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RESEARCH ARTICLES

Carnitine synthesis and uptake into cells are stimulated by fasting in pigs as a model of nonproliferating species

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Abstract

In rodents, fasting increases the carnitine concentration in the liver by an up-regulation of enzymes of hepatic carnitine synthesis and novel organic cation transporter (OCTN) 2, mediated by activation of peroxisome proliferator-activated receptor (PPAR) α. This study was performed to investigate whether such effects occur also in pigs which like humans, as nonproliferating species, have a lower expression of PPARα and are less responsive to treatment with PPARα agonists than rodents. An experiment with 20 pigs was performed, which were either fed a diet ad-libitum or fasted for 24 h. Fasted pigs had higher relative mRNA concentrations of the PPARα target genes carnitine palmitoyltransferase 1 and acyl-CoA oxidase in liver, heart, kidney, and small intestinal mucosa than control pigs, indicative of PPARα activation in these tissues ($P<.05$). Fasted pigs had a higher activity of γ-butyrobetaine dioxygenase (BBD), enzyme that catalyses the last step of carnitine biosynthesis in liver and kidney, and higher relative mRNA concentrations of OCTN2, the most important carnitine transporter, in liver, kidney, skeletal muscle, and small intestinal mucosa than control pigs ($P<.05$). Fasted pigs moreover had higher concentrations of free and total carnitine in liver and kidney than control pigs ($P<.05$). This study shows for the first time that fasting increases the activity of BBD in liver and kidney and up-regulates the expression of OCTN2 in various tissues of pigs, probably mediated by PPARα activation. It is concluded that nonproliferating species are also able to cover their increased demand for carnitine during fasting by an increased carnitine synthesis and uptake into cells.

Keywords: Fasting; Novel organic cation transporter; Carnitine; Peroxisome proliferator-activated receptor α; Pig

1. Introduction

Carnitine is an essential metabolite, which has a number of indispensable functions in intermediary metabolism. The most prominent function lies in its role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix where β-oxidation takes place [1–3]. Carnitine is derived from dietary sources and endogenous biosynthesis [4]. Carnitine biosynthesis involves a complex series of reactions involving several tissues. Lysine provides the carbon backbone of carnitine. Lysine in protein peptide linkages undergoes methylation of the ε-amino group to yield trimethyllysine, which is released upon protein degradation. The released trimethyllysine is further oxidised to γ-butyrobetaine (BB) by the action of trimethyllysine dioxygenase, 3-hydroxy-*N*-trimethyllysine aldolase and 4-*N*-trimethylaminobutyraldehyde dehydrogenase. BB is hydroxylated by γ-butyrobetaine dioxygenase (BBD) to form carnitine. In humans, this last reaction occurs primarily in liver and kidney [5].

Distribution of carnitine within the body and intracellular homeostasis of carnitine are controlled by novel organic cation transporters (OCTN) which belong to the solute carrier 22A family [6,7]. Three OCTN have been identified so far, OCTN1, OCTN2 and OCTN3, localised in the plasma membrane of cells [8–10]. OCTN are polyspecific; they transport several cations and L-carnitine [11,12]. OCTN1 and OCTN2 are expressed in several tissues such as kidney, intestine, skeletal muscle, heart, liver and brain [8,13,14].

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Due to its high binding affinity for carnitine and its wide tissue expression, OCTN2 is the physiologically most important carnitine transporter, operating for the reabsorption of carnitine from the urine as well as playing a major role in tissue distribution.

Recently, it has been shown that enzymes of hepatic carnitine synthesis as well as OCTN2 and OCTN3 in rodents are up-regulated by activation of peroxisome proliferator-activated receptor (PPAR) α, a transcription factor belonging to the nuclear hormone receptor superfamily [15–17]. In mammals, PPARα mediates the metabolic response to fasting. It is highly expressed in tissues with high rates of fatty acid oxidation such as liver, skeletal muscle or kidney [18]. During fasting, PPARα is activated by nonesterified fatty acids (NEFA) deriving from adipose tissue and taken up into tissues. PPARα target genes are mainly involved in cellular fatty acid uptake and intracellular fatty acid transport, mitochondrial and peroxisomal fatty acid oxidation, ketogenesis and gluconeogenesis [19]. In rodents, fasting or calorie restriction increased carnitine concentrations in several tissues by an increased carnitine synthesis and/or an increased uptake of carnitine into tissues by OCTN [15,20,21]. It has been postulated that up-regulation of OCTNs by PPARα activation in rodents is a means to supply liver cells with sufficient carnitine required for transport of excessive amounts of fatty acids into the mitochondrion and therefore plays an important role in the adaptive response of metabolism to fasting [22].

