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PPARα-activation results in enhanced carnitine biosynthesis and OCTN2-mediated hepatic carnitine accumulation

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Abstract

In fasted rodents hepatic carnitine concentration increases considerably which is not observed in PPARα−/− mice, indicating that PPARα is involved in carnitine homeostasis. To investigate the mechanisms underlying the PPARα-dependent hepatic carnitine accumulation we measured carnitine biosynthesis enzyme activities, levels of carnitine biosynthesis intermediates, acyl-carnitines and OCTN2 mRNA levels in tissues of untreated, fasted or Wy-14643-treated wild type and PPARα−/− mice. Here we show that both enhancement of carnitine biosynthesis (due to increased γ-butyrobetaine dioxygenase activity), extra-hepatic γ-butyrobetaine synthesis and increased hepatic carnitine import (OCTN2 expression) contributes to the increased hepatic carnitine levels after fasting and that these processes are PPARα-dependent.

Keywords: Carnitine; PPARα; Carnitine biosynthesis; OCTN2; γ-butyrobetaine dioxygenase; fasting

1. Introduction

Peroxisome Proliferator Activated Receptor α (PPARα) is a ligand-dependent transcription factor. Upon ligand binding, PPARα heterodimerizes with the Retinoid X Receptor and binds to specific response elements (PPARα response elements, PPREs) to stimulate expression of target genes. PPARα is expressed in tissues with a high rate of fatty acid oxidation (such as liver, heart, skeletal muscle, kidney and brown adipose tissue) [1] and several aspects of fatty acid metabolism are regulated by PPARα, including the cellular uptake of fatty acids, the activation of fatty acids, peroxisomal and mitochondrial β-oxidation, ketogenesis, ω-oxidation and lipoprotein metabolism [1–6].

Several observations suggest that PPARα is involved in carnitine homeostasis. Carnitine is an essential compound in fatty acid metabolism due to its role in the transfer of long-chain fatty acids into the mitochondrial matrix, where β-oxidation takes place [7,8]. Carnitine is also used to transport peroxisomal β-oxidation products to the mitochondria, to export accumulating acyl-groups and to modulate the level of free CoA in different subcellular compartments [9,10].

Carnitine can be absorbed from the diet and synthesized endogenously [10]. OCTN2 (SLC22A5) is the sodium-dependent carnitine transporter responsible for the dietary absorption of carnitine. This transporter also mediates cellular uptake and renal reabsorption of both carnitine and its precursor, 4-trimethylaminobutyric acid (γ-butyrobetaine, γ-BB) (Fig. 1) [10,11]. The first metabolite of the carnitine biosynthesis pathway (Fig. 1) is 6-N-trimethyllysine (TML), which is generated by lysosomal degradation of proteins containing trimethylated lysine residues (such as calmodulin, histones, actin and myosin). Free TML is hydroxylated by the enzyme TML-dioxygenase (EC 1.14.11.8, TMLD) to 3-hydroxy-6-N-trimethyllysine (HTML). HTML is

Abbreviations: PPARα, Peroxisome proliferator activated receptor α; TML, 6-N-trimethyllysine; TMLD, 6-N-trimethyllysine dioxygenase; HTML, 3-hydroxy-6-N-trimethyllysine; TMABA, 4-trimethylaminobutyraldehyde; TMABA-DH, 4-trimethylaminobutyraldehyde dehydrogenase; γ-BB, 4-trimethylaminobutyric acid; γ-BBD, 4-trimethylaminobutyric acid dioxygenase

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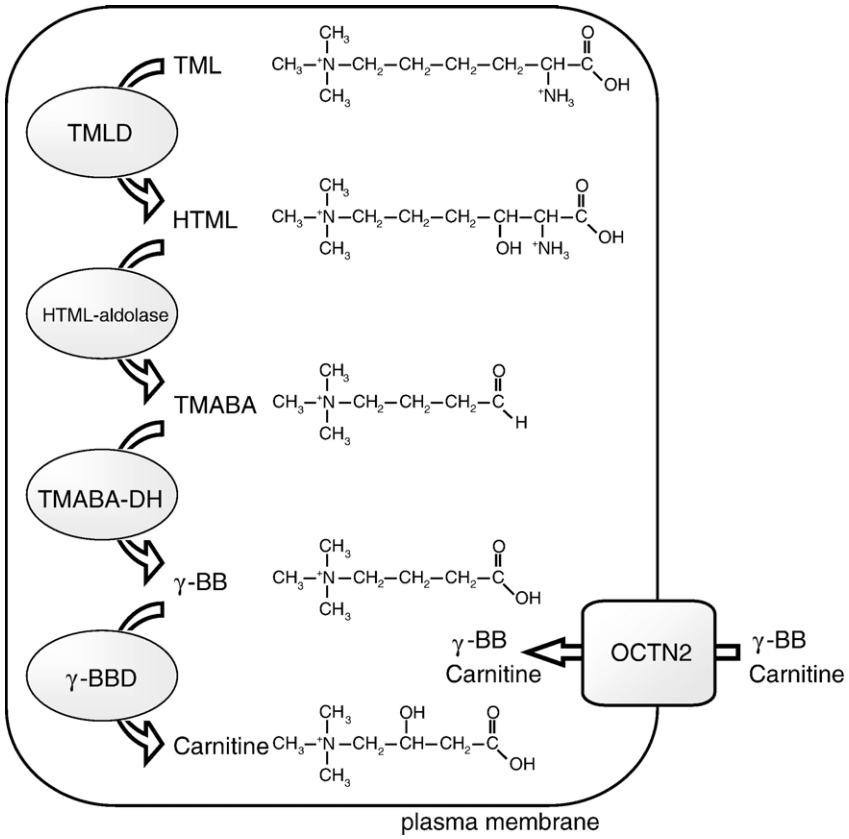

