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Clofibrate treatment up-regulates novel organic cation transporter (OCTN)-2 in tissues of pigs as a model of non-proliferating species

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Abstract

Recent studies have shown that treatment of rodents with agonists of peroxisome proliferator-activated receptor (PPAR)-α causes an up-regulation of novel organic cation transporter (OCTN)-2, a carnitine transporter, and increases carnitine concentration in the liver. This study was performed to investigate whether such effects occur also in pigs which like humans have a lower expression of PPARα and are less responsive to treatment with PPARα agonists than rodents. An experiment with 18 pigs was performed which were fed a control diet or the same diet supplemented with 5 g clofibrate/kg for 28 days. Pigs treated with clofibrate had higher relative mRNA concentrations of OCTN2 in liver (3.1-fold), skeletal muscle (1.5-fold) and epithelial cells from small intestine (1.8-fold) than control pigs ($P<0.05$). Pigs treated with clofibrate had also higher concentrations of free and total carnitine in the liver and a higher concentration of free carnitine in skeletal muscle than control pigs ($P<0.05$). Concentrations of γ-butyrobetaine, the precursor of endogenous formation of carnitine, in liver, muscle and plasma did not differ between both groups; the activity of γ-butyrobetaine dioxygenase, the rate limiting enzyme of carnitine synthesis, in the liver was lower in pigs treated with clofibrate than in control pigs ($P<0.05$). This study shows for the first time that treatment with a PPARα agonist causes an up-regulation of OCTN2 in liver, muscle and enterocytes from small intestine of pigs. This in turn increases carnitine concentrations in liver and muscle probably by enhancing carnitine uptake into cells.

Keywords: Clofibrate; 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 (McGarry and Brown, 1997; Brass, 2002; Steiber et al., 2004). Carnitine is derived from dietary sources and endogenous biosynthesis (Rebouche and Seim, 1998). 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 by the action of trimethyllysine dioxygenase, 3-hydroxy-*N*-trimethyllysine aldolase and 4-*N*-trimethylaminobutyraldehyde dehydrogenase. γ-Butyrobetaine is hydroxylated by γ-butyrobetaine dioxygenase to form carnitine. In humans, this last reaction occurs primarily in liver and kidney (Vaz and Wanders, 2002).

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 (Lahjouji et al., 2001; Tein, 2003). Three OCTN have been identified so far, OCTN1, OCTN2 and OCTN3, localised in the plasma membrane of cells (Tamai et al., 1997; Tamai et al., 1998; Tamai et al., 2000). OCTN are polyspecific; they transport several cations and L-carnitine (Ohashi et al., 1999; Ohashi et al., 2001). Carnitine transport by OCTN1 and OCTN2

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is sodium dependent whereas that by OCTN3 is sodium independent (Tamai et al., 2000). OCTN1 and OCTN2 are expressed in several tissues such as kidney, intestine, skeletal muscle, heart, liver and brain (Wu et al., 1999; Tamai et al., 2000; Slitt et al., 2002). In contrast, OCTN3 is expressed exclusively in testes and kidney (Tamai et al., 2000). Among the three OCTN, OCTN3 has the highest specificity for carnitine, OCTN1 has the lowest one (Tamai et al., 2000). OCTN operate on the intestinal absorption and renal reabsorption of carnitine and play a major role in tissue distribution by catalysing the uptake of carnitine into body cells. Due to its high binding affinity for carnitine and its wide 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. OCTN1 contributes less to carnitine transport than OCTN2 due to its low carnitine transport activity. OCTN3 may be important for carnitine uptake into testis, and may contribute to reabsorption of carnitine in kidney (Tamai et al., 2000).

Many years ago it has been shown that starvation or treatment of rats with clofibrate increases the concentration of carnitine in the liver (McGarry et al., 1975; Brass and Hoppel, 1978; Paul and Adibi, 1979). As both starvation and clofibrate treatment lead to an activation of peroxisome proliferator-activated receptor (PPAR)-α, a transcription factor belonging to the nuclear hormone receptor superfamily (Schoonjans et al., 1996), we have recently tested the hypothesis that activation of this nuclear receptor is responsible for the increased liver carnitine concentrations observed in those studies. We found that activation of PPARα by clofibrate increases mRNA concentrations of OCTN2 in liver and small intestine of rats (Luci et al., 2006; Ringseis et al., 2007). These data suggest that PPARα activation stimulates intestinal absorption of carnitine and the delivery of carnitine from blood into the liver which provides an explanation for the increased carnitine concentration in the liver of rats starved or treated with clofibrate. More recently, Van Vlies et al. (2007) have shown that treatment with WY-14,643, another synthetic PPARα agonist, increases gene expression of OCTN2 and the activity of γ-butyrobetaine dioxygenase in the liver in wild-type mice but not in PPARα-deficient mice. These findings clearly show that up-regulation of OCTN2 and hepatic carnitine synthesis are directly mediated by PPARα activation.

