vitro.

Experiment 1. Experiment 1 was designed to test specific aims 1) - 2)

The objective of the first study was to determine whether infusion of the long chain fatty acid oleic acid (OA) and the medium chain fatty acid caprylic acid (CA, 8-C) into the hepatic portal vein have different efficacies to reduce food intake.

Experiment 2. Experiment 2 was designed to test specific aims 1) - 3)

The aim of the second study was accordingly to determine whether delivery of the medium chain fatty acid caprylic acid (CA) directly to the liver, in comparison to the systemic circulation, would increase hepatic fatty acid oxidation and inhibit feeding in rats in a behaviorally specific fashion.
Experiment 3. Experiment 3 was designed to test specific aim 4)

In the third study we examined whether transiently transfection of 293 T cells with a doxycycline-dependent transcriptional activator and the rat CPT 1α gene under the control of a TRE-dependent promoter will allow an increased expression of CPT 1α resulting in an increased fatty acid oxidation rate. We also wanted to assess whether CPT 1 over-expression affects cell viability.
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4. Effect of hepatic portal vein infusion of caprylic acid and oleic acid on food intake in rats

4.1. Introduction

Several studies in both animals (Bray et al., 1980; Denbow et al., 1992; Furuse et al., 1992; Lavau and Hashim, 1978; Noguchi et al., 2002) and humans (Stubbs and Harbron, 1996) suggest that medium-chain triglycerides (MCT) enhance satiety and decrease food intake more than long-chain triglycerides (LCT). The reason for this difference is not known.

One important factor may be the differences in post-absorptive handling of MCT and LCT. First, medium-chain fatty acids (MCFA) derived from digestion of MCT are absorbed directly into the hepatic portal vein (HPV), whereas long-chain fatty acids (LCFA) from LCT are packaged into chylomicrons and are absorbed into the lymphatic system (St Onge and Jones, 2002). Second, the transport of MCFA into the mitochondria does not require carnitine palmitoyltransferase-1 (CPT-1), which is the rate-limiting step of mitochondrial transport of LCFA (Williamson et al., 1968). Both of these effects would tend to increase the rate of hepatic oxidation of MCFA. Consistent with this, oxidation of MCFA was reported to increase ketogenesis – an indicator of hepatic fatty acid oxidation – more so than the oxidation of LCFA in humans (Van Wymelbeke et al., 2001).

Because hepatic fatty acid oxidation (FAO) is thought to initiate a physiological inhibition of food intake, the more pronounced effect of MCFA on hepatic FAO could explain the superior satiating effect of MCT. However, Cox and his colleagues (Cox et al., 2004; Meyer et al., 1998) reported the opposite result: Intraintestinal infusion of the MCFA caprylic acid (CA) reduced food intake less, not more, than isoenergetic intraintestinal infusion of the LCFA oleic acid (OA). Possibly, the superior feeding-inhibitory potency of intraintestinal OA was due to pre-absorptive factors, and feeding-inhibitory signals arising from the metabolism of CA in the liver may in fact be more potent than those arising from hepatic metabolism of OA.

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To test this hypothesis, in these experiments we compared the feeding effects of CA and OA infused directly into the HPV.

4.2. Methods

4.2.1. Animals

Male rats weighing 400-590 g during tests were bred locally in SPF conditions using Sprague-Dawley founders from Charles River Germany. For tests, rats were individually housed in wire-mesh cages with ad libitum access to water and ground rat chow (No. 3433, Provimi Kliba NAFAG, Kaiseraugst, Switzerland) with a metabolizable energy content of 12.4 kJ/g. The colony room was temperature-controlled (22 ± 2° C) with a reversed 12:12-h light-dark cycle. Rats were adapted to these housing conditions and to daily handling and weighing for at least 8 d before surgery. All protocols for these experiments were approved by the Canton of Zurich’s Animal Use and Care Committee.

4.2.2. Surgery

Rats were anesthetized by intraperitoneal injection (1.25 ml/kg) of 18 mg/kg ketamine (Ketasol-100, Dr. E. Gräub AG, Bern, Switzerland), 5 mg/kg xylazine (Rompun; Bayer, Leverkusen, Germany), and 0.05 mg/kg acepromazine (Prequillan, Arovet AG, Zollikon, Switzerland). One or two intravascular catheters were implanted using a sterile technique, as described below. A non-steroidal anti-inflammatory (Rimadyl, 100 µg/kg, Carprofen, E. Gräub AG, Switzerland) was injected subcutaneously for analgesia at the time of surgery and on the first two days postoperatively.
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4.2.2.1. HPV catheters

Catheters and headsets were custom-made from silicon tubing (ID 0.51 mm, OD 0.94 mm, length 27 cm; Ulrich Swiss, St. Gallen, Switzerland), a 20-gauge Vacutainer cannula (Becton-Dickenson, Basel, Switzerland), and polypropylene surgical mesh (2.5 x 2.5 cm, Bard mesh, Oberrieden, Switzerland), implanted in the ileocolic vein and advanced into the HPV, exteriorized in the interscapular area, and fitted with caps as previously described (Surina-Baumgartner et al., 1995). Silk suture (Silkam, 3/0, Braun, Melsungen, Germany) and glue (Histoacryl, Braun) were used to fix the catheter to the ileocolic vein, and resorbable suture (3-0 Vicryl, Ethicon GmbH, Norderstedt, Germany) was used to close the skin and muscle. During the first postoperative week the catheters were flushed daily with 0.3 ml 0.9% sterile saline and filled with 80 µl heparinized saline (100 I.U./ml heparin, Braun), thereafter every second day.

4.2.2.2. Jugular vein catheters

Silicon (ID 0.51 mm, OD 0.94 mm, length 10.5 cm) catheters were implanted into the jugular vein and advanced to the right atrium using the original method of Steffens (Steffens, 1969), modified as previously described (Ferrari et. al, AM J PHYSIOL, in press), exteriorized 1 cm rostral to the HPV catheter, and capped as described for vena cava catheters. Catheters were flushed every day with 0.2 ml 0.9% saline and then filled with 70-100 µl PVP-Heparin (Polyvinylpyrrolidone, Sigma, Buchs, Switzerland).

4.2.3. Test Procedure

About one week after surgery, rats were placed in individual open-topped plexiglass infusion cages (37 x 21 x 41 cm) with stainless steel grid floors. Rats had ad
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libitum access to water, in sipper tubes hung outside the cage, and ground chow, which was in food cups placed under a 39 cm² hole at the end of a niche in the cage wall that was 4.6 cm wide, 7 cm deep, and 29.5 cm high, with the base 5 cm above the cage floor. The cups were mounted on electronic balances (Mettler PM 3000, Greifensee, Switzerland) that were interfaced with a computer (Olivetti M 300, Nuernberg, Germany) in an adjacent room. A custom-designed program (VZM; Krügel, Munich, Germany) recorded the weights of the food cups every 30 s, enabling continuous measurement of spontaneous feeding patterns. In addition, a video system (Enhanced Observation Kit VSS 2285 / OOT, Philips, Zurich, Switzerland) permitted continuous observation of the rats.

Each day the HPV catheter was attached to a syringe pump (model A99; Razel, Stamford, CT, USA) mounted above the cage via a segment of Tygon tubing (0.76 mm ID, 1.22 mm OD; Portex, Hythe, Kent; UK) sheathed with a stainless steel spring and connected to a swivel joint fixed ~45 cm above the cage floor, which allowed the rat to move freely. The infusion pumps were remotely controlled from the adjacent room. Six-hour infusions (22 μl/min) began one hour prior to dark onset. At the end of the infusions the catheter was detached and flushed, and the headset was capped. Rats were adapted to this procedure several times before tests.

4.2.4. Experimental Design

4.2.4.1. Effects of HPV CA infusion on food intake and plasma metabolites

The effects of HPV infusions of 2 different CA (Sodium caprylate, Sigma, Buchs, Switzerland; converted CA equivalents below) concentrations on feeding and plasma metabolites were tested at each test in 12 rats with HPV and jugular vein catheters. Rats were food-deprived for 2 h, and food was returned at the beginning of the dark phase. Rats had been adapted to the hook-up procedure several times. Six-hour
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Infusions of CA (1.5 and 7.5 μmol/min, 0.2 and 1.1 mg/min, respectively) and equimolar, equal pH saline were tested using a crossover design, with at least 2 d of ad libitum feeding between trials. Sterile infusion solutions of 70 mmol/l (10.1 g/l) and 350 mmol/l (50.5 g/l) CA were prepared daily by heating 5 min at 45° C until a clear solution was obtained, adjusting the pH to 7.4, and filtering (Nalgene 0.2 μm, Nalge Nunc International, Rochester, NY, USA).

At the end of the infusion, the jugular catheters were attached and a 900-μl blood sample was taken. An equal volume of saline was then infused, and the catheter was then detached and refilled with PVP. Blood was mixed with NaF (150 mg/ml, 3 mg/ml whole blood), stored on ice during the experiment, and centrifuged for 8 min at 8000 rpm at 4°C immediately afterwards. Plasma was aliquoted and stored at -20°C. Glucose, free fatty acids (FFA), and β-hydroxybutyrate (BHB) were analyzed as previously described (Langhans, 1991).

4.2.4.2. Effect of HPV OA infusion on food intake

The effects of HPV infusions of sodium oleate (Sigma, Buchs, Switzerland; converted OA equivalents below) on feeding were tested in a new group of 13 rats. Rats were food-deprived as described in Experiment 1. Six-hour infusions of OA (50 nmol/min, 14 μg/min) and equimolar, equal pH saline were tested using a crossover design, with at least 2 d of ad libitum feeding between trials. Sterile infusion solutions 2.3 mmol/l sodium oleate (corresponding to 0.65 g/l OA) were prepared daily by heating 15 min at 56° C until a clear solution was obtained, adjusting the pH to 7.4, and filtering as before.
4.2.4.3. Effects of equimolar HPV OA and CA infusion on plasma metabolites, corticosterone, liver enzymes and cytokines

Blood samples were taken in a situation that was similar to the feeding tests, i.e., during a crossover test of OA and CA (50 nmol/min), with at least 2 days between consecutive experiments, in food-deprived rats (n = 8, with HPV and jugular vein catheters). Catheters were attached just before lights out, at the beginning of the dark phase a 3.0 - 3.2 g chow pellet was offered, and 30 min later the remaining food was removed, intake was verified to be at least 2.8 g and 6 h infusions of OA or CA began. At the end of the infusion, the jugular catheters were attached and a 700-μl blood sample was taken. An equal volume of saline was infused afterwards, and the catheter was then detached and refilled with PVP. Blood was mixed with EDTA (90 mg/ml, 1.8 mg/ml whole blood), stored on ice during the experiment, and then centrifuged for 8 min at 8000 rpm at 4° C. Plasma aliquots were stored at -70° C. Corticosterone were analyzed by RIA (Linco Research, St. Charles, MI, USA); TNF-α and IL-6 were measured by immunoassay (Linco Research) in a Luminex100 (Linco Research); the other assays were performed as previously described (Langhans, 1991).

