Reduced hepatic fatty acid oxidation in fasting PPARα null mice is due to impaired mitochondrial hydroxymethylglutaryl-CoA synthase gene expression

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Abstract Glucose and fatty acid metabolism (oxidation versus esterification) has been measured in hepatocytes isolated from 24 h starved peroxisome proliferator-activated receptor-α (PPARα) null and wild-type mice. In PPARα null mice, the development of hypoglycemia during starvation was due to a reduced capacity for hepatic gluconeogenesis secondary to a 70% lower rate of fatty acid oxidation. This was not due to inappropriate expression of the hepatic CPT I gene, which was similar in both genotypes, but to impaired mitochondrial hydroxymethylglutaryl-CoA synthase gene expression in the PPARα null mouse liver. We also demonstrate that hepatic steatosis of fasting PPARα null mice was not due to enhanced triglyceride synthesis.

Key words: Gluconeogenesis; Fatty acid oxidation; Triglyceride synthesis; Mitochondrial hydroxymethylglutaryl-CoA synthase; Peroxisome proliferator-activated receptor-α null mouse

1. Introduction

The discovery of peroxisome proliferator-activated receptors (PPAR), members of the superfamily of nuclear hormone receptors, has provided new insights into the regulation of gene expression by fatty acids and peroxisome proliferators (reviewed in [1]). The PPAR family contains three distinct isoforms: α, β/δ and γ. Whereas the physiological impact of the ubiquitous PPARβ/δ is still unknown (reviewed in [1]), it has been shown that PPARγ is a major factor involved in adipocyte differentiation (reviewed in [2]). The phenotypic characterization of PPARα null mice suggested that this receptor is mainly involved in the control of hepatic fatty acid and glucose metabolism. For instance, a late onset sexually dimorphic phenotype has been observed in PPARα null mice. The 8-month-old females became obese whereas age-matched males developed a centrilobular steatosis [3]. In younger animals, the most striking difference in the phenotypes between wild-type and PPARα null mice was observed during starvation. During starvation, PPARα null mice developed severe hypoglycemia [4,5] which was attributed to a defect in hepatic glucose production [5]. As the expression of the gene encoding phosphoenolpyruvate carboxykinase (PEPCK, the rate limiting enzyme in gluconeogenesis) was normally increased in the liver of fasting PPARα null mice [5], it was suggested that hepatic gluconeogenesis was due to a limitation of fatty acid oxidation. This conclusion was only based upon the fact that the plasma β-hydroxybutyrate concentration was markedly reduced in fasting PPARα null when compared to wild-type mice [4,5]. Moreover, it was suggested that the development of fatty liver during starvation in PPARα knockout mice resulted from the impaired fatty acid oxidation. However, all these assumptions were deduced from the level of mRNA encoding specific proteins without measurement of enzyme activities or metabolic fluxes.

The aim of the present study was to determine (1) the rates of gluconeogenesis and fatty acid oxidation in isolated hepatocytes from fasting wild-type and PPARα null mice, (2) the expression genes encoding regulatory proteins of these metabolic pathways, and (3) the capacity for fatty acid esterification in hepatocytes isolated from fasting mice of both genotypes.

2. Materials and methods

2.1. Animals

Three- to five-month-old wild-type or homozygous PPARα null (−/−) mice on a C57BL/6 genetic background were used. They were housed at 23°C in individual plastic cages with light from 07.00 to 19.00 h. They had free access to water and food (in terms of energy: 73% carbohydrate, 5% fat, 22% protein; A03, UAR, Epinay, France). After intraperitoneal pentobarbital anesthesia (100 mg/kg body weight), livers from fed and 24 h starved mice of both genotypes were rapidly sampled, frozen in liquid nitrogen and kept at −80°C until RNA extraction. Mice were starved for 24 h before hepatocyte isolation.

2.2. Isolation and incubation of mouse hepatocytes

The livers from two wild-type and two PPARα null (−/−) mice were perfused simultaneously with Hanks' balanced salt solution (HBSS, KCl 5.4 mM, KH_2PO_4 0.45 mM, NaCl 138 mM, $NaHCO_3$ 4.2 mM, Na_2HPO_4 0.34 mM, glucose 5.5 mM, HEPES 1 M, EGTA 50 mM, $CaCl_2$ 50 mM, pH 7.4). Livers were washed at a rate of 5 ml/min using the portal vein. Then collagenase (0.025%) was added and the perfusion rate was decreased to 4 ml/min. Hepatocytes ($1–2\times10^6$ cells/ml) were incubated in 2 ml of oxygenated (O_2:CO_2 95:5) glucose-free HBSS for 1 h in a gyratory shaking water bath.

