Peroxisome-proliferator-activated receptor-α (PPARα) deficiency leads to dysregulation of hepatic lipid and carbohydrate metabolism by fatty acids and insulin

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The aim of the present study was to determine whether peroxisome-proliferator-activated receptor-α (PPARα) deficiency disrupts the normal regulation of triacylglycerol (TAG) accumulation, hepatic lipogenesis and glycogenesis by fatty acids and insulin using PPARα-null mice. In wild-type mice, hepatic TAG concentrations increased ($P < 0.01$) with fasting (24 h), with substantial reversal after refeeding (6 h). Hepatic TAG levels in fed PPARα-null mice were 2.4-fold higher than in the wild-type ($P < 0.05$), increased with fasting, but remained elevated after refeeding. PPARα deficiency also impaired hepatic glycogen repletion ($P < 0.001$), despite normal insulin and glucose levels after refeeding. Higher levels of plasma insulin were required to support similar levels of hepatic lipogenesis *de novo* (^3H_2O incorporation) in the PPARα-null mice compared with the wild-type. This difference was reflected by corresponding changes in the relationship between plasma insulin and the mRNA expression of the lipogenic transcription factor sterol-regulatory-element-binding protein-1c, and that of one of its known targets, fatty acid synthase. In wild-type mice, hepatic pyruvate dehydrogenase kinase (PDK) 4 protein expression (a downstream marker of altered fatty acid catabolism) increased ($P < 0.01$) in response to fasting, with suppression ($P < 0.001$) by refeeding. Although PDK4 up-regulation after fasting was halved by PPARα deficiency, PDK4 suppression after refeeding was attenuated. In summary, PPARα deficiency leads to accumulation of hepatic TAG and elicits dysregulation of hepatic lipid and carbohydrate metabolism, emphasizing the importance of precise control of lipid oxidation for hepatic fuel homoeostasis.

Key words: glycogen, lipogenesis, pyruvate dehydrogenase kinase, sterol-regulatory-element-binding protein, triacylglycerol.

INTRODUCTION

The mechanisms by which altered lipid metabolism might contribute to insulin resistance and dysregulation of fuel homoeostasis is currently the focus of intense research. Insulin-resistant subjects have been reported to exhibit elevated triacylglycerol (TAG) levels in muscle (reviewed in [1]). Similarly, high-fat feeding elicits excess tissue TAG accumulation, and is associated with development of insulin resistance and impaired fuel handling [2] (reviewed in [3]). Factors that influence tissue TAG accumulation include precursor supply, namely circulating non-esterified fatty acids (NEFA) and/or altered fatty acid oxidation (FAO), and their control by insulin. Recent studies [4] demonstrate that the adipose-tissue-derived hormone adiponectin reverses the insulin resistance associated with both lipoatrophy and obesity by decreasing TAG content in muscle and liver in obese mice, in part as a consequence of increased expression of FAO enzymes. Increased delivery of fatty acid (FA) via muscle- and liver-specific expression of lipoprotein lipase leads to increased tissue TAG accumulation [5]. In liver, increased TAG accumulation is associated with an impaired ability of insulin to suppress endogenous glucose production (EGP) [5]. It was suggested that there was a direct and causative relationship between the accumulation of intracellular FA-derived metabolites and control of hepatic metabolism by insulin [5].

Peroxisome-proliferator-activated receptor-α (PPARα) receptor agonists (e.g. WY14,643) are potent systemic lipid-lowering agents. Following its chronic administration to high-fat-fed rats, WY14,643 enhanced insulin sensitivity by reducing muscle lipid accumulation [2]. However, paradoxically, activation of PPARα by the selective agonist WY14,643 also leads to increased pyruvate dehydrogenase kinase isoform (PDK) 4 protein expression in skeletal muscle [6]. This response to WY14,643 is identical with that which results when the dietary lipid supply is increased by high-fat feeding [7], and mimics that observed when the ambient FA supply is increased through augmented adipose-tissue lipolysis in experimental diabetes [8] and in insulin-resistant man [9]. Increased PDK4 protein expression would be predicted to impair glucose tolerance by suppressing oxidative glucose disposal via the pyruvate dehydrogenase complex (PDC) and forcing fat oxidation [10].

PPARα is expressed at high levels in the liver [11–13]. The expression of many genes involved in hepatic FAO is regulated by PPARα, following its activation by exogenous ligands,

Abbreviations used: CPT I, carnitine palmitoyltransferase I; EGP, endogenous glucose production; FA, fatty acid; FAO, FA oxidation; FAS, FA synthase; HMG, 3-hydroxy-3-methylglutaryl; NEFA, non-esterified FA; PDC, pyruvate dehydrogenase complex; PDK, pyruvate dehydrogenase kinase; PPARα, peroxisome-proliferator-activated receptor-α; SREBP, sterol-regulatory-element-binding protein; TAG, triacylglycerol; TBS, Tris-buffered saline.

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including certain FA [14], the plasma concentrations of which increase in response to fasting due to increased adipose-tissue lipolysis. PPARα-deficient mice exhibit an impaired ability to up-regulate hepatic FAO in response to fasting, despite suppression of insulin levels and increases in FA supply [15,16]. In addition, the normal circadian rhythm of hepatic FA synthesis is disrupted in PPARα-deficient mice, such that the normal increase in FA synthesis that occurs during the dark phase is eliminated [17]. In normal mice, an antiparallel relationship exists between the hepatic expression of PPARα mRNA and FA synthase (FAS) mRNA [17]. It was suggested that PPARα deficiency might result in insensitivity of key enzymes of the lipogenic pathway to induction in response to the rise in insulin that accompanies increased food consumption [17].