4.2.5. Data Collection and Analysis

Meals were defined as weight changes ≥ 0.3 g, lasting ≥ 1 min, and separated by ≥ 15 min from other weight changes (Surina-Baumgartner et al., 1995). Meal duration was the time from the first to the last weight change in a single meal, and the intermeal interval (IMI) was the time from the last weight change in a meal to the first change in the next meal. Data are expressed as means ± SEM. Cumulative food intake data were analyzed with ANOVA, and post hoc comparisons between means were done with Tukey's HSD test. Meal pattern and plasma parameters were analyzed with t-tests. Minimum level of significance was p < 0.05.
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4.3. Results

4.3.1. Effect of HPV CA infusion on food intake and plasma metabolites

Six-h, HPV infusions of 1.5 μmol/min (0.2 mg/min) CA did not affect either cumulative food intake, $F(22,242) = 0.54$ (Figure 1A), or meal pattern (data not shown). HPV infusion or 7.5 μmol/min (1.1 mg/min) CA significantly reduced cumulative food intake (Figure 1B) beginning 3 h after the start of infusions and lasting throughout the test ($F(20,240) = 1.77$; $p < 0.05$). The maximal difference of 5.3 g occurred after 18 h. The interaction effect of drug and time was also significant, $F(22,242) = 9.36$; $p < 0.0001$). No reliable changes in meal size or meal number were detected (data not shown).

Neither dose of CA affected significantly the plasma concentrations of glucose, BHB or FFA (data not shown). Plasma FFA tended to be increased (SAL: $0.13 \pm 0.01$; CA: $0.30 \pm 0.09$ mmol/l, $t(9) = 1.873$; $p = 0.09$) after 6-h HPV infusions of 7.5 μmol/min (1.1 mg/min) CA.
Infusion of caprylic and oleic acid

![Graphs](image)

Fig. 1. Effects of HPV infusion (A) of CA (1.5 μmol/min and; 70 mmol/l; 22 μl/min) and (B) of CA (7.5 μmol/min; 350 mmol/l; 22 μl/min) and equimolar saline on cumulative food intake in 2-h food-deprived rats re-fed at dark onset. Infusions began one hour prior to dark onset and ended 6 h later (dotted line). Gray background indicates dark phase. Data are means ± SEM intakes of 12 rats tested using a crossover design. SAL, saline; CA, caprylic acid.

*Significantly different from SAL at 3-h and all subsequent time points; Tukey-Test after significant ANOVA, P < 0.05.

4.3.2. Effect of HPV OA infusion on food intake

Six-h, HPV infusions of 0.05 μmol/min (14 μg/min) OA reduced cumulative food intake beginning 3 h after the start of infusions and lasting throughout the test (Figure 2, interaction effect of drug and time, F(23,276) = 3.48; p < 0.0001). The maximal difference of 5.8 g occurred after 12 h. This reduction was due mainly to a decrease in the number of meals during the dark phase, (SAL: 7.2 ± 0.5 g; OA: 5.8 ± 0.5 g; t(12) = 2.77; p < 0.05), and a non-significant increase in IMI, (t(24) = 1.55). Average nocturnal meal size was not affected by HPV OA.
Infusion of caprylic and oleic acid

Fig. 2. Effects of HPV infusion of OA (50 nmol/min; 2.3 mmol/l; 22 μl/min) and equimolar saline on cumulative food intake in 2-h food-deprived rats re-fed at dark onset. Infusions began one hour prior to dark onset and ended 6 h later (dotted line). Gray background indicates dark phase. Data are means ± SEM intakes of 13 rats tested using a crossover design. SAL, saline; OA, oleic acid.

*Significantly different from SAL at 3-h and all subsequent time points; Tukey-Test after significant ANOVA, P < 0.05.

4.3.3. Effects of equimolar HPV OA and equimolar HPV CA infusion on plasma metabolites, corticosterone, liver enzymes and cytokines

No differences were detected in plasma concentrations of glucose, FFA, or BHB after HPV OA and HPV CA. Corticosterone, cytokines (TNF-α, IL-6) and liver enzymes (ALT, γ-GT) remained at basal levels in both groups (Table 1).
Infusion of caprylic and oleic acid

Table 1:
Effects of HPV CA and OA infusion on corticosterone, liver enzymes and cytokines

<table>
<thead>
<tr>
<th></th>
<th>CA</th>
<th>OA</th>
</tr>
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<tbody>
<tr>
<td>Cort (mg/l)</td>
<td>106.7 ± 10.8</td>
<td>115.1 ± 15.3</td>
</tr>
<tr>
<td>Liver Enzymes (U/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALT</td>
<td>39.6 ± 2.9</td>
<td>42.1 ± 2.6</td>
</tr>
<tr>
<td>γ-GT</td>
<td>1.1 ± 0.2</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Cytokines (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α</td>
<td>35.4 ± 15.0</td>
<td>27.8 ± 7.8</td>
</tr>
<tr>
<td>IL-6</td>
<td>860.6 ± 397.5</td>
<td>1072.3 ± 415.1</td>
</tr>
</tbody>
</table>

Data represents Mean ± S.E.M., n = 8.
Blood samples were taken after end of the infusion.
Cort, corticosterone; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; γ-GT, γ-glutamyl transferase; ALT, alanine aminotransferase.

4.4. Discussion

This study was designed to test the hypothesis that feeding-inhibitory signals arising from the hepatic metabolism of CA are more potent than those arising from the hepatic metabolism of OA. The results disconfirmed the hypothesis. Under the conditions tested, HPV infusion of 0.05 μmol/min (14 μg/min) OA produced a robust inhibition of feeding, whereas a dose of CA that was 30 time larger in μmol/min (1.5 μmol/min) and 14 times larger in mg/min (200 μg/min) failed to have any effect on feeding. Similar feeding-inhibitory effects were observed after HPV infusion of 7.5 μmol/min (1100 μg/min) CA, i.e., a dose 150 times the molar dose of OA (80 times the mg dose). Why HPV OA was so much more potent than HPV CA is not clear. The infused OA dose is so small that it is unlikely that hepatic fatty acid oxidation was increased. Therefore, these data do not support the hypothesis that the rate of hepatic FAO determines the feeding-inhibitory potency of circulating LCFA.
Infusion of caprylic and oleic acid

Our results are qualitatively similar to those of Meyer et al. (Meyer et al., 1998) and Cox et al. (Cox et al., 2004), who reported that 90-min intraintestinal infusions of 4.5 mg/min OA reduced food intake, whereas 3.5 mg/min CA was ineffective (Meyer et al., 1998) and that 7-h intraintestinal infusions of 3 mg/min OA, but not equicaloric infusions of CA, produced a long-lasting reduction of nocturnal intake (Cox et al., 2004). Also similar to the findings of Cox et al. (Cox et al., 2004) OA decreased food intake by reducing the number of meals without affecting meal size. Yet, different to our study the infused dose was much higher (1.26 g per 7 h instead of 5 mg per 6 h) and they infused OA into the jejunum and not into the HPV. Given the large number of pre-absorptive controls of eating related to fat (Beglinger and Degen, 2004; Berger et al., 2004; Strader and Woods, 2005; Tso and Liu, 2004), however, it seems unlikely that the same physiological mechanisms were responsible for the reduction of food intake after intraintestinal infusion of relatively larger amounts of OA and HPV infusion of much smaller amounts of OA.

Given the extensive evidence that hepatic FAO can control food intake (Friedman, 1998; Langhans, 2003; Leonhardt and Langhans, 2004), it is unclear why HPV OA, which should be metabolized slower than CA, inhibited feeding more potently. It does not appear that the infusions had toxic effects that could account for the decreased eating. Intraduodenal infusions of higher doses of sodium OA (7.2 mg/min and 14.4 mg/min; (Ramirez et al., 1997)) than used here have been reported to increase lactate dehydrogenase (LDH) activity, which is a marker for cell death, and to produce a conditioned taste aversion, whereas an equicaloric dose of Na-CA had no effect on LDH activity. Similarly, Benthem et al. (Benthem et al., 2000) reported that HPV infusion of 42 µg/min OA for 24 h markedly increased plasma concentration of the liver enzyme alanine aminotransferase, which is a marker of hepatotoxicity, and also increased plasma corticosterone levels, whereas in our study ALT and γ-GT, another marker for hepatotoxicity, as well as plasma corticosterone and plasma pro-inflammatory cytokine levels all remained at basal levels.
Infusion of caprylic and oleic acid

In conclusion, the present data provide evidence that the post-absorptive actions of OA inhibit feeding more potently than the post-absorptive actions of CA, but do not identify what those mechanisms are or whether they are more sensitive to all LCFA than MCFA or are specific to the two FA tested. Furthermore, although the lack of increase in plasma BHB here fails to support the hypothesis that FAO was involved in the feeding effects of HPV CA, it remains possible that a larger dose of CA indeed partly affects feeding by increasing FAO. In this context it is important to note that there is much less ketogenesis per mole of fatty acid oxidized to CO₂ in fasted than in ad libitum fed rats (McGarry and Foster, 1971; Yeh and Zee, 1976), so that ketogenesis production may have underestimated the degree of FAO in our experiments.
Infusion of caprylic acid

5. **Caprylic acid infusion acts in the liver to decrease food intake in rats**

5.1. **Introduction**

Numerous reports that peripheral administration of mercaptoacetate and other inhibitors of fatty acid oxidation stimulates feeding indicate that fatty acid metabolism contributes to the control of food intake in many species including humans, at least when moderate amounts of fat are consumed (Friedman et al., 1986; Friedman et al., 1990; Friedman et al., 1999; Horn et al., 2004; Kahler et al., 1999; Scharrer and Langhans, 1986; Singer-Koegler et al., 1996). The site of origin of the feeding-stimulatory effect of an inhibition of fatty acid oxidation, however, is not clear. The liver is a likely site. Fat is the major source of metabolic energy for hepatocytes, which is why the level of hepatic fatty acid oxidation is normally high (Seifter and England, 1994). More directly, metabolic measures during and after meals (Langhans and Scharrer, 1987b; Prete et al., 2001; Scharrer and Langhans, 1986), denervation studies (Langhans and Scharrer, 1987a; Ritter and Taylor, 1989b; Ritter and Taylor, 1990b), and electrophysiological recordings from hepatic afferent nerves (Lutz et al., 1997) all strongly indicate that hepatic fatty acid oxidation leads to a signal that is transmitted to the brain via hepatic vagal afferents and that contributes to the control of feeding (for reviews, see (Leonhardt and Langhans, 2004; Scharrer, 1999)).