Fig. 1. Cellular import and biosynthesis of carnitine: TML (6-N-trimethyllysine) is hydroxylated by TMLD (TML-dioxygenase) to HTML (3-hydroxy-6-N-trimethyllysine), which is converted to TMABA (4-trimethylaminobutyraldehyde) by HTML-aldolase. TMABA-DH (TMABA-dehydrogenase) oxidizes TMABA to γ-BB (4-trimethylaminobutyric acid), which is hydroxylated to carnitine by γ-BBD (γ-BB dioxygenase). OCTN2 imports carnitine and its precursor, γ-BB [10].

subsequently converted to 4-trimethylaminobutyraldehyde (TMABA), which is oxidized by TMABA-dehydrogenase (EC 1.2.1.47, TMABA-DH) to γ-BB. Finally, γ-BB is hydroxylated to carnitine by γ-BB dioxygenase (EC 1.14.11.1, γ-BBD), which in mice is only present in liver [12], (Fig. 1) [10,13,14].

In both rat and mouse, treatment with PPARα agonists has been shown to influence liver carnitine metabolism [5,15]. Upon treatment, hepatic carnitine concentration increased considerably in two independent studies [5,15]. Based on experiments with clofibrate-fed rats (a synthetic PPARα ligand) Paul et al. proposed that the increase was due to an increase in the rate of hepatic carnitine synthesis [15]. More recently, however, we could not demonstrate increased activity of carnitine biosynthesis enzymes in phytol-treated mice (phytanic acid and its precursor phytol, are natural PPARα ligands [16,17]), despite an obvious increase in hepatic carnitine concentration [5]. When mice or rats are fasted, which also results in PPARα activation, liver carnitine levels also rise considerably [6,18]. This is not observed in fasted or PPARα agonist-fed PPARα−/− mice, indicating that this effect is mediated via PPARα [5,6].

To investigate the role of PPARα in carnitine metabolism and determine what mechanism underlies the hepatic increase of carnitine concentration we used wild type and PPARα−/− mice which were either fed normal chow, chow containing the potent PPARα agonist Wy-14643 [1] or were fasted for 48 h. We measured carnitine biosynthesis enzyme activities, levels of carnitine biosynthesis intermediates, acylcarnitines and OCTN2 mRNA levels in selected tissues and compared the data from the different treatment groups. Our results show that both enhanced carnitine biosynthesis and differential OCTN2 expression contribute to the PPARα-dependent changes in carnitine metabolism.

2. Materials and methods

2.1. Chemicals

RNA*later* reagent was obtained from Qiagen, the Trizol reagent from Invitrogen, The first-strand cDNA synthesis kit and the LightCycler FastStart DNA Master SYBR Green I kit from Roche and Wy-14643 was purchased from Tocris Bioscience.

2.2. Animals

Control diet was produced by soaking pelleted mouse chow (CRM(pl), Technilab SDS) in ethanol (33 ml ethanol per 100 g pellets) and the pellets were left to dry. For the 0.1% (w/w) Wy-14643-diet, 100 g pellets were soaked in 33 ml ethanol containing 3 mg/ml (e.g. 100 mg) Wy-14643. Subsequently, the pellets were left to dry.

All mice were obtained from the Jackson Laboratory. Male 129 wild type and PPARα−/− mice were fed normal mouse chow until 9 weeks of age. Next, mice were divided into three groups. The first group (wild type $n=5$ and PPARα−/− $n=5$) was fed control food for 2 weeks at libitum. The second group (wild type $n=5$ and PPARα−/− $n=5$) received chow supplemented with 0.1% Wy-14643 for 2 weeks at libitum. The last group (wild type $n=5$ and PPARα−/−

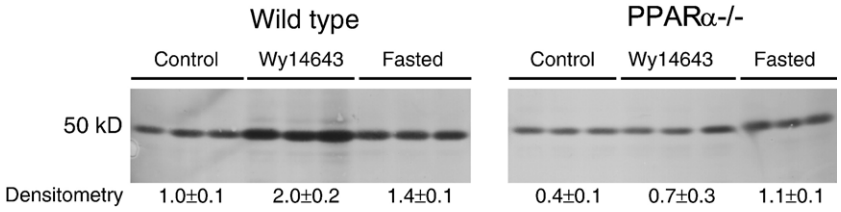

Fig. 2. Western blot analysis of peroxisomal straight-chain acyl-CoA oxidase in livers of 3 wild type and 3 PPARα−/− mice on control diet, on Wy-14643-diet or fasted for 48 h. Band intensities were quantified using ImageJ sofware (http://rsb.info.nih.gov/ij/index.html) [21]. Values are means±standard deviation of three mice.

$n=4$) was fed control diet for 2 weeks and subsequently fasted for 48 h. All mice had free access to water and were killed at the same time of day. At the end of the experiment, mice were anesthetized with isoflurane and blood was collected by cardiac puncture. Tissues (liver, heart, kidney and gastrocnemius muscle) were collected and a small piece was treated with RNA*later* reagent according to the manufacturer's instructions and stored at −80 °C until RNA