In the present investigation, we studied PPARα-null mice and age-matched wild-type controls under conditions (feeding *ad libitum*, 24 h fasting and refeeding after fasting) that produced wide variations in ambient plasma NEFA and insulin concentrations to examine the relationship between hepatic TAG levels and the regulation by insulin and fatty acids of hepatic fuel storage, synthesis and utilization.

MATERIALS AND METHODS

Materials

General laboratory reagents were from Roche Diagnostics (Lewes, East Sussex, U.K.) or from Sigma (Poole, Dorset, U.K.), with the following exceptions. Organic solvents were of analytical grade and obtained from BDH (Poole, Dorset, U.K.). ^3H_2O, ECL® reagents, Hyperfilm and secondary antibodies were purchased from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, U.K.). RNAzol kits were purchased from Biogenesis (Poole, Dorset, U.K.). Anti-PDK4 antibodies, generated in rabbits against recombinant PDK4 [18], were kindly provided by Professor Robert A. Harris (Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, IN, U.S.A.). Bradford reagents for protein estimation were purchased from BioRad (Hemel Hempstead, Hertfordshire. U.K.). Kits for determination of plasma insulin, glucose, 3-hydroxybutyrate and NEFA concentrations were from Mercodia (Uppsala, Sweden), Roche Diagnostics, Sigma and Alpha Labs (Eastleigh, Hants., U.K.) respectively.

Animals

All studies were conducted in adherence to the regulations of the United Kingdom Animal Scientific Procedures Act (1986). Male PPARα-null mice bred on to an Sv/129 genetic background were kindly provided by Dr J. M. Peters and Dr F. J. Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda, MD, U.S.A.). Wild-type male Sv/129 mice were used as controls. Mice were used between the ages of 14 and 20 weeks. To facilitate the sampling process, mice were maintained on a 12 h light/12 h dark cycle, with the 12 h light period beginning at 03:00 h. Mice were fed *ad libitum*, fasted for 24 h or refed (6 h) *ad libitum* after fasting for 24 h. The diet consisted of fat (4.3 %), carbohydrate (mainly starch, 51.2 %), protein (22.3 %), fibre (4.5 %) and ash (7.7 %). Fasted mice had food removed at the mid-point of the dark phase of the diurnal cycle. They were either killed 24 later (starved), or refed for 6 h during the remainder of the dark phase and killed at the beginning of the light phase of the cycle (refed). Mice fed *ad libitum* were also killed at this time point (fed). Previous studies [19] have reported increased mean body masses in male PPARα mice on a Sv/129 background compared with wild-type mice at 3 to 4 months of age, although differences were not observed at all ages. In the present study, there were no statistically significant differences in mean body masses between the control and PPARα-null mice in either the fed state (control, 29.2 ± 0.5 g; PPARα-null, 25.9 ± 2.1 g) or after refeeding (control, 30.0 ± 0.8 g; PPARα-null, 27.7 ± 1.8 g). However, body masses of the PPARα-null mice after 24 h starvation were 8 % lower than those of the wild-type controls (control, 27.4 ± 0.3 g; PPARα-null, 22.2 ± 0.8 g; $P < 0.01$). Food intake did not significantly differ between the control and PPARα-null mice (see [17]).

Tissue sampling

Mice were anaesthetized by injection of sodium pentobarbital (30 mg/ml in 0.9 % NaCl; 1.5 ml/kg of body mass, intraperitoneal) and, once locomotor activity had ceased, livers were rapidly excized and stored in liquid N_2 until analysis. Blood was sampled from the inferior vena cava, centrifuged for 5 min at 12000 ***g*** and the plasma was stored at −20 °C.

Measurement of rates of hepatic FA synthesis *in vivo*

Mice were injected intraperitoneally with ^3H_2O (1 mCi) and killed 2 h later. Samples of plasma were obtained to measure the specific radioactivity of plasma water. The livers were removed, and immediately frozen in liquid N_2. The labelled FA-containing fraction was obtained as described previously [20]. Rates of FA synthesis *in vivo* measured by this method were linear with time over a wide range [21,22].

Immunoblotting

Liver samples (approx. 100 mg) were homogenized using a Polytron Tissue homogenizer (PT 10 probe; position 5, 15 s) in 1 ml of ice-cold extraction buffer A [20 mM Tris, 137 mM NaCl, 2.7 mM KCl, 1 mM $CaCl_2$, 10 % glycerol, 1 % Igepal, 45 mM sodium orthovanadate, 0.2 mM PMSF, 10 μg/ml leupeptin, 1.5 mg/ml benzamidine, 50 μg/ml aprotinin and 50 μg/ml pepstatin A (in DMSO), pH 8.0]. Homogenates were placed on ice for 20 min, centrifuged (10000 ***g***, 20 min, 4 °C) and the supernatants were stored (−20 °C) until analysis. Protein concentrations were determined using the Bradford method using BSA as the standard. The assay was linear over the range of protein concentrations routinely used. Liver samples (20–50 μg of total protein) were subjected to SDS/PAGE using a 12 % resolving gel with a 6 % stacking gel. Resolved proteins were transferred electrophoretically to nitrocellulose membranes, blocked for 2 h at 22 °C with Tris-buffered saline (TBS) supplemented with 0.05 % Tween and 5 % (w/v) non-fat powdered milk, and incubated overnight at 4 °C with polyclonal antisera raised against PDK4. Nitrocellulose membranes were then washed with 0.05 % Tween in TBS (3 × 5 min) and incubated with horseradish peroxidase-linked secondary antibody anti-rabbit IgG (1:2000 dilution, in 1 % (w/v) non-fat milk in TBS with 0.05 % Tween) for 2 h at 22 °C. Bound antibody was visualized using ECL® according to the manufacturer's instructions. The blots were exposed to Hyperfilm and the signals quantified by scanning densitometry and analysed with Molecular Analyst software (BioRad). The amounts of extracts loaded on to gels were varied to establish that the relative densities of the bands corresponding to PDK4 were linear with concentration. Immunoblots were performed under conditions in which autoradiographic detection was in the linear response range. For each representative immunoblot presented, the results are from a single gel exposed for a uniform duration and each lane represents a preparation from a different animal.