2.3. Glucose production rates and fatty acid metabolism

2.3.1. Glucose production. The rates of glucose production were determined after 1 h incubation period in the absence (endogenous)

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or in the presence of lactate/pyruvate (10/1 mM). The incubations were ended by adding 0.2 ml $HClO_4$ (40% v/v).

2.3.2. Fatty acid oxidation and esterification. Long-chain and medium-chain fatty acid metabolism was studied using [1-^{14}C]oleate (0.3 mM; 0.5 μCi/μmol) plus carnitine (1 mM) and [1-^{14}C]octanoate (0.6 mM; 0.1 μCi/mmol), respectively. Both fatty acids were bound to 2% (w/v) defatted albumin. For studies of fatty acid oxidation and ketogenesis, incubations were ended by adding 0.2 ml $HClO_4$ (40% v/v). The production of $^{14}CO_2$ and labeled acid-soluble products was determined as previously described for [^{14}C]oleate [6] and [^{14}C]octanoate [7] respectively. For studies of oleate esterification, incubations were ended by centrifugation for 30 s at $3000 \times g$. The lipids from the chloroform/methanol (2:1 v/v) cell extracts were separated by thin layer chromatography, as described previously [8].

2.4. Metabolite analysis

Glucose, β-hydroxybutyrate and acetoacetate concentrations were measured in the neutralized perchloric filtrates by enzymatic methods as described previously [9].

2.5. Measurement of in vivo liver lipogenesis

Well fed and 24 h starved mice of both genotypes were injected intraperitoneally with (250 μCi in 200 μl) ^3H_2O (1 mCi/mmol). One hour later, mice were anesthetized with sodium pentobarbital (800 mg/kg body weight) and the livers were rapidly sampled. Saponification and extraction of radiolabeled fatty acids were performed on 1 g of liver according to Stansbie et al. [10].

2.6. Extraction and Northern blot analysis of total RNA

Total RNA from frozen tissues was extracted with guanidinium thiocyanate followed by purification through a CsCl cushion gradient according to Chirgwin et al. [11]. RNA was quantified by ultraviolet absorbance at 260 nm (260/280 ratio >1.8). Northern blot analysis of total RNA (20 μg) was performed after 1% agarose gel electrophoresis in 2.2 M formaldehyde as previously described [12]. Hybridization of the blots with an excess of [γ-^{32}P]ATP-labelled synthetic oligonucleotide specific for the 18S rRNA subunit [13] allowed us to correct for possible variations in the amount of RNA transferred onto the membranes. The hybridization probes are the 2.6 kb *Pst*I fragment from PCK 10 [14], the 1.1 kb *Pst*I fragment from glucose 6-phosphatase (Glc-6-Pase) [15], the *Eco*RI fragment from p61a CPT I [16] and the *Kpn*I fragment from pMS1-hydroxymethylglutaryl (HMG)-CoA synthase [17]. Probes were radiolabeled using the multiprime DNA labelling system (Amersham). Quantifications were performed by scanning densitometry of the autoradiographs.

2.7. Statistical analysis

Results are expressed as means ± S.E.M. Statistical analysis was performed using the rank-order test [18].

3. Results and discussion

The blood glucose concentration in 24 h starved PPARα null mice was 60% lower than in wild-type ones (2.8 ± 0.2 versus 7.4 ± 1.0 mM, $n=6$, $P<0.01$). These values were similar to those reported for fasting PPARα +/+ and PPARα −/− bred on a SV129 background [4,5]. This suggests that genetic background did not alter the glycemic response to fasting. In PPARα null mice the fasting hypoglycemia could be due, at least in part, to a reduced capacity for hepatic gluconeogenesis. Indeed, the rate of glucose production measured from lactate/pyruvate was 20% lower in hepatocytes isolated from PPARα null mice than in wild-type mice (Table 1). This reduced capacity for gluconeogenesis in the liver of PPARα null mice cannot be attributed to inappropriate induction of the two rate limiting enzymes of gluconeogenesis, PEPCK and Glc-6-Pase. As shown in Table 1 both PEPCK and Glc-6-Pase gene expression were normally induced after 24 h starvation in the liver of PPARα null mice. Such results were previously reported for PEPCK in SV129 background mice [5]. Once PEPCK and Glc-6-Pase genes have been fully induced, active hepatic gluconeogenesis is also dependent upon cofactors (acetyl-CoA, ATP and NADH) provided by mitochondrial fatty acid oxidation. Thus, we determined the rates of long- and medium-chain fatty acid oxidation in hepatocytes isolated from each genotype. As shown in Table 2 the rate of long-chain (oleate) fatty acid oxidation was 70% lower in PPARα null mice than in wild-type ones. This did not result from a limitation in the entry of long-chain fatty acids into mitochondria since the expression of the CPT I gene (the

