BJ www.biochemj.org

BJ Metabolism

Biochem. J. (2009) **417**, 43–54 (Printed in Great Britain) doi:10.1042/BJ20080740

online data

Unsaturated fatty acid regulation of cytochrome P450 expression via a CAR-dependent pathway

Robert D. FINN, Colin J. HENDERSON, Claire L. SCOTT and C. Roland WOLF[1]

Cancer Research UK Molecular Pharmacology Unit, Biomedical Research Centre, Level 5, Ninewells Hospital and Medical School, Dundee DD1 9SY, U.K.

The liver is responsible for key metabolic functions, including control of normal homoeostasis in response to diet and xenobiotic metabolism/detoxification. We have shown previously that inactivation of the hepatic cytochrome P450 system through conditional deletion of POR (P450 oxidoreductase) induces hepatic steatosis, liver growth and P450 expression. We have exploited a new conditional model of POR deletion to investigate the mechanism underlying these changes. We demonstrate that P450 induction, liver growth and hepatic triacylglycerol (triglyceride) homoeostasis are intimately linked and provide evidence that the observed phenotypes result from hepatic accumulation of unsaturated fatty acids, which mediate these phenotypes by activation of the nuclear receptor CAR (constitutive androstane receptor) and, to a lesser degree, PXR (pregnane X receptor). To our knowledge this is the first direct evidence that P450s play a major role in controlling unsaturated fatty acid homoeostasis via CAR. The regulation of P450s involved in xenobiotic metabolism by this mechanism has potentially significant implications for individual responses to drugs and environmental chemicals.

Key words: constitutive androstane receptor (CAR), cytochrome P450, linoleic acid, P450 oxidoreductase (POR), pregnane X receptor (PXR), steatosis.

INTRODUCTION

Cytochrome P450 enzymes (P450s) play a major role in the metabolism and disposition of chemicals and their activities are critical in determining the efficacy or side effects of drugs. These enzymes also have additional functions in adrenal steroid hormone biosynthesis and the hepatic synthesis of cholesterol and its degradation to bile acids [1]. The P450s involved in these different pathways are distinct and their regulation has conventionally been considered to be by different mechanisms. This is manifest in that many of the hepatic enzymes involved in foreign compound metabolism are expressed constitutively at low levels, but become highly induced on exogenous chemical exposure, resulting in an increased rate of excretion of the compound(s). Significant advances have been made in our understanding of this adaptive response through the identification of transcription factors, such as AhR (aryl hydrocarbon receptor), PXR (pregnane X receptor) and CAR (constitutive androstane receptor), which have the capacity to bind drugs and exogenous chemicals, resulting in transcriptional activation of genes that mediate their detoxification [2–5]. Although distinct classes of compounds bind to each receptor, owing to the diversity of these compounds' chemical structures, promiscuity exists in their specificity, particularly in the case of PXR and CAR [6,7].

We, and the group of Ding, have reported a mouse model where hepatic P450 activity has been reduced by $>95\,\%$ by the conditional deletion of the *Por* (P450 oxidoreductase) gene [8,9]. This was achieved by crossing mice carrying the *Por* gene flanked by loxP sites with mice expressing Cre recombinase under the control of the rat albumin promoter. This resulted in the specific hepatic deletion of POR neonatally with a concomitant reduction in P450 activity [8,9]. Although HRN (hepatic reductase-null) mice developed normally and were fertile, there were a number of intriguing phenotypic changes, including an enlarged liver and hepatic steatosis. Also, there was a profound increase in the expression of a range of P450s, with the most marked changes being in the expression of CYP2B10 (CYP is cytochrome P450) and CYP3A11 [10]. These enzymes, which are expressed at low levels in untreated animals, are highly inducible by exogenous chemicals such as barbiturates and synthetic glucocorticoids [11,12]. These data show that the P450 system is a key regulator of hepatic lipid homoeostasis and liver growth and that this metabolic homoeostasis can have a profound effect on the control of P450 gene expression and, as a consequence, on the metabolism/deposition of drugs. The HRN mouse therefore provides a model for investigating the endogenous factors involved in regulating P450 genes and the role of the P450 system in controlling hepatic steatosis and liver growth.

Studies of the temporal events that lead to the hepatic phenotype are, however, not possible in the HRN mice because the deletion of the *Por* gene occurs neonatally. To overcome this, we have created a model where the HRN genotype is regulated conditionally and in an inducible manner. This was achieved by crossing *Por*-floxed mice with mice where Cre recombinase is conditionally regulated by the rat *Cyp1a1* promoter. This promoter is activated through the AhR, and administration of a single dose of 3MC (3-methlycholanthrene) at 40 mg/kg results in a specific, time-dependent, hepatic deletion of POR and a phenotype identical with that observed in HRN mice [13].

In the present study, we have used the new model to study the temporal biochemical changes that control lipid accumulation and P450 overexpression. We provide evidence that dietary unsaturated fatty acids play a key role in both lipid accumulation and P450 induction. Also, using nuclear receptor/HRN

Abbreviations used: AhR, aryl hydrocarbon receptor; CAR, constitutive androstane receptor; Cpt1a, carnitine palmitoyltransferase 1a; CYP, cytochrome P450; FAS, fatty acid synthase; HRN, hepatic reductase-null; i.p., intraperitoneal; 3MC, 3-methylcholanthrene; P450, cytochrome P450; PB, phenobarbital; POR, P450 oxidoreductase; PPAR, peroxisome-proliferator-activated receptor; PUFA, polyunsaturated fatty acid; PXR, pregnane X receptor; TCPOBOP, 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene.

[1] To whom correspondence should be addressed (email c.r.wolf@dundee.ac.uk).

double- and triple-knockout mice, we show that P450 overexpression occurs predominantly through the activation of CAR.

EXPERIMENTAL

Reagents

All chemicals were purchased from Sigma–Aldrich, except where indicated.

Production of transgenic mice

$Por^{lox/lox}/Cre^{Alb}$ (HRN) and $Por^{lox/lox}/Cre^{CYP1A1}$ mice on a C57/BL6 genetic background were generated as described previously [9,13]. Littermates with the $Por^{lox/lox}$ genotype were used as controls. PXR-null ($Pxr^{-/-}$) and CAR-null ($Car^{-/-}$) mice on a C57/BL6 genetic background were crossed on to the HRN line to generate $Pxr^{-/-}/Por^{lox/lox}Cre^{Alb}$ ($Pxr^{-/-}$/HRN) and $Car^{-/-}/Por^{lox/lox}Cre^{Alb}$ ($Car^{-/-}$/HRN) double-knockout mice respectively. $Pxr^{-/-}$/HRN and $Car^{-/-}$/HRN were crossed to generate $Car^{-/-}/Pxr^{-/-}/Por^{lox/lox}Cre^{Alb}$ ($Car^{-/-}/Pxr^{-/-}$/HRN) triple-knockout mice. $Pxr^{-/-}/Por^{lox/lox}$, $Car^{-/-}/Por^{lox/lox}$ and $Car^{-/-}/Pxr^{-/-}/Por^{lox/lox}$ littermates were used as controls. $Pxr^{-/-}$ and $Car^{-/-}$ mice were identified by PCR as described previously [14,15].

All mice were maintained under standard animal house conditions with a 12 h light/12 h dark cycle and free access to water and RM1 (control) diet (Special Diets Services). All studies were carried out on 12-week-old male mice, except where indicated, in accordance with the Animal Scientific Procedures Act (1986) and after local ethical review.