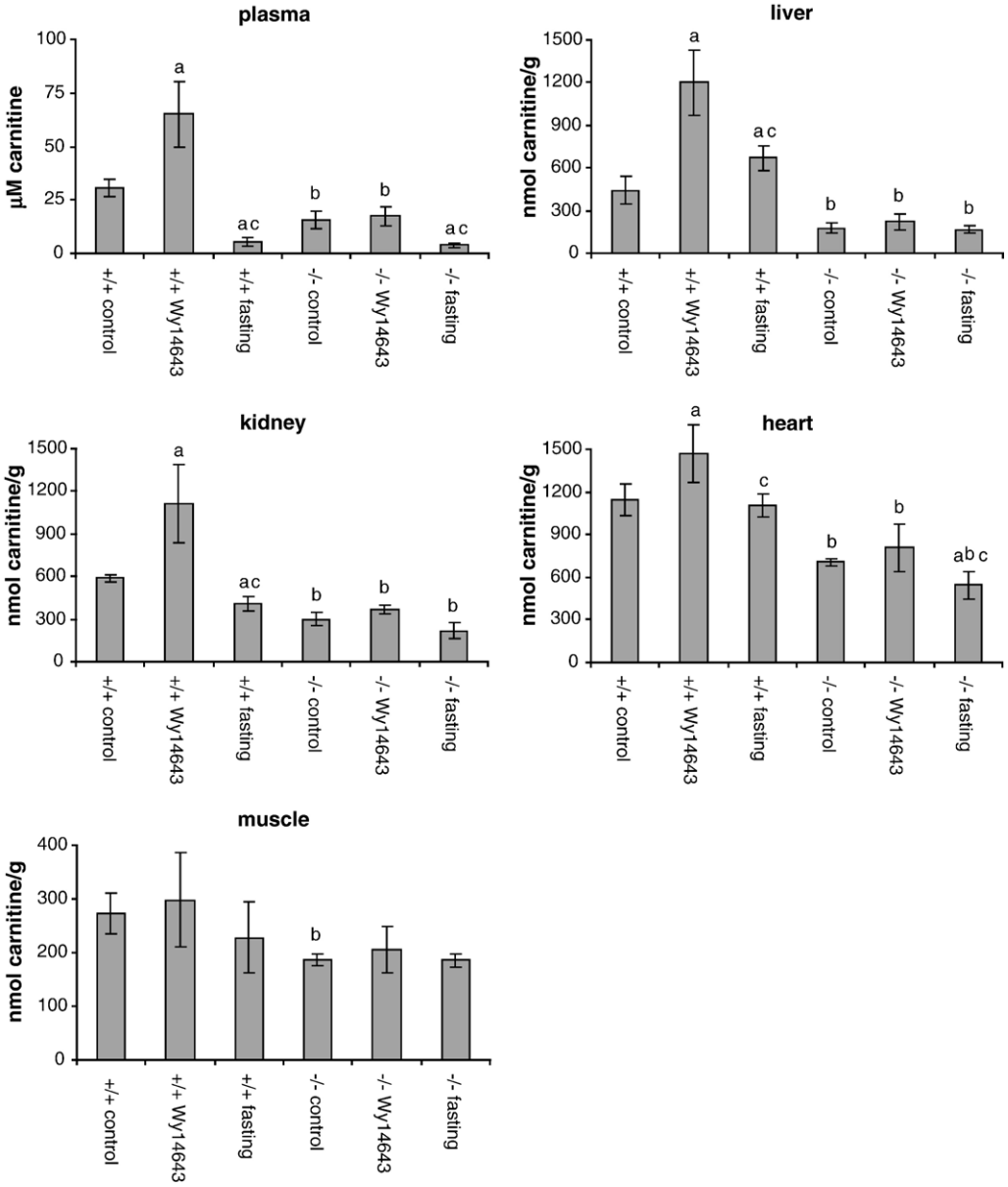

Fig. 3. Carnitine levels in plasma and different tissues of wild type (+/+) and PPARα−/− mice (−/−) on control diet, on Wy-14643-diet or fasted for 48 h. Values are means±standard deviation. [a] indicates a significant difference ($p<0.05$) between mice with the same genotype on the control diet and another treatment. [b] indicates a significant difference ($p<0.05$) between mice with a different genotype but the same treatment. [c] indicates a significant difference ($p<0.05$) between Wy-14643-treated mice and fasted mice with the same genotype.

isolation, the rest was immediately frozen in liquid nitrogen and stored at −80 °C until further use. All experiments were approved by the local ethical committee.

2.3. Carnitine metabolites and biosynthesis enzymes

Levels of carnitine biosynthesis intermediates, acyl-carnitines and activities of carnitine biosynthesis enzymes were determined as described previously [12,19]. In short, carnitine biosynthesis intermediates and acyl-carnitines were extracted from lyophillized tissue pieces using acetonitrile. The extracted acyl-carnitines were derivatized with a 4:1 mixture of propanol and acetylchloride and analyzed with tandem-MS. The extracted biosynthesis intermediates were derivatized with methylchloroformiate and analyzed with tandem-MS [20]. To determine the activities of the carnitine biosynthesis enzymes, tissue homogenates were incubated with the appropriate substrates and co-factors. After incubation the samples were deproteinized and derivatized with methylchloroformiate. Product formation was analyzed with tandem-MS [12].

2.4. Immunoblot analysis

Immunoblot analysis of peroxisomal straight-chain acyl-CoA oxidase was performed as described previously [5]. The bands were quantified using the ImageJ sofware (http://rsb.info.nih.gov/ij/index.html) [21].

2.5. Quantitative real-time RT-PCR analysis

Total RNA was isolated from RNA*later*-treated tissues using Trizol extraction. cDNA was produced using a first-strand cDNA synthesis kit. For the quantitative real-time PCR the LightCycler FastStart DNA Master SYBR Green I kit was used and the following primers: 5′GTGGGCCGCTCTAGGCACCAA3′ and 5′CTCTTTGATGTCACGCACGATTTC3′ for β-actin [22], 5′ ACCCCACTGTGGTCAGAAAC3′ and 5′CACCAAAGCTCTCAGGGAAG3′ for OCTN2 and 5′CACCTGGGGTTCAGCTTCTG3′ and 5′ CACATCACCTGGATTCATCTTG3′ for γ-BBD. All samples were analyzed in duplicate. To confirm that a single product was formed during PCR, melting curve analysis and agarose gel electrophoresis were performed. The data were analyzed using linear regression calculations as described previously [23] and values for OCTN2 and γ-BBD mRNA levels were normalized against the values for the housekeeping gene β-actin to adjust for variations in the amount of input RNA.