Measurement of mRNA

Frozen livers were ground to a powder under liquid N_2 and total RNA was extracted from portions of powder using the RNAzol kit. The amount of mRNA was assayed by reverse transcription [23], followed by real-time PCR using an ABI PRISM Sequence Detection System (PE Applied Biosystems, Foster City, CA, U.S.A.). Primers and probes for assaying FAS and β-actin mRNA have been described previously [17]. Primers for sterol-regulatory-element-binding protein (SREBP)-1c mRNA were 5′-ATCGGCGCGGAAGCTGTCGGGGTAGCGTC-3′ (forward) and 5′-ACTGTCTTGGTTGTTGATGAGCTGGAGC-AT-3′ (reverse), with 5′-FAM-CGGAGCCATGGATTGCAC-ATTTGA-TAMRA-3′ as the probe (where FAM is 6-carboxy-fluorescein and TAMRA is 6-carboxy-tetramethyl-rhodamine). Reactions were carried out in triplicate in 30 μl of TaqMan Universal PCR Master Mix containing 300 nM of each primer and 200 nM of the appropriate probe under the standard conditions recommended by the manufacturer. Primers (67 nM) and probe (85 nM) for β-actin were included to provide an internal standard. All values were related to a curve generated by a standard liver preparation and were corrected for β-actin mRNA content.

Measurement of plasma and liver lipid content

Plasma NEFA and 3-hydroxybutyrate concentrations were measured by enzymic methods using commercial kits. The total lipid fractions of livers were obtained by solvent extraction as described previously [24]. The extracted lipid fraction was dissolved in a small volume of ethanol, and duplicate aliquots were removed for enzymic measurement of TAG.

Statistical analysis

Results are expressed as the means ± S.E.M. with the numbers of mice in parentheses. Statistical analysis was performed by ANOVA, followed by Fisher's post-hoc tests for individual comparisons or Student's *t* test as appropriate (Statview, Abacus Concepts, Berkeley, CA, U.S.A.). $P < 0.05$ was considered to be statistically significant.

RESULTS AND DISCUSSION

Hepatic FAO is not limited by FA delivery in PPARα-null mice *in vivo*

The predominant product of FAO in liver is ketone body formation (reviewed in [25]). Since the rate of ketone body utilization by extra-hepatic tissues is determined by ambient ketone body concentrations, plasma concentrations of the ketone body 3-hydroxybutyrate were measured to provide an indication of hepatic FAO. After prolonged fasting, regulation of FA esterification and oxidation is exerted at the branch point represented by the partitioning of acetyl-CoA (formed from acyl-carnitine) between the synthesis of citrate and aceto-acetyl-CoA and by the reaction catalysed by mitochondrial 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase (reviewed in [26]). Confirming previous reports [15,16,27] of milder ketonaemia after fasting in PPARα-null mice that has been attributed to impaired induction of mitochondrial HMG-CoA synthase [27], plasma 3-hydroxybutyrate concentrations were significantly suppressed (by 83 %; $P < 0.001$) by PPARα deficiency after fasting (Table 1). Fasting plasma NEFA levels were modestly, but nevertheless significantly, higher (by 27 %; $P < 0.001$) in the PPARα-deficient mice (Table 1). Thus the relationship between circulating NEFA and 3-hydroxybutyrate concentrations is greatly altered in fasting PPARα-null mice compared with age-matched wild-type controls.

Table 1 Plasma 3-hydroxybutyrate and NEFA concentrations in wild-type (PPARα$^{+/+}$) and PPARα-null (PPARα$^{-/-}$) mice after fasting for 24 h

Details of the fasting protocol are given in the Materials and Methods section. Blood was sampled from the inferior vena cava. Plasma metabolite concentrations were determined using commercial kits. Results are expressed as the means ± S.E.M., with the numbers of mice in parentheses. Statistically significant effects of PPARα deficiency are indicated by $^{**}P < 0.01$.

	PPARα$^{+/+}$	PPARα$^{-/-}$
3-Hydroxybutyrate (mM)	1.19 ± 0.14 (4)	0.20 ± 0.05** (4)
NEFA (mM)	0.79 ± 0.04 (5)	1.00 ± 0.04** (5)

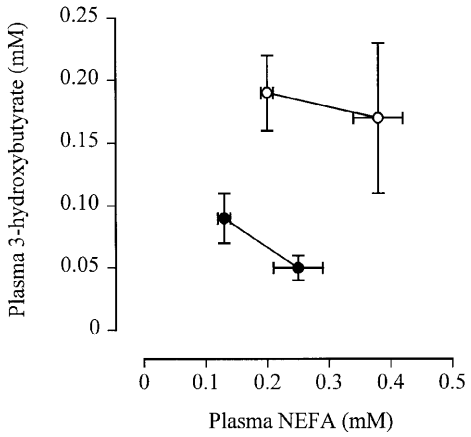

Figure 1 Relationship between plasma NEFA and 3-hydroxybutyrate concentrations in wild-type (PPARα$^{+/+}$, ○) and PPARα-deficient (PPARα$^{-/-}$, ●) mice in the fed state and following refeeding after starvation

Details of the protocols for starvation are given in the Materials and Methods section. Results are expressed as the means ± S.E.M. for 4–5 mice in each experimental group.