Drug treatments

3MC in corn oil was administered to $Por^{lox/lox}/Cre^{CYP1A1}$ and $Por^{lox/lox}$ mice by i.p. (intraperitoneal) injection at 40 mg/kg of body weight, and animals were killed at the indicated times.

Dietary restrictions

$Por^{lox/lox}/Cre^{CYP1A1}$ and $Por^{lox/lox}$ mice were weaned and maintained on a control diet until 8 weeks of age before being transferred to either a fat-deficient diet (Special Diets Services, 821459) or a fatty-acid-supplemented fat-deficient diet (Supplementary Table S1 at http://www.BiochemJ.org/bj/417/bj4170043add.htm), for an additional 6 weeks. As controls, both $Por^{lox/lox}/Cre^{CYP1A1}$ and $Por^{lox/lox}$ mice were weaned and maintained on the control diet for the duration of the experiment. All diets were isocaloric and free from cholesterol. At 12 weeks of age, mice were administered either a single i.p. dose of 3MC (40 mg/kg of body weight) or corn oil, and killed 14 days later.

For linoleic acid treatment, $Por^{lox/lox}/Cre^{CYP1A1}$ mice were maintained on the fat-deficient diet as described above. At 12 weeks of age, mice were administered a single i.p. dose of 3MC (40 mg/kg of body weight), followed by an oral dose of linoleic acid of 65 mg/day from day 5 after 3MC administration until being killed 9 days later.

Immunoblotting and biochemical analysis

Microsomal fractions were prepared by differential centrifugation [12], and protein concentrations were determined using the Bio-Rad Protein Assay Reagent. Western blot analysis was carried out as described previously [16] using 5 μg of microsomal protein per lane and polyclonal antisera raised against human POR [17], murine CYP7A1 [13] or rat P450s [16]. Purified human POR, rat CYP4A1 and the following murine P450s, CYP2B10, His_6–CYP3A11 and His_6–CYP7A1, were used as standards. Immunoreactive proteins were detected using horseradish-peroxidase-conjugated donkey anti-(rabbit IgG) as a secondary antibody (Dako) and visualized by ECL® Plus (enhanced chemiluminescence (GE Healthcare). The relative protein content of individual P450s in microsomal fractions was determined by direct scanning of ECL® membranes with Bio-Rad Quantity One densitometry software. POR activity was determined by NADPH-dependent reduction of cytochrome *c* as described previously [17].

RNA isolation and real-time quantitative PCR analysis

Total RNA was isolated from snap-frozen liver samples using TRIzol® (Invitrogen). Genomic DNA was removed using RQ1 DNase (Promega), and RNA (600 ng) was reverse-transcribed using random hexamers and Superscript II RNase H reverse transcriptase polymerase (Invitrogen). Real-time quantitative PCR analysis was carried out with gene-specific primers for CYP2B10, CYP3A11 and CAR as described previously [10] and CD36 (Mm01135198_m1), FAS (fatty acid synthase) (Mm01253292_m1) and Cpt1a (carnitine palmitoyltransferase 1a) (Mm00550438_m1) were supplied by Applied Biosystems. Reactions were carried out in triplicate and monitored by measuring fluorescence at 518 nm with excitation at 494 nm. mRNA levels were quantified using the Prism 7700 associated software and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as an internal standard.

Blood chemistry

Blood was collected by cardiac puncture into heparinized tubes, and plasma was analysed for non-fasting total cholesterol and non-fasting triacylglycerols (triglycerides) using assay kits (Thermo Trace) on a Cobas Fara II centrifugal analyser (Roche).

Histopathology

Tissue samples were snap-frozen in Cryo-M-Bed (Bright Instrument Co.), cryo-sectioned (10 μm) and stained with Oil Red O (lipid content) or haematoxylin and eosin (morphological analysis).

Lipid isolation and analysis

A small piece of liver was homogenized in PBS containing 0.1 % Triton X-100, and hepatic triacylglycerol and cholesterol levels were determined using assay kits on a Cobas Fara II centrifugal analyser [18].

Detailed fatty acid analysis of the various diets was carried out by Special Diet Services (Supplementary Tables S2 and S3 at http://www.BiochemJ.org/bj/417/bj4170043add.htm).

Plasmid construction

The plasmids pCMX Gal4-hPXR-LBD and p4*4xGal4-UAS-luciferase were a gift from Luisella Vignati (Department of Pharmacokinetics, Dynamics and Metabolism, Gruppo Pfizer Inc., Italy) [19] and the former was used as a source of the pCMX Gal4 vector to facilitate the generation of further reporter constructs. The mouse CAR ligand-binding domain from amino acids 118–358 (mCAR-LBD) was amplified using 5′-GCGGAATTCGGTACTAAAGCATCTTTGCAACTGAA-3′ and 5′-GCGGATCCTCAACTGCAAATCTCCCCGAGC-3′ as forward

and reverse primers respectively, incorporating 5′ EcoRI and 3′ BamHI sites to facilitate cloning. The resulting 738 bp PCR product was cloned as an EcoRI/BamHI fragment into pCMX Gal4-hPXR-LBD replacing the hPXR-LDB section and generating the vector pCMX Gal4-mCAR-LBD.

Cell culture, transient transfection and cell treatments

All cell culture media and supplements were supplied by Invitrogen. HepG2 cells were cultured in DMEM (Dulbecco's modified Eagle's medium) containing 10 % (v/v) heat-inactivated foetal bovine serum, 1× non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine at 37 °C and 5 % CO_2.

HepG2 cells were transiently transfected with pCMX Gal4-mCAR-LBD/p4*4xGal4-UAS-luciferase using the method of Vignati et al. [19], with the following modifications: FuGENE™ 6 (Roche) was used instead of Lipofectamine™ Plus (Invitrogen), assays were carried out in serum-containing medium and 0.2 μg of the plasmid pRL-TK was included to allow normalization of luciferase reporter gene activity. Cells were treated with 0.25–0.5 μM TCPOBOP {1,4-bis-[2-(3,5-dichloropyridyloxy)]-benzene} and 1 mM PB (phenobarbital) as positive controls and DMSO as a further control. Linoleic acid, complexed to fatty-acid-free BSA at a molar ratio of 2:1, was tested at various concentrations ranging from 0 to 12.5 μM, with the addition of 20 μM α-tocopheryl succinate to prevent lipid peroxidation. Luciferase reporter activity was determined using the Dual-Luciferase Reporter Assay System (Promega).

RESULTS

Temporal analysis of the phenotypic changes observed in the HRN mice

$Por^{lox/lox}/Cre^{CYP1A1}$ mice, in the absence of a chemical inducer, were phenotypically normal and indistinguishable from wild-type animals. Treatment with a single dose of 3MC (40 mg/kg) led to a time-dependent complete deletion of the hepatic POR and the development of a phenotype that was identical with that of the original HRN model [13]. We therefore used this model to study the sequential development of the HRN phenotypic traits over a 28 day period following 3MC treatment.