Regarding the expression of PPARα in tissues and the effects of PPARα activation on transcription of its target genes, there are, however, great differences between various species. In rodents, PPARα is highly expressed, and activation of PPARα not only induces many genes involved in various metabolic pathways but also causes severe peroxisome proliferation in the liver [23]. In contrast to rodents, PPARα agonists do not induce peroxisome proliferation in the liver of many other species, such as guinea pigs, swine, monkeys, and humans. These non-proliferating species have a lower expression of PPARα in the liver and the response of many genes to PPARα activation is much weaker than in proliferating species [24]. For that reason, effects related to PPARα activation observed in rodents cannot be directly applied for non-proliferating species such as humans. We have recently shown that pigs have a similar mRNA concentration of PPARα in the liver as humans, which is approximately 10-fold lower than in rats [25]. Therefore, the pig may be a useful model to study biochemical effects induced by treatment with PPARα agonists.

It has not yet been investigated whether nonproliferating species such as pigs or humans are also able, like rodents, to meet their increased demand of carnitine during fasting required for the transport of excessive amounts of fatty acids into the mitochondrion in tissues with a high rate of β-oxidation by an increased synthesis of carnitine or transport of carnitine from blood into tissues. Therefore, we studied the effect of fasting on concentrations of carnitine in tissues in the pig as a nonproliferating species. To find out whether fasting stimulates the uptake of carnitine from blood into tissues, we determined gene expression of OCTN2 in various tissues. To study whether fasting enhances carnitine synthesis, we determined mRNA concentration and activity of BBD in liver and kidney, the sites of carnitine synthesis, and, moreover, determined the concentration of BB, the precursor of carnitine synthesis, in these tissues.

2. Materials and methods

All experimental procedures described followed established guidelines for the care and use of laboratory animals according to law on animal welfare and were approved by the local veterinary office [Halle (Saale), Germany].

2.1. Animals and treatments

Twenty male, 10-week-old crossbred pigs [(German Landrace×Large White)×Pietrain] were kept in a room under controlled temperature at 23±2°C and 55±5% relative humidity with light from 0600 to 1800 h. Prior to the experiment, all the pigs received a nutritionally adequate diet [26] containing (in g/kg): corn (300), wheat (200), barley (200), soybean meal (120), whey powder (60), wheat bran (30), soybean oil (25) and mineral and vitamin premix including L-lysine, DL-methionine, L-threonine, L-isoleucine, L-leucine, L-valine, L-phenylalanine, L-tryptophan, and glutamic acid (65). This diet contained 13.6 MJ metabolisable energy and 164 g crude protein per kg. One day before the start of the experiment, at an average body weight of 25 kg, the pigs were divided into two groups of 10 animals each. At the day of the experiment, the first group (control group) received the diet ad libitum for the next 24 h. The second group received their last meal at 8 a.m. and was then fasted for the next 24 h. Pigs were then killed at the morning of the next day either in the fed status (control group) or after a 24-h fasting period (treatment group).

2.2. Sample collection

After killing under light anaesthesia, blood was collected into heparinised polyethylene tubes. Plasma was obtained by centrifugation of the blood (1100 g, 10 min, 4°C). Samples of liver, heart, kidney and skeletal muscle (musculus longissimus dorsi) were taken and immediately snap-frozen. In addition, a 35-cm small intestinal segment was dissected starting at 30-cm distal to the pyloric sphincter and washed with ice-cold 0.9% NaCl (w/v). Afterward, the small intestinal segment was opened length-wide, the mucosa scraped off, and snap-frozen. All tissue samples were stored at -80°C, pending further analysis.