Evidence from studies of fatty acid oxidation antagonists in favor of a role for hepatic fatty acid oxidation in the control of feeding is not paralleled by similar support from studies of stimulation of fatty acid oxidation. Recently, we (Jambor de Sousa et al., unpublished data) could demonstrate that infusion of both the long-chain fatty (>12 carbon chain length) acid (LCFA) oleic acid (C18) and the medium-chain (i.e., 6-12 carbon chain length) fatty acid (MCFA) caprylic acid (C8) into the hepatic portal vein (HPV) reduced food intake in male rats. However, to achieve a similar feeding-inhibitory effect, a caprylic acid (CA) dose that was 150 times the molar dose of oleic acid was
Infusion of caprylic acid necessary. The reason why HPV oleic acid (OA) at a much lower dose as HPV CA was able to reduce food intake is unknown, however, the infused OA dose was so small that it is unlikely that hepatic fatty acid oxidation was increased. Therefore, these data do not support the hypothesis that the rate of hepatic fatty acid oxidation determines the feeding-inhibitory potency of circulating LCFA. Whether an increased hepatic fatty acid oxidation caused the feeding suppressive effect of the MCFA CA is still unclear. The finding that plasma levels of the ketone body β-hydroxybutyrate (indicator of hepatic β-oxidation) were not increased after HPV CA does not exclude this possibility, because an unlimited consumption of a carbohydrate rich diet suppresses ketogenesis (McGarry and Foster, 1971; Yeh and Zee, 1976).

In view of these data, our aims in this study were several fold: First, we wanted to test whether the feeding suppressive effect of HPV CA was liver-specific. Second, we sought to determine whether HPV CA under changed experimental conditions would increase ketogenesis – as an indicator for hepatic fatty acid oxidation - and third, whether HPV CA inhibits feeding in rats in a behaviorally specific fashion without inducing aversion or liver damage.

5.2. Methods

5.2.1. Animals

Male rats were bred locally in SPF conditions using Sprague-Dawley founders from Charles River Germany. For tests, rats were individually housed in wire-mesh cages. The colony room was temperature-controlled (22 ± 2°C) with a reversed 12:12-h light-dark cycle (lights on 2200) and offered ad libitum access to water and ground rat chow (No. 3433, Provimi Klika NAFAG, Kaiseraugst, Switzerland) with a metabolizable energy content of 12.4 kJ/g. Rats were adapted to these housing conditions and to daily handling and weighing (between 0900-1000) for at least 8 d before surgery. All
Infusion of caprylic acid protocols for these experiments were approved by the Canton of Zurich’s Animal Use and Care Committee.

5.2.2. Surgery

Rats were anesthetized by intraperitoneal injection (1.25 ml/kg) of 18 mg/kg ketamine (Ketasol-100, Dr. E. Gräub AG, Bern, Switzerland) and 5 mg/kg xylazine (Rompun, Bayer, Leverkusen, Germany), and 0.05 mg/kg acepromazine (Prequillan, Arovet AG, Zollikon, Switzerland). One or two intravascular catheters were implanted using sterile technique as described below. A non-steroidal anti-inflammatory (Rimadyl, 100 µg/kg, Carprofen, E. Gräub AG, Switzerland) was subcutaneously injected for analgesia immediately after surgery and on each of the two following days.

5.2.2.1. HPV catheters

Catheters and headsets were custom-made from silicon tubing (ID 0.51 mm, OD 0.94 mm, length 27 cm; Ulrich Swiss, St. Gallen, Switzerland), a 20-gauge Vacutainer cannula (Becton-Dickenson, Basel, Switzerland), and polypropylene surgical mesh (2.5 x 2.5 cm; Bard mesh, Oberrieden, Switzerland), implanted in the ileocolic vein and advanced into the HPV, exteriorized in the interscapular area, and fitted with caps as previously described (Surina-Baumgartner et al., 1995). Silk suture (Silkam 3/0, Braun, Melsungen, Germany) and glue (Histoacryl, Braun) were used to fix the catheter to the ileocolic vein, and resorbable suture (3-0 Vicryl, Ethicon GmbH, Norderstedt, Germany) was used to close the skin and muscle. During the first postoperative week the catheters were flushed daily with 0.2 ml 0.9% sterile saline and filled with 80 µl heparinized saline (100 I.U. heparin/ml saline; Heparin, Braun), thereafter every second day.
5.2.2.2. Vena cava catheters

Silicon (ID 0.635 mm, OD 1.1938 mm, length 27 cm; Ulrich Swiss) catheters were implanted into the inferior vena cava using Kaufman's (Kaufman, 1980) method and exteriorized 1 cm rostral to the HPV catheter and fitted with the same caps as for the HPV cannula. The cannula was placed into the vena cava just rostral to the renal veins and advanced 3-4 cm so its tip lay near the junction of the hepatic vein and inferior vena cava. The cannula was anchored to the right psoas muscles with 3/0 silk suture. Catheters were flushed as described above.

5.2.2.3. Jugular vein catheters

Silicon (ID 0.51 mm, OD 0.94 mm, length 10.5 cm) catheters were implanted into the jugular vein and advanced to the right atrium using the original method of Steffens (Steffens, 1969), modified as previously described (Ferrari et al., AM J PHYSIOL, in press), exteriorized 1 cm rostral to the HPV catheter. These were capped with a smaller (8 mm) cap than the HPV catheters, leaving exposed a 4 mm length of the steel 20-gauge Vacutainer cannula (Becton Dickenson, Basel, Switzerland) from which the internal part of the assembly was made (Surina-Baumgartner et al., 1995). The cannula was sealed between experiments with a 3-4 mm length of polyethylene tubing (ID 0.76 mm, OD 1.22 mm; Sims Portex Ltd, Hythe, UK) that was flame sealed at one end. Catheters were flushed every day with 0.2 ml 0.9% saline and then filled with 80 μl PVP-Heparin (500 I.U. heparin/ml PVP; Polyvinylpyrrolidone, Sigma, Buchs, Switzerland).

5.2.3. Test Procedure

About one week after surgery, rats were placed in individual open-topped plexiglass infusion cages (37 x 21 x 41 cm) with stainless steel grid floors. Rats had ad libitum access to water, in sipper tubes hung outside the cage, and ground chow, which
Infusion of caprylic acid

was in food cups placed under a 39 cm² hole at the end of a niche in the cage wall that was 4.6 cm wide, 7 cm deep, and 29.5 cm high, with the base 5 cm above the cage floor. The cups were mounted on electronic balances (Mettler PM 3000, Greifensee, Switzerland) that were interfaced with a computer (Olivetti M 300, Nuremberg, Germany) in an adjacent room. A custom-designed program (VZM; Krügel, Munich, Germany) recorded the weights of the food cups every 30 s, enabling continuous measurement of spontaneous feeding patterns. In addition, a video system (Enhanced Observation Kit VSS 2285 / OOT, Philips, Zurich, Switzerland) permitted continuous observation of the rats.

At each experimental day between 0930-0950 the HPV catheter was attached to a syringe pump (model A99; Razel, Stamford, CT, USA) mounted above the cage via a segment of Tygon tubing (ID 0.58 mm, OD 0.965 mm; Becton Dickinson, Basel, Switzerland) sheathed with a stainless steel spring and connected to a swivel joint fixed ~45 cm above the cage floor, which allowed the rat to move freely. The infusion pumps were remotely controlled from the adjacent room. Ninety-min infusions (40 μl/min) began at dark onset (1000). At 1130 the catheter was detached and flushed, and the headset was capped.

For all experiments, except experiment 5, new groups of animals were used.

5.2.4. Experimental Design

5.2.4.1. Infusion of CA into the HPV

The effects of HPV infusions of CA (Sodium caprylate, Sigma, Buchs, Switzerland) on feeding were tested in 13 rats (weight range during tests, 411-522 g). Rats were food-deprived for 18 h, and food was returned at the beginning of the infusion. Rats had been adapted to the hook-up procedure several times and to the deprivation once. Ninety-minute infusions of CA (2.3 mg/min) and equivolemic (40 μl/min) equimolar (0.35 mol/l), equal pH saline were tested using a crossover design, with at least 2 d of ad libitum feeding between trials. Sterile infusion solutions were prepared daily by
Infusion of caprylic acid

heating 5 min at 45° C until a clear solution was obtained, adjusting the pH to 7.4, and filtering (Nalgene 0.2 μm, Nalge Nunc International, Rochester, NY, USA).

5.2.4.2. Infusion of CA into the vena cava compared to the HPV CA infusion

The effects of HPV and vena caval infusions of CA on feeding were compared in seven rats (weight range during tests, 303-425 g), using the method described above. Rats received saline and CA (207 mg/90 min) by each route, using two crossover tests, which were done in random order. All trials were at least 2 d apart.

5.2.4.3. HPV CA infusion and blood sampling of the jugular vein

Blood samples were taken in a situation that mimicked the feeding tests, i.e., during a crossover test of CA and saline, with at least 2 days between consecutive experiments, in food-deprived rats (n = 10, weight range during tests, 436-476 g) with HPV and jugular vein catheters. Different to the experiment 1 and 2, rats received a defined meal 30 minutes before the infusion started and during the infusion, rats had no access to food. The reasons for these changes were 1) an unlimited access to a carbohydrate rich diet would suppress ketogenesis (McGarry and Foster, 1971; Yeh and Zee, 1976) and under these conditions β-hydroxybutyrate (BHB) would not be a useful indicator for hepatic β-oxidation and 2) we wanted to exclude the possibility that the expected differences in food intake would affect the levels of the analyzed plasma parameters.

At 0800, the jugular vein catheters were attached. As an anticoagulant, 30 μl 7% Na-citrate, separated from the saline by small air bubbles, was advanced to near the tip of the catheter. This was withdrawn and replaced every 15 min. Between 0845 and 0850, a basal 700 μl blood sample was withdrawn and 700 μl saline was infused. The catheter was then detached and refilled with PVP. At 0945 catheters were attached, at 1000 a 3.8 - 4.0 g chow pellet was offered, and at 1030 remaining food was removed. Intake was verified to be at least 3.5 g and 90-min infusions of saline or
Infusion of caprylic acid

CA (207 mg/90 min) began. Jugular vein blood samples of 800, 300 and 900 µl were taken at 1100, 1130 and 1200, respectively. Equal volumes of saline were infused after each sample. After the last blood sample was taken, blood was replaced by infusion of fresh blood (containing 15% acid citrate dextrose (ACD) Solution, USP Formula A, Kantonsapotheke Zürich, Zurich, Switzerland) taken from donor Sprague-Dawley rats via heart puncture. Finally the jugular and HPV catheters were filled with 80 µl PVP-heparin and 80 µl of saline-heparin, respectively, and food was returned to the rats.

