Disturbances in the normal regulation of SREBP-sensitive genes in PPARα-deficient mice

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Abstract Peroxisome proliferator-activated receptor α (PPARα)-null mice were used to investigate the nature of the relationship between the normal circadian rhythm of hepatic PPARα expression and the expression of the lipogenic and cholesterogenic sterol regulatory element-binding protein (SREBP)-regulated genes, acetyl-CoA carboxylase, fatty acid synthase (FAS), and 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR). The expression of FAS and HMG-CoAR varied rhythmically over the diurnal cycle in the normal mice, with patterns that were the opposite of that of PPARα. The diurnal variation of lipogenic and cholesterogenic gene expression was attenuated or abolished in the PPARα-null mice. This resulted in decreased expression compared with normal mice, but only during the dark phase of the cycle, when food intake was high. The diurnal variation in hepatic fatty acid and cholesterol synthesis was also abolished in the PPARα-null animals and the variations in the concentration of plasma triacylglycerol, nonesterified fatty acids, and cholesterol were all attenuated. The failure of HMG-CoAR expression to increase during the feeding period in the PPARα-null mice was associated with a decrease in hepatic nonesterified cholesterol content and an increase in cholesteryl ester compared with normal mice. There was no defect in the downregulation of hepatic HMG-CoAR mRNA in response to dietary cholesterol in the PPARα-null mice. Under these conditions, hepatic PPARγ expression increased in both the control and PPARα-deficient mice. The results suggest that PPARα-deficiency disturbs the normal circadian regulation of certain SREBP-sensitive genes in the liver, but does not affect their response to dietary cholesterol.—Patel, D. D., B. L. Knight, D. Wiggins, S. M. Humphreys, and G. F. Gibbons. **Disturbances in the normal regulation of SREBP-sensitive genes in PPARα-deficient mice.** ***J. Lipid Res.*** **2001.** 42: **328–337.**

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The expression of many genes involved in hepatic lipid and lipoprotein metabolism is regulated by the transcription factor peroxisome proliferator-activated receptor α (PPARα), a member of the nuclear hormone receptor gene family [for reviews see refs. (1, 2)]. Transcriptional control of these genes is mediated through response elements in their promoter regions, which bind PPARα that has been activated by various exogenous and endogenous ligands, including fibrates and certain fatty acids. Target genes in the liver include apolipoproteins A-I and A-II (apoA-I and apoA-II), which regulate the synthesis and plasma secretion of high density lipoprotein, apoC-III, which is an inhibitor of lipoprotein lipase, and several genes encoding enzymes of fatty acid activation and oxidation. Co-ordinated changes in the expression of these genes make a major contribution to hepatic lipid homeostasis by regulating, either directly or indirectly, the extent of lipid flux into and out of the liver. The expression of PPARα exhibits a circadian variation (3) that is probably regulated by cyclical changes in the concentration of plasma glucocorticoids (4). Hepatic PPARα mRNA levels are highest in the postabsorptive phase, when food intake is low (3), and remain high if the period of food deprivation is prolonged (5, 6). In humans, the postabsorptive phase shows many of the metabolic characteristics of short-term starvation (7) and so it is possible that a major function of PPARα is to orchestrate compensatory changes in hepatic lipid metabolism in response to a reduction in food consumption.

A variety of genes that are not apparently direct targets for PPARα are also regulated by hepatic lipid balance in a co-ordinated manner, and one of the major objectives of the present work was to identify those genes whose expression might be expected to be influenced by the indirect,

Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; ACC, acetyl-CoA carboxylase; FAS, fatty acid synthase; HMG-CoAR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDLR, low density lipoprotein receptor; NEFA, nonesterified fatty acids; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol regulatory element-binding protein.

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metabolic consequences of changes in PPARα activity. These include the genes for acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) (8) in the fatty acid synthesis pathway, and for 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoAR) (9, 10) in the cholesterol synthesis pathway. As well as responding to changes in hepatic lipid flux, the expression of these genes is dependent on insulin (11–13), and this relationship forms an integral part of the overall lipogenic response to changes in food intake. In rodents, variations in food intake over the diurnal cycle result in a circadian periodicity of fatty acid synthesis (14, 15) and cholesterogenesis (16), which is thought to be due, at least in part, to cyclical variations in the activities of ACC, FAS, and HMG-CoAR (17–19). Rates of cholesterogenesis in humans show a similar circadian periodicity, which is also dependent on food intake (20). Under certain circumstances, changes in the expression of ACC, FAS, and HMG-CoAR in liver are co-ordinately regulated by the sterol-sensitive transcription factor sterol regulatory element-binding protein (SREBP) (21, 22), although it is not known whether this is responsible for the variations observed over the diurnal cycle.

Mice in which the PPARα gene has been disrupted (PPARα-null) are hypercholesterolemic (23), hypoketonemic, and have an elevated concentration of plasma nonesterified fatty acids (NEFA) (5). They are also unable to respond to the normal lipid-lowering effects of fibrate drugs (23). Because of their inability to activate genes that normally mediate the utilization of fatty acids as an energy source, PPARα-null mice are unable to mount an adequate response to the challenge of starvation (5, 6). Because there are several metabolic similarities in terms of fuel selection between the postabsorptive phase of the diurnal cycle and short-term starvation (7), we have used PPARα-null mice as a model with which to study the normal role of PPARα in the lipogenic and cholesterogenic responses to circadian variations in nutritional status and hepatic lipid flux.