At 6 days after 3MC administration a >85 % reduction in the expression of POR protein, determined both by immunoblotting and cytochrome *c* activity, had occurred (Figures 1A and 1B, i), increasing to approx. 96 % by day 14. These data were confirmed by immunohistochemistry where staining was reduced in all hepatocytes by day 3 and undetectable on day 14 and remained absent for at least 28 days (results not shown). Treatment with 3MC resulted in the anticipated induction of CYP1A1 as a consequence of the activation of the AhR. By days 7–9, CYP1A1 levels, presumably due to the metabolism and elimination of 3MC, were undetectable, indicating that the level of CYP1A1 was not induced by the POR deletion (Figure 1A). The transient changes observed in CYP1A1 expression imply that Cre recombinase expression would follow the same pattern and suggest that the deletion of POR is reversible. Cre recombinase is indeed expressed transiently, similarly to CYP1A1; however, return of POR is dependent on cell turnover, which, in the case of hepatocytes, takes several weeks. In $Por^{lox/lox}/Cre^{CYP1A1}$ mice, no return of POR was observed for 60 days after 3MC treatment (results not shown). In contrast, very marked increases in the expression of other P450s were detected (Figure 1A). CYP2B, CYP2C, CYP3A and CYP7A1 levels increased profoundly as a function of time and in association with the decrease in POR expression. Particularly marked changes were obtained in P450s identified as CYP2B10 (corresponding to the upper band and CYP2B9 to the lower band according to the findings of Li-Masters and Morgan [20]) and CYP3A11. Interestingly, a stepwise increase in the level of CYP2B proteins occurred between days 9 and 11, whereas the increase in CYP2C and CYP3A proteins appeared to occur more gradually. An induction of CYP7A1 occurred on day 7 and continued to increase until day 21 (Figure 1A). CYP4A levels decreased from day 11 in both control ($Por^{lox/lox}$) as well as HRN mice, after which time they remained slightly higher in HRN mice (results not shown). No changes were observed in the expression levels of CYP2D proteins in any samples. In control $Por^{lox/lox}$ animals, 3MC treatment had no effect on any of the P450s measured other than on the induction of CYP1A1 (results not shown).

Associated with the time-dependent reduction in POR were changes in liver size and non-fasting plasma total cholesterol and triacylglycerols levels (Figure 1B, ii, iii and iv). Significant consistent changes in liver/body weight ratio were observed from day 11, suggesting that other phenotypic changes must precede this effect (Figure 1B, ii). A significant decrease in non-fasting plasma triacylglycerol levels also occurred at this time (Figure 1B, iv). A reduction in non-fasting total plasma cholesterol levels was also observed which reduced steadily from day 3, in parallel with POR reduction (Figure 1B, iii), indicating that the reduction in triacylglycerols occurs independently of the changes in cholesterol, but in parallel with liver enlargement.

Loss of POR also led to a profound increase in hepatic lipids (Figure 1C). This accumulation occurred rapidly after 3MC administration and paralleled the loss of POR. At early time points, increases in lipid appeared to be localized to the perivenous region, the major site of POR and P450 expression, and not to the periportal region [21,22]. At later time points, this zonal localization was lost, leading to lipid accumulation throughout the liver. Quantification of hepatic lipid content demonstrated that the hepatic steatosis was due to increases in hepatic triacylglycerols by day 11 (Figure 2A). No significant changes in hepatic cholesterol levels were observed over the entire time course of the experiment (Figure 2B). The changes in hepatic triacylglycerol content were closely correlated with changes in both liver/body weight ratio and the reduction in plasma triacylglycerols (Figures 2C and 2D). This suggests that the level of hepatic triacylglycerols determines the circulating triacylglycerol levels and that they are related to liver enlargement.

Changes in hepatic lipid homoeostasis mediate P450 induction

Hepatic deletion of POR led to a profound time-dependent induction of several P450s, including members of the CYP2B and CYP3A families. This appeared to occur at both the protein and the mRNA level [9,10]. Changes in CYP2B10 expression were first seen on day 3, with a very marked increase between days 9 and 11 (Figure 1A). The increases in CYP2B10 protein and mRNA were not correlated with the decrease in POR activity (Supplementary Figure S1A, i and ii, at http://www.BiochemJ.org/bj/417/bj4170043add.htm) and therefore did not appear to be due to changes in the metabolism of an endogenous inducing agent. However, CYP2B10 protein and mRNA levels were correlated with each other, indicating that the induction of the *Cyp2b10* gene occurs mainly at a transcriptional level (Supplementary Figure S1A, iii). In addition, *Cyp2b10* mRNA levels were correlated with hepatic triacylglycerol concentration (Supplementary Figure S1A, iv). Similar analysis for CYP3A11 indicated that the increased protein expression occurred more rapidly than for