In the transition from the fed state to the fasted state, relief of inhibition of carnitine palmitoyltransferase I (CPT I) by decreasing malonyl-CoA concentrations provides a potent mechanism in the liver whereby low rates of FA synthesis result in increased rates of ketogenesis [28]. After refeeding, there is a slow reversal of the fasting-induced increase in CPT I activity and its decreased sensitivity to inhibition by malonyl-CoA (reviewed in [28]). Plasma 3-hydroxybutyrate concentrations were suppressed by PPARα deficiency both in the fed state (by 71 %) and on refeeding after fasting (by 51 %), conditions where adipose-tissue lipolysis is suppressed. Even at these low rates of delivery of FA to the liver, the results indicate that ketonaemia for a given level of FA delivery is much lower in PPARα-null mice than in the wild-type controls (Figure 1).

Effect of PPARα deficiency on the relationship between plasma NEFA and hepatic TAG concentration

In transgenic mice with hepatic-specific over-expression of lipoprotein lipase, an increase in intracellular TAG content from approx. 20 μmol/g to approx. 30 μmol/g does not affect endogenous glucose production (EGP) in the basal state, but greatly impairs suppression of EGP by insulin [5]. Hepatic TAG concentrations in wild-type and PPARα-deficient mice in the fed state, the fasted state and following refeeding after fasting are

Table 2 Hepatic TAG and glycogen concentrations in wild-type (PPARα$^{+/+}$) and PPARα-deficient (PPARα$^{-/-}$) mice in the fed state, the fasted state and following refeeding after fasting

Details of the protocols for fasting and refeeding are given in the Materials and Methods section. Hepatic TAG and glycogen concentrations were determined as described in the Materials and methods section. Results are expressed as the means ± S.E.M., with the numbers of mice in parentheses. Statistically significant effects of PPARα deficiency are indicated by $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant effects of fasting are indicated by †$P < 0.05$, ††$P < 0.01$ and †††$P < 0.001$. Statistically significant effects of refeeding are indicated by ‡‡$P < 0.01$ and ‡‡‡$P < 0.001$.

	Hepatic TAG concentration (μmol/g of liver wet mass)		Hepatic glycogen concentration (mg/g of liver wet mass)	
	PPARα$^{+/+}$	PPARα$^{-/-}$	PPARα$^{+/+}$	PPARα$^{-/-}$
Fed	11.3 ± 1.8 (7)	26.9 ± 6.2* (8)	62.9 ± 9.8 (5)	69.2 ± 6.3 (5)
Fasted (24 h)	77.8 ± 10.2†† (8)	90.5 ± 19.8† (8)	3.7 ± 2.3††† (5)	1.2 ± 0.9††† (5)
Refed (6 h)	32.6 ± 8.7‡‡‡ (7)	98.6 ± 16.2** (9)	130.5 ± 10.4‡‡‡ (5)	85.5 ± 5.1***‡‡‡ (5)

shown in Table 2. In wild-type mice, hepatic TAG concentrations, low in the fed state, increased significantly (by 6.9 fold; $P < 0.01$) with 24 h fasting to levels that greatly exceed those predicted to induce hepatic insulin resistance with respect to suppression of EGP (see [5]). Hepatic TAG accumulation was substantially reversed by 6 h refeeding (Table 2). Thus liver TAG concentrations in wild-type mice increased in fasting, where hepatic NEFA delivery is increased due to a lack of restraint on adipose-tissue lipolysis, but this effect of fasting is reversed when adipose-tissue lipolysis is suppressed. The contribution of *de novo* lipogenesis to total hepatic TAG is probably relatively low [29]. Thus hepatic FA delivery exceeds the hepatic capacity for FA disposal via oxidation, and surplus FAs are re-esterified and stored as hepatic TAG. On refeeding, the rate of FA delivery to the liver decreases and is now exceeded by FA removal as very-low-density lipoprotein–TAG and oxidized products. Hepatic TAG stores thus become depleted. PPARα-deficient mice exhibited hepatic TAG levels in the fed state that were comparable with those found in fasted wild-type mice (Table 2) and, importantly, hepatic TAG concentrations exhibited a refractory response to both fasting and refeeding after fasting (Table 2). Thus, hepatic TAG concentrations after refeeding were significantly higher (by 3.0 fold: $P < 0.01$) in PPARα-deficient mice compared with wild-type mice (Table 2). Measurement of hepatic lipid levels in livers sampled at 4 h intervals during the diurnal cycle showed that, at each time point, hepatic TAG was increased by 2–3-fold in the PPARα-deficient compared with the wild-type mice (results not shown). These results support recent findings by others [19] of increased lipid content, assessed by haematoxylin and eosin staining, in livers of 6-month-old fed male PPARα-null mice in an Sv/129 genetic background compared with wild-type controls. A previous study [16] reported that fasting of 8–12-week-old PPARα-null mice induced a fatty liver, as assessed by visual inspection and Oil Red O staining, but differences in hepatic lipid content between PPARα-null and wild-type mice were not obvious in the fed state. The present results suggest that increased hepatic TAG accumulation in PPARα-null mice predominantly reflects altered FA partitioning between oxidation and esterification. TAG accumulation in PPARα-null mice in the fed and refed states did not reflect increased delivery of systemic FA, as plasma NEFA concentrations were unaffected in the fed and refed states and only modestly elevated after fasting by PPARα deficiency.