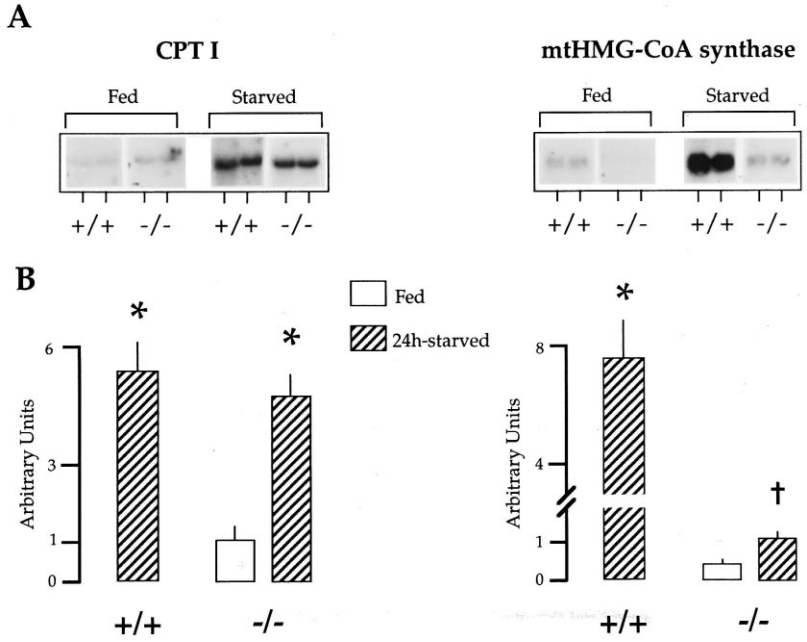

Fig. 1. A: Representative Northern blot analysis of mitochondrial CPT I and HMG-CoA synthase gene expression in the liver of fed and 24 h starved PPARα null (−/−) and wild-type (+/+) mice. B: Densitometric quantification of mRNA levels. Results are means ± S.E.M. of four wild-type mice and six PPARα null mice, the reference value (1) being the level of mRNA in the liver of fed wild-type mice. *$P<0.01$ compared to fed wild-type mice, †$P<0.01$ compared to 24 h starved wild-type mice.

Table 1
Rates of glucose production in mice of both genotypes

	PPARα (+/+)	PPARα (−/−)
Glucose production (nmol/h/10^6 hepatocytes)	540 ± 10	430 ± 20*
PEPCK mRNA (% of fed level)	260 ± 30†	310 ± 30†
Glc-6-Pase mRNA (% of fed level)	190 ± 20†	160 ± 10†

The rates of glucose production were measured from a mixture of lactate/pyruvate (10/1 mM) in 1 h incubated hepatocytes isolated from 24 h starved mice of both genotypes. Endogenous glucose production (absence of gluconeogenic substrate) was subtracted from each value. Liver PEPCK and Glc-6-Pase mRNA levels were measured in well fed and 24 h starved mice of both genotypes. Results are means ± S.E.M. of four to six different mice. *$P < 0.05$ compared to wild-type mice (+/+); †$P < 0.01$ compared to fed mice of the respective genotype.