Regarding the expression of PPARα in tissues and the effects of PPARα activation on transcription of its target genes, there are 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 (Peters et al., 2005). 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 (Holden and Tugwood, 1999). 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 ten-fold lower than in rats. Therefore, we proposed that the pig may be a useful model to study biochemical effects induced by treatment with PPARα agonists (Luci et al., 2007). The aim of the present study was to find out whether treatment with PPARα activators influences carnitine homeostasis in the pig as a non-proliferating species. Therefore, we determined gene expression of OCTN2 in enterocytes of small intestine, liver and muscle and carnitine concentrations in plasma, liver and muscle of these pigs treated with clofibrate. We also determined concentrations of γ-butyrobetaine in these tissues and examined mRNA concentration and activity of γ-butyrobetaine dioxygenase in the liver in order to explore whether clofibrate treatment enhances carnitine biosynthesis in the liver.

2. Materials and methods

2.1. Animals and treatments

Eighteen male 8 weeks 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. One day before the beginning of the experimental feeding period, the pigs were weighted and randomly allocated to two groups with body weights of 12.0 ± 1.1 kg in control group and 11.9 ± 0.6 kg in treatment group. Both groups of pigs received a nutritionally adequate diet (National Research Council, 1998) for growing pigs containing (in g/kg) wheat (400), soybean meal (230), wheat bran (150), barley (100), sunflower oil (90) and mineral premix including L-lysine, DL-methionine and L-threonine (30). This diet contained 14.4 MJ metabolisable energy and 185 g crude protein per kg. The native carnitine concentration of the diet was low (<5 mg/kg as determined by tandem mass spectrometry, see Section 2.3). The diet of the treatment group was supplemented with 5 g clofibrate per kg. Diet intake was controlled, and each animal in the experiment was offered an identical amount of diet per day. The amount of diet administered was about 15% below that consumed ad-libitum by pigs of a similar weight (as assessed in a previous study). Therefore, the diet offered was completely taken in by all pigs in the experiment. During the feeding period, the amount of diet offered each day was increased continuously from 400 to 1200 g. The intake of metabolisable energy was in clear excess of the requirement for maintenance (as given by National Research Council, 1998). Therefore, all the pigs had a normal growth rate. The pigs had free access to water via nipple drinking systems. The experimental diets were administered for 28 days. All experimental procedures described followed established guidelines for the care and use of laboratory animals and were approved by the local veterinary office.

2.2. Sample collection

After completion of the feeding period the animals were killed under light anaesthesia. Four hours before euthanasia each pig was fed its respective diet. After killing, blood was

collected into heparinised polyethylene tubes. Plasma was obtained by centrifugation of the blood (1100 g, 10 min, 4 °C). Samples of liver and *musculus longissimus dorsi* were taken and stored at −80 °C until analysis. For isolation of enterocytes of small intestine, the abdomen was immediately opened after killing, and a 35 cm intestinal segment was dissected starting at 30 cm distal to the pyloric sphincter, and flushed two times with ice-cold wash buffer (phosphate-buffered saline containing 0.2 mM phenylmethylsulphonyl fluoride and 0.5 mM dithiothreitol, pH 7.4). The isolation of porcine enterocytes of small intestine was performed by the modified distended intestinal sac technique according to Fan et al. (2004). After isolation, enterocytes of small intestine were washed two times with ice-cold phosphate-buffered saline, retained by centrifugation (400 g, 4 min, 4 °C), and immediately snap-frozen in liquid nitrogen.

2.3. Analysis of carnitine and γ-butyrobetaine

Concentrations of free carnitine, acetyl carnitine and γ-butyrobetaine in plasma and tissues were determined by tandem mass spectrometry with deuterated carnitine-d_3 (Larodane Fine Chemicals, Malmö, Sweden) as internal standard as described recently in detail (Ringseis et al., 2007).

2.4. Activity of γ-butyrobetaine dioxygenase

Activity of γ-butyrobetaine dioxygenase in liver homogenates was determined as described previously in detail by Van Vlies et al. (2006). Liver homogenates were prepared by homogenising hepatic tissue in 10 mM Mops buffer (pH 7.4) containing 0.9% (w/v) NaCl, 10% (w/v) glycerol, and 5 mM dithiothreitol.