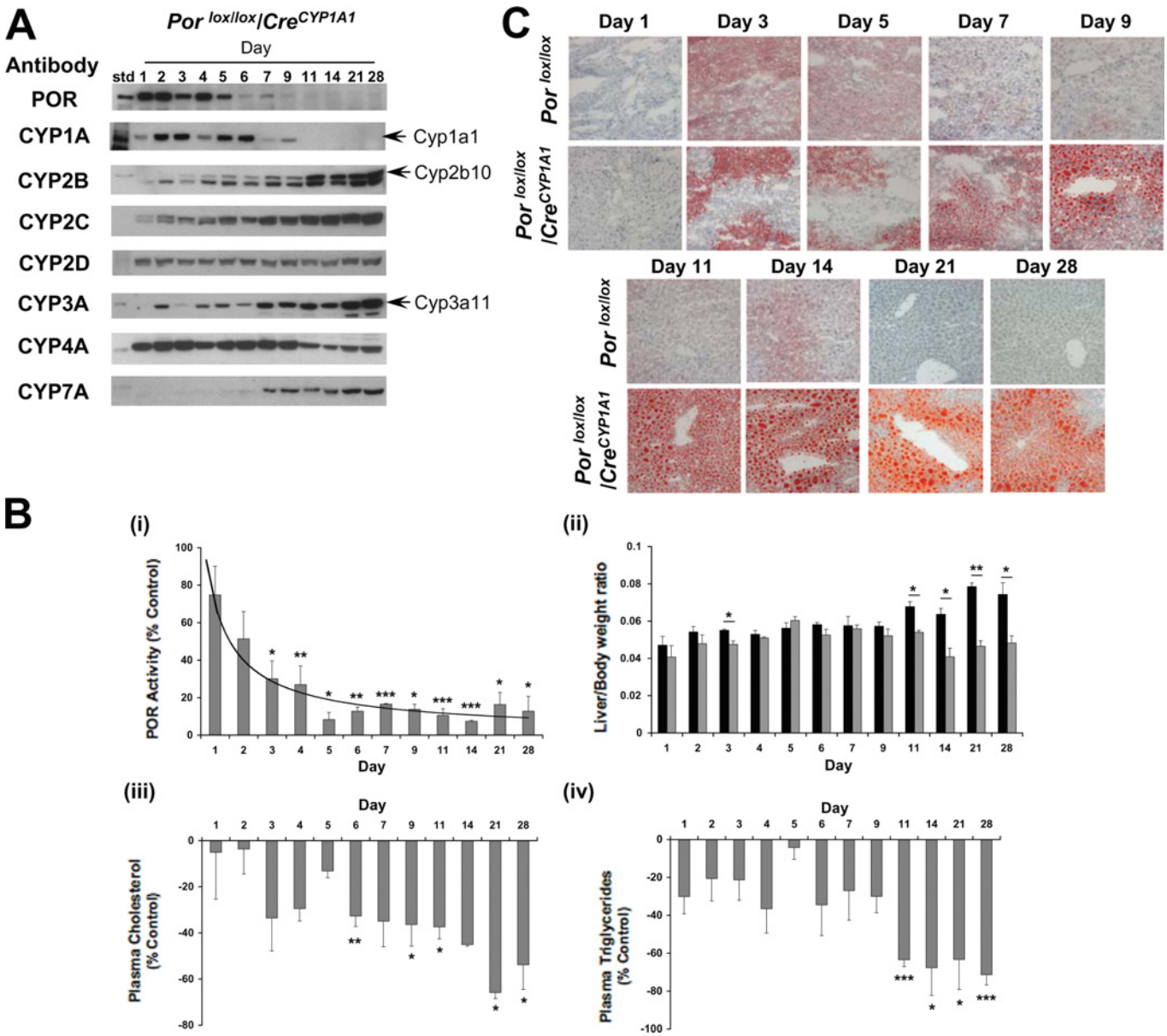

Figure 1 Time course of the phenotypic changes in conditional HRN mice

Por$^{lox/lox}$/*Cre*^{CYP1A1} and *Por*$^{lox/lox}$ mice were treated with a single dose of 3MC (40 mg/kg of body weight) before plasma and liver samples were collected at the time points shown. (**A**) POR and P450 protein expression. Samples represent a pool of three animals per time point and 5 μg of hepatic microsomal protein per lane. Purified human POR and murine P450s were used as standards. (**B**) (i) Hepatic microsomal POR activity. (ii) Liver/body weight ratios. Black bars: *Por*$^{lox/lox}$/*Cre*^{CYP1A1}. Grey bars: *Por*$^{lox/lox}$. (iii) Non-fasting plasma total cholesterol. (iv) Non-fasting plasma triacylglycerol (triglyceride) levels. Results are means $\pm$ S.E.M. for three animals per time point, and in (i), (iii) and (iv) are expressed as a percentage compared with *Por*$^{lox/lox}$ mice at the same time point. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$ compared with *Por*$^{lox/lox}$ mice. (**C**) Hepatic lipid content was visualized by Oil Red O staining of snap-frozen sections. Photomicrographs were taken at 10$\times$ magnification, bright field, and are representative of the results from at least three mice per group.

CYP2B10, steadily increasing from day 2, reaching a maximum by day 21, but also did not directly correlate with changes in POR activity (Figure S1B). Although *Cyp3a11* mRNA levels were increased in POR mice, this did not correlate with the level of CYP3A11 protein, indicating a more complex mechanism of regulation of this gene (Supplementary Figure S1B, iii), possibly involving both transcriptional and post-translational mechanisms. Unlike *Cyp2b10*, only a weak correlation between the increases in *Cyp3a11* mRNA levels and hepatic triacylglycerol levels was observed (Supplementary Figure S1B, iv). Thus differences observed in the induction kinetics of CYP2B10 and CYP3A11, at both the protein and the mRNA level, suggests that the mechanisms regulating their overexpression are, at least in part, distinguishable.

To determine the contributions of lipid uptake, oxidation and synthesis to the development of the fatty liver phenotype and the induction of P450s, changes in the expression levels of CD36, FAS and Cpt1a were determined in 3MC-treated *Por*$^{lox/lox}$/*Cre*^{CYP1A1} mice compared with *Por*$^{lox/lox}$ mice (Supplementary Figure S2 at http://www.BiochemJ.org/bj/417/bj4170043add.htm). No significant changes in the expression levels of these genes were observed, except for in Cpt1a on day 28 which showed a minor,

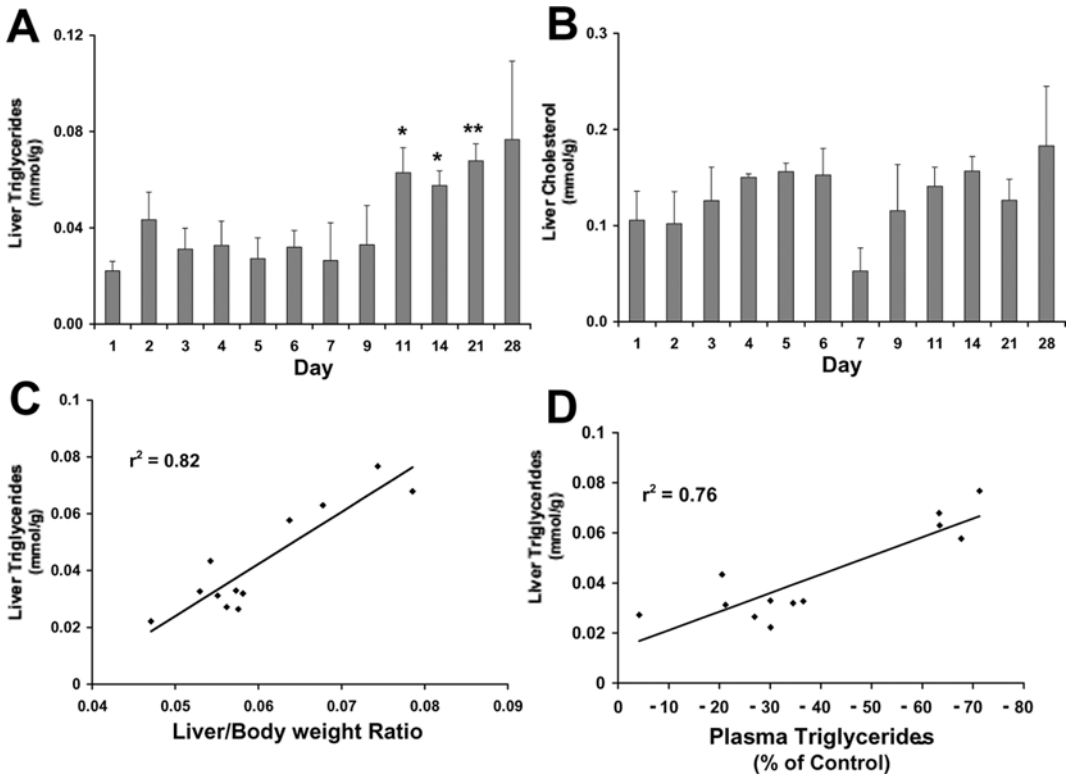

Figure 2 Hepatic lipid accumulation and its relationship with organ weight and plasma lipid levels in conditional HRN mice

Por$^{lox/lox}$/*Cre*^{CYP1A1} and *Por*$^{lox/lox}$ mice were treated with a single dose of 3MC (40 mg/kg of body weight), and killed on the indicated days. (**A**) Hepatic triacylglycerol (triglyceride) concentrations. (**B**) Hepatic cholesterol concentrations. Correlation of hepatic triacylglycerol concentration with (**C**) liver/body weight ratio and (**D**) non-fasting plasma triacylglycerol levels. Results are means ± S.E.M. for three animals per time point. $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$ compared with lipid levels on day 1.

but significant, increase and may possibly be an attempt to reverse lipid accumulation by increasing β-oxidation. Increased expression of CD36 was observed on days 21 and 28 in *Por*$^{lox/lox}$/*Cre*^{CYP1A1} mice, which may contribute to the increase in hepatic lipid at these time points; however, no correlation could be made before day 21. There was no correlation between the expression levels of these genes and the increases in P450 expression. They therefore do not appear to contribute to the lipid accumulation.