In normal mice, increased intracellular TAG accumulation is paralleled by a comparable fold increase in long-chain acyl-CoA concentration [5]. A high rate of FA synthesis does not occur under conditions of increased plasma NEFA *in vivo*, owing to the inhibitory effects of long-chain acyl-CoA on acetyl-CoA carboxylase activity. Since up-regulation of several FAO enzymes is impaired in response to fasting in PPARα-null mice, leading to the accumulation of long-chain acyl-CoA, it would be anticipated that this regulatory interaction would be disturbed as a consequence of PPARα deficiency. A non-linear inverse relationship was observed between plasma NEFA and hepatic lipogenesis in both wild-type and PPARα-null mice (Figure 2). However, a higher plasma concentration of NEFA was required to produce a similar degree of inhibition of hepatic lipogenesis for the wild-type mice than the livers of PPARα-null mice (Figure 2), and rates of hepatic lipogenesis in the fed state were significantly higher (44%; $P < 0.05$) in wild-type mice compared with PPARα-null mice.

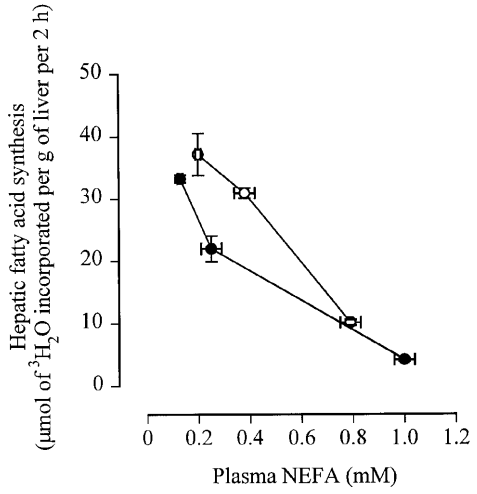

Figure 2 Relationship between plasma NEFA concentrations and rates of hepatic FA synthesis in wild-type (PPARα$^{+/+}$, ○) and PPARα-deficient (PPARα$^{-/-}$, ●) mice in the fed state, the fasted state and following refeeding after starvation

Details of the protocols for starvation are given in the Materials and Methods section. Results are expressed as the means ± S.E.M. for 3–4 mice in each experimental group.

PPARα deficiency and regulation of PDC

Inhibition of flux via the PDC is an important component of the hepatic response to starvation and to the initial period of

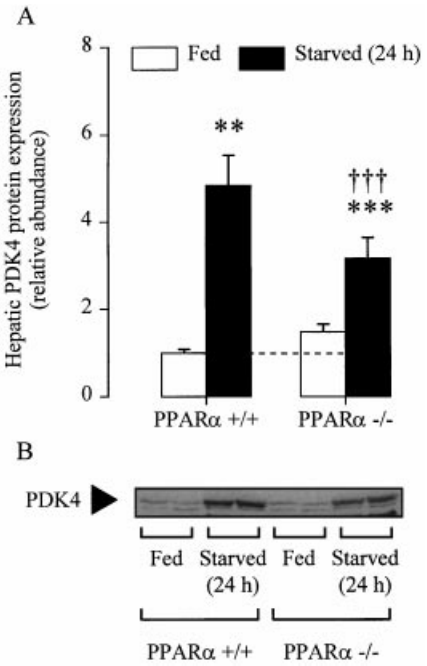

Figure 3 Effects of 24 h starvation on PDK4 protein expression in livers of wild-type (PPARα$^{+/+}$) and PPARα-deficient (PPARα$^{-/-}$) mice

Rabbit polyclonal antisera raised against recombinant PDK4 were used to detect PDK4 protein using Western-blot analysis. Each lane corresponds to 50 μg of liver protein. Western blots were analysed by scanning densitometry and quantified using Molecular Analyst 1.5 software. (**A**) Quantification of Western-blot analysis of hepatic PDK4 protein expression in fed or 24 h fasted wild-type or PPARα-deficient mice. Results are expressed as the means ± S.E.M. for 5 mice in each experimental group. (**B**) Representative immunoblots of PDK4 protein expression are shown for individual liver preparations from two fed versus two 24 h fasted mice. Statistically significant effects of 24 h starvation are indicated by $^{**}P < 0.01$ and $^{***}P < 0.001$. Statistically significant differences between wild-type mice and PPARα-null mice are indicated by $\dagger\dagger\dagger P < 0.001$.

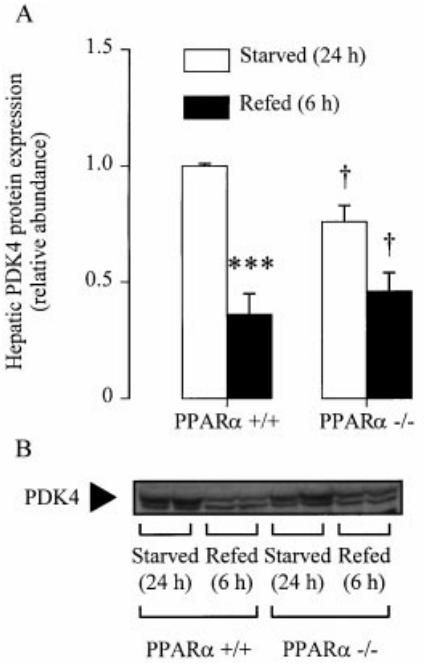

Figure 4 Effects of 6 h refeeding after 24 h starvation on PDK4 protein expression in livers of wild-type (PPARα$^{+/+}$) and PPARα-deficient (PPARα$^{-/-}$) mice