2.5. RNA isolation and real-time detection PCR analysis

For the determination of mRNA expression levels of OCTN2, liver and muscle isoforms of carnitine palmitoyltransferase-1 (L-CPT-1, determined in liver and enterocytes from small intestine; M-CPT, determined in muscle), γ-butyrobetaine dioxygenase, acyl-CoA oxidase (ACO), liver and intestinal isoforms of fatty acid-binding protein (L-FABP, determined in liver and muscle; I-FABP, determined in enterocytes from small intestine), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) for normalisation total RNA were isolated from liver, skeletal muscle and enterocytes using Trizol™ reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. GAPDH served as an appropriate reference gene in this experiment since the cycle threshold (Ct)-values of GAPDH did not differ between both groups. RNA concentration and purity were estimated from the optical density at 260 and 280 nm, respectively. 1.2 μg of total RNA was subjected to cDNA synthesis using M-MuLV Reverse Transcriptase (MBI Fermentas, St. Leon-Rot, Germany). For determination of mRNA expression levels real-time detection RT-PCR using a MJ Research Opticon System (Biozym Diagnostik, Oldendorf, Germany) was applied. 2 μL cDNA templates were amplified in 200 μL PCR tubes in a final volume of 20 μL containing 500 μmol/L dNTP (Roth, Karlsruhe, Germany), 3.5 mmol/L $MgCl_2$, 1.25 U GoTaq® Flexi DNA Polymerase, 4 μL 5× buffer (all from Promega, Mannheim, Germany), 0.5 μL 10× Sybr Green I (Sigma-Aldrich, Taufkirchen, Germany), and 26.7 pmol of each primer pair. Characteristics of gene-specific primers obtained from Operon (Köln, Germany) are shown in Table 1. The PCR protocol comprised an initial denaturation at 95 °C for 3 min and 20–35 cycles of amplification comprising denaturation at 95 °C for 25 s, annealing at primer-specific temperatures (60 °C) for 30 s and elongation at 72 °C for 55 s. Subsequently melting curve analysis was performed from 50 °C to 99 °C with continuous fluorescence measurement. The amplification of a single product of the expected size was confirmed using 1% agarose gel electrophoresis. Amplification efficiencies for all primers were determined by template dilution series. Calculation of the relative mRNA concentration was made using the amplification efficiencies and the Ct values (Pfaffl, 2001). Relative expression ratios are expressed as fold changes of mRNA concentration in the clofibrate group compared to the control group.

2.6. Statistical analysis

Student's t test was used to compare means of treatments with those of control. Differences with $P<0.05$ were considered to be significant. Data in the text are given as means ± SD.

3. Results

3.1. Food intake and body weights

Due to the controlled feeding system, food intake throughout the feeding period was the same for each pig in the experiment, averaging 696 ± 7 g/day. Body weight after 28 days of

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
ACO	CTCGCAGACCCAGATGAAAT	TCCAAGCCTCGAAGATGAGT	AF185048
BBD	AGTCACTGGGGGTGATTCAG	GTTTGGATTGGACGGAGAAA	Partial sequence (Ruan et al., 2007)
GAPDH	AGGGGCTCTCCAGAACATCATCC	TCGCGTGCTCTTGCTGGGGTTGG	AF017079
I-FABP	TACAGCCTCGCAGACGGAACTG	TGCTTGATGAGGAGAGGAAAACAG	AY960624
L-CPT-1	GCATTTGTCCCATCTTTCGT	GCACTGGTCCTTCTGGGATA	AF288789
L-FABP	TTCGGTGCATGTCTAAGCTG	TGAGAGGGAGAGGATGAGGA	DQ182323
M-CPT-1	ACTGTCTGGGCAAACCAAAC	CTTCTTGATGAGGCCTTTGC	NM_001007191
OCTN2	TGACCATATCAGTGGGCTA	AGTAGGGAGACAGGATGCT	Partial sequence (Ruan et al., 2007)

experiment did not differ between control pigs and pigs treated with clofibrate (control: 26.0±1.5 kg; clofibrate: 25.2±1.2 kg, $P>0.05$).

3.2. mRNA concentrations of PPARα target genes in liver, skeletal muscle and enterocytes of small intestine

To assess activation of PPARα, mRNA concentrations of three PPARα target genes FABP, CPT-1 and ACO were determined in liver, skeletal muscle and enterocytes of small intestine. In the liver, relative mRNA concentrations of L-CPT-1, L-FABP and ACO were 2.2-, 2.4- and 1.7-fold, respectively, higher in pigs treated with clofibrate than in control pigs ($P<0.05$, Fig. 1). In muscle, none of the three genes was upregulated in pigs treated with clofibrate compared to control pigs (Fig. 1). In enterocytes of small intestine, mRNA concentration of L-CPT-1 was 2.0-fold higher in pigs treated with clofibrate compared to control pigs ($P<0.05$); mRNA concentrations of ACO and I-FABP in enterocytes of small intestine, however, did not differ between both groups of pigs.

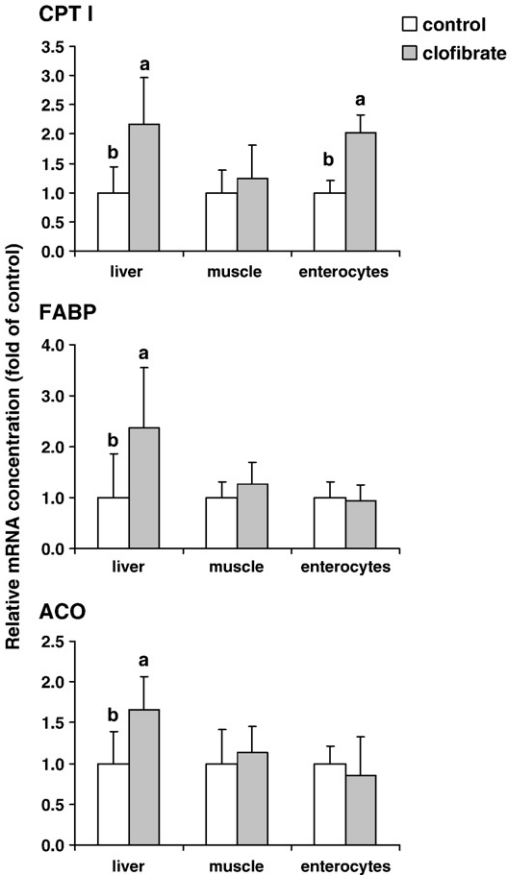

Fig. 1. Relative mRNA concentrations of ACO, CPT-1 and FABP in liver, skeletal muscle and enterocytes of small intestine in pigs fed a control diet (control) or a diet supplemented with 5 g clofibrate per kg (clofibrate) for 28 days. mRNA concentrations were determined by real-time RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase. Bars represent means±S.D. ($n=9$/group) and are expressed as fold increase compared to control. Bars marked without a common superscript letter differ ($P<0.05$).