2.3. Analysis of carnitine and BB

Concentrations of free carnitine, acetyl carnitine, and BB in plasma and tissues were determined by tandem

mass spectrometry with deuterated carnitine-d_3 (Cambridge Isotype Laboratories, Andover, MA, USA) as internal standard as described recently in detail [27]. Total carnitine was calculated as the sum of free carnitine and acetyl carnitine.

2.4. Determination of NEFA and γ-hydroxybutyrate in plasma

Plasma concentration of NEFA was determined using the enzymatic NEFA C kit from Wako Chemicals (Neuss, Germany, Ref. 99975406) plasma. Plasma concentration of β-hydroxybutyrate was measured using a kit from R-Biopharm (Darmstadt, Germany).

2.5. Activity of BBD

Activity of BBD in liver and kidney homogenates was determined as described previously in detail by Van Vlies et al. [28]. Liver and kidney homogenates were prepared by homogenising hepatic tissue in 10 mM 3-morpholinopropanesulfonic acid buffer (pH 7.4) containing 0.9% (w/v) NaCl, 10% (w/v) glycerol, and 5 mM dithiothreitol.

2.6. RNA isolation and reverse transcriptase-polymerase chain reaction analysis

For the determination of mRNA expression levels of OCTN2, OCTN1, liver and muscle isoforms of carnitine palmitoyltransferase-1 (L-CPT-1, determined in liver, small intestinal mucosa, kidney and heart; M-CPT-1, determined in skeletal muscle), BBD, acyL-CoA oxidase (ACO), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalisation total RNA was isolated from liver, skeletal muscle, heart, kidney, and small intestinal mucosa using Trizol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. Synthesis of cDNA and reverse transcriptase-polymerase chain reaction (RT-PCR) with real-time detection for determination of OCTN2, CPT-1 and ACO were performed as recently described in detail [27]. OCTN1 mRNA concentration was determined by means of semi-quantitative RT-PCR using a polymerase chain reaction (PCR) thermocycler (Biometra, Göttingen, Germany) as described previously [29]. The number of PCR cycles applied for semiquantitative RT-PCR was determined in preliminary experiments ensuring that relative quantification of mRNA expression was performed within the linear range of amplification of PCR products. Relative expression ratios are expressed as fold changes of mRNA abundance in the treatment group (fasted) compared to the control group (fed). Characteristics of gene-specific primers obtained from Operon (Köln, Germany) are shown in Table 1.

2.7. Statistical analysis

Student's *t* test was used to compare means of treatments with those of control. Differences with $P<.05$ were considered to be significant. Data in the text are given as means±S.E.M.

3. Results

3.1. Body weights of the pigs

Initial body weights of the pigs were similar in both groups (control group: 25.1±0.6 kg; treatment group: 25.5±0.6 kg). During the experimental period of 24 h, the control group consumed 1.2±0.1 kg food and gained 0.8±0.1 kg body weight. The final body weight of these pigs was 25.9±0.6 kg. The treatment group lost 1.5±0.1 kg body weight during the 24-h fasting period. The final body weight of these pigs was 24.0±0.7 kg.

3.2. Concentrations of NEFA and β-hydroxybutyrate in plasma

Pigs fasted for 24 h had higher concentrations of NEFA (0.74±0.06 vs. 0.04±0.01 mmol/L, $P<.05$) and β-hydroxybutyrate (48±11 vs. 13±1 μmol/L, $P<.05$) in plasma than control pigs.