Dietary fatty acids mediate P450 induction in HRN mice

In view of the finding that changes in hepatic fatty acid homoeostasis correlated with the increases in P450 expression, detailed fatty acid analysis was carried out on livers from HRN and *Por*$^{lox/lox}$ (control) mice (Supplementary Tables S4 and S5 at http://www.BiochemJ.org/bj/417/bj4170043add.htm). Changes in all fatty acid classes occurred; however, the most significant changes were in mono-unsaturated and polyunsaturated fatty acids (Supplementary Table S5), with $C_{16:1}$, $C_{18:1}$ and $C_{20:3}$ increasing 6.78-, 14.06- and 6.19-fold respectively (Supplementary Table S4). These data are in close agreement with the report of Weng et al. [23], who, for their HRN model, reported additional fold changes of 7.3 and 14.9 for $C_{18:3}$ and $C_{20:1}$ unsaturated fatty acids respectively. Together, these findings suggest that the accumulated hepatic triacylglycerols preferentially contain unsaturated fatty acid chains and that these fatty acid classes may mediate the P450 induction.

In the light of this possibility, *Por*$^{lox/lox}$/*Cre*^{CYP1A1} mice were placed on a fat-deficient diet for 4 weeks before 3MC administration. This treatment completely prevented the increases in P450 protein and mRNA expression as a consequence of POR deletion (Figure 3A). Maintenance of control *Por*$^{lox/lox}$ mice on a fat-deficient diet caused slight reductions in the constitutive expression of CYP7A and CYP2C protein, and did not affect the levels of the other P450s (results not shown). In addition to the changes in hepatic P450 expression, maintenance of *Por*$^{lox/lox}$/*Cre*^{CYP1A1} mice on a fat-deficient diet prevented the accumulation of lipid in the livers of these animals (Figure 3C), resulting in an Oil Red O staining pattern that was indistinguishable from that of *Por*$^{lox/lox}$ mice on a control diet. Also no significant changes in total plasma cholesterol or triacylglycerol levels were measured on deletion of POR in *Por*$^{lox/lox}$/*Cre*^{CYP1A1} mice fed a fat-deficient diet (Supplementary Figure S3). These data indicate that hepatic triacylglycerol accumulation in POR mice originates from the diet and that as a consequence of the inactivation of the P450 system an inability to maintain hepatic lipid homoeostasis occurs which triggers increased P450 expression (possibly in an attempt to restore normal hepatic lipid levels). It is worth noting that the fat-deficient diet also prevented the liver enlargement seen on POR deletion, thus providing a metabolic link to all three phenomena.

In order to identify the fatty acids that mediated P450 induction, various fatty acids were added back to the fat-deficient diet as outlined in Supplementary Table S1. *Por*$^{lox/lox}$/*Cre*^{CYP1A1} and *Por*$^{lox/lox}$ were maintained on the supplemented diets and analysed 14 days after 3MC administration. In diets containing unsaturated fatty acids, the loss of P450 gene expression observed with the fat-deficient diet was reversed. This was particularly the case for diets containing sunflower oil (which is composed of approx. 88 % unsaturated fatty acids) (Figure 3B, Supplementary Table S2). The

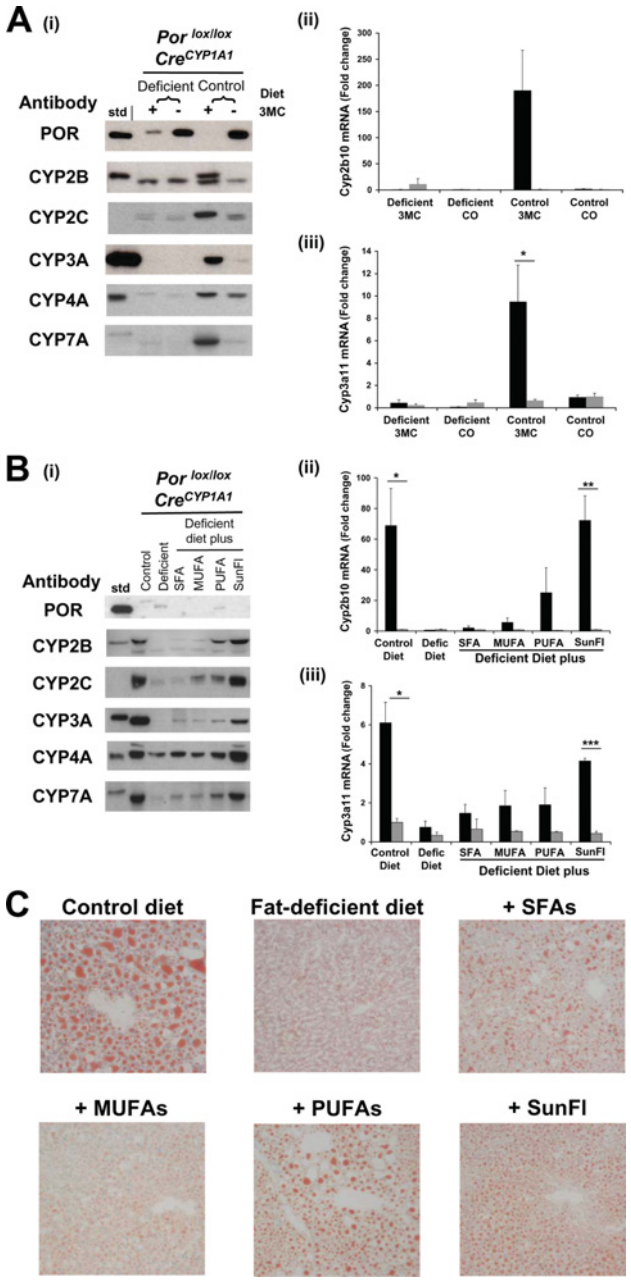

Figure 3 Development of phenotypic changes in conditional HRN mice on restricted fatty acid diets

$Por^{lox/lox}/Cre^{CYP1A1}$ mice were maintained on a control diet until 8 weeks of age, and then either placed on a fat-deficient or fatty-acid-supplemented diet, or continued on the control diet, for a further 6 weeks. At 12 weeks of age, they were treated with a single dose of 3MC (40 mg/kg of body weight) and killed 14 days later. (**A**) POR and P450 expression levels in $Por^{lox/lox}/Cre^{CYP1A1}$ and $Por^{lox/lox}$ mice maintained on a control or fat-deficient diet treated with 3MC (+) or corn oil (CO) (−). (i) Protein expression determined by Western blotting. Hepatic *Cyp2b10* (ii) and *Cyp3a11* (iii) mRNA levels expressed as a fold change compared with $Por^{lox/lox}$ mice. (**B**) POR and P450 expression levels in $Por^{lox/lox}/Cre^{CYP1A1}$ mice maintained on fatty-acid-supplemented diets and treated with 3MC. (i) Protein expression determined by Western blotting. Hepatic *Cyp2b10* (ii) and *Cyp3a11* (iii) mRNA levels expressed as a fold change compared with $Por^{lox/lox}$ mice. (**C**) Hepatic lipid content determined by Oil Red O staining of snap-frozen liver sections of $Por^{lox/lox}/Cre^{CYP1A1}$ mice maintained on fatty-acid-supplemented diets and treated with 3MC. Photomicrographs were taken at 10× magnification, bright field, and are representative of the results from at least three mice per group. For mRNA analysis in (**A**) and (**B**), results are means ± S.E.M. ($n = 3$). * $P \leqslant 0.05$; ** $P \leqslant 0.01$; *** $P \leqslant 0.001$ compared with $Por^{lox/lox}$ mice. std, standard. Black bars: $Por^{lox/lox}/Cre^{CYP1A1}$; grey bars: $Por^{lox/lox}$. In all panels: SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; SunFl, 1% sunflower oil.