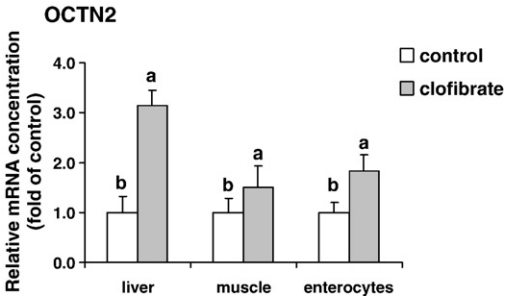

Fig. 2. Relative mRNA concentration of OCTN2 in liver, skeletal muscle and enterocytes of small intestine in pigs fed a control diet (control) or a diet supplemented with 5 g clofibrate per kg (clofibrate) for 28 days. mRNA concentrations were determined by real-time RT-PCR and normalized to glyceraldehyde-3-phosphate dehydrogenase. Bars represent means±S.D. ($n=9$/group) and are expressed as fold increase compared to control. Bars marked without a common superscript letter differ ($P<0.05$).

3.3. mRNA concentration of OCTN2 in liver, skeletal muscle and enterocytes of small intestine

Pigs treated with clofibrate had higher mRNA concentrations of OCTN2 in liver (3.1-fold), enterocytes of small intestine (1.8-fold) and skeletal muscle (1.5-fold) than control pigs ($P<0.05$, Fig. 2).

3.4. mRNA concentrations and activity of γ-butyrobetaine dioxygenase in the liver

The mRNA concentration of γ-butyrobetaine dioxygenase in the liver did not differ between both groups of pigs (clofibrate: 0.87±0.24; control: 1.00±0.33). The activity of γ-butyrobetaine dioxygenase in the liver was reduced in pigs treated with clofibrate compared to control pigs (clofibrate:

Table 2
Concentrations of carnitine (free, acetyl and total) and γ-butyrobetaine in plasma, liver and skeletal muscle of pigs fed a control diet (control) or a diet supplemented with 5 g clofibrate per kg (clofibrate) for 28 days

	Control	Clofibrate
Plasma (μmol/L)		
Free carnitine	6.34±1.33	7.52±1.68
Acetyl carnitine	1.17±0.65	1.15±0.48
Total carnitine	7.54±1.95	8.97±2.04
γ-Butyrobetaine	0.93±0.24	0.94±0.22
Liver (nmol/g)		
Free carnitine	42.6±6.8[b]	51.3±7.7[a]
Acetyl carnitine	0.80±0.22[a]	0.36±0.09[b]
Total carnitine	43.4±6.9[b]	51.7±7.8[a]
γ-Butyrobetaine	4.19±1.17	3.34±0.85
Skeletal muscle (nmol/g)		
Free carnitine	222±32[b]	273±59[a]
Acetyl carnitine	216±57	218±65
Total carnitine	441±66	493±103
γ-Butyrobetaine	61±19	55±16

Data are means±S.D. with nine animals per group. Means with different superscript letters (a, b) are significantly different ($P<0.05$).

0.29±0.05 nmol×min^{-1}×mg protein^{-1}; control: 0.42±0.08 nmol×min^{-1}×mg protein^{-1}, $P<0.05$).

3.5. Concentrations of carnitine and γ-butyrobetaine in liver, muscle and plasma

Pigs treated with clofibrate had a higher concentration of free carnitine and a lower concentration of acetyl carnitine in the liver than control pigs ($P<0.05$, Table 2). The concentration of total carnitine in the liver was higher in pigs treated with clofibrate than in control pigs ($P<0.05$, Table 2). Pigs fed clofibrate had also higher concentration of free carnitine in the skeletal muscle than control pigs; the concentration of acetyl carnitine in skeletal muscle did not differ between both groups (Table 2). The concentration of total carnitine in skeletal muscle was 12% higher in pigs treated with clofibrate than in control pigs; this difference was however not statistically significant (Table 2). Concentrations of free, acetyl and total carnitine in plasma did not differ between the two groups of rats (Table 2). The concentrations of γ-butyrobetaine in liver, muscle and plasma did also not differ between both groups of pigs (Table 2).