3.3. Relative mRNA abundance of PPARα target genes in tissues

To study whether fasting caused activation of PPARα, we determined gene expression of the classical PPARα target genes CPT-1 and ACO in tissues of the pigs. Fasted pigs had significantly higher relative mRNA concentrations of CPT-1 in liver (7.6-fold), skeletal muscle (2.3-fold), heart (2.1-fold), kidney (6.1-fold) and small intestinal mucosa (2.9-fold) and of ACO in liver (1.5-fold), kidney (2.4 fold) and small intestinal mucosa (2.8-fold) than

Table 1
Characteristics of the primers used for real-time reverse transcriptase polymerase chain reaction analysis

Gene	Forward primer (from 5′to 3′)	Reverse primer (from 5′to 3′)	NCBI GenBank/transcript ID (Ensembl)
ACO	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	AF185048
BBD	AGTCACTGGGGGTGATTCAG	GTTTGGATTGGACGGAGAAA	ENST00000263182 (ensembl)
GAPDH	AGGGGCTCTCCAGAACATCATCC	TCGCGTGCTCTTGCTGGGGTTGG	AF017079
L-CPT-1	GCATTTGTCCCATCTTTCGT	GCACTGGTCCTTCTGGGATA	AF288789
M-CPT-1	ACTGTCTGGGCAAACCAAAC	CTTCTTGATGAGGCCTTTGC	NM_001007191
OCTN2	TGACCATATCAGTGGGCTA	AGTAGGGAGACAGGATGCT	ENST00000245407 (ensembl)
OCTN1	CATGGGTCTGGCCATGATA	AAGTTATTAGCACTTTGGGAC	ENST00000200652 (ensembl)