rate limiting enzyme in long-chain fatty acid oxidation, reviewed in [19]) was increased to the same extent in the liver of 24 h starved wild-type and PPARα null mice (Fig. 1). Long-chain fatty acid oxidation is controlled not only by transcriptional activation of the CPT I gene but also through allosteric regulation by malonyl-CoA, a potent inhibitor of CPT I activity (reviewed in [19]). However, it seems unlikely that such a regulation could explain the reduced rate of long-chain fatty acid oxidation in PPARα null mice, since the oxidation of octanoate (which is independent of CPT I activity) was also 65% lower in hepatocytes from PPARα null mice than in wild-type ones (Table 2). Whatever the fatty acid used, the decrease in mitochondrial oxidation resulted from a reduction in the synthesis of acid-soluble products (Krebs cycle intermediates+ketones) due to an 80% inhibition of ketone body production (Table 2). This low rate of ketogenesis was due to an impaired induction of the mitochondrial form of HMG-CoA (mtHMG-CoA) synthase (the rate limiting enzyme of the ketogenic pathway [20]) in the liver of 24 h starved PPARα null mice when compared to wild-type mice (Fig. 1). It is noteworthy that mtHMG-CoA synthase gene expression was already decreased in the liver of fed PPARα null mice (Fig. 1). Such a metabolic defect was previously described in hepatoma cell lines [21] and in newborn pig liver [22] and was shown to be responsible for an inhibition of mitochondrial fatty acid oxidation. Indeed, an impaired utilization of acetyl-CoA in the mtHMG-CoA synthase pathway induced the accumulation of acetyl-CoA and/or β-oxidation intermediates that are strong inhibitors of acyl-CoA dehydrogenases [23]. In addition, octanoate oxidation could be blunted in PPARα null mice as the result of an impaired stimulation of medium-chain acyl-CoA dehydrogenase (a PPARα target gene) gene expression during starvation [4]. Whether oleate oxidation could also be limited in PPARα null mouse liver by inappropriate expression of long-chain acyl-CoA dehydrogenase gene remains to be determined.

Our data clearly show that mtHMG-CoA synthase gene expression was impaired in PPARα null mouse liver despite an increase in circulating free fatty acid concentration [5]. As fatty acids induced mtHMG-CoA synthase gene expression (reviewed in [20]), this suggested that transcriptional effects of fatty acids required PPARα as previously shown in vitro [24]. Hepatic mtHMG-CoA synthase gene expression is also controlled by cAMP and glucocorticoids during starvation (reviewed in [20]). The absence of induction of liver mtHMG-CoA synthase in fasting PPARα null mice could be due to an insensitivity of the gene to cAMP or glucocorticoids for unknown reasons. By contrast, starvation-induced CPT I, Glc-6-Pase and PEPCK gene expression seems to be completely independent of PPARα. This was expected for the PEPCK gene whose expression was not controlled by fatty acid in the liver (Pégorier and Girard, unpublished data), but was more surprising for Glc-6-Pase and CPT I which are both regulated by long-chain fatty acids [25,26]. Glc-6-Pase and CPT I are also tightly regulated by changes in pancreatic hormones [25,26]. Thus, the induction of PEPCK, Glc-6-Pase and CPT I genes during starvation could be mainly due to the fall in plasma insulin and a rise in plasma glucagon. Another possibility regarding the Glc-6-Pase and CPT I genes is that their induction by long-chain fatty acids is a PPARα-independent mechanism. Such a possibility has been recently discussed [27].

The last aim of this work was to determine whether the steatosis that develops during starvation in the liver of PPARα null mice [4,5] could be due to any of the non-exclusive following mechanisms: (1) an increase in endogenous fatty acid synthesis; (2) an increase in fatty acid uptake by the liver, (3) an increase in the capacity for hepatic triglyceride synthesis, (4) a decrease in the processing and/or exportation of very low density lipoprotein (VLDL).

Fatty acids used as precursors of triglycerides could theoretically come from endogenous fatty acid synthesis from glu-

Table 2
Long- and medium-chain fatty acid metabolism in hepatocytes isolated from 24 h starved PPARα (+/+) and PPARα (−/−) mice

	[1-^{14}C]Oleate converted (nmol/h/10^6 hepatocytes)		[1-^{14}C]Octanoate converted (nmol/h/10^6 hepatocytes)	
	PPARα +/+	PPARα −/−	PPARα +/+	PPARα −/−
CO_2	2.9 ± 0.6	2.4 ± 0.4	22.6 ± 2.2	25.5 ± 1.7
ASP	25.0 ± 1.3	6.6 ± 0.6*	166 ± 29	43 ± 8*
Total oxidized	27.8 ± 1.7	9.0 ± 1.0*	188 ± 31	68 ± 6*
Triglycerides	20.6 ± 1.6	22.4 ± 1.4		
Phospholipids	4.8 ± 0.5	3.0 ± 0.5		
Total esterified	25.4 ± 2.0	25.5 ± 1.3		
Acetoacetate	157 ± 28	24 ± 6*	271 ± 24	55 ± 6*
β-Hydroxybutyrate	175 ± 5	40 ± 3*	342 ± 36	90 ± 12*
Total ketones	331 ± 36	64 ± 8*	613 ± 50	145 ± 23*