MATERIALS AND METHODS

Animals

PPARα-null mice bred onto an SV/129 genetic background were kindly provided by J. M. Peters and F. J. Gonzalez (National Institutes of Health, Bethesda, MD) (24). Wild-type SV/129 mice were used as controls. All mice were male, and were used between the ages of 14 and 20 weeks. Animals were maintained in temperature-controlled rooms (22–24°C) on a 12-h light/12-h dark cycle. To facilitate the sampling process over the whole 24-h cycle, mice were housed in two separate rooms, one with a 12-h light period beginning at 08:00 h and the other with a 12-h light period beginning at 03:00 h. All mice were fed a commercially available pelleted diet (Special Diet Service, Witham, Essex, UK) containing 4.3% fat, 51.2% carbohydrate (mainly starch), 22.3% protein, 4.5% fiber, and 7.7% ash. Food consumption for both genotypes during a 6-h period of the dark phase (D6–D12) was similar (normal, 9.6 ± 0.4 g per six mice; PPARα-null, 8.4 ± 0.8 g per six mice) as was food consumption during the light phase (normal, 2.5 ± 0.5 g per six mice; PPARα-null, 2.0 ± 0.4 g per six mice). For each genotype, therefore, food consumption was significantly higher during the dark phase than during the light phase ($P < 0.001$ in each case). For cholesterol-feeding experiments, the diet was supplemented with 2% cholesterol (greater than 99% purity; Sigma-Aldrich, Dorset, UK), which was fed for 7 days. Mice were anesthetized and blood samples were taken from the inferior vena cava at the times indicated, which are abbreviated as the number of hours into the light (L) or dark (D) phase. The livers were removed at the same time and immediately either placed on ice (for measurement of enzyme activities) or frozen in liquid nitrogen. Portions of the frozen liver were used to prepare total RNA by the acid guanidinium thiocyanate method (25), or for the subsequent extraction of lipids.

Measurement of mRNA

HMG-CoAR, low density lipoprotein receptor (LDLR), and β-actin mRNAs were assayed by RNase protection assay. Probes were obtained by the polymerase chain reaction (PCR) with the primers shown in **Table 1** and first-copy cDNA was obtained from mouse liver as template. This gave products of 301, 386, and 110 bp for HMG-CoAR, LDLR, and β-actin, respectively. The products were subcloned into pGEM-T vector (Promega, Madison, WI) and sequenced. After linearization, antisense-labeled RNA was synthesized with a MAXIscript labeling kit from Ambion (Austin, TX) with bacteriophage SP6 or T7 RNA polymerase as appropriate, and biotin 14-CTP as label. Samples of total RNA (25 μg) were hybridized with the labeled probes (4 ng) and treated with RNase A + T1, and the protected fragments were separated by electrophoresis on nonreducing gels, using an RPA11 assay kit (Ambion) according to the manufacturer instructions. Fragments were transferred by electroblotting to BrightStar Plus nylon membranes (Ambion) and fixed with UV light, and the bands were visualized with streptavidin-alkaline phosphatase (AP) and CDP-STAR (Ambion), again exactly as described by the manufacturer. The films were scanned with a Bio-Rad (Hercules, CA) imaging densitometer and the density of each band was determined with the Molecular Analyst software provided with the instrument. Probes for β-actin were included in each assay and the films were exposed as necessary to bring the densities into the linear range. Values for HMG-CoAR and LDLR mRNA were corrected for the recovery of β-actin mRNA and expressed within each experiment as a percentage of the mean value from samples obtained at D6.

ACC, FAS, PPARα, and PPARγ mRNAs were assayed by reverse transcription (26) followed by real-time PCR, using an ABI PRISM sequence detection system (PE Biosystems, Foster City, CA) with the oligonucleotide primers and fluorescent probes as shown in Table 1. Reactions were carried out in 30 μl of TaqMan Universal PCR master mix containing a 300-nM concentration 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 rates of hepatic cholesterol and fatty acid synthesis in vivo

Mice were injected intraperitoneally with ^3H_2O (1 mCi) and killed 2 h later. Samples of blood plasma were obtained to measure the specific radioactivity of the plasma water. The livers were removed, and immediately frozen in liquid nitrogen. Labeled cholesterol was isolated as described by Marco de la Calle et al. (27) and the labeled fatty acid-containing fraction was obtained as described by de Vasconcelos et al. (28).

Measurement of plasma and hepatic lipid content

Blood samples collected in heparinized syringes from the vena cava were immediately centrifuged and the resulting

TABLE 1. Sequences of oligonucleotides used to prepare probes for RNase protection assays or as primers and labeled probes for real-time polymerase chain reaction (TaqMan) assays