Blood was gently mixed with EDTA (90 mg/ml, 1.8 mg/ml whole blood), stored on ice during the experiment, and centrifuged 8 min at 5000 g at 4° C immediately afterwards. Plasma was aliquoted and stored at -70° C. Glucose, free fatty acids (FFA), and BHB were measured in each sample. Insulin, corticosterone, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), γ-glutamyl transferase (γ-GT) and alanine aminotransferase (ALT) were measured at selected times (see Results). Insulin and corticosterone were analyzed by RIA (Linco Research, St. Charles, MI, USA); TNF-α and IL-6 were measured by immunoassay (Linco Research) in a Luminex100 (Linco Research); the other assays were performed as previously described (Langhans, 1991).

5.2.4.4. Two bottle choice test

The ability of HPV infusions of CA (207 mg/90 min) to serve as the unconditioned stimulus for a conditioned taste aversion was tested in 16 rats (weight range during tests: 412-486 g). On the conditioning day, rats that had been food and water deprived for 15.5 h were offered access to a 0.125% saccharin solution (conditioned stimulus) from 0730-0930 and the amount consumed was recorded. Between 0930 and 1000 the catheters were attached, and at 1000 food was returned and 90-min infusions of CA (n = 6, infused as previously), equimolar saline (n = 5), or, as a positive control, equimolar LiCl (n = 5) began. Catheters were detached from the catheters at 1130. Two days later water and saccharin were presented at dark onset, and intakes were measured 12 h later.
Infusion of caprylic acid

5.2.4.5. Measurement of gastric emptying

The effects of CA (207 mg/90 min) on gastric emptying were tested during terminal experiments. After 18-h food deprivation, 27 rats with HPV catheters (weight range during tests: 413-591 g) were attached to the catheters between 0930 and 1000 and offered 4 g food at 1000. At 1030 the feeding cups were removed, intake was verified to be at least 3.5 g, and 90-min infusions of CA (n = 15) or saline (n = 12), as in the previous experiments, were begun. At 1200 the rats were euthanized with CO₂, and the stomachs were removed. Stomach contents were desiccated for 4 h at 100° C, weighed, and expressed as a percent of the dry weight of the food ingested.

5.2.5. Data Collection and Analysis

Meals were defined as weight changes ≥ 0.3 g, lasting ≥ 1 min, and separated by ≥ 15 min from other weight changes (Surina-Baumgartner et al., 1995). The intermeal interval (IMI) was the time from the last weight change in a meal to the first change in the next meal. Data are expressed as means ± SEM. Data were analyzed with t-tests or ANOVA with drug, time, and route of administration as factors, as appropriate. Simple post hoc comparisons between means were done with Tukey’s HSD test, and complex comparisons were done with the sequentially-rejective Bonferroni test (Holm, 1979). One outlier was detected in the FFA with the Dean and Dixon test and was excluded. In Experiment 4, the data did not meet the requirements for ANOVA and were analyzed by a Kruskal-Wallis test followed by Mann-Whitney U-test. The level of significance was P < 0.05 throughout.
Infusion of caprylic acid

5.3. Results

5.3.1. Effect of HPV CA infusion on food intake

Ninety-min, HPV infusions of CA (207 mg, 0.35 mol/l) rapidly inhibited feeding. Cumulative food intake was significantly reduced by 1 h and remained so throughout the 24-h test (Fig. 1, interaction effect of drug and time, $F(23,276) = 2.85; P < 0.0001$). Most of this reduction was due to a marked decrease in the size of the first post-deprivation meal (Fig. 1, $t(12) = 3.34; P < 0.01$). That is, rats began feeding immediately after food was presented, but ate a 40% smaller first meal during infusion of CA in comparison to saline. Nocturnal food intake was also smaller after CA infusion (Table 1, $t(12) = 2.22, P < 0.05$). No changes in meal number were detected. The maximal difference of the food intake namely 5.8 g occurred after 13 h.

Table 1. Effects of hepatic portal vein infusion of caprylic acid (CA) on nocturnal feeding

<table>
<thead>
<tr>
<th></th>
<th>SAL</th>
<th>CA</th>
</tr>
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<tbody>
<tr>
<td><strong>First Meal</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Latency (min)</td>
<td>2.2 ± 0.3</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>Size (g)</td>
<td>7.4 ± 0.8</td>
<td>4.5 ± 0.6 **</td>
</tr>
<tr>
<td>IMI (min)</td>
<td>115.8 ± 16.3</td>
<td>121.2 ± 23.6</td>
</tr>
</tbody>
</table>

| **Subsequent Meals** |         |        |
| Number              | 5.3 ± 0.5 | 5.4 ± 0.5 |
| Size (g)            | 3.9 ± 0.2 | 3.5 ± 0.2 |
| IMI (min)           | 104.7 ± 7.3 | 107.6 ± 9.6 |

| **Total Nocturnal FI (g)** | 27.1 ± 1.4 | 23.0 ± 1.5 * |

Values are means ± S.E.M.; $n = 13$.
SAL, saline; IMI, intermeal interval; FI, food intake.

*P < 0.05, **P < 0.01 SAL vs. CA.
Fig. 1. Effects of hepatic portal vein infusion of CA (2.3 mg/40 μl/min; 0.35 mol/l) and equimolar saline on cumulative food intake and the size of the first nocturnal meal (inset) in 18-h food-deprived rats re-fed at dark onset. Infusions began at dark onset (t = 0) and ended 1.5 h later (dotted line). Gray background indicates dark phase. Data are means ± SEM intakes of 13 rats tested using a crossover design. SAL, saline; CA, caprylic acid.

*Significantly different from SAL at 1-h and all subsequent time points; Tukey-Test after significant ANOVA, P < 0.05.

**Significantly different from SAL, t-test, P < 0.01.

5.3.2. Effect of vena caval CA infusion on food intake compared to HPV CA infusion

HPV CA infusion reduced the size of the first meal about 38%, but VC CA infusion had no significant effect on meal size (Fig. 2). As in experiment 1, cumulative food
Infusion of caprylic acid intake was significantly reduced from 1-24 h in the rats receiving HPV infusions of CA, but was not reduced in the rats receiving vena caval infusions (interaction effect of time, drug and route of administration, F(69,414) = 1.86, P < 0.001; data not shown).

Fig. 2. Effects of hepatic portal vein (HPV) and vena cava (VC) infusions of CA (2.3 mg/40 μl/min for 90 min; 0.35 mol/l) and equimolar SAL on the size of the first nocturnal meal in 18-h food-deprived rats re-fed at dark onset. Meal sizes are mean ± SEM intake of 7 double-cannulated rats tested in two crossover tests in random order. SAL, saline; CA, caprylic acid.

*Significantly different from HPV SAL, sequentially-rejective Bonferroni test after significant ANOVA, P < 0.05.

#Effect of HPV CA (HPV SAL - HPV CA) significantly different from effect of VC CA (VC SAL - VC CA), sequentially-rejective Bonferroni test after significant ANOVA, P < 0.05.

5.3.3. Effect of HPV CA infusion on jugular vein plasma metabolites and hormones

HPV CA infusion significantly attenuated the postprandial decreases in FFA (F(1,8) = 17.32, P < 0.05) and BHB (F(3,27) = 4.24, P < 0.05) during the whole infusion period (Fig. 3). HPV CA infusion did not affect the postprandial increases in
Infusion of caprylic acid

plasma concentrations of glucose and insulin measured at different time points during infusion (30, 60 and 90 min for glucose and 30 and 90 min for insulin, respectively). No changes were detected in plasma concentrations of the stresshormone corticosterone, neither after 30 min (data not shown) nor after 90 min (Table 2) indicating that neither the hypothalamus-pituitary-adrenal axis (HPA axis) was activated nor that the pro-inflammatory cytokines TNF-α and IL-6, nor the liver enzyme ALT (Table 2) were increased. Plasma concentration of γ-GT, another liver enzyme, was in contrast increased by CA infusion (t(9) = -2.51, P < 0.05) (Table 2).

Fig. 3. Effects of hepatic portal vein infusion of CA (2.3 mg/40 μl/min; 0.35 mol/l) and equimolar saline on plasma metabolites in 18-h food-deprived rats. With dark onset rats had access to a meal of 3.8
Infusion of caprylic acid

- 4.0 g for 30 min, thereafter the infusion started for 90 min. Blood samples were taken 45 min before (basal) and 30, 60 and 90 min after the start of the infusion. SAL, saline; CA, caprylic acid.

A: Free fatty acids (FFA), overall treatment effect SAL vs. CA, *P< 0.01. B: β-hydroxybutyrate (BHB), **significantly different from SAL at 30 min and all subsequent time points; Tukey-Test after significant ANOVA, P< 0.01.

Table 2. Effects of hepatic portal vein caprylic acid (CA) infusion on plasma pro-inflammatory cytokine, liver enzyme and corticosterone (Cort) concentrations

<table>
<thead>
<tr>
<th></th>
<th>SAL</th>
<th>CA</th>
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<tbody>
<tr>
<td>TNF-α (pg/ml)</td>
<td>17.3 ± 7.7</td>
<td>24.7 ± 8.6</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>1027 ± 362</td>
<td>759 ± 164</td>
</tr>
<tr>
<td>γ-GT (U/l)</td>
<td>0.64 ± 0.1</td>
<td>0.91 ± 0.1*</td>
</tr>
<tr>
<td>ALT (U/l)</td>
<td>33.41 ± 6.7</td>
<td>36.87 ± 7.5</td>
</tr>
<tr>
<td>Cort (μg/l)</td>
<td>91.25 ± 11.41</td>
<td>100.70 ± 12.98</td>
</tr>
</tbody>
</table>

Values are means ± SEM; n = 10. Blood samples were taken at the end of the infusion. SAL, saline; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; γ-GT, γ-glutamyl transferase; ALT, alanine aminotransferase. *P < 0.05, SAL vs. CA.

5.3.4. Effect of HPV CA infusion on aversion testing

On the conditioning day, each group of rats consumed similar amounts of saccharine (SAL: 10.1 ± 3.4; CA: 9.4 ± 1.0; LiCl: 11.4 ± 2.0 ml). During the HPV infusions, CA and LiCl produced similar, insignificant decreases in the size of the first meal (Fig. 3A). Comparable to experiment 1, the rats that received HPV CA ate a 37% smaller first meal in comparison to the control group; however, this did not reach significance because the number of rats per group was only 5 or 6, whereas in
experiment 1, 13 rats were tested. LiCl decreased 12-h cumulative food intake in comparison to saline (F(2,13) = 6.09, P < 0.05); 12-h intakes after CA were intermediate between saline and LiCl and not significantly different from either (Fig. 3A), however, as in experiment 1 HPV CA suppressed the 12-h cumulative food intake by 17%.