2.6. Statistical analyses

Data are expressed as means±standard deviation. Statistical significance was evaluated using an unpaired Student's *t*-test. The results were considered significant at $p \leq 0.05$.

3. Results

3.1. Mice

At the age of 9 weeks, there was no significant difference in body weight between wild type and PPARα−/− mice (21.3±1.9 g and 24.2±3.3 g). Also, after the diet period both groups had similar body weights (22.3±1.5 g and 25.8±3.5 g). After the 48-h fasting period wild type and PPARα−/− mice lost 4.6±0.5 g, which was approximately 17% of their total body weight.

3.2. Activation of PPARα

In order to confirm activation of PPARα and enhanced expression of its target genes by the 0.1% Wy-14643 diet and the 48-h fasting period, we used immunoblot analysis of peroxisomal straight-chain acyl-CoA oxidase, a known PPARα target. Compared to wild type mice on the control diet, straight-chain acyl-CoA oxidase was much more abundant in livers of fasted wild type mice and wild type mice on the Wy-14643-diet (Fig. 2), demonstrating that both treatment protocols activate PPARα and induce expression of this target gene. As expected, the Wy-14643-diet had no effect in PPARα−/− mice. Fasting does appear to increase the quantity of straight-chain acyl-CoA oxidase in PPARα−/− mice, indicating that the abundance of this protein is also regulated in a PPARα-independent manner. This effect has been observed previously [5].

3.3. Carnitine levels

Measurement of plasma and tissue carnitine levels showed that they differ profoundly between wild type and PPARα−/− mice and between the different treatment groups (Fig. 3). Because the differences in free carnitine levels between the groups were very similar when compared to the differences observed in total carnitine levels, we only show results for free carnitine. This also implies that the degree of acylation is not different, which was confirmed by analysis of the acyl-carnitine spectra (results not shown).

Table 1
Activity of carnitine biosynthesis enzymes in tissues of wild type and PPARα−/− mice

		Wild type mice			PPARα−/− mice		
		Control diet	Wy-14643 diet	Fasting	Control diet	Wy-14643 diet	Fasting
Liver	TMLD	21.7±6.9	19.4±5.3	19.0±3.4	37.4±8.7 [b]	37.7±4.2 [b]	40.0±7.2 [b]
	TMABA-DH	4654±712	5137±981	4015±587	4218±368	4340±608	3259±271 [a, c]
	γ-BBD	141±17	208±19 [a]	269±34 [a, c]	151±21	177±14 [b]	148±19 [b]
Kidney	TMLD	45.1±6.0	52.2±8.0	43.5±7.2	44.0±6.0	51.2±15.8	52.0±17.7
	TMABA-DH	2241±314	3753±93 [a]	2838±120 [a, c]	1632±167 [b]	1732±189 [b]	2109±315 [a, b]
Heart	TMLD	30.5±5.5	18.1±6.0 [a]	21.2±4.4 [a]	33.9±3.3	34.9±5.5 [b]	33.0±4.6 [b]
	TMABA-DH	189±87	225±86	166±20	250±70	207±48	156±31 [a]
Muscle	TMLD	3.6±2.1	3.6±1.1	1.8±0.3 [c]	0.6±0.4 [b]	0.9±0.7 [b]	1.4±1.2
	TMABA-DH	102±61	64±13	36±18	ND	ND	ND

All activities are given in pmol×min^{-1}×mg protein^{-1}. Values are means±standard deviation. ND=not detectable.
[a] With significant difference ($p<0.05$) between mice with the same genotype on the control diet and another treatment.
[b] With significant difference ($p<0.05$) between mice with a different genotype but the same treatment.
[c] With significant difference ($p<0.05$) between Wy-14643-treated mice and fasted mice with the same genotype.

On the control diet, there is already a significant difference in carnitine levels between wild type and PPARα−/− mice. Compared to wild type mice, PPARα−/− mice had lower plasma carnitine concentration (16±4 μM vs. 30±4 μM) and lower carnitine levels in all investigated tissues (Fig. 3).

When wild type mice were fed Wy-14643, the carnitine levels in plasma, liver, kidney, and heart rose significantly when compared to mice fed the control diet (Fig. 3). In fasted wild type mice, only the hepatic carnitine level was elevated (1.5-fold) compared to mice on the control diet. In these animals, plasma carnitine level was decreased when compared to fed mice.

Neither the Wy-14643-diet nor fasting led to an elevation of plasma or tissue carnitine levels in PPARα−/− mice (Fig. 3).

3.4. Carnitine biosynthesis enzymes and intermediates

In order to determine if the observed differences in carnitine levels resulted from differences in carnitine biosynthesis we measured the activities of carnitine biosynthesis enzymes (Table 1) and the levels of biosynthesis intermediates (Fig. 4).