cDNA	Primer or Probe	Oligonucleotide Sequences
		For RNase protection assay
LDLR	Forward	5′-CGGAATTCCGGAACGAGTTCCAGTGTAGAGACGG-3’
	Reverse	5’-GCTCTAGAGCATGATTTGCAGCGGAAGTGGGC-3′
HMG-CoAR	Forward	5′-CGGAATTCCGCCTGACATGCAGATTCTGGCAG-3′
	Reverse	5′-GCTCTAGAGCTTGAACTCCCCACATTCTGTGC-3′
β-Actin	Forward	5′-CGGAATTCCGTGATGGTGGGAATGGGTCAG-3′
	Reverse	5′-GCTCTAGAGCCCAGTTGGTAACAATGCCATGTTC-3′
		For TaqMan assay
ACC	Forward	5′-TGTGTGGAAGTGGATGTGCA-3′
	Probe	5′-FAM-CGGCTGAGTGATGGTGGCCTGC-TAMRA-3′
	Reverse	5′-ACTGCTGCCGTCATAAGACAAG-3′
FAS	Forward	5′-TACCAAGCCAAGCACATTCG-3′
	Probe	5′-FAM-CCAGGGCATCCAAGTGCTCGTGTC-TAMRA-3′
	Reverse	5′-TGGCTTCGGCGATGAGA-3′
PPARα	Forward	5′-CCTCTTCCCAAAGCTCCTTCA
	Probe	5′-FAM-CACGGAGCATGCGCAGCTCGTA-TAMRA-3′
	Reverse	5′-CGTCGGACTCGGTCTTCTTG-3′
PPARγ	Forward	5′-GGTTGACACAGAGATGCCATTCT-3′
	Probe	5′-FAM-CTTCGGAATCAGCTCTGTGGACCTCTCC-TAMRA-3′
	Reverse	5′-AATGCGAGTGGTCTTCCATCA-3′
β-Actin	Forward	5′-GAGCTATGAGCTGCCTGACG-3′
	Probe	5′-VIC-CATCACTATTGGCAACGAGCGGTTCC-TAMRA-3′
	Reverse	5′-AGTTTCATGGATGCCACAGGA-3′

Abbreviations: FAM, carboxy fluorescein; TAMRA, *N′,N′,N′,N′*-tetramethyl-6-rhodamine.

plasma samples were stored at −20°C. NEFA, triacylglycerol (TAG), and 3-hydroxybutyrate were measured in duplicate samples with enzymatic methods as previously described (29–31). Total cholesterol was measured by the enzymatic method of Trinder (32) after treatment of the plasma with cholesterol esterase. Livers were ground to a fine powder under liquid N_2. The total lipid fractions were obtained by solvent extraction as described by Folch, Lees, and Sloane-Stanley (33). The extracted lipid fraction was dissolved in a small volume of ethanol and duplicate aliquots were removed for enzymatic measurement of TAG as described above. Nonesterified and total (nonesterified plus esterified) cholesterol were determined by the method of Trinder (32) before or after treatment with cholesterol esterase, respectively. The concentration of cholesteryl ester was calculated by the difference between the two values.

Measurement of acyl-CoA:cholesterol acyltransferase (ACAT) activity

Microsomes were prepared from fresh liver as described by Marco de la Calle et al. (27) and were suspended in 20 mM imidazole-chloride buffer (pH 7.4) containing dithiothreitol (5 mM). Assays were carried out according to the method of Gavigan and Knight (34), in which endogenous microsomal cholesterol was used as a cosubstrate with [1-^{14}C]oleoyl-CoA (20 μM, 125 dpm/pmol) as the labeled substrate. Incubations were carried out in a total volume of 50 μl at 37°C for 20 min. The reaction was stopped by addition of a mixture of chloroform–methanol 2:1 (v/v) and the labeled cholesteryl ester was separated by thin-layer chromatography (34).

Statistical analysis

Data were analyzed with SPSS for Windows, release 9.0 (SPSS, Chicago, IL). All the data are presented as means ± SEM. Distribution of the data was checked by the Shapiro-Wilks test. When data were normally distributed (LDLR mRNA, PPARα mRNA, and plasma metabolites), analysis of variance was used with Tukey's honestly significant difference test for post-hoc analysis. For all other variables, nonparametric tests were used as follows. A Kruskal-Wallis test was used for differences between the control and PPARα-null mice and for time effects (diurnal changes). A Mann-Whitney test was used to test for differences between genotypes during the whole of the light phase or during the whole of the dark phase.

RESULTS

The normal circadian rhythms of hepatic lipogenic gene expression and in vivo fatty acid synthesis are disturbed in PPARα-deficient mice

Groups of normal and PPARα knockout mice were killed at 4-h intervals over a 24-h period and the livers were excised and immediately frozen in liquid nitrogen. Total RNA was extracted and the concentration of mRNA for ACC and FAS was determined as described in Materials and Methods. In the livers of normal mice there was a significant variation in the content of FAS mRNA over the 24-h period ($P < 0.001$, **Fig. 1**), with a peak at the midpoint of the dark phase. There was no such cyclical variation in the PPARα-null animals. In this case, the expression of FAS mRNA failed to increase at the onset of the dark phase, when food intake increased. Overall, FAS mRNA expression was greater in the control animals ($P < 0.001$) and this difference was accentuated during the dark phase of the cycle, when food consumption was highest. Expression of ACC over the whole of the diurnal cycle was significantly greater in the control than in the PPARα-null mice ($P < 0.001$). Similar to