Rabbit polyclonal antisera raised against recombinant PDK4 were used to detect PDK4 protein using Western blot analysis. Each lane corresponds to 50 μg of liver protein. Western blots were analysed by scanning densitometry and quantified using Molecular Analyst 1.5 software. (**A**) Quantification of Western-blot analysis of hepatic PDK4 protein expression in 24 h fasted (open bars) or 6 h refed (closed bars) wild-type (PPARα$^{+/+}$) or PPARα-deficient (PPARα$^{-/-}$) mice. Results are expressed as the means ± S.E.M. for 4 mice in each experimental group. (**B**) Representative immunoblots of PDK4 protein expression are shown for individual liver preparations from two 24 h fasted versus two 6 h refed mice. Statistically significant effects of 6 h refeeding are indicated by $^{***}P < 0.001$. Statistically significant differences between wild-type mice and PPARα-null mice are indicated by $\dagger P < 0.05$.

refeeding after starvation, since it directs available pyruvate towards gluconeogenesis. Enhanced PDK4 protein expression is an important mechanism underlying inactivation of hepatic PDC in response to fasting [30], possibly secondary to high rates of FAO. The use of PPARα-null mice, in which the capacity for removal of incoming FA through oxidation is blunted, is therefore a valuable tool with which to dissect out the critical mechanisms that underlie the control of hepatic PDK4 expression in relation to hepatic FA supply *in vivo*. Since FAO is impaired in the PPARα-null compared with the wild-type mice, we anticipated that hepatic PDK4 protein expression in starvation might be decreased as a consequence of the decreased FAO associated with PPARα deficiency. As expected, we detected a marked (4.8 fold; $P < 0.01$) increase in the amount of PDK4 protein in the wild-type mouse liver after 24 h fasting, when plasma NEFA levels are elevated (Figure 3). However, although fasting increased hepatic PDK4 protein expression (2.6-fold increase; $P < 0.01$) in PPARα-null mice, the magnitude of the response to fasting was greatly attenuated (by 47%) in the PPARα-null mice compared with that observed in wild-type mice. As a consequence, hepatic PDK4 protein expression in the PPARα-null mice after 24 h fasting was only 66% ($P < 0.001$) of corresponding wild-type values. Protein expression of pyruvate dehydrogenase E1α was similar in wild-type and PPARα-null mice (results not shown), indicating that the differences between wild-type and PPARα-null mice in terms of the response of PDK isoform protein expression to fasting did not reflect changes in total PDC protein. The present results therefore indicate that PPARα participates in the response of hepatic PDK4 protein expression to fasting. However, as yet, there have been no reports that the promoter region of PDK4 contains a PPAR response element. A possible explanation for the regulatory link between enhanced PDK4 protein expression and PPARα activity may therefore be that hepatic PDK4 protein expression is regulated by FAO and that PPARα influences hepatic PDK4 protein expression indirectly.

In cultured hepatocytes from fasted rats, the addition of insulin suppresses PDK activity within 4–6 h [31]. In the present study, elevation of plasma insulin levels and suppression of plasma NEFA levels by refeeding a standard high-carbohydrate low-fat diet for 6 h after fasting significantly suppressed hepatic PDK4 protein expression in wild-type mice (64%; $P < 0.001$) (Figure 4). However, similar elevation of plasma insulin levels, by 6 h refeeding, led to more modest suppression of hepatic PDK4 protein expression (40% respectively; not significant) in PPARα-null mice compared with control wild-type mice (Figure 4). As a consequence, hepatic PDK4 protein expression in PPARα-null mice at 6 h after refeeding exceeded that of 6 h refed wild-type controls by 28% ($P < 0.05$). Thus, overall, the large excursions of PDK4 protein expression during starvation and

Table 3 Plasma insulin and glucose concentrations in wild-type (PPAR$\alpha^{+/+}$) and PPARα-null (PPAR$\alpha^{-/-}$) mice after fasting for 24 h

Details of the fasting protocol are given in the Materials and Methods section. Blood was sampled from the inferior vena cava. Plasma insulin and glucose concentrations were determined using commercial kits. Results are expressed as the means $\pm$ S.E.M., with the numbers of mice in parentheses. Statistically significant effects of PPARα deficiency are indicated by $^{*}P < 0.05$ and $^{**}P < 0.01$.

	PPAR$\alpha^{+/+}$	PPAR$\alpha^{-/-}$
Insulin (μ-units/ml)	9 ± 3 (9)	$26 \pm 3^{**}$ (8)
Glucose (mM)	8.8 ± 0.8 (9)	8.7 ± 0.9 (7)
Insulin/glucose concentration ratio (units/mol)	1.2 ± 0.4 (9)	$3.3 \pm 0.7^{*}$ (7)
Glucose $\times$ insulin (mmol/units per litre)	76 ± 31 (9)	$232 \pm 36^{**}$ (7)

refeeding of wild-type mice were blunted in the livers of the PPARα-deficient mice.