4. Discussion

We have recently found that treatment of rats with clofibrate leads to an up-regulation of OCTN2 in liver and small intestine of rats (Luci et al., 2006, Ringseis et al., 2007). To find out whether clofibrate causes an up-regulation of OCTN2 also in a non-proliferating species we performed an experiment with relatively young pigs with a body weight slightly in excess of 10 kg. We used such young pigs as pigs with a similar weight have been used in other studies to characterise PPARα expression and activation in pigs (Yu et al., 2001; Cheon et al., 2005; Peffer et al., 2005). The concentration of clofibrate in the diet of 5 g per kg diet was adopted from these studies (Yu et al., 2001; Cheon et al., 2005; Peffer et al., 2005), resulting in a daily dose of 220 mg per kg body weight. This dose is relatively high compared with doses used in humans for treatment of hyperlipidaemia, which are usually in the range between 25 and 30 mg per kg body weight.

To examine activation of PPARα, we determined mRNA concentrations of three classical PPARα target genes, namely ACO, CPT-1 and FABP, in the relevant tissues. As expected, clofibrate treatment caused an up-regulation of these genes in the liver, indicative of PPARα activation in this tissue. The extent of up-regulation, was however, relatively small compared to rodents in which treatment with PPARα agonists increases hepatic mRNA concentrations of PPARα target genes typically 10 to 20-fold compared to control (He et al., 2002; Frederiksen et al., 2004; Knight et al., 2005; König et al., 2007). The reason for the comparatively low up-regulation of these enzymes in pigs by clofibrate might be the lower hepatic PPARα expression in pigs compared to rodents. Furthermore, the presence of an alternative spliced PPARα isoform, which lacks the ligand-binding domain, could contribute to the lower responsiveness of the pig to clofibrate (Sundvold et al., 2001). The finding that clofibrate causes a moderate up-regulation of PPARα target genes agrees well with recent studies conducted in piglets that were treated with clofibrate. In these studies mRNA concentrations and activities of ACO and CPT-1 were two to four times higher in livers of piglets treated with clofibrate in doses similar to that used in the present study than in untreated piglets (Yu et al., 2001; Peffer et al., 2005). The finding that PPARα target genes in muscle and enterocytes were not up-regulated by clofibrate treatment (with the only exception of CTP-1 in enterocytes) indicates that there was no or at least a very small activation of PPARα in these tissues. The reason for the lacking PPARα activation in these tissues may be the fact that expression of PPARα is much lower in muscle and small intestine than in liver (Braissant et al., 1996; Kliewer et al., 1994). In addition, a differential expression of PPARα coactivators, which are required for induction of PPARα-dependent gene transcription, between different organs, might be also responsible for the cell- and tissue-specific induction of PPARα-responsive genes (Cook et al., 2000). Recent studies reporting tissue-specific expression of coactivators of PPARα (Zoete et al., 2007) are indeed supportive of this assumption. In rats, up-regulation of PPARα target genes by clofibrate in liver was also much stronger than in small intestine or skeletal muscle (Ringseis et al., 2007).

We found for the first time that clofibrate treatment causes an up-regulation of OCTN2 in liver, muscle and epithelial cells of the small intestine of pigs. According to recent studies in rodents and in rat liver cells (Luci et al., 2006; Ringseis et al., 2007, Van Vlies et al., 2007) we propose that up-regulation of OCTN2 in pigs may be at least in part due to PPARα activation. The finding that OCTN2 was also up-regulated in muscle and in enterocytes, although there was no indication of PPARα activation in these tissues, suggests that clofibrate could stimulate expression of OCTN2 also by a mechanism independent of PPARα. Clofibrate is a relatively unspecific PPARα agonist and exerts also a PPARγ binding activity (Krey et al., 1997). Whether transcription of OCTN2 could be also influenced by PPARγ has not yet been investigated.

Van Vlies et al. (2007) have recently shown that treatment with WY-14,643 increases the activity of γ-butyrobetaine dioxygenase in wild-type mice but not in PPARα knockout mice. They proposed that PPARα activation stimulates also carnitine biosynthesis in the liver. These findings are in contrast to our studies with rats in which clofibrate treatment did not increase mRNA concentration of γ-butyrobetaine dioxygenase in the liver of rats (Luci et al., 2006; Ringseis et al., 2007). The present study shows that clofibrate treatment does not increase mRNA concentration of γ-butyrobetaine dioxygenase and even reduces its activity in the liver of pigs. Although we cannot explain the observation that activity of γ-butyrobetaine dioxygenase was even reduced by clofibrate, these findings indicate that the capacity of the liver to synthesize carnitine was obviously not increased by clofibrate treatment. It has been shown that γ-butyrobetaine which is formed in most tissues is also a substrate of OCTN2 (Tamai et al., 2000). An up-regulation of OCTN2 in the liver therefore could increase the uptake of γ-butyrobetaine from plasma into the liver. As, however, γ-butyrobetaine concentrations in liver and plasma