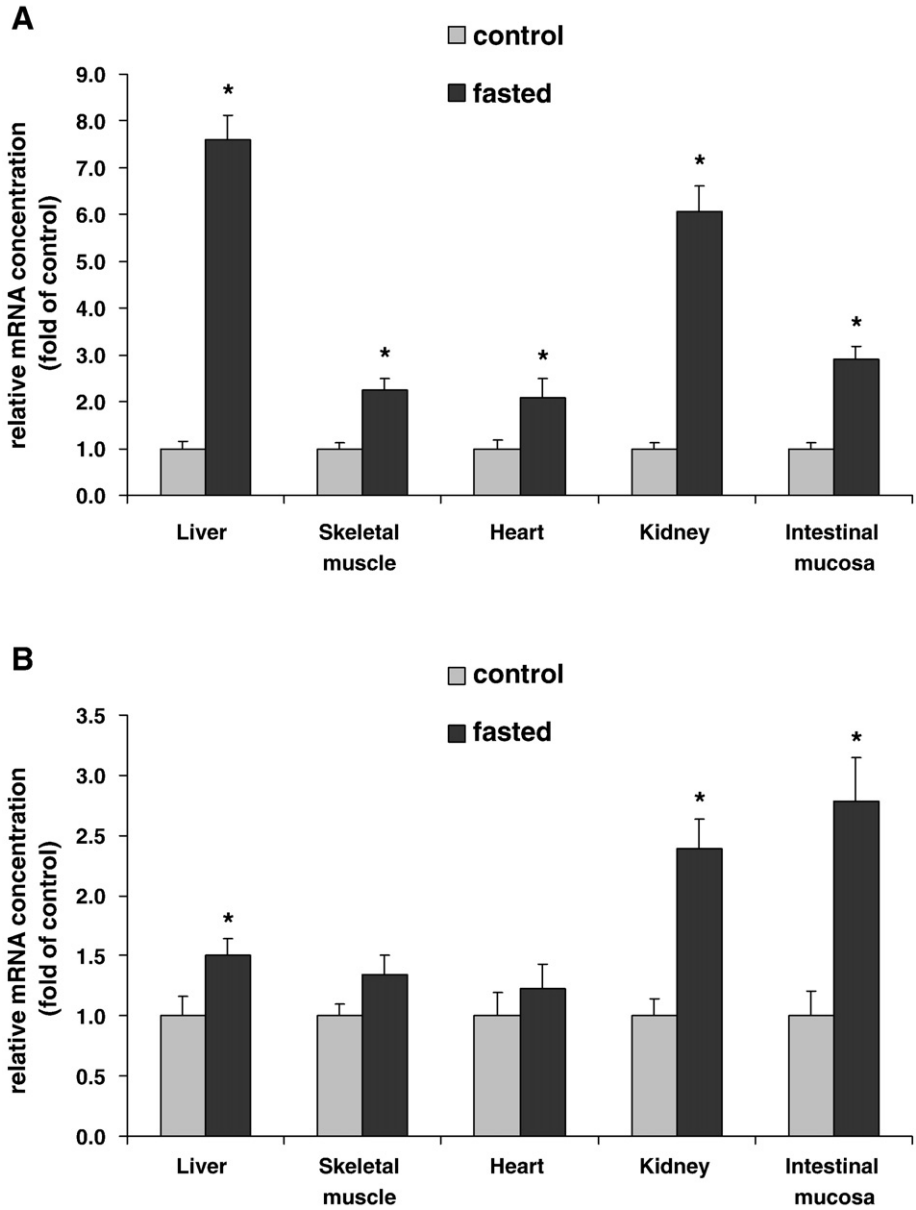

Fig. 1. Relative mRNA concentrations of the PPARα target genes CPT-1 (A) and ACO (B) in tissues of control (fed) and fasted pigs. Total RNA was isolated from tissues, 1.2 μg total RNA reverse transcribed, and cDNA was subjected to RT-PCR with real-time detection as described in methods and materials. Glyceraldehyde-3-phosphate dehydrogenase was used for normalization. Bars represent means±S.E.M. (n=10/group) and are expressed as fold increase of relative mRNA concentration compared to control. *P< .05 compared to control group.

control pigs (P<.05, Fig. 1). Relative mRNA concentrations of ACO in skeletal muscle and heart did not differ between both groups of pigs (Fig. 1).

3.4. Activity of BBD in liver and kidney

Fasted pigs had a higher activity of BBD in liver and kidney than control pigs (P<.05, Fig. 2).

3.5. Relative mRNA abundance of OCTN1 and OCTN2 in tissues

Fasted pigs had higher relative mRNA concentrations of OCTN2 in liver (1.6-fold), skeletal muscle (1.3-fold), kidney (3.9-fold) and small intestinal mucosa (2.0-fold) than control pigs (P<.05, Fig. 3). Relative mRNA concentration of OCTN2 in the heart was 1.7-fold higher in fasted pigs than in control pigs, but the difference was not statistically significant (Fig. 3). Relative mRNA concentrations of OCTN1 in liver, skeletal muscle, heart, kidney and small intestinal mucosa did not differ between both groups of pigs (relative mRNA concentration: liver: control: 1.00±0.18, fasted: 0.87±0.17; skeletal muscle: control: 1.00±0.14, fasted: 0.86±0.20; heart: control: 1.00±0.03, fasted: 1.05±0.03; kidney: control: 1.00±0.09, fasted: 0.97±0.06; small intestinal mucosa: control: 1.00±0.10, fasted: 1.19±0.11).