The lower carnitine levels observed in the tissues and plasma of PPARα−/− mice on the control diet, compared to wild type mice on control diet, were probably not due to diminished carnitine biosynthesis, since the carnitine biosynthesis enzyme activities and the levels of carnitine biosynthesis intermediates are similar in both groups. The activity of the different enzymes involved in carnitine biosynthesis only was lower in skeletal

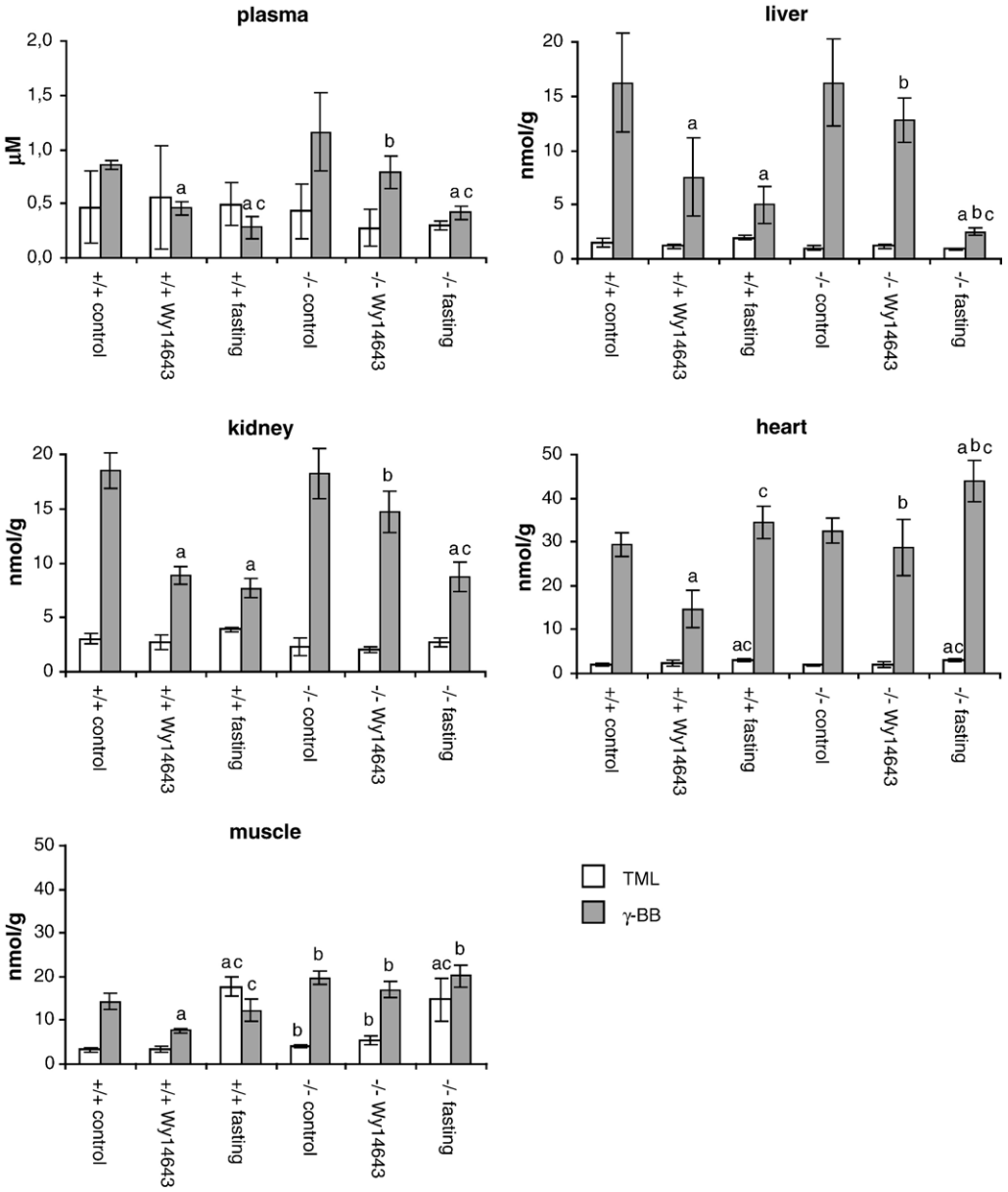

Fig. 4. TML (open bars) and γ-BB (filled bars) levels in plasma and different tissues of wild type (+/+) and PPARα−/− (−/−) mice on control diet, on Wy-14643-diet or fasted for 48 h. Values are means±standard deviation. [a] indicates a significant difference ($p<0.05$) between mice with the same genotype on the control diet and another treatment. [b] indicates a significant difference ($p<0.05$) between mice with a different genotype but the same treatment. [c] indicates a significant difference ($p<0.05$) between Wy-14643-treated mice and fasted mice with the same genotype.

muscle and kidney of the PPARα−/− mice (Table 1). In skeletal muscle, however, the γ-BB production probably is minimal even in wild type mice, given the very low TMLD and TMABA-DH activity in this tissue. TMABA-DH activity is always much higher than the TMLD activity, which suggests that the lower TMABA-DH activity observed in PPARα−/− kidney probably has no effect on γ-BB production in this tissue. This is supported by the fact that the γ-BB levels in muscle and kidney do not differ significantly between wild type and PPARα−/− mice (Fig. 4). As shown previously [12], HTML is not detectable in any of the investigated mouse tissues and plasma.

The increase in plasma and tissue carnitine levels after Wy-14643 treatment of wild type mice is accompanied by a decrease of γ-BB levels in all tissues and plasma compared to the control diet (Fig. 4). This is most likely caused by enhanced conversion of γ-BB into carnitine, because there is a significant increase in liver γ-BBD activity after the Wy-14643-diet (208±19 vs. 141±17 pmol × min^{-1} × mg^{-1}, Table 1). The lower γ-BB levels in the Wy-14643 group were probably not caused by a decrease in γ-BB synthesis, because TML levels (Fig. 4) and TMLD activity (Table 1) were similar to those of the control group in all tissues except heart, which showed a lower TMLD activity. Tissue TMABA-DH activity was similar in both groups except in kidney, where a higher TMABA-DH was measured after the Wy-14643-diet.

When wild type mice were fasted, the hepatic γ-BBD activity almost doubled, compared to fed mice (Table 1), most likely resulting in enhanced carnitine production. This was supported by the fact that γ-BB levels were decreased in plasma, liver and kidney of fasted mice (Fig. 4), whereas γ-BB synthesis did not appear to be reduced given the normal plasma, kidney and liver TML levels (Fig. 4) and TMLD / TMABA-DH activity (Table 1).