were not altered by clofibrate, it is unlikely that more γ-butyrobetaine was available in the liver for carnitine biosynthesis. Although we did not directly quantify carnitine biosynthesis, we assume that increased carnitine concentrations in liver and muscle of pigs treated with clofibrate were not due to an increased carnitine biosynthesis but rather to an up-regulation of OCTN2. OCTN2 is localized in the plasma membrane of cells and delivers carnitine from the blood into cells (Lahjouji et al., 2001). An up-regulation of OCTN2 in liver and skeletal muscle indicates that more carnitine was transported from the blood into these tissues which might be a plausible explanation for the increased carnitine concentrations in these tissues of pigs treated with clofibrate. The importance of OCTN2 for the supply of cells with carnitine is evident by the fact that inborn or acquired defects of this transporter lead to primary or secondary systemic carnitine deficiency (Tein, 2003). OCTN1 is another system able to transport carnitine which was not considered in this study. In rats, hepatic OCTN1 was also up-regulated by clofibrate, half as much as OCTN2 (Luci et al., 2006; Ringseis et al., 2007). Therefore, the possibility exists that OCTN1 was also up-regulated in tissues of pigs treated with clofibrate. However, as OCTN1 plays a minor role for carnitine transport compared to OCTN2 due to its low carnitine transport activity (Tamai et al., 2000), it is likely that the effects on tissue carnitine concentrations were predominately mediated by OCTN2. OCTN3, another carnitine transporter, is not expressed in liver and muscle, and therefore does not contribute to changes in carnitine concentrations in these tissues (Tamai et al., 2000). In small intestine, OCTN2 is involved in absorption of carnitine from the diet (Sekine et al., 1998; Tamai et al., 1998). An up-regulation of OCTN2 in enterocytes of the small intestine therefore could improve carnitine absorption from the diet. As the diet used in the present study contained less carnitine, an improvement of the carnitine absorption probably had however less effect on whole body carnitine status.

The effects observed in this study are similar to those observed in our recent rat studies (Luci et al., 2006; Ringseis et al., 2007). The comparison of the present study with those recent rodent studies however shows that the up-regulation of OCTN2 in the liver and the increase of hepatic carnitine concentration are clearly stronger in rodents than in pigs. This is another indication that these effects were mediated by PPARα which is less responsive in pigs than in rats.

Activation of PPARα causes up-regulation of many genes involved in hepatic mitochondrial and peroxisomal β-oxidation. CPTs are rate limiting for β-oxidation of fatty acids (Brandt et al., 1998; Mascaro et al., 1998). The up-regulation of CPTs which is essential for the metabolic adaptations induced by activation of PPARα might increase the demand of carnitine in the respective tissue. We postulate that up-regulation of OCTN2 by PPARα activation is a means to supply 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 cells to PPARα activation.

The observed up-regulation of OCTN2 in tissues due to treatment with clofibrate 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 (Wu et al., 1999; Koepsell and Endou, 2004; Lahjouji et al., 2004; Grube et al., 2006; Hirano et al., 2006; Kato et al., 2006). Therefore, it is possible that fibrates and other PPARα agonists influence intestinal absorption and tissue distribution of such compounds.

In conclusion, the present study shows for the first time that clofibrate treatment causes an up-regulation of OCTN2 in liver, muscle and enterocytes of pigs, a model of non-proliferating species, probably by activation of PPARα. Up-regulation of OCTN2 may enhance carnitine uptake from blood into tissues which may be the reason for the increased carnitine concentrations in liver and muscle of pigs treated with clofibrate. As there exists a similarity in the gene response to PPARα agonists between pigs and humans, clofibrate or other PPARα agonists could exert similar effects in humans.

References

Braissant, O., Foufelle, F., Scotto, C., Dauca, M., Wahli, W., 1996. Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -β, and -δ in the adult rat. Endocrinology 137, 354–366.

Brandt, J.M., Djouadi, F., Kelly, D., 1998. Fatty acids activate transcription of the muscle carnitine palmitoyltransferase I gene in cardiac myocytes via the peroxisome proliferator-activated receptor alpha. J. Biol. Chem. 273, 23786–23792.

Brass, E.P., 2002. Pivalate-generating prodrugs and carnitine homeostasis in man. Pharmacol. Rev. 54, 589–598.

Brass, E.P., Hoppel, C.L., 1978. Carnitine metabolism in the fasting rat. J. Biol. Chem. 253, 2688–2693.

Cheon, Y., Nara, T.Y., Band, M.R., Beever, J.E., Wallig, M.A., Nakamura, M.T., 2005. Induction of overlapping genes by fasting and a peroxisome proliferator in pigs: evidence of functional PPAR alpha in nonproliferating species. Am. J. Physiol., Regul. Integr. Comp. Physiol. 288, R1525–R1535.

Cook, W.S., Yeldandi, A.V., Rao, M.S., Hashimoto, T., Reddy, J.K., 2000. Less extrahepatic induction of fatty acid β-oxidation enzymes by PPARα. Biochem. Biophys. Res. Commun. 278, 250–257.

Fan, M.Z., Matthews, J.C., Etienne, N.M., Stoll, B., Lackeyram, D., Burrin, D.G., 2004. Expression of apical membrane L-glutamate transporters in neonatal porcine epithelial cells along the small intestinal crypt–villus axis. Am. J. Physiol.: Gastrointest. Liver Physiol. 287, G385–G398.

Frederiksen, K.S., Wulff, E.M., Sauerberg, P., Mogensen, J.P., Jeppesen, L., Fleckner, J., 2004. Prediction of PPAR-alpha ligand-mediated physiological changes using gene expression profiles. J. Lipid Res. 45, 592–601.