Hepatocytes were incubated for 1 h in the presence of [1-^{14}C]oleate (0.3 mM) plus carnitine (1 mM), or in the presence of [1-^{14}C]octanoate (0.6 mM). Both fatty acids were bound to 2% fat-free albumin. ASP means acid-soluble products, representing citric acid cycle intermediates plus ketone bodies. Results are means ± S.E.M. of four different mice of each genotype. *$P < 0.01$ compared to wild-type mice (PPARα +/+).

cose (lipogenesis) and/or from fatty acid uptake by the liver. Hepatic lipogenesis is markedly reduced during starvation due to the inhibition of the transcription of key regulatory enzyme by long-chain fatty acids (reviewed in [28]). Therefore, if the fatty acid-induced inhibition of lipogenic enzyme gene transcription was dependent upon PPARα, then lipogenesis would not be down-regulated during starvation in PPARα null mouse liver. The rates of lipogenesis were indeed similar in the livers of fasting wild-type and PPARα null mice (respectively 2.6 ± 0.7 and 1.8 ± 0.5 μmol/h/g wet weight, $n = 3$). This suggest that the regulation of genes encoding key lipogenic enzymes (fatty acid synthase, acetyl-CoA carboxylase) by long-chain fatty acids is independent of PPARα as previously suggested [29].

Once inside the hepatic cell, long-chain fatty acids may undergo metabolism via two major pathways: (1) esterification to build up fatty acid esters (triglycerides, phospholipids) and (2) oxidation to CO_2 and ketone bodies. If we assume that the sum of radiolabeled oleate recovered in different fractions (CO_2, ASP, esterified products) is a good index of oleate uptake, then the capacity for oleate uptake is 35% lower in PPARα null mouse hepatocytes (34 nmol/h/10^6 hepatocytes) than in wild-type ones (53 nmol/h/10^6 hepatocytes). Although the mechanism by which fatty acids are taken up by liver cells is still a mater of debate (reviewed in [30]), a part of the uptake could be mediated by a fatty acid transport protein (FATP). As FATP is a PPARα target gene [31,32], the disruption of the PPARα gene could have reduced the amount of FATP in hepatocytes of PPARα null mice and thus the oleate uptake. Such a decreased capacity for fatty acid uptake by the liver would explain the higher plasma free fatty acid concentration in fasting PPARα null mice than in wild-type ones [5]. Although a greater proportion of fatty acid is converted into triglycerides in hepatocytes from PPARα null mice (73%) than in wild-type mouse hepatocytes (47%) as the consequence of the lower fatty acid uptake, this does not explain why triglycerides accumulate in PPARα null mice liver. Therefore, we tested the possibility that hepatic steatosis could be due to an enhanced capacity for esterification of fatty acids. Since octanoate cannot be incorporated directly into cell triglycerides [7], the rate of oleate esterification was solely investigated. As shown in Table 2 the rates of oleate esterification were similar whatever the genotype studied. Altogether these results clearly showed that hepatic steatosis in fasting PPARα null mice was neither due to an increase in maximal capacity for fatty acid esterification nor to an increase in fatty acid availability which was either similar (endogenous lipogenesis) or decreased (fatty acid uptake) when compared to wild-type mice.

As the expression of the genes encoding apolipoproteins E, B [5] or CIII [33] was not affected by PPARα knockout, this suggests that the packaging of triglycerides into VLDL could be normal and thus not responsible for the hepatic steatosis. This does not exclude the possibility that triglyceride accumulation in PPARα null mouse liver could be due to a defect in the process of VLDL secretion.

In conclusion, our data clearly demonstrate that fatty acid oxidation was markedly reduced in fasting PPARα null mouse liver secondary to an impaired induction of mtHMG-CoA synthase gene. This low rate of mitochondrial fatty acid oxidation could have two major consequences on hepatic metabolism: (1) a lower efficiency of gluconeogenesis due to a reduced availability in obligatory cofactors (acetyl-CoA, ATP and NADH) and (2) a reduced uptake of long-chain fatty acid by liver cells. This work also shows that the development of hepatic steatosis in fasting PPARα null mice is not due to an increased capacity for lipogenesis or fatty acid esterification.

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