Like the carnitine levels, carnitine biosynthesis enzyme activities and biosynthesis intermediate levels were not altered by either the Wy-14643 treatment or fasting in the PPARα−/− mice.

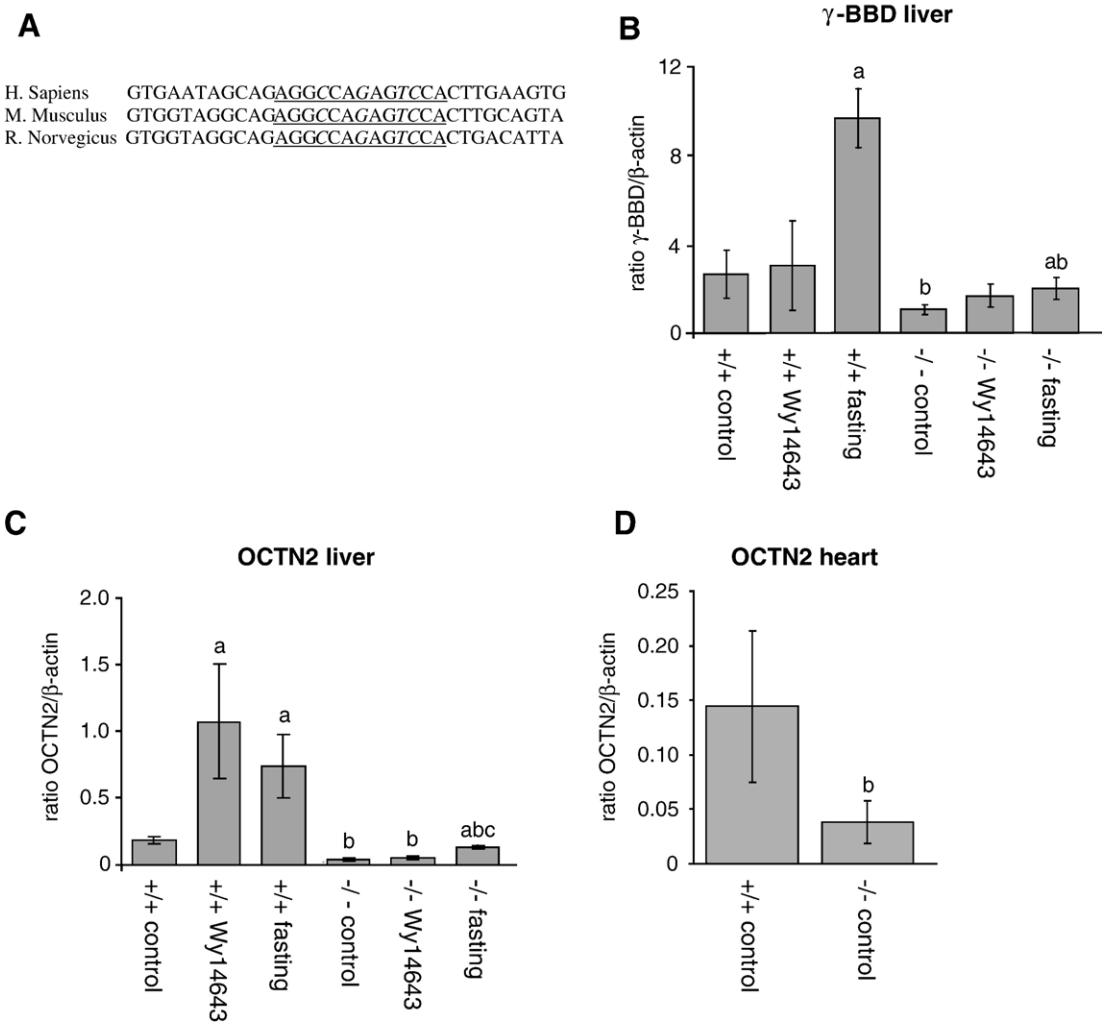

Fig. 5. (A) Alignment of DR1 sequences (underlined) of the mouse, rat and human *BBOX1* genes. The four nucleotides which are different from the consensus PPRE sequence are printed in italics. (B) γ-BBD mRNA levels in livers of wild type (+/+) and PPARα−/− (−/−) mice on control diet, on Wy-14643-diet or fasted for 48 h. (C) OCTN2 mRNA levels in livers of wild type (+/+) and PPARα−/− (−/−) mice on control diet, on Wy-14643-diet or fasted for 48 h. (D) OCTN2 mRNA levels in hearts of wild type (+/+) and PPARα−/− (−/−) mice on control diet. Values are means ± standard deviation. [a] indicates a significant difference ($p<0.05$) between mice with the same genotype on the control diet and another treatment. [b] indicates a significant difference ($p<0.05$) between mice with a different genotype but the same treatment. [c] indicates a significant difference ($p<0.05$) between Wy-14643-treated mice and fasted mice with the same genotype.

3.5. γ-BBD and OCTN2 mRNA levels

Because of the apparent PPARα-dependent increase in γ-BBD activity, we examined the promotor of the γ-BBD gene (*bbox1*) of mouse, rat and man for PPREs and measured the mRNA levels of γ-BBD in liver. Using the NUBIScan software [24] we analyzed a 1000-bp sequence upstream of the ATG of the *BBOX1* gene from mouse, rat and man. We found three conserved DR1 sequences in all three species located approximately 110 bp upstream of the start-codon. Fig. 5A shows an alignment of the region containing the DR1 sequences. The NUBIScan score and *p*-value for all three analyses were 0.81647 (with a maximum of 1.0) and <0.002, respectively.

We also determined the hepatic γ-BBD mRNA levels in the different groups using quantitative PCR. The γ-BBD mRNA levels are lower in the PPARα−/− mouse when compared to wild type (Fig. 5B). Fasting induces the expression of γ-BBD in the wild type mice but not in PPARα−/− mice. Surprisingly, no enhanced expression is observed in Wy-14643-treated wild type mice.