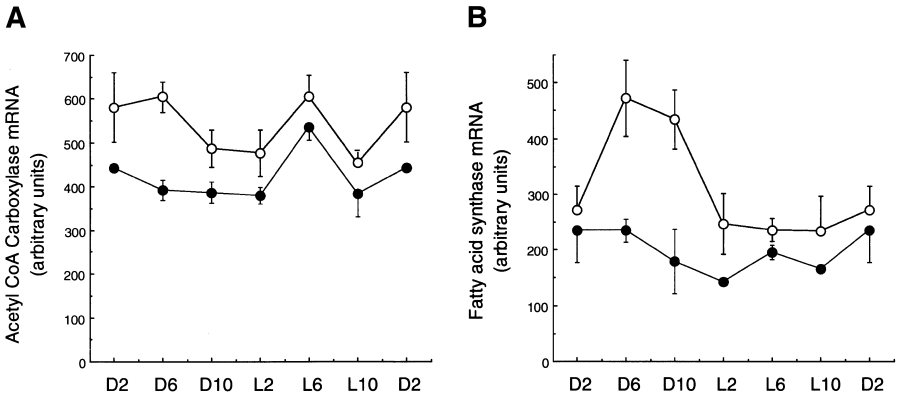

Fig. 1. Diurnal changes in the content of ACC mRNA (A) and FAS mRNA (B) in the livers of normal mice (open circles) and PPARα-null mice (solid circles). Results shown are the means ± SE of values from 10 animals at D6 or L6 and from 4 animals at all other times. For ACC there was a significant overall difference between normal and PPARα-null mice ($P < 0.001$). This difference occurred during the dark phase ($P < 0.001$) but not during the light phase ($P = 0.097$) of the cycle. For FAS there was a significant overall diurnal variation in the normal mice ($P < 0.001$) and a significant difference between normal and PPARα-null mice ($P < 0.001$). There was no significant difference in expression between the dark and light phase in the PPARα-null mice.

the expression of FAS mRNA, this difference was due mainly to an increased expression during the dark phase (values at D2, D6, and D10; $P < 0.001$) rather than during the light phase (values at L2, L6, and L10; $P = 0.097$). These differences in content of the mRNA for ACC and FAS were reflected by changes in total carbon flux through the lipogenic pathway as measured by ^3H_2O incorporation into hepatic fatty acids in vivo (**Table 2**). Thus, in the normal mice, fatty acid synthesis was significantly greater at the midpoint of the dark phase than at the midpoint of the light phase. This increase was abolished in the PPARα-null animals, so that the apparent rate of fatty acid synthesis remained low throughout the cycle.

The normal rise in expression of HMG-CoAR mRNA during the diurnal cycle is abolished in the livers of PPARα-null mice

The concentration of HMG-CoAR mRNA in the livers of the normal mice showed a significant circadian variation, in which expression was increased 3-fold at the peak in the mid-dark phase compared with the trough at the beginning of the light phase (**Figs. 2** and **3A**). In the PPARα-null animals the content of HMG-CoAR mRNA was also lowest at the beginning of the light phase, with a gradual rise over the next 12 h, but did not show the dramatic increase during the dark phase that occurred in the normal mice. Thus, the expression of HMG-CoAR mRNA was 2- to 3-fold higher in normal animals than in the PPARα-null animals during the dark phase, when food intake was high, but was similar in normal and PPARα-null animals during the light phase, when food intake was low.

The rate of hepatic cholesterol synthesis, as measured by incorporation of ^3H_2O, did not faithfully reflect the amount of HMG-CoAR mRNA in the tissue. It is known that the rate of cholesterol synthesis in rodent liver follows a diurnal rhythm with a peak in the dark phase (16), and this was observed here with the normal mice (Table 2). The rhythm was abolished in the PPARα-null mice, as would be expected from their expression of HMG-CoAR mRNA. However, whereas the mRNA expression remained low in the PPARα-null animals, the rate of cholesterol synthesis remained high. This resulted in a higher rate of

TABLE 2. Rates of hepatic fatty acid and cholesterol synthesis in vivo in normal and PPARα-null mice

	Dark Phase		Light Phase	
	Normal	PPARα-Null	Normal	PPARα-Null
Fatty acids	28.8 ± 2.3[a,b]	18.9 ± 1.5	20.8 ± 1.5	18.9 ± 1.8
Cholesterol	2.12 ± 0.26[a]	2.40 ± 0.24	0.91 ± 0.09[b]	1.86 ± 0.21

Values are expressed as micromoles of ^3H_2O incorporated per gram of liver per 2 h (average ± SEM). There were 12 animals in each group. Mice were injected with ^3H_2O at D6 (dark phase) or L6 (light phase) and killed 2 h later.

[a] Significantly different ($P < 0.01$) from the corresponding light phase.

[b] Significantly different ($P < 0.01$) from the corresponding PPARα-null mice.

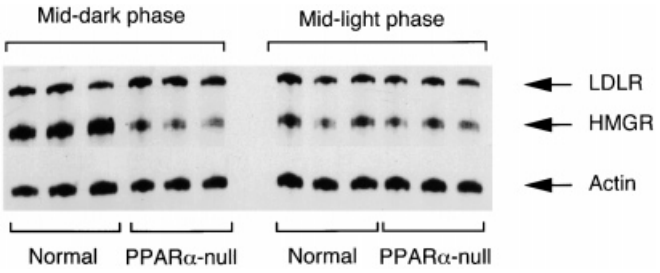

Fig. 2. RNase protection assay for HMG-CoAR (HMGR) mRNA and LDLR mRNA. Bands shown are for RNA from three separate livers from normal and PPARα-null mice at D6 and L6, with bands for actin mRNA as a loading control.