inclusion of saturated fatty acids in the diet was essentially without effect. None of the treatments had effects on POR or P450 gene expression in the control $Por^{lox/lox}$ animals, with the exception of CYP4A which was induced by all three unsaturated fatty acid diets (results not shown). Analysis of *Cyp2b10* and *Cyp3a11* mRNA levels confirmed that the changes in protein expression were at least in part a result of transcriptional activation, the highest increases in mRNA being found in mice on the sunflower-oil-supplemented diet (Figure 3B, ii and iii). Analysis of plasma lipid levels revealed some minor decreases in both total cholesterol and triacylglycerol levels between $Por^{lox/lox}/Cre^{CYP1A1}$ and $Por^{lox/lox}$ for all dietary treatments; however, these were only significant for the sunflower-oil-supplemented diet where a 1.5- ($P = 0.036$) and 1.8- ($P = 0.013$) fold decrease was observed respectively (Supplementary Figures S3A and S3B at http://www.BiochemJ.org/bj/417/bj4170043add.htm). Hepatic lipid accumulation was observed for all diets, except for the fat-deficient diet, indicating that lipid accumulation alone is not sufficient to cause P450 induction (Figure 3C). In addition, no significant increases in liver/body weight ratio were observed for the diets used, except that containing sunflower oils (Supplementary Figure S3C). These data indicate that lipid accumulation alone was not the cause of liver enlargement, but that unsaturated fatty acids had the capacity to induce the effect. Comparison of the fatty acid composition of the diets (Supplementary Tables S2 and S3), identified $C_{18:2}$ (linoleic acid), an $n-6$ PUFA (polyunsaturated fatty acid), as a candidate fatty acid mediator of both P450 induction and liver growth as it was the only fatty acid present in the diets that correlated with the effects observed. This hypothesis suggests that the addition of linoleic acid as the sole dietary fatty acid should be sufficient to induce P450 expression in $Por^{lox/lox}/Cre^{CYP1A1}$ mice maintained on a fat-deficient diet. This was indeed found to be the case, with oral administration of linoleic acid (65 mg/day for 9 days) to $Por^{lox/lox}/Cre^{CYP1A1}$ mice on a fat-deficient diet, resulting in induction of CYP2B10, CYP3A11 and CYP2C proteins (Figure 4A). Analysis of *Cyp2b10* and *Cyp3a11* mRNA levels showed this induction to occur at the transcription level (Figure 4B). In addition, administration of linoleic acid was found to promote hepatic lipid accumulation (Figure 4C). This lipid accumulation was reduced compared with $Por^{lox/lox}/Cre^{CYP1A1}$ mice maintained on a control diet and appeared to be concentrated around the blood vessels, a pattern similar to that observed at early time points following 3MC administration (Figure 1C); however, in this case, it is probably due to differences in the amount of fat intake. These results therefore indicate that linoleic acid, an $n-6$ dietary essential fatty acid, is a key regulator of P450 expression in HRN models and that members of the CYP2B, CYP3A and CYP2C, in a normal physiological situation, are involved in its metabolism.

Role of nuclear receptors in P450 induction in HRN mice

Nuclear receptors that have been shown to mediate the regulation of many P450 genes in response to exogenous chemicals [24,25] have also been shown to play an important role in controlling liver growth [26,27]. Of particular interest with regards to this study were CAR and PXR, which have been intimately linked to the regulation of CYP2B and CYP3A proteins in response to both exogenous and endogenous inducing agents [28–30], and PXR has been linked to triacylglycerol homoeostasis [27]. In order to investigate whether these nuclear receptors mediate the P450 overexpression in the HRN mice, we crossed the HRN animals with mice nulled at either the *Car* or *Pxr* locus alone or in combination to generate $Car^{-/-}$/HRN, $Pxr^{-/-}$/HRN and $Pxr^{-/-}/Car^{-/-}$/HRN genotypes.

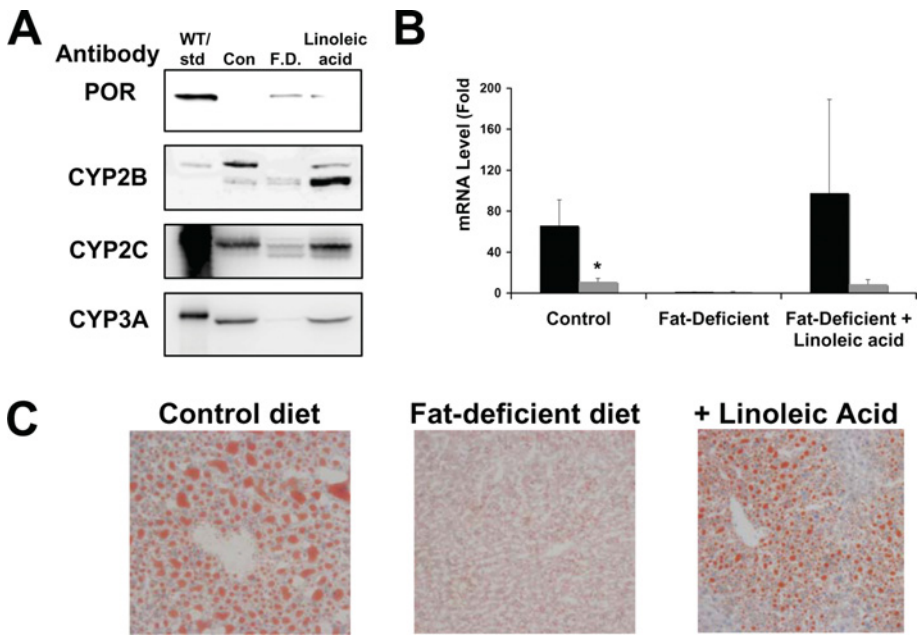

Figure 4 Induction of hepatic P450 expression in conditional HRN mice by linoleic acid

Por$^{lox/lox}$/*Cre*^{CYP1A1} mice were maintained on a control diet until 8 weeks of age, and then placed on a fat-deficient diet for a further 6 weeks. At 12 weeks of age, they were treated with a single dose of 3MC (40 mg/kg of body weight) and 5 days later administered linoleic acid (65 mg/day) for 9 days before being killed. (**A**) POR and P450 expression levels were determined by Western blotting. WT, wild-type; std, standard; Con, control; F.D., fat-deficient diet. (**B**) Hepatic *Cyp2b10* (black bars) and *Cyp3a11* (grey bars) mRNA levels expressed as a fold change compared with *Por*$^{lox/lox}$/*Cre*^{CYP1A1} mice maintained on a fat-deficient diet. Results are means ± S.E.M. ($n = 3$). *$P \leqslant 0.05$ compared with *Por*$^{lox/lox}$/*Cre*^{CYP1A1} mice maintained on a fat-deficient diet. (**C**) Hepatic lipid content determined by Oil Red O staining of snap-frozen liver sections. Photomicrographs were taken at 10× magnification, bright field, and are representative of the results from at least three mice per group.

Loss of PXR did not affect POR deletion or the induction of P450 proteins observed in HRN mice (Figure 5A). In agreement with previous findings, *Pxr*$^{-/-}$ mice exhibited slight increases in CYP3A11 protein [14], and also CYP2B and CYP2C proteins (Figure 5A, i); *Cyp3a11* mRNA was also elevated relative to levels in control *Por*$^{lox/lox}$ mice (Figure 5A, ii). Basal expression of *Cyp2b10* mRNA was also very slightly increased (Figure 5A, ii), although this was not reflected in detectable protein expression (Figure 5A, i). The deletion of PXR did not affect the increases in both CYP2B10 and CYP3A11 protein in HRN animals; however, a reduction in both *Cyp2b10* and *Cyp3a11* mRNA levels was observed, but this was not significant compared with HRN mice (Figure 5A, ii). No changes in plasma and hepatic lipid levels were observed in *Pxr*$^{-/-}$/HRN mice compared with HRN mice (Supplementary Table S6 at http://www.BiochemJ.org/bj/417/bj4170043add.htm and Figure 5D); however, in *Pxr*$^{-/-}$ mice, non-fasting plasma total cholesterol and triacylglycerol levels were reduced, the former significantly, in comparison with wild-type mice (Supplementary Table S6).