Apart from the rate of carnitine biosynthesis, carnitine levels can also be influenced by alterations in carnitine transport. To investigate this, we determined OCTN2 mRNA levels in various tissues by quantitative PCR. In liver and heart, PPARα−/− mice had considerably lower levels of OCTN2 mRNA (5-fold and 3.5-fold, respectively) compared to the wild type mice (Fig. 5C). This could contribute to the lower carnitine levels observed in these tissues of PPARα−/− mice. Surprisingly, no significant difference in kidney or skeletal muscle OCTN2 expression was found between wild type and PPARα−/− mice (results not shown).

In the wild type mice both the Wy-14643 diet and fasting led to an significant increase in hepatic OCTN2 expression (Fig. 5C). This most likely contributes directly to the increased hepatic carnitine level, but also indirectly given that OCTN2 also transports γ-BB, which (via the elevated γ-BBD activity) also yields carnitine. Surprisingly, OCTN2 expression was unchanged in kidney, heart and skeletal muscle after fasting or Wy-14643 treatment (results not shown). Unfortunately, neither urine nor intestine was collected, so we cannot exclude the possibility that renal carnitine reabsorption or increased intestinal carnitine uptake also contributes to the increased carnitine levels. However, because the kidney OCTN2 mRNA levels (the main transport system for carnitine reabsorption) were unchanged, compared to the control diet, it is unlikely that there is enhanced renal reabsorption of carnitine contributing to the higher tissue carnitine levels.

As expected, the Wy-14643-diet had no effect in PPARα−/− mice. Hepatic OCTN2 mRNA levels, however, did increase upon fasting, when compared to fed PPARα−/− mice (Fig. 5C). Nevertheless, expression of OCTN2 in the liver of fasting PPARα−/− mice was still lower (~2-fold) than in wild type mice on control diet and therefore probably too low to increase the liver carnitine concentration.

In contrast to the apparent PPARα-dependency of OCTN2, NUBIScan analysis of the OCTN2 promotor (1000 bp upstream of the start-codon) did not reveal a DR1 sequence with a low *p*-value.

4. Discussion

Fasting or treatment with PPARα agonists increases the carnitine concentration in liver. The reason for this increase in hepatic carnitine content is still a matter of speculation, however, it seems logical to increase the liver carnitine levels during fasting when β-oxidation is enhanced, since carnitine is an important buffer of acyl-groups and thereby ensures that there is enough free CoA to sustain the high metabolic flux.

We have shown that in fasted and Wy-14643-fed wild type mice the elevation of the liver carnitine level is caused by an enhancement of both hepatic carnitine and γ-BB uptake (OCTN2 expression) and biosynthesis (primarily by increasing γ-BBD activity). In PPARα−/− mice, neither the Wy-14643-treatment nor fasting led to an elevation of the hepatic carnitine concentration or enhancement of the liver γ-BBD activity or mRNA levels. These results indicate that γ-BBD activity is regulated by PPARα. In contrast, hepatic γ-BBD mRNA levels of wild type mice were not elevated by 2 weeks of Wy-14643 treatment. This discrepancy has also been observed for short-chain hydroxyl-acyl-CoA dehydrogenase [2], where 2 weeks of Wy-14643 treatment resulted in a clear induction of short-chain hydroxyl-acyl-CoA dehydrogenase protein but not in elevation of the mRNA levels. The observed differences between γ-BBD activity and mRNA levels could result from the relatively long period of Wy-14643 treatment (2 weeks). Wy-14643 treatment for 6 h or 5 days did result in an increase in γ-BBD mRNA levels (1.95-fold and 1.7-fold, respectively) as was shown in recent experiments (data available at http://nutrigene.4t.com/microarray/ppar2007/, Kersten, S., personal communication). It is possible that after an initial stimulation of γ-BBD expression by PPARα the mRNA levels slowly return to normal while the protein levels remain elevated. γ-BBD activity, however, does not appear to correspond with its mRNA levels. γ-BBD activity is similar in livers of both wild type and PPARα−/− mice on the control diet, but mRNA levels are much lower in the PPARα−/− mice compared to wild type animals. The presence of a putative PPRE in the promoter of the *bbox1* gene supports the role of PPARα in the regulation of γ-BBD. Although 4 nucleotides in this DR1 sequence differ from the consensus PPRE sequence, the fact that this sequence is conserved between mouse, rat and man and that these alternative nucleotides are also found in PPRE sequences of other genes indicates that this sequence is likely to act as a functional PPRE. Further investigations are needed to confirm this.

Previously, we did not observe higher γ-BBD activity in phytol treated mice [5], despite a clear elevation of the liver carnitine content. Although we did not measure OCTN2 mRNA levels in these mice, it is very likely that enhanced hepatic carnitine uptake was the cause of the increased liver carnitine concentration after the phytol diet. Because phytol metabolites are much weaker PPARα agonists than Wy-14643, a difference in PPARα sensitivity between OCTN2 and γ-BBD might explain the fact that we did not find increased γ-BBD activity in our phytol-treated mice. This is supported by recent experiments (data available at http://nutrigene.4t.com/microarray/ppar2007/, Kersten, S., personal communication), where Wy-14643 treatment for 5 days

resulted in a 16- to 60-fold increase in OCTN2 expression but only in a 1.7-fold increase in γ-BBD mRNA levels. OCTN2 appears to be more sensitive to stimulation by PPARα than γ-BBD.