On the test day, total fluid intakes were about 50 ml/12 h for all rats, but the relative choice of saccharine and water differed significantly among the treatment groups (H(2) = 8.62, P < 0.05). Saline-treated rats ingested mainly saccharine, whereas LiCl-treated rats ingested very little saccharine (Fig. 3B). Three of the six selected CA-treated rats ingested mainly saccharine and three ingested very little saccharine, with the result that their average percent saccharine selection was intermediate between those of saline- and LiCl-treated rats and not significantly different from either (Fig. 3B). There was no consistent relation between the effects of CA on first meal size on the conditioning day and on saccharine preference on the test day (Fig. 3C).
Infusion of caprylic acid

Fig. 4. Effects of hepatic portal vein infusion of CA (2.3 mg/min; n = 6), saline (n = 5) and LiCl (n = 5) on chow and saccharine intakes and saccharine selection in a conditioned taste aversion test. All infusions were 40 μl/min for 90 min and 0.35 mol/l. SAL, saline; LiCl, lithium chloride; CA, caprylic acid.

A: On the conditioning day, LiCl significantly decreased 12-h nocturnal food intake; data are means ± SEM (*P < 0.05, Tukey's test after significant ANOVA; data are means ± SEM). B: On the test
Infusion of caprylic acid.

day, LiCl, but not CA, significantly reduced 12-h saccharine selection; horizontal lines indicate median 12-h saccharine intakes as a percent of total fluid intake; symbols are individual data (*P < 0.05, Mann-Whitney U-test following overall Kruskal-Wallis;). C: Lack of association between 12-h food intake on the conditioning day and saccharine intake on the test day in CA-infused rats; individual data.

5.3.5. Effect of HPV CA infusion on gastric emptying

HPV CA infusion reduced 90-min gastric emptying from 67.7 ± 4.1% of meal size in saline-infused rats to 48.7 ± 3.1% in CA-infused rats (t(25) = -3.76, P < 0.001).

5.4. Discussion

The most important new finding resulting from these experiments is that the feeding-inhibitory effect of CA originated in the liver because vena caval infusions of identical doses of CA did not affect feeding (aim 1). Under the present experimental conditions, HPV infusion of the MCFA CA inhibited deprivation-induced feeding in rats by limiting the size of the first post-deprivation meal, whereas in our previous study (Jambor de Sousa et al., unpublished data) HPV CA had no clear effect on meal pattern although food intake was also significantly suppressed. Possibly, a different experimental design, such as 18 h food deprivation instead of 2 h and infusion of 2.3 mg CA/min for 1.5 h instead of 1.1 mg CA/min for 6 h, was responsible for this discrepancy. These data suggest that some consequence of increased free fatty acid concentration in the portal vein or liver is sensed by the brain and contributes to the control of meal ending satiation. This signal appears not to be calibrated to compensate for energy content of the CA because both the reduction in the energy content of the first meal after CA infusion (35.3 kJ) as well as the net reduction in 24-h food intake (51.9 kJ) far exceeded the energetic content of the infused CA (a mean of 1.3 kJ up to the end of the first meal and 7.0 kJ for the entire 90-min infusion). This is a larger response than has been reported for the satiating effects of fats (tricaprylin, triolein)
Infusion of caprylic acid intragastrically infused during meals, which tend to lead to reductions in feeding that are energetically smaller than the infused fats (Maggio and Koopmans, 1982).

We did not identify what hepatic action of CA inhibited feeding. The apparent sufficiency of an inhibition of fatty acid oxidation to increase feeding in rats, as reviewed in the introduction, suggests that CA infused into the HPV may have inhibited feeding by increasing hepatic fatty acid oxidation. Two observations were consistent with this. First, the high plasma concentrations of fatty acids induced by food deprivation dropped less during the CA infusion than during control infusion, presumably reflecting the addition of exogenous CA to the endogenous free fatty acid pool. Second, and more importantly, the deprivation-induced high plasma concentrations of the ketone body BHB also dropped less during CA infusion, indicating a continued increased level of hepatic fatty acid oxidation (aim 2). Indeed, Yeh and Zee (Yeh and Zee, 1976) demonstrated that in MCT-fed rats ketogenesis was suppressed by administration of an inhibitor of fatty acid oxidation indicating that an increased ketogenesis by MCT feeding is caused by a rapid hepatic oxidation of MCFA. Thus, our data are in line with the hypothesis that CA inhibited feeding at least in part by increasing hepatic fatty acid oxidation, however, they are not sufficient to prove that increased hepatic fatty acid oxidation elicited satiation.

It is also possible that CA produced aversive or toxic effects that caused the inhibition of feeding. We assessed this possibility in several ways (aim 3). First, we conducted a conditioned taste aversion test. This test, however, produced ambiguous results, with half the rats displaying a strong aversion and half no aversion, and with no association between the aversive and feeding-inhibitory effects of CA. Therefore, it appears unlikely that an aversion of the sort that produces a conditioned taste aversion accounts for the entire feeding-inhibitory effect of CA. We also looked for signs of inflammation or HPA axis activation because under some conditions fatty acid infusions have led to tissue damage (Ramirez et al., 1997) or increases in plasma corticosterone and other HPA axis hormones (Benthem et al., 2000). Our tests produced little
Infusion of caprylic acid
evidence for any such effect. Neither plasma concentrations of the stress hormone corticosterone nor of the pro-inflammatory cytokines IL-6 and TNF-α were affected by HPV CA infusion. Plasma concentration of the liver enzyme γ-GT, which is a clinical marker of liver damage, was increased, but not to pathophysiological levels (Hagar, 2004), and plasma concentration of ALT, another indicator of liver damage, did not increase at all.

The idea that HPV CA increased hepatic fatty acid oxidation and thereby reduces food intake is in line with the finding that ingestion of medium chain triglycerides (MCT) increases hepatic fatty acid oxidation (i.e. plasma ketone levels) more than long chain triglycerides (LCT) (Krotkiewski, 2001; Nakamura et al., 1994; Van Wymelbeke et al., 1998). Several animal (Bray et al., 1980; Denbow et al., 1992; Furuse et al., 1992) and human (Stubbs and Harbron, 1996; Van Wymelbeke et al., 1998) studies suggest that MCT reduce food intake more compared to LCT. The main difference between MCT and LCT is that medium-chain fatty acids (MCFA) are absorbed into the portal vein, whereas fatty acids from dietary LCT are packed into chylomicrons and bypass the liver via the lymphatic system (St Onge and Jones, 2002), and that the transport of MCFA into the mitochondria does not require carnitine palmitoyltransferase-1 (CPT-1) (Williamson et al., 1968). Other than the LCFAs, MCFAs are rapidly taken up and oxidized by the liver. The assumption that the liver is at least partly responsible for the different feeding suppressive effects of LCT and MCT is in line with findings of Denbow et al. (Denbow et al., 1992). They demonstrated that infusion of tributyrin or tricaprylin into the portal vein of cockerels decreases food intake faster compared to HPV triolein. Yet not in all the studies did MCT reduce food intake more than LCT (Hill et al., 1993; Maggio and Koopmans, 1982). Maggio and Koopmans (Maggio and Koopmans, 1982) reported equivalent reductions of food intake in rats in response to equicaloric intragastric infusions of MCT and LCT. In this experiment only short-chain triglycerides (SCT, two to six carbons) induced a higher feeding suppressing effect during the first hour of feeding than MCT or LCT. Greenberg et al. (Greenberg et al., 1993) infused Intralipid (2.5 to 10 kcal / 60 min) in either the hepatic portal vein or the inferior vena cava of sham feeding
Infusion of caprylic acid

rats. Intravenous infusion of Intralipid by either route had no effect on sham feeding, whereas duodenal infusion of Intralipid rapidly inhibited sham feeding (Greenberg et al., 1990). These data indicate that the availability of free fatty acids in the portal vein is possibly necessary for the feeding suppressive effect of HPV CA.

Although our data are in line with the hypothesis that an increased hepatic oxidation of CA caused the feeding suppressive effect of HPV CA, we cannot exclude the possibility that other mechanisms may have also contributed to the inhibition of feeding. Ketone bodies alone can inhibit feeding (Carpenter and Grossman, 1983; Langhans et al., 1983; Langhans et al., 1985b; Langhans et al., 1985a) consequently the higher levels of ketones might inhibit feeding independent of the fatty acid oxidation that produced them. We consider this mechanism unlikely because 1) only very high plasma ketone concentrations affect food intake (Scharrer, 1999) and 2) we assume that under normal feeding conditions plasma ketone levels will not or only slightly increase (McGarry and Foster, 1971). Yeh and Zee (Yeh and Zee, 1976) demonstrated that MCT feeding increased plasma insulin and decreased plasma glucose, therefore a modified glucose metabolism might have also contributed to the feeding suppressive effect of HPV CA. This is also unlikely, because in our experiment HPV CA did neither affect plasma glucose nor plasma insulin.

We also observed a decrease in gastric emptying at the end of the CA infusion. If gastric emptying also was slowed earlier, during the first meal, it may have contributed to the reduction in food intake. Finally, in our previous study (Jambor de Sousa et al., unpublished data) HPV infusions of OA inhibited feeding much more potently than HPV infusion of CA. The infused OA dose was so small that it is unlikely that hepatic fatty acid oxidation was increased. Therefore we cannot exclude the possibility, that HPV OA and CA infusions affected feeding by the same unidentified mechanism and that the different potency of these two fatty acids to reduce food intake is related to a higher sensitivity for LCFA compared to MCFA.

In summary, in the present study we could demonstrate that CA acts in the liver to reduce food intake. As yet we have not ascertained whether the metabolism of the medium chain fatty acid is necessary to elicit the feeding suppressive effect or whether
Infusion of caprylic acid

the fatty acid itself is somehow sensed to mediate this effect. Therefore further studies are necessary to investigate this aspect.
6. **CPT 1α over-expression increases long-chain fatty acid oxidation and reduces cell viability**

6.1. **Introduction**

Several demonstrations that inhibition of peripheral fatty acid oxidation in rodents (Del Prete et al., 1998; Friedman et al., 1986; Friedman et al., 1990; Friedman and Tordoff, 1986; Scharrer and Langhans, 1986) and humans (Kahler et al., 1999) stimulates eating support the hypothesis that hepatic fatty acid oxidation contributes to the control of food intake and energy balance (reviewed in (Leonhardt and Langhans, 2004)). In contrast so far there is little evidence that stimulation of hepatic fatty acid oxidation inhibits eating. One approach to investigate the contribution of increased hepatic fatty acid oxidation to the control of food intake and energy balance would be to develop a transgenic animal with an inducible increase in the activity of key enzymes of fatty acid oxidation in the liver. CPT 1α appears to be an appropriate target for such a manipulation because CPT 1α catalyses the primary rate-limiting step in fatty acid oxidation, transfer of long-chain fatty acids from CoA to carnitine for translocation across the mitochondrial inner membrane (Park and Cook, 1998). Rubi et al. (Rubi et al., 2002) showed that infection of cultured pancreatic β-cells with recombinant adenovirus CPT 1α increased fatty acid oxidation. Given the complex interactions and redundancies in the physiological control of food intake and energy balance, an acutely inducible over-expression of CPT 1α is presumably required to avoid the adaptive counter regulatory responses that would probably be stimulated by constitutive transgenic manipulations. Previously, such a transgenic model has not been reported.