Glucose flux to glycogen *in vivo* in PPARα-deficient mice

Long-term adaptations of the liver to prolonged fasting include decreases in the maximal activities of glucokinase [32] and the lipogenic enzymes acetyl-CoA carboxylase [33] and FAS [34]. In contrast, the activities of the liver isoform of CPT I [28] and phosphoenolpyruvate carboxykinase [35] are increased. As a consequence, the predominant direction of hepatic carbon flux is towards gluconeogenesis and glucose output rather than glycolysis, and towards FAO and ketogenesis rather than FA synthesis. This pattern of carbon flux continues for the first few hours of refeeding after prolonged fasting, except that the gluconeogenic product (glucose-6-phosphate) is directed towards glycogen synthesis, and glucose output is suppressed [36–39] (reviewed in [40]). Hepatic glycogen storage in the fed state and hepatic glycogen depletion in response to fasting were unaffected by PPARα deficiency (Table 2). In contrast, net hepatic glycogen synthesis at 6 h after refeeding was significantly impaired (by 34 %; $P < 0.001$) in the PPARα-null group (Table 2). It has been hypothesized that impaired FAO, secondary to PPARα deficiency, contributes to impaired hepatic gluconeogenesis in fasting in the PPARα-null mouse [27]. Our present results argue that impaired FAO during the fasted-to-fed transition in the PPARα-null mice may also contribute to impaired hepatic glycogen repletion by virtue of the component contributed by the indirect (gluconeogenic) pathway.

PPARα deficiency leads to insulin resistance with respect to hepatic glycogen storage on refeeding after fasting

An equally plausible explanation of impaired glycogen deposition after refeeding would be that flux to glucose-6-phosphate is unimpaired, but impaired insulin action at the level of the disposition of glucose-6-phosphate between glycogen synthesis and glucose output leads to continued EGP at the expense of glycogen storage. This could arise through impaired insulin secretion or action. Whole-body insulin action, as assessed from intraperitoneal glucose tolerance, is not impaired in PPARα-null mice in the immediately post-absorptive state [16]. However, although steady-state fasting glucose levels were similar, fasting plasma insulin concentrations were 2.9-fold higher ($P < 0.01$) in PPARα-null mice compared with the age-matched wild-type controls (Table 3). Thus, both the insulin-to-glucose ratio and the product of steady-state fasting glucose levels and steady-state fasting insulin levels are significantly increased [by 2.8-fold ($P < 0.05$) and 3.1-fold ($P < 0.01$) respectively] in the 24 h fasted PPARα-null mice compared with the age-matched wild-type controls. Neither plasma insulin (PPAR$\alpha^{+/+}$, 88 ± 15 μ-unit/ml; PPAR$\alpha^{-/-}$, 79 ± 9 μ-unit/ml) nor plasma glucose (PPAR$\alpha^{+/+}$, 20.7 ± 1.8 mM; PPAR$\alpha^{-/-}$, 19.2 ± 0.9 mM) levels at 6 h after refeeding differed significantly between the two groups of mice. Our results therefore imply that PPARα deficiency may lead to the phenotype of hepatic insulin resistance with respect to net hepatic glycogen deposition on refeeding after starvation.

PPARα deficiency impairs the normal relationship between plasma insulin, hepatic lipogenesis and the mRNA expression of SREBP-1c and FAS

In normal rodents, diurnal fluctuations in plasma insulin levels resulting from the pattern of food intake over the 24 h cycle give rise to a circadian periodicity of hepatic lipogenesis *de novo* [17,21,22,41,42]. This relationship is abolished in PPARα-deficient mice, and lipogenesis does not increase following the increase in food intake and plasma insulin during the dark phase of the cycle [17]. To assess further the possible impairment in insulin action due to PPARα deficiency, we therefore analysed the relationship between ambient insulin concentrations and lipogenic rates during starvation and after refeeding, manipulations that led to marked excursions in insulin levels. An approximately linear positive relationship existed between rates of hepatic FA synthesis and ambient insulin levels in wild-type mice (Figure 5). Rates of hepatic FA synthesis in PPARα-null mice did not differ significantly from those of wild-type controls at insulin concentrations in the high-physiological range (approx. 80 μ-unit/ml), but were suppressed relative to wild-type controls at insulin concentrations in the lower physiological range (Figure 5).

The mature forms of the transcription factors SREBP-1a and SREBP-1c are potent transactivators of the FAS gene [43,44]. Their overexpression in the liver massively stimulates lipogenesis *de novo* [45]. It is now also clear that expression of SREBP-1c mRNA is stimulated by insulin [46–48], and this relationship forms part of the overall mechanism by which insulin stimulates lipogenesis [44]. A transcriptional increase in SREBP-1c is observed when isolated hepatocytes are treated with insulin [49,50]. Furthermore, hepatic SREBP-1c mRNA levels are reduced *in vivo* when rats are made diabetic by streptozotocin administration, but can be restored by insulin administration [51]. To test the possibility that the decreased response of lipogenesis to insulin in the PPARα-deficient mouse livers reflected impaired regulation of SREBP-1c mRNA expression by insulin, we measured the response of SREBP-1c mRNA expression to changes in plasma insulin caused by starvation and refeeding. Figure 5 shows that, at all concentrations of plasma insulin, mRNA expression of SREBP-1c was lower in the PPARα-deficient compared with the wild-type mouse livers. In wild-type mice, SREBP-1c mRNA expression augmented with increasing ambient insulin concentration in the low physiological range, but a plateau was attained at an insulin concentration of approx. 50 μ-unit/ml. In PPARα-deficient mice, SREBP-1c mRNA expression increased with increasing ambient insulin in the low physiological range, but maximal expression was approx. 50 % that of wild-type. Hepatic SREBP-1c expression is not lowered and lipogenesis is elevated in the *ob*/*ob* mouse, the lipodystrophic mouse and the fatty Zucker rat, models of insulin resistance that are characterized by chronic hyperinsulinaemia in the fed state [48]. Importantly, in the present experiments, SREBP-1c mRNA expression in livers of PPARα-deficient mice (which do not exhibit hyperinsulinaemia in the fed state) did not