Fasting (but not Wy-14643-treatment) did lead to a relatively large increase (3-fold) in liver OCTN2 expression in PPARα−/− mice, although OCTN2 mRNA levels only increased to half of wild type levels on control diet. This indicates that OCTN2 expression is regulated both by a PPARα-dependent and independent mechanism. In fed rats (where levels of endogenous PPARα ligands are low) treatment with glucagon or anti-insulin serum (which mimics the levels of these hormones during fasting) led to an increase in liver carnitine [18]. Although this experiment does not differentiate between elevation of the carnitine concentration by biosynthesis or by hepatic carnitine uptake, these results together with the observed increase in OCTN2 expression in the fasted PPARα−/− mice suggest that liver OCTN2 levels could be influenced by high glucagon or low insulin levels. More studies are needed to investigate whether this relation indeed exists.

While γ-BBD and OCTN2 are upregulated by PPARα activation, TMLD activity in wild type mice was not altered by Wy-14643-treatment, except in heart (Table 1). In this tissue TMLD activity was decreased in both fasted and Wy-14643-fed wild type mice when compared to the control diet (Table 1). This was not observed in the PPARα−/− mice, indicating that the reduction in heart TMLD activity is PPARα-dependent. In contrast, liver TMLD activity is not altered by Wy-14643-treatment or fasting in both wild type and PPARα−/− mice (Table 1). PPARα−/− mice, however, have an almost 2-fold higher basal TMLD activity as compared to wild type mice, indicating that like in heart, TMLD activity is negatively influenced by PPARα. Oddly, the opposite was observed in skeletal muscle, where the TMLD activity in PPARα−/− mice on control or on Wy-14643-diet was lower than in wild type mice. Given these discrepancies, it seems that TMLD is not only regulated by PPARα, but also by other factors.

Although Wy-14643-supplemented chow and fasting both stimulate PPARα, these treatments have different effects on carnitine homeostasis. Wy-14643 very specifically stimulates PPARα, while during fasting carnitine metabolism is also influenced by other factors. PPARα activation in fed Wy-14643 treated mice, however, does not reflect the physiological situation in which PPARα is normally stimulated by endogenous ligands, which are released upon fasting. The differences between these treatments are apparent in Fig. 4. Heart and skeletal muscle TML and γ-BB levels are elevated in fasted animals compared to Wy-14643 treated mice (Fig. 4). These differences are PPARα-independent because they are also observed in PPARα−/− mice. TML originates from protein breakdown [13,25], and for this reason the increase in TML levels most likely reflects fasting-induced proteolysis. The elevated levels of TML indirectly, via extra-hepatic conversion to γ-BB, provide the liver with additional carnitine precursors. In heart, the higher TML levels are accompanied by an increase in γ-BB, but this is not observed in skeletal muscle (Fig. 4). This could be explained by the very low muscle TMLD activity and the almost complete absence of TMABA-DH activity (Table 1).

In Wy-14643-treated wild type mice, plasma, liver, kidney and heart carnitine levels were higher when compared to fasted wild type mice (Fig. 3). Since there was no significant difference in carnitine acylation (results not shown) or carnitine biosynthesis enzyme activities between these two groups, the difference in free carnitine level is probably due to the difference in length of the treatment. Mice were treated with Wy-14643 for 2 weeks but fasted for "only" 48 h. In both situations carnitine synthesis was enhanced, γ-BBD activity after fasting was even more increased than after Wy-14643-treatment (Table 1), but Wy-14643-treated mice had more time to accumulate carnitine than fasted mice. Possibly, carnitine first accumulates in the liver and later, after a prolonged period of carnitine synthesis, also in other tissues. This may result in the observed low plasma carnitine level of fasted wild type mice and the elevated plasma carnitine level in Wy-14643 treated wild type mice. We cannot exclude, however, that differences in urinary carnitine excretion contribute to the observed carnitine levels.

During preparation of this manuscript Luci et al. reported that OCTN2 expression is increased in the liver of clofibrate-fed rats [26]. The authors did not find an increase in mRNA levels coding for carnitine biosynthesis enzymes after PPARα agonist treatment. Based on this observation and the fact that TML levels in medium of rat hepatoma cells did not decrease upon Wy-14643 treatment, they concluded that the PPARα-dependent increase in liver carnitine levels was solely mediated by enhanced OCTN2 expression and not by enhanced carnitine biosynthesis. This is in contrast to our results with Wy-14643-treated and fasted wild type mice, where an increase in both γ-BBD activity (and γ-BBD mRNA levels after fasting) and OCTN2 expression was found, which both contribute to elevated carnitine levels. These differences could be the result of the difference in species (mice vs. rats) or that 4 days of clofibrate treatment is less effective in stimulating PPARα target genes than Wy-14643 (a 5-day treatment of Wy-14643 does result in upregulation of γ-BBD mRNA).

Although the studies of Luci et al. are well designed, the authors forgot to take into account several aspects of carnitine homeostasis. Firstly, γ-BB is an excellent substrate for OCTN2 [11]. For this reason, upregulation of this transporter also enhances the delivery of carnitine equivalents to the liver and given the very large capacity of the liver to convert γ-BB into carnitine [27], the upregulation of OCTN2 enhances the flux through the carnitine biosynthesis pathway. Secondly, TML is poorly absorbed by the liver [28]. The kidney converts most of the circulatory TML into γ-BB, which is subsequently imported into the liver and converted into carnitine [29]. The fact that TML levels in medium of rat hepatoma cells remain the same after Wy-14643 treatment [26] does not disprove that the increase of the carnitine concentration in livers of rodents treated with PPARα agonists is caused by an increased hepatic carnitine synthesis. In general, experiments in cell culture systems are not suited to draw conclusions concerning whole organism metabolism.

In conclusion, we show that the observed increase in liver carnitine levels upon fasting or PPARα agonist treatment is mediated by a PPARα-dependent increase in hepatic transport

of both carnitine and its precursor γ-butyrobetaine, as well as enhanced systemic carnitine biosynthesis.

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