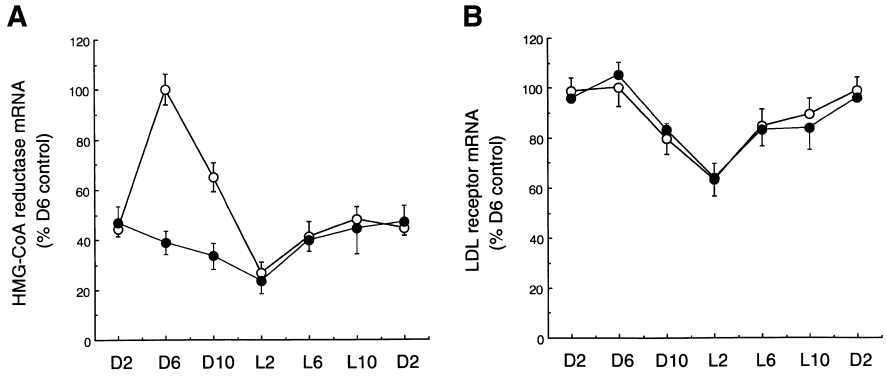

Fig. 3. Diurnal rhythm in the content of HMG-CoAR mRNA (A) and LDLR mRNA (B) in the livers of normal mice (open circles) and PPARα-null mice (solid circles). Results shown are the means ± SE of values from 10 animals at D6 or L6 and from 4 animals at all other times. Overall, the expression of HMG-CoAR was significantly greater in the normal mice ($P = 0.002$). This difference occurred only during the dark phase ($P < 0.001$). In the normal mice there was a significant difference between the light and dark phases ($P < 0.001$). There was no such difference in the PPARα-null mice ($P = 0.521$). For LDLR, there was a significant diurnal rhythm in both genotypes ($P < 0.001$).

cholesterol production in the livers of the PPARα-null animals than in those of the normal animals during the light phase of the cycle (Table 2), when there was no observable difference between the genotypes in HMG-CoAR mRNA content (Fig. 3A).

Simultaneous measurements of the content of LDLR mRNA over the diurnal cycle also revealed a significant rhythmicity in the livers of the normal mice (Fig. 3B). Although the amplitude of the rhythm was much smaller, the general pattern was similar to that of the HMG-CoAR mRNA (Fig. 3A), with a trough at the beginning of the light phase. A similar diurnal variation in the amounts of LDLR protein in membranes of rodent livers has been observed previously (35). Rather surprisingly, in view of the large differences in HMG-CoAR mRNA expression, the values for LDLR mRNA in the normal and the PPARα-null mice were virtually identical.

An antiparallel relationship exists between the expression of PPAR genes and that of lipogenic genes over the diurnal cycle

In the livers of normal mice, the concentration of PPARα mRNA exhibited a pronounced diurnal rhythmicity, with a peak during the light phase, when food consumption was low, and a trough during the dark phase, when the animals were eating (**Fig. 4A**). As expected, PPARα mRNA was essentially absent from the livers of the PPARα-null mice. The concentration of mRNA for a second member of the PPAR family, PPARγ, showed a diurnal pattern similar to that of PPARα (Fig. 4B). There was no difference in the pattern of expression of PPARγ in the PPARα-null mice compared with that in the normal mice, but the values were much higher at each time point studied. Our method does not give an accurate assessment of the relative concentrations of PPARα and PPARγ. However, assuming that the efficien-

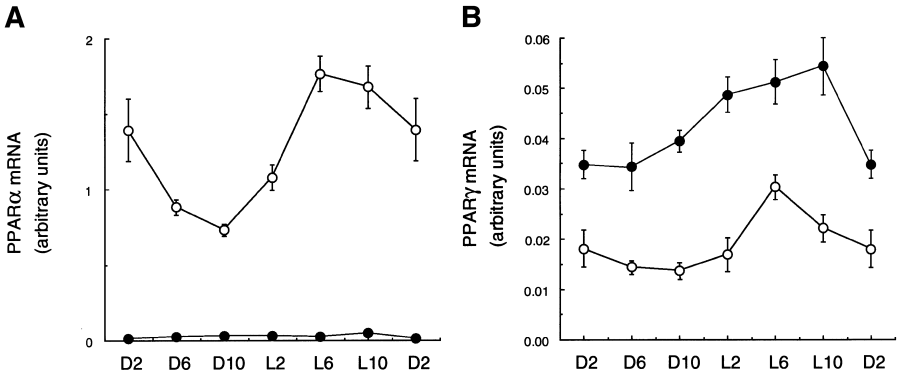

Fig. 4. Diurnal rhythm in the content of PPARα mRNA (A) and PPARγ mRNA (B) in the livers of normal mice (open circles) and PPARα-null mice (solid circles). Results shown are the means ± SE of values from 10 animals at D6 or L6 and from 4 animals at all other times. There was a significant diurnal variation in PPARα mRNA in normal mice ($P < 0.001$). For PPARγ there were significant diurnal rhythms of expression for both the normal and PPARα-null mice ($P < 0.05$). Values for PPARγ mRNA were significantly higher overall in PPARα-null mice than in normal mice ($P > 0.001$). In both the light phase and the dark phase PPARγ expression was higher in the PPARα-null mice ($P > 0.001$).

cies of the PCR and the affinities of the probes were similar for the two assays, it can be estimated that the concentration of PPARγ in the normal livers was less than 5% of that of PPARα. A similar relationship has been reported previously (36). Thus the 2- to 3-fold increase in PPARγ expression in the livers of the PPARα-null animals would not be expected to compensate for the absence of PPARα.