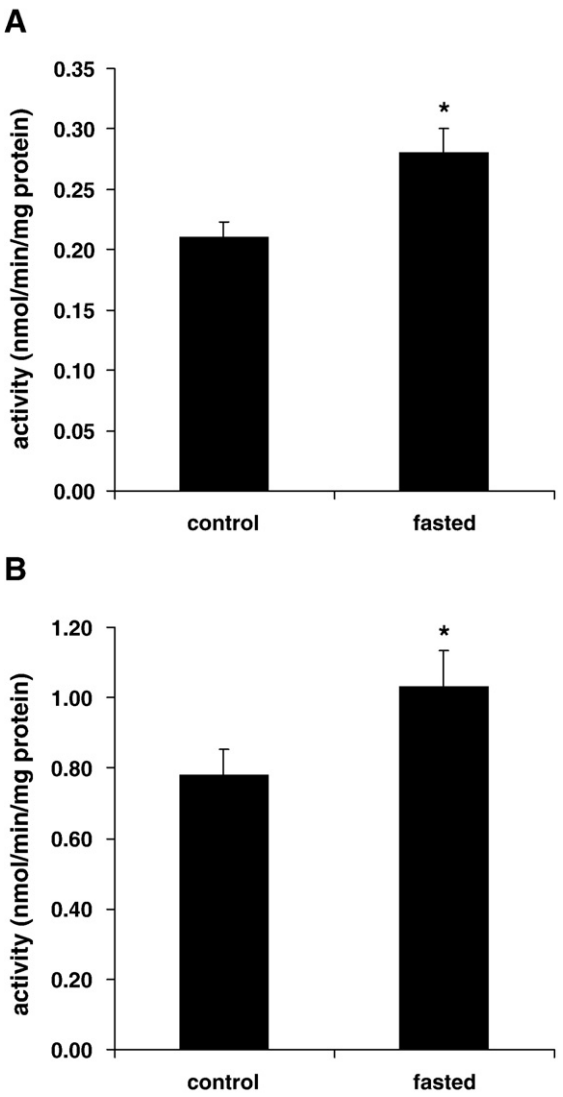

Fig. 2. Activity of γ-BBD in liver (A) and kidney (B) of control (fed) and fasted pigs. Bars represent means±S.E.M. (n=10/group). *P< .05 compared to control group.

3.6. Concentrations of carnitine and BB in plasma and tissues

Fasted pigs exhibited a higher level of free and total carnitine in liver and kidney than control pigs (P<.05, Table 2). Fasted pigs moreover had a slightly increased concentration of total carnitine in plasma compared with control pigs (P<.15), whereas the concentration of free carnitine did not differ between both groups of pigs (Table 2). Concentrations of free and total carnitine in skeletal muscle and heart did not differ between both groups of pigs (Table 2). Concentrations of BB in plasma, liver, kidney, skeletal muscle and heart did not differ between both groups of pigs (Table 2).

4. Discussion

In rodents, it has been shown that fasting or calorie restriction causes an increase of tissue carnitine concentrations [15,20,21]. It has been moreover shown that this effect is mediated by activation of PPARα through an up-regulation of enzymes of carnitine synthesis and OCTN2 [15,16]. It is well known that the response of many genes to PPARα activation is much weaker in nonproliferating than in proliferating species [24]. Therefore, it was unknown whether a similar effect with respect to carnitine homeostasis due to fasting would also occur in nonproliferating species. The present study shows for the first time that fasting for a short term of only 24 h causes an increase of carnitine concentrations in liver and kidney in pigs, a model of a nonproliferating species. The finding that the activity of BBD was increased in liver and kidney of fasted pigs suggests that increased carnitine concentrations in these tissues may have been at least in part the result of an increased carnitine biosynthesis. As gene expression of OCTN2 was also increased, it is possible that increased carnitine concentrations in liver and kidney were in part also mediated by an increased uptake of carnitine from blood into these tissues. In contrast to liver and kidney, concentrations of carnitine in skeletal muscle and heart were not changed by 24-h fasting, although mRNA concentrations of OCTN2 were increased in these tissues, too. An increased expression of OCTN2 is expected to enhance the uptake of carnitine from the blood into these tissues. However, skeletal muscle and heart have the highest carnitine concentrations in the body and are regarded as carnitine storage sites since they contain more than 95% of the whole body carnitine [2]. It is therefore likely that the short time period of 24 h was too short to detect an accumulation of carnitine in muscle which may be seen during a longer period of fasting. Indeed, in rats, fasting or calorie restriction led to an increase of carnitine concentrations in skeletal muscle and heart due to an increased expression of OCTN2 in these tissues [21]. The present study shows, moreover, that gene expression of OCTN1 in pig tissues is not up-regulated by fasting. This finding matches with our recent study performed with mice which showed that expression of OCTN1 is not regulated by PPARα [16].