Grube, M., Meyer zu Schwabedissen, H.E., Präger, D., Haney, J., Möritz, K.U., Meissner, K., Rosskopf, D., Eckel, L., Böhm, M., Jedlitschky, G., Kroemer, H.K., 2006. Uptake of cardiovascular drugs into the human heart: expression, regulation, and function of the carnitine transporter OCTN2 (SLC22A5). Circulation 113, 1114–1122.

He, W.S., Nara, T.Y., Nakamura, M.T., 2002. Delayed induction of delta-6 and delta-5 desaturases by a peroxisome proliferators. Biochem. Biophys. Res. Commun. 299, 832–838.

Hirano, T., Yasuda, S., Osaka, Y., Kobayashi, M., Itagaki, S., Iseki, K., 2006. Mechanism of the inhibitory effect of zwitterionic drugs (levofloxacin and grepafloxacin) on carnitine transporter (OCTN2) in Caco-2 cells. Biochim. Biophys. Acta 1758, 1743–1750.

Holden, P.R., Tugwood, J.D., 1999. Peroxisome proliferator-activated receptor alpha: role in rodent liver cancer and species differences. J. Mol. Endocrinol. 22, 1–8.

Kato, Y., Sugiura, M., Sugiura, T., Wakayama, T., Kubo, Y., Kobayashi, D., Sai, Y., Tamai, I., Iseki, S., Tsuji, A., 2006. Organic cation/carnitine transporter

OCTN2 (Slc22a5) is responsible for carnitine transport across apical membranes of small intestinal epithelial cells in mouse. Mol. Pharmacol. 70, 829–837.

Kliewer, S.A., Forman, B.M., Blumberg, B., Ong, E.S., Borgmeyer, U., Mangelsdorf, D.J., Umesono, K., Evans, R.M., 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. Proc. Natl. Acad. Sci. U. S. A. 91, 7355–7359.

Knight, B.L., Hebbach, A., Hauton, D., Brown, A.M., Wiggins, D., Patel, D.D., 2005. A role for PPARα in the control of SREBP activity and lipid synthesis in the liver. Biochem. J. 389, 413–421.

Koepsell, H., Endou, H., 2004. The SLC22 drug transporter family. Pflugers Arch. 447, 666–676.

König, B., Koch, A., Spielmann, J., Hilgenfeld, C., Stangl, G.I., Eder, K., 2007. Activation of PPARalpha lowers synthesis and concentration of cholesterol by reduction of nuclear SREBP-2. Biochem. Pharmacol. 73, 574–585.

Krey, G., Braissant, O., L'Horset, F., Kalkhoven, E., Perroud, M., Parker, M.G., Wahli, W., 1997. Fatty acids, eicosanoids, and hypolipidemic agents identified as ligands of peroxisome proliferator-activated receptors by coactivator-dependent receptor ligand assay. Mol. Endocrinol. 11, 779–791.

Lahjouji, K., Elimrani, I., Lafond, J., Leduc, L., Qureshi, I.A., Mitchell, G.A., 2004. L-Carnitine transport in human placental brush-border membranes is mediated by the sodium-dependent organic cation transporter OCTN2. Am. J. Physiol. Cell Physiol. 287, C263–C269.

Lahjouji, K., Mitchell, G.A., Qureshi, I.A., 2001. Carnitine transport by organic cation transporters and systemic carnitine deficiency. Mol. Genet. Metab. 73, 287–297.

Luci, S., Geissler, S., König, B., Koch, A., Stangl, G.I., Hirche, F., Eder, K., 2006. PPARα agonists up-regulate organic cation transporters in rat liver cells. Biochem. Biophys. Res. Commun. 24, 704–708.

Luci, S., Giemsa, B., Kluge, H., Eder, K., 2007. Clofibrate causes an upregulation of PPARα target genes but does not alter expression of SREBP target genes in liver and adipose tissue of pigs. Am. J. Physiol., Regul. Integr. Comp. Physiol. 293, R70–R77.

Mascaro, C., Acosta, E., Ortiz, J.A., Marrero, P.F., Hegardt, F.G., Haro, D., 1998. Control of human muscle-type carnitine palmitoyltransferase I gene transcription by peroxisome proliferator-activated receptor. J. Biol. Chem. 273, 8560–8563.

McGarry, J.D., Brown, N.F., 1997. The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur. J. Biochem. 244, 1–14.

McGarry, J.D., Robles-Valdes, C., Foster, D.W., 1975. Role of carnitine in hepatic ketogenesis. Proc. Natl. Acad. Sci. U. S. A. 72, 4385–4388.

National Research Council, 1998. Nutrient Requirement of Swine, Tenth Revised Edition. National Academie of Sciences, Washington DC.

Ohashi, R., Tamai, I., Nezu Ji, J., Nikaido, H., Hashimoto, N., Oku, A., Sai, Y., Shimane, M., Tsuji, A., 2001. Molecular and physiological evidence for multifunctionality of carnitine/organic cation transporter OCTN2. Mol. Pharmacol. 59, 358–366.

Ohashi, R., Tamai, I., Yabuuchi, H., Nezu, J.I., Oku, A., Sai, Y., Shimane, M., Tsuji, A., 1999. Na^+-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. J. Pharmacol. Exp. Ther. 291, 778–784.