PPARα deficiency is associated with a decreased hepatic cholesterol content and an increased plasma cholesterol concentration

The decreased concentration of HMG-CoAR mRNA in the livers of the PPARα-null mice during the dark phase suggested that this might have arisen as a compensatory response to an increased content of nonesterified cholesterol (37). However, serial measurements of hepatic nonesterified cholesterol over the 24-h period revealed the opposite (**Fig. 5B**). The content of nonesterified cholesterol in the livers of the PPARα-null mice was lower than normal throughout the diurnal period ($P = 0.001$), and particularly during the early part of the light phase. There was no significant diurnal variation in nonesterified cholesterol content in either the normal or the PPARα-null mice. In contrast to nonesterified cholesterol, the hepatic content of cholesteryl ester was higher in the PPARα-null mice throughout the 24-h cycle except at L6 (Fig. 5C) ($P = 0.008$). This increase was not due to an increased ACAT activity, which was decreased in the liver microsomes of the PPARα-null mice, at least at D6 (normal, 1.33 ± 0.15 pmol/20 min/mg protein; PPARα-null, 0.78 ± 0.05 pmol/20 min/mg protein, n = 3; $P = 0.025$), probably as a result of a deficiency of microsomal nonesterified cholesterol in the ACAT substrate pool (34). The concentration of total cholesterol in plasma was about 20% higher in the PPARα-null mice than in the normal mice (Fig. 5A) ($P < 0.001$).

PPARα deficiency is associated with an attenuated diurnal rhythmicity of plasma lipids

The lower plasma cholesterol content in the normal mice (Fig. 5A) showed a significant diurnal variation ($P = 0.010$) but this rhythmicity was abolished in the PPARα-null mice ($P = 0.109$). The diminished capacity for long-chain fatty acid oxidation in the PPARα-null mice (38) was reflected in a generally decreased concentration of 3-hydroxybutyrate, but this did not reach statistical significance (**Fig. 6C**). There was also a small increase in the plasma concentration of NEFA ($P = 0.07$) (Fig. 6B). This was accompanied by a small increase in plasma TAG ($P = 0.06$) (Fig. 6A). There were significant diurnal variations in the plasma concentration of NEFA and TAG in both the normal and the PPARα-null mice ($P < 0.001$ in all cases). The amplitudes of these rhythms in the PPARα-null mice were, however, decreased compared with those in the normal mice.

Cholesterol feeding suppresses HMG-CoAR mRNA expression in the livers of both normal and PPARα-deficient mice

In view of the abnormal relationship between hepatic nonesterified cholesterol content and HMG-CoAR mRNA expression in the livers of the PPARα-null mice, we determined the response of HMG-CoAR mRNA to an increased hepatic delivery of cholesterol resulting from an increased dietary cholesterol content. After consumption of a diet supplemented with 2% cholesterol for 7 days, the relative decrease in the expression of HMG-CoAR mRNA in the livers of PPARα-null mice was almost identical to that observed in the normal animals (**Table 3**), suggesting that there was no abnormality in the response to dietary cholesterol. The decline in carbon flux into hepatic cholesterol in vivo resulting from cholesterol feeding was also similar in the two genotypes, but in each case the decrease was considerably greater

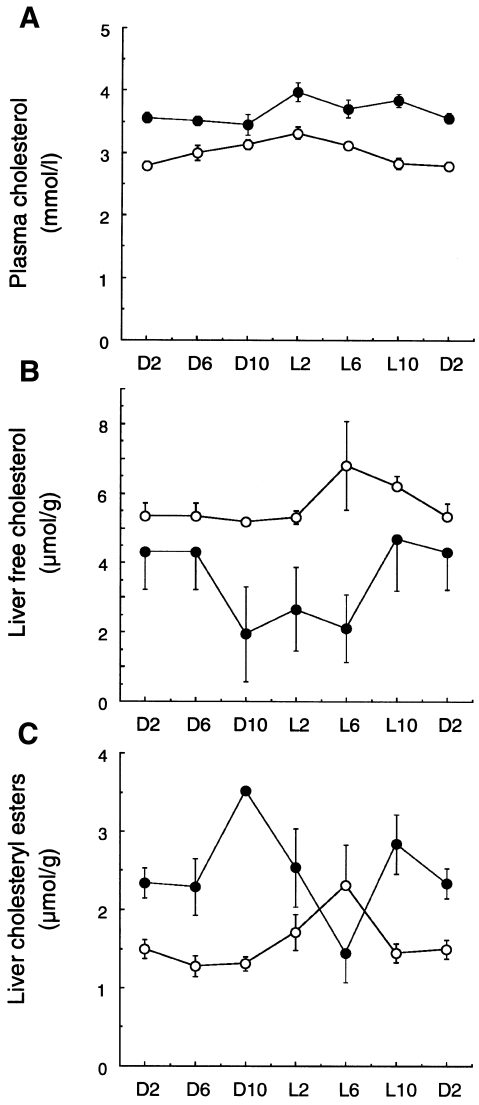

Fig. 5. Diurnal variation in the plasma cholesterol concentration (A), and in the hepatic nonesterified cholesterol (B), and esterified cholesterol (C) content of normal mice (open circles) and PPARα-null mice (solid circles). Results are the means ± SE of values from 4 animals at each time. The plasma cholesterol concentration had a significant diurnal rhythm only in the control mice ($P = 0.010$). The cholesterol concentration was significantly different between the two genotypes ($P < 0.001$). The content of hepatic cholesteryl ester was greater ($P = 0.008$), and the content of nonesterified hepatic cholesterol was lower ($P < 0.001$), in the PPARα-null mice than in the normal mice.