We do not have direct evidence for this, but it is highly likely that the up-regulation of enzymes of carnitine biosynthesis and OCTN2 in fasted rats was mediated by activation of PPARα. Studies using PPARα$^{-/-}$ and wild-type mice have shown that genes encoding these proteins are directly up-regulated by PPARα activation [15–17]. Fasting is known to cause an activation of PPARα. NEFA released from adipose tissue during fasting are taken up into tissues by fatty acid transporters where they bind to and activate PPARα [30,31]. Cheon et al. [32] have recently shown that fasting causes an up-regulation of several classical PPARα target genes involved in mitochondrial and peroxisomal β-oxidation, ketogenesis and gluconeogenesis in the liver of pigs. These authors concluded that PPARα plays a central role in the adaptation of hepatic metabolism to fasting in pigs like in rats and

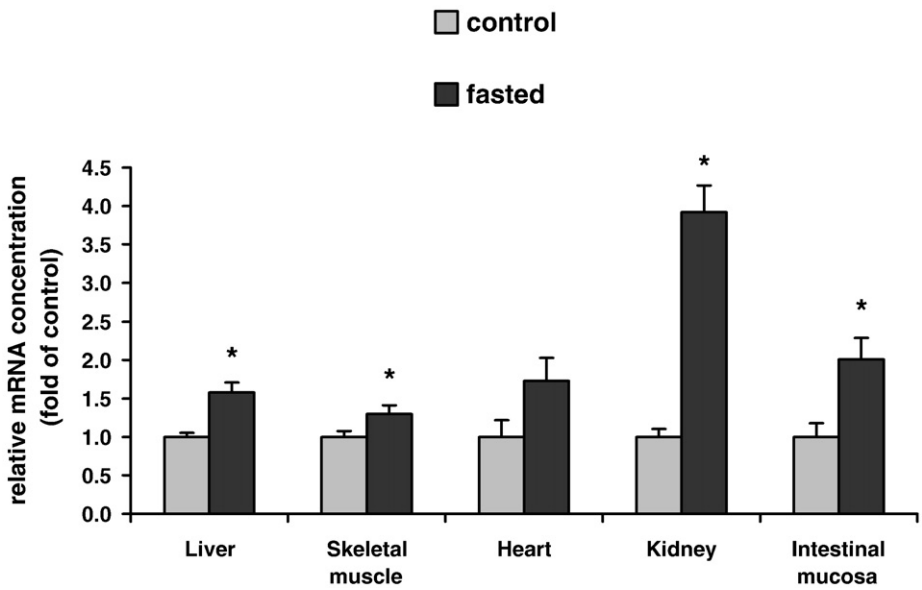

Fig. 3. Relative mRNA concentration of novel OCTN2 in tissues of control (fed) and fasted pigs. Total RNA was isolated from tissues, 1.2 μg total RNA reverse transcribed, and cDNA was subjected to RT-PCR with real-time detection as described in methods and materials. Glyceraldehyde-3-phosphate dehydrogenase was used for normalization. Bars represent means±S.E.M. (n=10/group) and are expressed as fold increase of relative mRNA concentration compared to control. *P< .05 compared to control group.

mice. In the present study, fasted pigs had markedly increased concentrations of NEFA in plasma due to the hydrolysis of triglycerides in adipose tissue and subsequent release of fatty acids into the circulation during the fasting period. To confirm that fasting caused an activation of PPARα, we determined the mRNA concentrations of ACO and CPT-1 in the relevant tissues. Up-regulation of these classical PPARα target genes which possess functional PPAR-response elements in their promoter regions is considered as an indirect proof of PPARα activation [19]. Therefore, the finding that mRNA concentrations of these enzymes were increased in all tissues investigated of fasted pigs compared to control pigs clearly indicates that PPARα was activated in these tissues by fasting. The finding that the extent of up-regulation by fasting was greater for CPT-1 than for ACO agrees with the study of Cheon et al., indicating that the transcription of CPT-1 in pigs is more sensitive towards PPARα activation than that of ACO. An increased concentration of β-hydroxybutyrate in plasma of fasted pigs is another indication that fasting caused an activation of PPARα in the liver as hepatic ketogenesis is directly up-regulated by PPARα [19].