We had three aims. First, we wanted to develop a protocol for inducible transgenic over-expression of CPT 1α. Second, we wanted to measure the metabolic efficacy of CPT 1α over-expression by using a novel metabolic assay for fatty acid oxidation. Third, we wanted to measure the influence of the transgene on cell viability,
because changes in CPT 1 activity have been shown to affect apoptosis (de Pablo et al., 1999; Kong and Rabkin, 2002; Mutomba et al., 2000; Paumen et al., 1997; Shimabukuro et al., 1998) and cell proliferation (Berge et al., 2003) in-vitro. To fulfill these aims, a tet-on gene expression system for the liver specific CPT 1 isoform \([\text{CPT} \, 1\alpha \text{ or } \text{L-CPT} \, 1]\) (Britton et al., 1995) was transfected into the 293 T human embryonic kidney cell line. To measure fatty acid oxidation, transfected cells were incubated with a fatty acid that does not occur naturally in mammals, C17:1n-7, and the unique oxidation product C15:1n-7 was measured. Finally, viability was measured in transfected cells incubated with different concentrations of palmitic acid (PA).

6.2. Materials and Methods

6.2.1. Cells and Media

The cell culture media and reagents were purchased from Life Technologies (Basel, Switzerland) or Sigma (Buchs, Switzerland) unless otherwise indicated. The human embryonic kidney cell line 293 T was chosen because these cells can be transfected very efficiently. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g / L glucose. The medium was supplemented with 10% fetal bovine serum (FBS), 100 U / ml penicillin and 100 μg / ml streptomycin using a humidified incubator (37°C and 5% CO₂). The cultures were split or used when they were approximately 80% confluent.

6.2.2. Plasmids

A tet-on gene expression system based on the E. coli tetracycline-resistance operon was employed. The plasmid pUHrT62-1M2 (Urlinger et al., 2000) contains an improved Dox-inducible transactivator (rtTA) under the control of the ubiquitously active CMV promoter. In the presence of Dox, the transactivator protein changes its configuration and can bind to a tetracycline-response element (TRE), where it acts as an
CPT 1α over-expression

A second plasmid PUHD-CPT 1α bears this TRE upstream of a minimal CMV promoter and the gene of interest, namely CPT 1α. This was isolated by RT-PCR from rat liver polyA+ RNA and cloned downstream the TRE-minimal CMV promoter. This permits a Dox-inducible expression of CPT 1α. The insert was sequenced and checked by comparing the DNA sequence to the rat mRNA sequence from NCBI data base (U88294) (de Vries et al., 1997). The transfection efficiency was assessed with the plasmid pENFG1, a mammalian expression construct coding for enhanced GFP under the control of the CMV promoter.

6.2.3. Fatty acids

Cis-10-heptadecenoic acid (C17:1n-7) was bound to fatty acid-poor bovine serum albumin (20% w/v) at 37°C overnight. This mixture was then added to the cell culture medium to give a final concentration of 0.25 mM C17:1n-7 and 2% BSA. PA (Fluka, Buchs, Switzerland) was dissolved in ethanol at 50°C, and then diluted with PBS to 20x final concentration. Firstly it was mixed with an equal volume of fatty acid-poor bovine serum albumin (20% w/v in PBS, BSA, Calbiochem, Switzerland; PBS, Gibco, Basel, Switzerland) at 37°C for 30 min and secondly with the cell culture medium to give final concentrations of 1% ethanol, 1% BSA, and the PA concentrations described in the MTT assay.

6.2.4. RT-PCR analysis

293 T cells were seeded into 60 mm dishes at 1.5x10⁶ cells per dish. At about 50-60% confluency the cells were transfected with either 4 μg pUHR-T62-1M2 plus 4 μg PUHD-CPT 1α or 4 μg pUHR-T62-1M2 using PolyFect as a transfection reagent (Qiagen, Basel, Switzerland), according to the manufacturer’s protocol. Non-transfected controls were not treated with PolyFect. Twenty four hours after the transfection, the supernatant was removed and fresh cell culture medium containing 1 μg / ml Dox, 4
mM L-carnitine, and C17:1n-7 was added. After 24 hours the cells were washed, harvested and counted.

Total RNA was isolated by adsorption to a silica gel membrane according to the manufacturer's instructions (RNeasy Mini Kit, Qiagen). Total RNA concentration was measured with a photometer (at 260 and 280 nm). Possible contamination with genomic DNA was eliminated by digesting with RNase-free DNase (Protocol of DNase I, Invitrogen Life Technologies, Basel, Switzerland). Then two-step RT-PCRs with oligo dT primers and specific PCR-primers for human CPT 1α, rat CPT 1α or rtTA were done. The PCR protocols were optimized to allow a semi-quantitative comparison of mRNA concentrations in differently transfected cells. The PCR products were analyzed on a 2% agarose gel and visualized with ethidium bromide.

6.2.5. Fatty acid oxidation assay

The transfection and incubation protocols were the same as above and β-oxidation of C17:1n-7 was measured to assess CPT 1α functionality after transfection. The cells were seeded in 175 cm² flasks at 1.7x10⁷ cells. The transfections were performed either with 18 μg pUHRt62-1M2 or with 18 μg pUHRt62-1M2 plus 18 μg PUHD-CPT 1α. The cells were treated with 0.25 mM C17:1n-7 for 24 h. The medium was removed and the cells were resuspended in methanol and stored at -70 °C. The cell suspensions were homogenized by sonication (Branson sonifier, Model 250, Branson Ultrasonics Corporation, Banbury, CT, USA; output 33 sec), mixed with 2 ml transmethylation mix (methanol with 10% v/v concentrated sulfuric acid and 10% v/v hexane) and 20 μl internal standard (hexane with heptanoic acid, C 7:0), and incubated at 50°C overnight. The fatty acid methyl esters (FAME) were extracted with 1 ml hexane and 1M NaCl ((Lewis et al., 2000), method was modified for analysis). The FAME composition was analyzed using a gas-chromatograph (HP 6890, Hewlett-Packard, Philadelphia, Pennsylvania, USA) equipped with a Supelcowax–10 column (30 m x 0.32 mm, 0.25 m; Supelco Inc., Bellefonte, USA). The relative amount of C15:1n-7
formed was determined by dividing the ratio of the area under the C15:1n-7 gas-
chromatograph curve to the area under the (C7:0) curve by the total number of cells
and multiplying by 10^7 cells.

6.2.6. MTT assay

The MTT (3-[4.5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assay,
an index of cell viability and cell proliferation, is based on the ability of viable cells to
reduce MTT from a yellow water soluble dye to a dark blue insoluble formazan
compound (Mosmann, 1983). 293 T cells were seeded in 96-well tissue culture plates
(15,000 cells/well). These were placed in an incubator for 24 h. The cells were then
transfected with 69 ng pUHrT62-1M2 and 69 ng PUHD-CPT 1α. The control cells were
transfected with 69 ng pUHrT62-1M2 and 69 ng pENFG1 and incubated for 48 h. The
cell culture medium used for transfection was supplemented with 1 μg/ml Dox and
double the amount of PolyFect. The medium was changed and the cells were treated
with various concentrations of PA for 24 h. For the last 2 h of treatment MTT was
added to each well. The medium was aspirated and a freshly prepared lysis solution
(5% formic acid in isopropanol) was added. The absorbance at 570 nm was determined
with a multiwell plate reader (Dynatech, MR 5000, Bioconcept, Allschwill, Switzerland)
with the background absorbance at 630 nm subtracted.

6.2.7. Trypan blue staining

293 T cells were transfected in the same way as for the MTT assay. The cells
were incubated with 0.0 and 0.5 mM PA for 6 h. Then harvested and stained with
Trypan blue (0.5%). The unstained (alive) and the blue cells (dead) were counted and
the viability calculated.
6.2.8. **Annexin V Labeling and Propidium Iodide (PI) staining**

293 T cells were seeded and transfected as described in the MTT assay. An exception was pENFG1 which was replaced with pUHrT62-1M2 because GFP and Annexin V Fluos emit similar wavelength lights. After transfection (48 h) the cells were incubated with 0 or 0.5 mM PA for 6 h, harvested, resuspended in 25 μl of Annexin V-PI staining buffer (Annexin-V-FLUOS Staining Kit, Roche Molecular Biochemicals, Basel, Switzerland), and were incubated for 15 min at 20 °C. The aliquots were counted using a Neubauer chamber, and Annexin V and PI staining were detected by fluorescence microscopy (Axioplan Imaging 2, Zeiss, Jena, Germany) using FITC and Rhodamine filters.

6.2.9. **Statistical analysis**

Data were analyzed with ANOVA (Statview, SAS Institute, Cary, NC, USA), with expressed gene (CPT α or GFP) and PA concentration as independent factors. When significant differences were found, post-hoc analyses were performed with the sequentially rejective Bonferroni procedure (Holm, 1979). C15:ln-7 formation was assayed with t-tests. The differences were considered significant when P < 0.05.

6.3. **Results**

6.3.1. **CPT α expression and functionality**

Human 293 T cells co-transfected with rtTA and rat CPT α vector were confirmed to express rat CPT α. RT-PCR of double transfected cells revealed a band at the expected size of 263 bp (Fig. 1A, lane 3) that was not found in control groups transfected only with rtTA or without transfection (lanes 1 and 2). Human CPT α was detected in all three preparations (lane 4 to 6). Although this technique does not permit an absolute quantification, there was clearly less human CPT α mRNA in co-transfected
Cells (lane 6). Perhaps this was due to down regulation of the endogenous CPT 1α gene. We also demonstrated that the transactivator was expressed in the single- and double-transfected cells, but as expected not in non-transfected cells (data not shown).

Double-transfected cells incubated with C17:1n-7 contained about 6-fold higher concentrations of C15:1n-7 than single or non-transfected cells (Fig. 1B). Because C15:1n-7 is a unique product of C17:1n-7 β-oxidation, this finding confirms that mitochondrial β-oxidation was increased in double-transfected cells over-expressing CPT 1α.

Fig. 1. CPT 1α mRNA expression and β-oxidation rates in non-transfected, rtTA transfected and rtTA plus CPT 1α double-transfected 293 T cells.

A. RT-PCR reveals a 263 bp band for transgenic rat CPT 1α in double-transfected cells (lane 3), but not in non- (lane 1) or single- (lane 2) transfected cells. Endogenous human CPT 1α could be detected in all three cell types (lanes 4-6). M: 100 bp molecular weight ladder.

B. β-oxidation rate as indicated by the relative oxidation of C17:1n-7 to C15:1n-7 in non-transfected (n.t.), single-transfected (rtTA), and double-transfected (rtTA+CPT 1α) cells. Relative amounts of C15:1n-7 formation were calculated as described in Methods. Data are mean ± S.E.M; n = 5, except n = 1 for non-transfected cells.