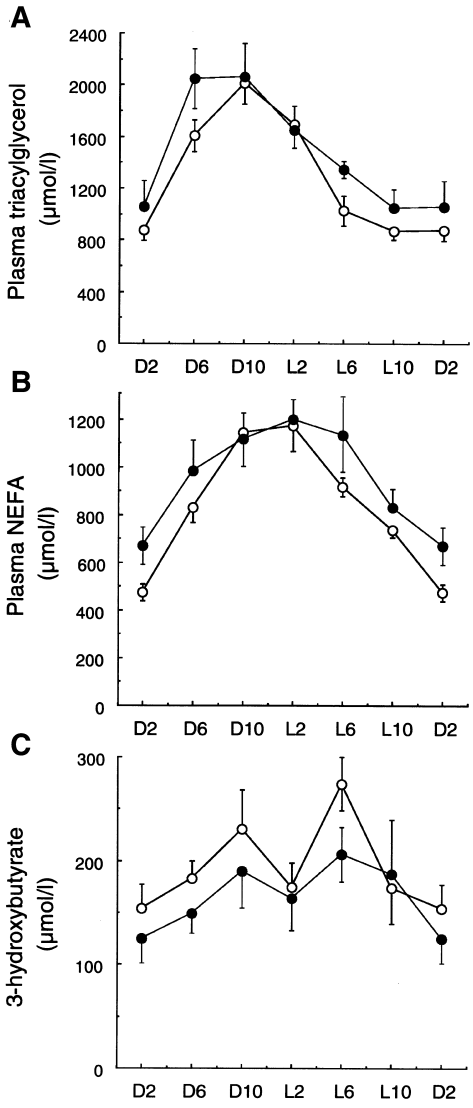

Fig. 6. Diurnal variations in the concentration of TAG (A), NEFA (B), and 3-hydroxybutyrate (C) in the plasma of normal mice (open circles) and PPARα-null mice (solid circles). Results are the means ± SE of values from four animals at each time point. For TAG and NEFA there were significant ($P < 0.001$) diurnal variations in concentration in both the PPARα-null and control mice. The concentrations were also different between the two genotypes ($P = 0.071$ and $P = 0.059$, respectively).

TABLE 3. Effects of cholesterol feeding on hepatic gene expression, lipogenesis, and cholesterogenesis

Parameter	Normal Mice	PPARα-Null Mice
	% changes after cholesterol feeding	
HMG-CoAR mRNA	63.4 ± 5.2 (−)[a]	62.5 ± 7.5 (−)[a]
LDLR mRNA	13.1 ± 12.0 (−)	12.4 ± 19.6 (−)
ACC mRNA	4.7 ± 5.8 (+)	17.5 ± 10.9 (+)
FAS mRNA	8.9 ± 11.1 (−)	6.3 ± 10.1 (+)
PPARα mRNA	13.6 ± 3.7 (+)[b]	—
PPARγ mRNA	128.7 ± 37.0 (+)[b]	94.7 ± 17.4 (+)[c]
Cholesterol synthesis	94.7 ± 1.0 (−)[a]	97.8 ± 0.3 (−)[a]
Nonsaponifiable lipid	58.8 ± 7.1 (−)[a]	81.4 ± 3.5 (−)[a]
Fatty acid synthesis	47.2 ± 35.5 (+)	1.9 ± 12.1 (+)

Mice were fed cholesterol (2%, w/w) for 1 week and killed at the midpoint of the dark phase of the diurnal cycle. The above values are expressed as the percentage change (increase or decrease) relative to the corresponding mice fed chow alone. (−) or (+) following each value refers to a decrease or increase, respectively. Values are expressed as the mean ± SEM of 6 normal mice and 6 PPARα-null mice in each dietary group (a total of 12 mice in each genotype).

[a] $P < 0.001$, significantly different from the respective chow-fed control group.

[b] $P < 0.05$, significantly different from the respective chow-fed control group.

[c] $P < 0.01$, significantly different from the respective chow-fed control group.

than that observed for HMG-CoAR mRNA expression. There was no decrease in the expression of ACC and FAS mRNAs and no decline in carbon flux into fatty acids in vivo after cholesterol feeding in either the normal or the PPARα-null livers. Interestingly, cholesterol feeding resulted in an increased expression of PPARγ mRNA in the livers of both the control and PPARα-null mice, and in a small increase in the expression of PPARα in the normal mice (Table 3).

DISCUSSION

Diurnal fluctuations in the rates of hepatic fatty acid (14, 15) and cholesterol (16) synthesis in rodents were discovered many years ago and were ascribed to variations in food consumption over the 24-h cycle. Such diurnal changes are thought to stem primarily from the regulation of the lipogenic enzymes FAS and ACC (8, 17) and the cholesterogenic enzyme HMG-CoAR (16), and the expression of their mRNAs (39–42). Normal mice showed the expected diurnal rhythm in hepatic PPARα mRNA content, with the peak around the midpoint of the light cycle, slightly earlier than it occurs in rats (3). We have also shown a similar variation in the hepatic content of mRNA for PPARγ, which was greatly increased at all time points in the PPARα-null animals. While the levels were still low in mice, such a compensatory balance between the two forms of PPAR could be more important in humans, where the relative hepatic content of PPARα is much lower (43).

It has been shown that PPARα makes an important contribution to the co-ordinated changes in hepatic lipid metabolism that enable mammals to adapt to the challenge of starvation (5, 6). Under these extreme conditions of food deprivation, ACC, FAS, and HMG-CoAR activities remain low (8, 18, 44). Our observations with the PPARα-null mice presented here suggest that PPARα has a wider general involvement in transducing hormone messages that signal changes in dietary status and indicate that it plays a significant part in the regulation of cholesterogenesis and lipogenesis during the rhythmic changes in food intake that occur over the diurnal cycle. The amplitudes of the diurnal rhythms in mRNA for FAS and HMG-CoAR were greatly attenuated in the livers of the PPARα-null mice (Figs. 1 and 3), leading to a persistently low level of expression. These observations were strongly suggestive of a requirement for functional PPARα in the control of circadian food-dependent fluctuations in gene expression.