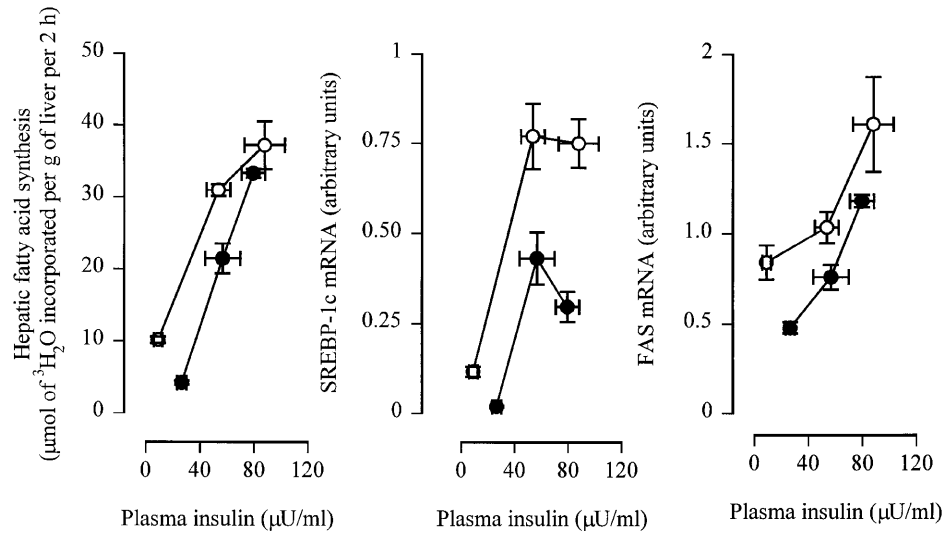

Figure 5 Relationship between plasma insulin concentrations and rates of hepatic FA synthesis, SREBP-1c and FAS mRNA expression in wild-type (PPARα$^{+/+}$, ○) and PPARα-deficient (PPARα$^{-/-}$, ●) mice in the fed state, the fasted state and following refeeding after starvation

Details of the protocols for starvation are given in the Materials and Methods section. Results are expressed as the means ± S.E.M. for 3–4 mice in each experimental group.

further increase when insulin levels achieved values in the high physiological range.

SREBP-1c is integral to increased liver fatty acid synthesis as shown by the failure of SREBP-1$^{-/-}$ mice to up-regulate the expression of lipogenic enzymes when subjected to a fasting/refeeding treatment [52]. The response of one of the SREBP-1c target genes, FAS, was determined by simultaneous measurement of changes in its mRNA concentration in the present experiments. FAS mRNA expression in wild-type mice increased in response to an increase in ambient insulin between 50 and 80 μ-units/ml (i.e. in a higher physiological range than that of SREBP-1c). However, as for FA synthesis, hepatic FAS mRNA expression in PPARα-null mice, although generally lower than that of wild-type livers, differed less markedly from those of wild-type controls at insulin concentrations in the high physiological range (approx. 80 μ-units/ml). Overall, our results suggest that impaired FA synthesis and FAS mRNA expression reflect hepatic insulin resistance secondary to PPARα deficiency. In contrast, PPARα deficiency primarily leads to impaired overall insulin responsiveness of SREBP-1c mRNA expression, rather than lowered insulin sensitivity. The finding that FAS mRNA expression at high insulin is relatively unaffected, despite markedly impaired SREBP-1c mRNA expression, supports the concept that FAS mRNA expression may be influenced by insulin via mechanism(s) in addition to increased SREBP-1c activity.

Concluding remarks

Using PPARα-deficient mice, we have demonstrated that control of hepatic lipid and carbohydrate flux is intimately connected to PPARα-linked functions. Our results support the concept that PPARα deficiency disrupts the normal control of hepatic metabolism by insulin during nutritional transitions (fed → fasted; fasted → fed). Importantly, impaired stimulation of hepatic lipogenesis and glycogen repletion, and impaired suppression of hepatic PDK4 protein expression, are consistent with dysregulation of insulin's actions to co-ordinate hepatic fuel handling during the fasted-to-fed transition. Furthermore, the failure to clear hepatic TAG when hepatic FA delivery is suppressed after refeeding supports the concept that impaired control of hepatic fuel handling by insulin induced by PPARα deficiency possibly reflects defective insulin signalling elicited by accumulation of intracellular FA-derived metabolites. Finally, the present study demonstrates that functional PPARα is required for the complete up-regulation of hepatic PDK4 protein expression in response to fasting and down-regulation of hepatic PDK4 expression on refeeding after fasting. Overall, the results establish, for the first time, the critical role of hepatic PPARα action, either directly or indirectly through modification of insulin's actions, in co-ordinating hepatic fuel handling at the level of lipid and carbohydrate synthesis and storage.

This study was supported in part by project grants from the Diabetes UK (RD98/1625 and RD01/2249) to M.C.S. and M.J.H., and an M.R.C. Programme Grant (G9224993) to G.F.G. We thank Dr Jeffrey M. Peters and Dr Frank J. Gonzalez for their kind gift of mating pairs of the PPARα-knockout mice. We also thank Professor Robert A. Harris for the gift of anti-PDK4 antibody.

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Received 21 November 2001/8 February 2002; accepted 20 March 2002