In relation to HRN mice nulled for CAR, profound changes in the HRN phenotype were observed. The elevated expression of CYP2B, CYP7A and CYP2C was markedly attenuated; however, CYP3A11 expression appeared to be unaffected (Figure 5B). Analysis of mRNA levels also showed that no induction of *Cyp2b10* mRNA occurred in *Car*$^{-/-}$/HRN mice, indicating that the increase in the expression of this P450 observed in HRN mice was a consequence of CAR activation (Figure 5B, ii). Interestingly, deletion of CAR in both control and HRN mice further increased *Cyp3a11* mRNA levels (Figure 5B, ii). The reduction in non-fasting plasma total cholesterol and triacylglycerol levels observed in the HRN mice were, however, not reversed on the *Car*$^{-/-}$ background (Supplementary Table S6); however, a significant reduction in hepatic lipid content for *Car*$^{-/-}$/HRN mice compared with HRN mice was observed (Figure 5D). These data suggest that, in addition to mediating P450 induction in HRN mice, CAR plays a role in hepatic lipid accumulation.

Finally, owing to the cross-talk between PXR and CAR [31], the effect of deleting both receptors on the development of the HRN phenotype was analysed. In *Pxr*$^{-/-}$/*Car*$^{-/-}$/HRN mice, consistent with the *Car*$^{-/-}$ mice, the expression of CYP2B and CYP2C proteins was profoundly reduced, whereas, in contrast, CYP7A expression levels were only slightly affected (Figure 5C). In mice nulled at both the *Car* and *Pxr* gene loci, a marked decrease in CYP3A11 protein was also observed (Figure 5C, i). The change in CYP2B protein expression was also reflected in a reduction in *Cyp2b10* mRNA levels (Figure 5C, ii). A slight decrease in *Cyp3a11* mRNA was observed in *Pxr*$^{-/-}$/ *Car*$^{-/-}$/HRN mice, although this was not significantly different from that of HRN or *Pxr*$^{-/-}$/*Car*$^{-/-}$ mice (Figure 5C, ii). Deletion of both CAR and PXR on an HRN background resulted in an increase in non-fasting plasma total cholesterol level that was not significantly different from control (*Por*$^{lox/lox}$) mice. Although an increase in plasma triacylglycerols was also observed, this was not significantly different from the HRN value (Supplementary Table S6). Hepatic lipid content was reduced in *Pxr*$^{-/-}$/*Car*$^{-/-}$/HRN mice compared with HRN mice, similar to levels observed in *Car*$^{-/-}$/HRN mice, but still elevated compared with controls (Figure 5D); however, this reduction did result in a change in liver size (Supplementary Table S6).

Direct activation of CAR by linoleic acid in HRN mice

In order to establish whether PUFAs could activate CAR directly, we carried out transactivation assays using a Gal4-CAR-LBD

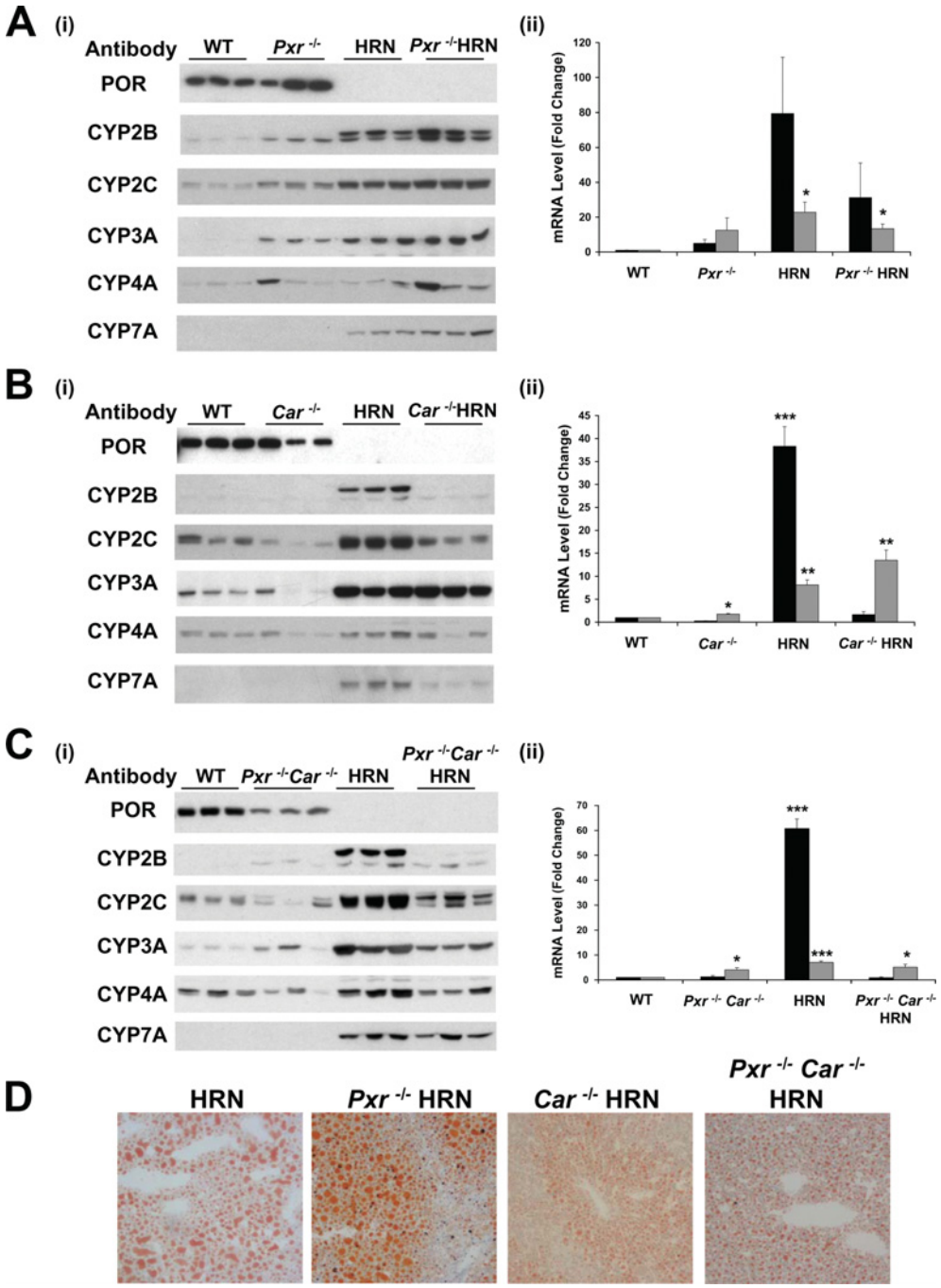

Figure 5 Induction of hepatic P450 expression and other phenotypes in HRN mice on nuclear receptor-null backgrounds