There have been no reports to indicate that the promoter regions of the ACC, FAS, or HMG-CoAR genes contain PPAR response elements, so it is unlikely that PPARα exerts its effects on these genes by direct transcriptional activation. There are two remaining general explanations for the link between PPARα and the circadian rhythms of lipogenic and cholesterogenic gene expression. First, because the expression of the genes is known to be regulated by the flux of fatty acids and cholesterol, respectively, through the liver, it is possible that PPARα indirectly influences ACC, FAS, and HMG-CoAR expression by controlling metabolic processes that affect hepatic lipid flux and, therefore, hepatic lipid concentration. Although this explanation may account for the effects of long-term starvation (5, 6) there is little evidence that it is responsible for the effects seen here. Plasma 3-hydroxybutyrate concentrations were only slightly decreased in the PPARα-null animals and there was only a small effect on plasma TAG and NEFA levels. It is also unlikely that the decrease in HMG-CoAR mRNA expression in the PPARα-null mice arose as a result of a persistent increase in cholesterol net flux into the liver, because steady-state levels of hepatic total and nonesterified cholesterol in these animals were not increased at any point throughout the 24-h cycle.

A more plausible explanation for the abolition of the diurnal rhythms in the PPARα-null mice is that PPARα is required for the expression of genes that mediate the response of HMG-CoAR, ACC, and FAS to dietary signals. In this regard, insulin is known to be an important regulator of lipogenic gene expression (8, 12) and has been shown to be at least partly responsible for the food-dependent nocturnal increase in hepatic ACC catalytic activity (17). It has also been implicated in the control of the circadian variation of HMG-CoAR activity, which was abolished in streptozotocin-diabetic rats (18) . Therefore, it is possible that HMG-CoAR, ACC, and FAS mRNA expression in the PPARα-deficient mice is insensitive to the rise in the plasma concentration of insulin that accompanies the increase in food consumption after the onset of the dark phase of the diurnal cycle. The mechanism by which insulin exerts its effects has not yet been elucidated, but evidence suggests that it involves an increase in the expression of SREBP transcription factors (13, 45), which are known to be implicated in the regulation of all three of the genes studied here (21, 22). If so, this increase in SREBP expression might be abolished under conditions of PPARα deficiency.

The lack of an insulin-mediated, SREBP-dependent increase in HMG-CoAR in the livers of the PPARα-null mice would also explain the paradoxical decrease in HMG-CoAR mRNA expression under conditions of hepatic nonesterified cholesterol depletion (Fig. 5). The microsomal activity of ACAT was decreased in the PPARα-null mice, confirming a reduced availability of nonesterified cholesterol in the substrate pool (34). It could be argued that the low expression of HMG-CoAR mRNA in the livers of the PPARα-null mice resulted from an inability to respond to changes in the availability of nonesterified cholesterol. To investigate this possibility we fed both genetic strains of mice a cholesterol-containing diet for 7 days and measured hepatic HMG-CoAR mRNA at the midpoint of the dark phase of the cycle. Table 3 shows that the substantial decline in HMG-CoAR expression was similar for both the normal and the PPARα-null mice, indicating that PPARα deficiency did not blunt the regulatory potency of dietary cholesterol. It should be noted, however, that in rats a dietary cholesterol-mediated decrease in HMG-CoAR mRNA is not a consistent finding (46). There was no inhibitory effect of cholesterol feeding on the expression of other SREBP-sensitive genes such as those encoding LDLR, ACC, and FAS (Table 3), at least not at this point in the diurnal cycle.

The reduced content of HMG-CoAR mRNA in the livers of the PPARα-null mice during the dark phase of the cycle was not accompanied by a reduction in the rate of carbon flux into cholesterol (Table 2). This apparent uncoupling of HMG-CoAR expression and cholesterol synthesis did not persist after cholesterol feeding, when HMG-CoAR mRNA content and rates of cholesterogenesis declined by similar amounts in both the normal and PPARα-null mice (Table 3). Again, during the light phase of the cycle there was no difference in hepatic HMG-CoAR expression between the two genotypes, yet cholesterol synthesis was significantly higher in PPARα-null mice (Table 2). This is not the first example of an apparent lack of concordance between HMG-CoAR activity and the rate of cholesterogenesis. Glucocorticoids lead to an uncoupling of HMG-CoAR activity and cholesterogenesis by virtue of their inhibition of cytosolic HMG-CoA synthase (47, 48), and we have previously shown that, under certain physiological conditions, cholesterogenesis is limited primarily, not by changes in HMG-CoAR, but by changes in the availability of prereductase cholesterogenic precursors (49). Most recently, Peet et al. (50) have reported an uncoupling between the expression of cholesterogenic genes and actual rates of cholesterol synthesis in the livers of mice lacking the oxysterol receptor LXRα. Thus it seems probable that there are a variety of separate regulatory pathways for these enzymes, which can apparently uncouple them from cholesterol synthesis. In particular, the present work suggests that PPARα may contribute to the regulation of SREBP-sensitive gene expression by a mechanism that is independent of hepatic cholesterol content but that is dependent on the hormonal changes that reflect dietary status.

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