**P < 0.01, rtTA versus and rtTA+CPT 1α t-test.
6.3.2. Cell viability

Transfection of the 293 T cells with GFP yielded a 70-80% transfection rate (data not shown). We estimated that the percentage of cells which were co-transfected with two different plasmids was at least 50%. Cell viability, as assayed by MTT, was not reduced in the control cells over-expressing only GFP, but was reduced in a concentration-dependent manner by 0.25 and 0.50 mM PA in cells over-expressing CPT 1α (significant interaction between gene [CPT 1α and GFP] and PA concentration, (F(1,496) = 8.319, P < 0.001; Fig. 2). Because an increased rate of cell death or a reduced rate of cell proliferation can each reduce cell viability, we also assessed cell death. CPT 1α over-expression and 0.5 mM PA each increased cell death, as measured by trypan blue staining (main effects of PA, F(1,1) = 22.01, P<0.001, and of gene, F(1,76) = 4.12, P < 0.05; interaction effect n.s.; Table 1). In contrast, 0.5 mM PA decreased total cell number only in cells over-expressing CPT 1α (interaction between gene and PA, (F(1,76) = 4.80, P < 0.05; Table 1). Annexin V labelling revealed that PA significantly increased the percentage of apoptotic cells (main effect of PA, F(1,20) = 9.13; P < 0.01, without a significant main or interaction effect of gene; the mean percent of apoptotic cells were for 0.0 mM PA, rtTA transfected cells, 6.9 ± 0.9%, and CPT 1α transfected cells, 10.5 ± 2.1%, and for 0.5 mM PA 15.3 ± 1.8% and 13.3 ± 2.3%, respectively). Finally, no differences in the necrotic rate were detected with PI staining (data not shown).
Fig. 2. Effects of CPT 1α over-expression and palmitic acid (PA) on cell viability assessed by an MTT assay. 293 T cells were transiently transfected either with rtTA plus GFP (rtTA+GFP) or rtTA plus CPT 1α (rtTA+CPT1α). Cell viability is expressed as percent 0 mM PA value. Data are mean ± S.E.M; n = 63/group.

**P < 0.001, versus 0 mM PA in same genotype.

## P < 0.001, rtTA+CPT 1α versus rtTA+GFP.
Table 1

Palmitic acid (PA) and CPT1α over-expression on cell number and death assessed by trypan blue staining.

<table>
<thead>
<tr>
<th></th>
<th>Cell Death (%)</th>
<th>Total Cell Number</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>trypan positiv/</td>
<td></td>
</tr>
<tr>
<td></td>
<td>total cell number</td>
<td></td>
</tr>
<tr>
<td>rtTA+GFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0 mM PA</td>
<td>4.2 ± 0.7</td>
<td>47663 ± 2379</td>
</tr>
<tr>
<td>0.5 mM PA</td>
<td>7.9 ± 1.0</td>
<td>52713 ± 2723</td>
</tr>
<tr>
<td>rtTA+CPT1α</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0 mM PA</td>
<td>5.7 ± 0.6</td>
<td>48775 ± 2594</td>
</tr>
<tr>
<td>0.5 mM PA</td>
<td>9.7 ± 0.9</td>
<td>43088 ± 2052</td>
</tr>
</tbody>
</table>

Data are mean ± S.E.M., n = 20/group

aMain effect of PA, P<0.001

bMain effect of gene, P<0.05

c0.5 mM PA: rtTA+GFP vs. rtTA+CPT1α, P<0.05

d(0-0.5 mM PA) difference in rtTA+GFP vs. rtTA+CPT1α, P<0.05

6.4. Discussion

This study yielded clear answers to the three aims defined in the introduction. First, we showed that human 293 T cells that were transfected with a Dox-dependent transcriptional activator and the rat CPT1α gene under the control of a TRE-dependent promoter expressed rat CPT1α in the presence of Dox. This demonstrates that the gene constructs and the Dox-dependent activation permit inducible over-expression of CPT1α in living cells (Aim 1). Second, incubation of the co-transfected cells with the long-chain fatty acid C17:1n-7 led to an approximately 6-fold increase in C15:1n-7 (the first product of C17:1n-7 β-oxidation) in double-transfected cells compared to single- or non-transfected cells. This demonstrates that over-expression of CPT1α in 293 T cells
CPT1α over-expression has the desired physiological effect (Aim 2). The finding that transgenic manipulation of CPT1α can increase fatty acid oxidation in living cells encourages the development of such techniques to test the roles of hepatic fatty acid oxidation in the control of metabolism, food intake and energy balance. It remains to be clarified whether the present in-vitro findings can be extrapolated to liver cells in-vivo. Indeed, the observation that incubation with ≥ 0.25 mM PA decreased the viability of CPT1α over-expressing cells (Aim 3) might present a possible complication in transgenic animals.

The substantial increase in mitochondrial β-oxidation in co-transfected 293 T cells is similar to a previous report of an increased β-oxidation rate of AdCA-CPT 1-infected INS1E cells (Rubi et al., 2002) and confirms that CPT1α is rate limiting for the β-oxidation of long-chain fatty acids, at least under the present conditions. The transfection rate of 293 T cells and hence the stimulatory effect of CPT1α over-expression on β-oxidation may be higher than what can be expected in liver cells in-vivo (Seifter and England, 1994). The in-vivo situation is more complex than cell culture because insulin, glucagon, thyroid hormones, and other hormones can affect CPT1α expression and activity in-vivo (Louet et al., 2001). Transgenic animals with an inducible over-expression of CPT1α in hepatocytes are therefore necessary to examine whether an increase in hepatic fatty acid oxidation occurs and how it is involved in the control of metabolism, food intake and energy balance.

Based on our in-vitro results, portal vein infusion of the C17:1n-7 and the measurement of C15:1n-7, neither of which occurs naturally, might be employed to detect differences in hepatic fatty acid oxidation between transgenic and control rats' in-vivo. Further pilot work; however, will be necessary to optimize this method for in-vivo use.

PA caused a concentration-dependent decrease in cell viability in CPT1α over-expressing cells, but not in GFP expressing control cells. CPT1α over-expression increased cell death in 293 T cells, and this effect was independent of PA and not clearly
attributable to an increase in apoptosis or necrosis. PA also increased cell death based on increased apoptosis and independent of \textit{CPT 1\alpha} expression. Together, these findings suggest that, unlike the control cells, \textit{CPT 1\alpha} over-expressing 293 T cells were unable to compensate for the PA-induced cell death by enhanced proliferation. This assumption is in line with the finding that the number of GFP expressing cells incubated with PA was higher than the number of \textit{CPT 1\alpha} over-expressing cells incubated with PA. The demonstration that the combination of PA exposure and \textit{CPT 1\alpha} over-expression reduced the viability of 293 T cells is in line with previous findings (Kong and Rabkin, 2002). Increased transport of activated long-chain fatty acids into the mitochondria may depolarize mitochondrial membranes and induce cell death through the release of cytochrome c (Kong and Rabkin, 2000; Mattson and Kroemer, 2003; Tronstad et al., 2003). Interestingly, other groups have reported that pharmacological inhibition of CPT 1\alpha, rather than increased CPT 1 activity or expression, enhanced PA-induced cell death (Paumen et al., 1997; Shimabukuro et al., 1998) and PA's antiproliferative effect (Berge et al., 2003). The reasons for such discrepant results are unclear. To our knowledge our report is the first to test the effects of transgenic \textit{CPT 1\alpha} over-expression on cell viability, in contrast to the pharmacological manipulations of CPT 1\alpha activity (Berge et al., 2003; de Pablo et al., 1999; Kong and Rabkin, 2002).

Whether delivery of fatty acids to \textit{CPT 1\alpha} over-expressing hepatocytes might also decrease cell viability \textit{in-vivo} remains unclear. The following issues should be considered in this context: First, as mentioned above, lower transfection rates are to be expected for liver cells than for the fast growing 293 T cell line. Second, because liver cells primarily metabolize fatty acids (Seifter and England, 1994), hepatocytes are presumably adapted to a high CPT 1\alpha activity and fatty acid oxidation rate, and therefore may be less susceptible to possible damage by the effects of \textit{CPT 1\alpha} over-expression. In fact some evidence suggests that only fast growing cancer cells are sensitive to the cytostatic and cytotoxic effects of CPT 1\alpha over-activity: C75, a fatty acid synthase inhibitor that also activates CPT 1\alpha (Kim et al., 2004), is cytostatic and cytotoxic for tumor cells that over-express fatty acid synthase, but not for normal cells
(Menendez et al., 2004). Also, as we used transiently transfected cells, it cannot be excluded that an interaction between the transfection procedure and CPT 1α over-expression contributed to the observed increase in cell death. CPT 1α over-expressing liver cells in-vivo may therefore be less sensitive to negative effects of fatty acids than 293 T cells.

All in all, this study shows that transfection of cells for inducible CPT 1α over-expression is a viable method to increase the cellular fatty acid oxidation. This provides a starting point for the use of such transgenic techniques in in-vivo tests, e.g., to investigate the role of fatty acid oxidation in the control of food intake and energy balance. It still needs to be clarified whether this approach is limited by negative effects of high fatty acid concentrations on the viability of the transfected cells.
7. General Discussion

The liver in the control of food intake

Hypotheses concerning the metabolic control of eating posit that eating is inversely related to the rate of fuel oxidation (Friedman, 1998; Langhans, 1996). So far it is not clear which organs can sense fuel utilization or how this information is transferred to the brain. The liver, which is directly exposed to the flow of absorbed nutrients and has a key role in metabolism (Seifter and England, 1994), is an ideal place for metabolic sensors involved in control of energy balance.

The first evidence directly implicating the liver in the metabolic control of food intake was a series of experiments done by Russek that involved only glucose and its metabolites. In one important study, Russek (Russek, 1963) reported that intraportal, but not intrajugular, infusion of glucose suppressed food intake in 22-h fasted dogs. Tordoff et al. (Tordoff et al., 1989) confirmed this finding in another species, e.g. in rats. Recently we (Langhans et al., 2001) found that in nondeprived rats, intra-meal hepatic portal vein (HPV) infusions of at least some doses of glucose reduced the size of spontaneous meals in rats.

Furthermore, HPV infusion of the metabolite L-lactate also inhibited food intake, by increasing the satiety ratio (intermeal interval (min) / g food intake) (Silberbauer et al., 2000). In addition, selective hepatic branch vagotomy blocked this effect of HPV lactate, suggesting that vagal afferents are necessary for the transfer of the signal to the brain.