Table 2
Concentrations of free and total carnitine and γ-butyrobetaine in plasma and tissues of control (fed) and fasted pigs

	Control (fed)	Fasted
Free Carnitine		
Liver (nmol/g)	32.1±1.7	53.6±5.1*
Kidney (nmol/g)	95±3	140±12*
Skeletal muscle (nmol/g)	315±13	351±30
Heart (nmol/g)	300±12	277±14
Plasma (μmol/L)	4.69±0.31	4.90±0.64
Total carnitine		
Liver (nmol/g)	33.7±1.6	53.8±5.1*
Kidney (nmol/g)	110±3	152±12*
Skeletal muscle (nmol/g)	628±25	594±25
Heart (nmol/g)	329±12	309±13
Plasma (μmol/L)	5.91±0.41	7.93±1.12
γ-Butyrobetaine		
Liver (nmol/g)	3.47±0.22	3.24±0.29
Kidney (nmol/g)	6.17±0.35	5.90±0.48
Skeletal muscle (nmol/g)	87.0±4.2	79.3±6.1
Heart (nmol/g)	56.6±2.6	56.2±4.1
Plasma (μmol/L)	0.67±0.05	0.73±0.09

Results are means±S.E.M. (n=10/group).
* P< .05 compared to control group.

In humans, it has been shown that not the activity of BBD but the availability of carnitine precursors, particularly BB, is rate limiting for carnitine synthesis [33]. In pigs, the rate-limiting factor of carnitine synthesis, either activity of BBD or availability of BB, has not yet been elucidated. In order to find out whether carnitine synthesis could have been stimulated by an increased availability of BB we determined concentrations of this carnitine precursor in plasma and tissues. Unchanged BB concentrations in liver and kidneys suggest that the amount of BB available for carnitine synthesis in these tissues was not changed by fasting. Most of the BB is produced in skeletal muscle which accordingly has the highest BB concentration in the body [5]. The finding that BB concentrations in skeletal muscle and plasma were also not different between both groups of pigs indicates that the production of BB in pigs was not influenced by fasting.

During fasting or energy restriction, activation of PPARα up-regulates many genes involved in hepatic mitochondrial and peroxisomal β-oxidation of fatty acids to supply acetyl-

CoA used for the generation of adenosine triphosphate (ATP) via citrate cycle and for the generation of ketone bodies—an important fuel for the brain during fasting [30]. These metabolic adaptations during fasting or energy restriction, triggered by PPARα, aim to minimize the use of protein and carbohydrates as fuel and allow mammals to survive long periods of energy deprivation. CPTs are rate limiting for β-oxidation of fatty acids [34]. The up-regulation of CPTs, which is essential for the metabolic adaptations occurring during fasting, might increase the demand of carnitine in cells. Up-regulation of enzymes of carnitine synthesis and OCTN2 during fasting, as recently shown in rodents [15,21], can therefore be regarded as a means to supply tissues with sufficient carnitine required for transport of excessive amounts of fatty acids into the mitochondrion and therefore plays an important role in the adaptive response of the metabolism to fasting. The present study shows that pigs as a nonproliferating species are also able to cover their increased demand for carnitine during fasting by an increased carnitine synthesis and uptake into cells, probably mediated by PPARα, although their expression of PPARα in tissues is much lower than in rodents. Noteworthy, the adaptations in carnitine homeostasis during fasting proceeded very fast. Even within the short-period of 24 h, carnitine concentrations in liver and kidney, the tissues with the highest rate of β-oxidation, were markedly increased compared to non-fasted control animals.

The observed up-regulation of OCTN2 in tissues due to fasting or calorie restriction may be not only relevant with respect to carnitine homeostasis but also to tissue distribution and intestinal absorption of various other compounds. OCTN2 is polyspecific and is able to bind other monovalent cations and various drugs such as verapamil, spironolactone or mildronate [13,35–39]. The effect of fasting or energy restriction on distribution and metabolism of such compounds in the body therefore deserves further investigation.

In conclusion, this study shows for the first time that fasting increases the activity of BBD which catalyses the last step of carnitine biosynthesis and up-regulates gene expression of OCTN2. These effects in turn probably lead to increased carnitine concentrations in liver and kidney of pigs as a model of a nonproliferating species such as recently shown in rodents. It is concluded that nonproliferating species are also able to cover their increased demand for carnitine during fasting by an increased carnitine synthesis and uptake into cells. These effects are probably mediated by PPARα, although their expression of PPARα in tissues is much lower than in rodents.

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