Paul, H.S., Adibi, S.A., 1979. Paradoxical effects of clofibrate on liver and muscle metabolism in rats. Induction of myotonia and alteration of fatty acid and glucose oxidation. J. Clin. Invest. 64, 405–412.

Peffer, P.L., Lin, X., Odle, J., 2005. Hepatic beta-oxidation and carnitine palmitoyltransferase I in neonatal pigs after dietary treatments of clofibric acid, isoproterenol, and medium-chain triglycerides. Am. J. Physiol., Regul. Integr. Comp. Physiol. 288, R1518–R1524.

Peters, J.M., Cheung, C., Gonzalez, F.J., 2005. Peroxisome proliferator-activated receptor-alpha and liver cancer: where do we stand? J. Mol. Med. 83, 774–785.

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, e45.

Rebouche, C.J., Seim, H., 1998. Carnitine metabolism and its regulation in microorganisms and mammals. Annu. Rev. Nutr. 18, 39–61.

Ringseis, R., Pösel, S., Hirche, F., Eder, K., 2007. Treatment with pharmacological peroxisome proliferator-activated receptor α agonist clofibrate causes upregulation of organic cation transporter 2 in liver and small intestine of rats. Pharmacol. Res. 56, 175–183.

Ruan, J., Guo, Y., Li, H., Hu, Y., Song, F., Huang, X., Kristiensen, K., Bolund, L., Wang, J., 2007. PigGIS: Pig Genomic Informatics System. Nucleic Acids Res. 35 (Database issue), D654–D657.

Schoonjans, K., Peinado-Onsurbe, J., Lefebvre, A.M., Heyman, R.A., Briggs, M., Deeb, S., Staels, B., Auwerx, J., 1996. PPARα and PPARγ activators direct a distinct tissue-specific transcriptional response via a PPRE in the lipoprotein lipase gene. Embo J. 15, 5336–5348.

Sekine, T., Kusuhara, H., Utsunomiya-Tate, N., Tsuda, M., Sugiyama, Y., Kanai, Y., Endou, H., 1998. Molecular cloning and characterization of high-affinity carnitine transporter from rat intestine. Biochem. Biophys. Res. Commun. 251, 586–591.

Slitt, A.L., Cherrington, N.J., Hartley, D.P., Leazer, M.T., Klaassen, C.D., 2002. Tissue distribution and renal developmental changes in rat organic cation transporter mRNA levels. Drug Metab. Dispos. 30, 212–219.

Steiber, A., Kerner, J., Hoppel, C.L., 2004. Carnitine: a nutritional, biosynthetic, and functional perspective. Mol. Asp. Med. 25, 455–473.

Sundvold, H., Grindflek, E., Lien, S., 2001. Tissue distribution of porcine peroxisome proliferator-activated receptor α: detection of an alternatively spliced mRNA. Gene 273, 105–113.

Tamai, I., Ohashi, R., Nezu, J., Yabuuchi, H., Oku, A., Shimane, M., Sai, Y., Tsuji, A., 1998. Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. J. Biol. Chem. 273, 20378–20382.

Tamai, I., Ohashi, R., Nezu, J.I., Sai, Y., Kobayashi, D., Oku, A., Shimane, M., Tsuji, A., 2000. Molecular and functional characterization of organic cation/carnitine transporter family in mice. J. Biol. Chem. 275, 40064–40072.

Tamai, I., Yabuuchi, H., Nezu, J., Sai, Y., Oku, A., Shimane, M., Tsuji, A., 1997. Cloning and characterization of a novel human pH-dependent organic cation transporter, OCTN1. FEBS Lett. 419, 107–111.

Tein, I., 2003. Carnitine transport: pathophysiology and metabolism of known defects. J. Inherit. Metab. Dis. 26, 147–169.

Van Vlies, N., Wanders, R.J., Vaz, F.M., 2006. Measurement of carnitine biosynthesis enzyme activities by tandem mass spectrometry: differences between the mouse and the rat. Anal. Biochem. 354, 132–139.

Van Vlies, N., Ferdinandusse, S., Turkenburg, M., Wanders, R.J., Vaz, F.M., 2007. PPARα-activation results in enhanced carnitine biosynthesis and OCTN2-mediated hepatic carnitine accumulation. Biochim. Biophys. Acta 1767 (9), 1134–1142.

Vaz, F.M., Wanders, R.J., 2002. Carnitine biosynthesis in mammals. Biochem. J. 361, 417–429.

Wu, X., Huang, W., Prasad, P.D., Seth, P., Rajan, D.P., Leibach, F.H., Chen, J., Conway, S.J., Ganapathy, V., 1999. Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. J. Pharmacol. Exp. Ther. 290, 1482–1492.

Yu, X.X., Odle, J., Drackley, J.K., 2001. Differential induction of peroxisomal beta-oxidation enzymes by clofibric acid and aspirin in piglet tissues. Am. J. Physiol., Regul. Integr. Comp. Physiol. 281, R1553–R1561.

Zoete, V., Grosdidier, A., Michielin, O., 2007. Peroxisome proliferator-activated receptor structures: ligand specificity, molecular switch and interactions with regulators. Biochim. Biophys. Acta 1771, 915–925.