The first evidence implicating hepatic fat oxidation in the control of feeding came in 1986, when Langhans and Scharrer (Scharrer and Langhans, 1986) showed that blocking of the fatty acid oxidation increases eating. Stimulation of increased feeding after blockade of fatty acid oxidation by peripheral administration of mercaptoacetate (MA) and other inhibitors of fatty acid oxidation has now been reported in many species, including humans (Friedman et al., 1986; Friedman et al., 1990; Friedman et al., 1999; Horn et al., 2004; Kahler et al., 1999; Leonhardt and Langhans, 2004; Scharrer and...
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Langhans, 1986; Scharrer, 1999; Singer-Koegler et al., 1996). Although it is not yet definitively proven that the liver mediates the feeding stimulatory effect of inhibition of fatty acid oxidation, several findings support this conclusion: 1) hepatic branch vagotomy markedly attenuated the eating-stimulatory effect of MA (Langhans and Scharrer, 1987d); 2) intraportal MA infusion increased afferent activity in the common hepatic branch of the vagus (Lutz et al., 1997), and capsaicin pretreatment, subdiaphragmatic vagotomy, and lesion of the vagal sensory terminal fields in the area postrema (AP) and in the nucleus of the solitary tract (NTS) all abolished the effect of inhibition of fatty acid oxidation on food intake eating (Ritter and Taylor, 1989a; Ritter and Taylor, 1990a).

**Hepatic portal vein infusion of fatty acids (Experiments 1 and 2)**

In contrast to the strong case for an eating-stimulatory effect of reduced fatty acid oxidation, the evidence for an eating-inhibitory effect of increased of fatty acid oxidation, in particular in the liver, is inconsistent and comparatively weak. Therefore, the general goal of this thesis was to investigate certain possibilities to enhance hepatic acid oxidation in order to test the hypothesis that an increased hepatic fatty acid oxidation will reduce food intake.

MCFAs, as described in the Introduction, are readily oxidized in the liver and are not dependent on CPT 1α and FA-binding protein to enter β-oxidation (Williamson et al., 1968), whereas fatty acids from dietary LCT are packed into chylomicrons and bypass the liver via the lymphatic system (St Onge and Jones, 2002) and do require FA-binding proteins and CPT 1 to enter β-oxidation (Seifter and England, 1994). We assumed that because of their faster uptake into the liver, HPV infusion of MCFA would reduce food intake more potently than compared to HPV LCFA. We tested this hypothesis in Experiment 1 by comparing the feeding effects of HPV infusions of the MCFA caprylic acid (C8:0) and the long chain fatty acid (LCFA) oleic acid (OA; C18:1). Infusions started one hour prior to dark onset and lasted for over 6 h; infusion rate was 0.05
μmol/min. The rats were food-deprived 2 h before dark onset and given access to food at dark onset. Contrary to our hypothesis, OA produced a robust inhibition of feeding, whereas a dose of CA that was 30 times larger (1.5 μmol/min) failed to have any effect on feeding. A feeding-inhibitory effect similar to that of OA was observed after HPV infusion of 7.5 μmol CA/min, i.e., a dose 150 times the dose of OA. It seems unlikely that increased hepatic oxidation of OA caused the feeding-inhibitory effect of HPV OA because the infused amount was so small (15 μmol, or 5 mg, over 6 h) and was so much more potent than CA. Therefore, our data leave open the question of how the liver senses LCFAs.

But for the feeding suppressive effect of HPV CA we could not exclude the possibility that an increased hepatic fatty acid oxidation was responsible, even though after HPV CA (7.5 μmol/min CA) BHB was not significantly increased, since carbohydrates significantly diminish the production of ketone bodies from MCFA without substantially depressing their oxidation (McGarry and Foster, 1971; Yeh and Zee, 1976).

In view of these data, in Experiment 2 we examined 1) whether the feeding suppressive effect of HPV CA is liver-specific, 2) whether the effect is associated with increased ketogenesis, an indicator of hepatic fatty acid oxidation, and 3) whether HPV CA inhibits feeding in rats in a behaviorally specific fashion without inducing aversion or liver damage. Rats were food-deprived for 18 h prior to dark onset, and CA infusion started at dark onset and lasted for 90 min, further the infusion rate was more than doubled (from 1.1 mg/min to 2.3 mg/min).

This study produced several important results. First, HPV infusion of CA elicited a lasting inhibition of deprivation-induced feeding (24 h cumulative food intake was decreased 13%), similar to its effect on spontaneous feeding in Experiment 1. Second, the effect was mediated mainly by a reduction in meal size; indeed, CA reduced the size of the first post-deprivation meal by 40%. Third, the feeding-inhibitory effect of CA originated in the liver because vena caval infusions of identical doses of CA did not affect feeding. Fourth, as described below, the data suggest that the feeding-inhibitory
effect of CA was produced by increased hepatic fatty acid oxidation. Fifth, the effect CA on feeding appeared behaviorally specific.

We conclude that the feeding-inhibitory effect of CA was probably produced by increased hepatic fatty acid oxidation because the high plasma concentrations of the ketone body BHB dropped significantly less during CA infusion than during control infusion, suggesting a higher rate of hepatic fatty acid oxidation. These data, however, are not sufficient to prove that the increased fatty acid oxidation elicited satiation. Several other mechanisms could be involved. Increased plasma ketone body concentration alone, for example ketone can sometimes inhibit feeding (Carpenter and Grossman, 1983). This explanation seems unlikely, because ketone infusion inhibited feeding by a smaller amount than the energy value of the infused ketones, whereas in our experiment the inhibition of energy intake was far greater than the energy value of the fat infused, much less than the ketones that could have been produced from it. Ketone bodies might contribute to the satiety signal, but the signal is not so strong to prevent eating after a long fasting period.

We conclude that the feeding-inhibitory effect of CA was behaviorally specific for several reasons. In a separate trial, a conditioned taste aversion (CTA) test was done. Although the overall result was ambiguous, with half the rats displaying a strong aversion and half displaying no aversion, there was no association between the aversive and the feeding-inhibitory effects of CA in individual rats. The inconsistent appearance of a CTA and the lack of association between aversion and feeding inhibition suggest that aversion of the type that supports formation of a CTA cannot account for the entire feeding inhibitory effect. In addition, HPV CA infusion did not increase plasma concentrations of either corticosterone or of the pro-inflammatory cytokines IL-6 and TNF-α indicating that neither inflammation nor HPA axis activation caused the feeding-inhibitory effect of HPV CA. Finally, plasma concentrations of the liver enzymes γ-GT and ALT, which are standard clinical markers of liver damage, did not increase to clinically relevant levels (Hagar, 2004), indicating that the infusions did not have hepatotoxic effects. All in all, these data suggest, but do not prove, that increased hepatic fatty acid
oxidation can decrease food intake, as predicted by the theory that eating is inversely related to the rate of fuel oxidation.

**Over-expression of CPT 1α and fatty acid oxidation (Experiment 3)**

The apparently low potency of HPV fatty acid infusions to reduce feeding and the difficulty of proving in such studies that increased fatty acid oxidation per se inhibits feeding suggested to us that it would be useful to develop an alternative means to test the relation between increased fatty acid oxidation and feeding. An attractive target to increase hepatic fatty acid oxidation is the manipulation of the CPT 1α, the enzyme that catalyzes the rate limiting step of β-oxidation in the mitochondria (Eaton, 2002). Therefore we were interested to determine whether transgenic over-expression of CPT 1α in hepatocytes increases hepatic fatty acid oxidation and decreases feeding. Given the complex interactions and redundancies in the physiological control of food intake, we assumed that an acutely inducible over-expression of CPT 1α might be required to avoid counter-regulatory responses that might be stimulated by constitutive transgenic manipulations. Therefore, as explained in the Introduction, we decided to attempt to develop a tissue-specific, inducible transgenic methodology, the tetra- or doxycycline (Dox) - regulated gene expression. The goal of the last experiment of this thesis was to contribute to the development of a new rat model with liver specific over-expression of CPT 1α by testing in an in-vitro system whether the constructs we designed 1) would lead to inducible over-expression of CPT 1α in double transfected cells, 2) whether CPT 1α over-expression would result in increased fatty acid oxidation, and 3) whether changes in CPT 1α activity would influence cell viability, as several reports indicate (Berge et al., 2003; de Pablo et al., 1999; Kong and Rabkin, 2002; Paumen et al., 1997).

To answer these questions, we transiently transfected 293T cells with a Dox-inducible transcription activator (rtTA) under the control of a ubiquitous promoter CMV and the target rat CPT 1α gene under the control of the tet operator. The 293 T human
embryonic kidney cell line was chosen because it is permanently transfected with the SV40 T-antigen, which allows for facilitated transfection. We also developed a novel method to measure fatty acid oxidation, based on measurement of the first oxidation product (C15:ln1) of C17 fatty acid that does not occur in nature. Our results 1) confirmed the successful induction of rats CPT 1α by RT-PCR, 2) indicated that activation of the CPT 1α transgene increased mitochondrial long-chain fatty acid oxidation approximately 6-fold, and 3) indicated that the viability of CPT 1α overexpressing cells was decreased in the presence of increased PA concentrations. We conclude from these results that continued development of this transgenic system for in-vivo studies of the role of fatty acid oxidation in the control of metabolism and energy balance is warranted.

The system used here, however, should be further refined for in-vivo rat use. First, the CMV promoter should be replaced by a more exclusively liver-specific promoter. One good choice is the albumin promoter. Therefore, we obtained a plasmid containing the mouse albumin promoter, which, in view of the strong conservation between mice and rats, should give rise to a liver-specific expression in rats. Thus, rats with a Dox-dependent albumin transcription activator (rtTA) of CPT 1α should have a liver-specific and Dox-inducible gene expression of the CPT 1α. Successful production of such animals will allow us to critically examine the hypothesis that an increased hepatic fatty acid oxidation will reduce food intake, which so far we could not prove definitively.

Although the strategy of using transgenic over-expression of CPT 1α to investigate the role of hepatic fatty acid oxidation in the control of feeding and energy balance seems promising, several potential drawbacks will have to be carefully considered. First, as the in-vitro data suggest, it is possible that CPT 1α overexpression in hepatocytes might increase cell death. Because hepatocytes depend mainly on fatty acid oxidation to cover their energy needs and are therefore adapted to a high rate of fatty acid oxidation, this risk seems lower than might be the case for other tissues. Second, the general physiological consequences of increased hepatic
fatty acid oxidation, especially in obesity and type 2 diabetes mellitus (T2DM), are not yet clear. On the one hand, increased fatty acid oxidation might help to prevent obesity and metabolic syndrome, but on the other hand, some (Diraison et al., 1998), but not all (Foley, 1992) data suggest that increased fatty acid oxidation might contribute to elevated hepatic glucose production and fasting hyperglycemia in T2DM, which would be problematic in subjects who already have reduced glucose tolerance. All in all, transgenic rats with inducible over-expressing \textit{CPT 1\alpha} in the liver will provide a useful tool to answer these questions.
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Publications

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