(**A**) POR and P450 expression levels in *Pxr*$^{-/-}$/HRN mice: (i) protein expression determined by Western blotting; and (ii) hepatic *Cyp2b10* (black bars) and *Cyp3a11* (grey bars) mRNA levels expressed as a fold change compared with *Por*$^{lox/lox}$ mice. (**B**) POR and P450 expression levels in *Car*$^{-/-}$/HRN mice: (i) protein expression determined by Western blotting; and (ii) hepatic *Cyp2b10* (black bars) and *Cyp3a11* (grey bars) mRNA expressed as a fold change compared with *Por*$^{lox/lox}$ mice. (**C**) POR and P450 expression levels in *Car*$^{-/-}$/*Pxr*$^{-/-}$/HRN mice: (i) protein expression determined by Western blotting; and (ii) hepatic *Cyp2b10* (black bars) and *Cyp3a11* (grey bars) mRNA expressed as a fold change compared with *Por*$^{lox/lox}$ mice. (**D**) Hepatic lipid content determined by Oil Red O staining of snap-frozen liver sections. Photomicrographs were taken at 10× magnification, bright field, and are representative of the results from at least three mice per group. For mRNA analysis in (**A**), (**B**) and (**C**), results are means ± S.E.M. ($n = 3$). $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$; $^{***}P \leqslant 0.001$ compared with *Por*$^{lox/lox}$ mice. WT, wild-type.

reporter assay in HepG2 cells [32]. In these experiments, both TCPOBOP and linoleic acid could activate CAR directly (Figure 6). PB, which is known to activate CAR by an indirect mechanism, was not active in this assay. In view of reports that the fatty-acid-sensitive transcription factor, PPARα (peroxisome-proliferator-activated receptor α) can effect CAR expression [33,34] and that the effects in the POR mice may be indirect, we measured *Car* mRNA expression in *Por*$^{lox/lox}$/*Cre*^{CYP1A1} mice. Expression levels of CAR were found to be unchanged (Supplementary Figure S2D).

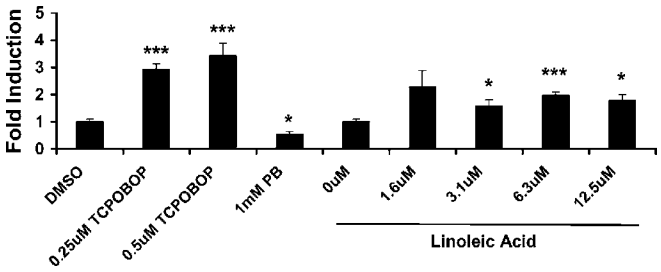

Figure 6 Activation of CAR by linoleic acid

HepG2 cells were transfected with pCMX Gal4-mCAR-LBD and p4*4xGal4-UAS4-luciferase as outlined in the Experimental section. Transfected cells were subsequently treated with linoleic acid at a concentration ranging from 0 to 12.5 μM, and luciferase activity was analysed 24 h later. Transfected cells were treated with 0.25–0.5 μM TCPOBOP or 1 mM PB as positive and negative controls respectively. Results are means $\pm$ S.E.M. ($n = 6$ determinations) and are expressed as a fold change compared with cells treated with either DMSO in the case of TCPOBOP and PB or the 0 μM sample in the case of linoleic acid. $^{*}P \leqslant 0.05$; $^{***}P \leqslant 0.001$.

DISCUSSION

The liver is the major organ controlling metabolic homoeostasis due to dietary change. It is also the major tissue responsible for drug and dietary chemical detoxification through the actions of phase I and phase II drug metabolism in which the P450 system plays a pivotal role. Although it has been known for some time that alterations in diet and disease states such as diabetes affect the P450 system, there has been little evidence that P450s can play a direct role in metabolic control [35–37]. However, there is a growing body of evidence, as demonstrated in the present paper, that these pathways are intimately linked. This has a wide range of implications not only for how alterations in P450 function may influence the pathogenesis of disease, but also for how diet and disease states may influence the metabolism and disposition of drugs and environmental agents; for example, obesity in rats has been shown to reduce expression levels of CAR and impaired CYP2B induction by PB [38].

The metabolic dialogue between the pathways of detoxification and metabolic homoeostasis are manifest in the findings that, when the P450 system is inactivated, profound changes in lipid homoeostasis and liver size occur which are paralleled by the marked induction of P450s associated with drug metabolism in the absence of an exogenous inducing agent. We have demonstrated that P450s from a range of gene families are induced and that this induction parallels the accumulation of hepatic triacylglycerols. We have also shown that the regulation of different P450s is, in part, distinguishable, as exemplified by CYP2B10 and CYP3A11. CYP2B10 induction is almost entirely controlled at the transcriptional level and occurs at a time point when there is a stepwise increase in triacylglycerol accumulation, whereas, in the case of CYP3A11, there is only a poor correlation between mRNA induction and protein levels, indicating that both transcriptional and post-translational mechanisms are involved. The latter mechanism could possibly occur via phosphorylation or ubiquitination [39–41]. This may also be the case for CYP2C proteins. Evidence for distinct pathways of regulation is also reflected in the finding that the overexpression of these proteins is, at least in part, mediated by different transcription factors (see below). We have also demonstrated that hepatic steatosis is not sufficient to induce the hepatic P450 expression, but this effect is mediated by dietary unsaturated fatty acids such as linoleic acid, which we have found is sufficient on its own to reverse the fat-deficient phenotype.

P450s of the CYP2B family are the major enzymes induced in the liver by barbiturate drugs such as PB [42] and have the capacity to metabolize fatty acids such as arachidonic acid [43]. Interestingly, Fulco and co-workers demonstrated over 20 years ago that a fatty acid mono-oxygenase involved in fatty acid oxidation in *Bacillus megaterium* is highly inducible by PB [44,45]. In fact, studies on this pathway have demonstrated that its induction provides an adaptive response against unsaturated fatty acid toxicity [46]. Whether the induction of enzymes such as CYP2B10 and CYP3A11 in mammals are part of a similar detoxification pathway against dietary unsaturated fatty acids which are not produced endogenously, such as linoleic acid, remains to be determined. However the data in the present study do raise the possibility that, under certain circumstances, diets containing unsaturated fatty acids may influence hepatic detoxification systems.

A wide range of transcription factors have now been discovered which control fatty acid homoeostasis and liver growth, including PPARα, CAR, PXR, LXR (liver X receptor) and HNF4α (hepatocyte nuclear factor 4α) [26,27,47–49]. For example, it has been demonstrated that PPARα mediates liver growth induced by perturbations in fatty acid homoeostasis as a consequence of peroxisome proliferation and that PXR plays a role in controlling hepatic triacylglycerol homoeostasis [27,50,51]. It was therefore intriguing that the majority of the phenotypes resulting from inactivation of the hepatic P450 system and the downstream effects on fatty acid homoeostasis were mediated by the transcription factor CAR. It has been postulated for some time that CAR function is regulated by both endogenous and exogenous inducing agents including metabolic control through AMPK (AMP-activated protein kinase) [52], interaction with the forkhead transcription factor FoxO1 (forkhead box O1) [53] and activation during fasting [54]. It has also been postulated that CAR function can be modulated by the fatty-acid-sensitive transcription factor PPARα [33,34]. However, this mechanism of CAR activation would not appear to explain the hepatic phenotype, as no changes in CAR expression were found and also linoleic acid activated CAR directly. However, to our knowledge, the finding that CAR may play a role in linking the control of hepatic lipid homoeostasis and endogenous P450 expression has not been reported previously. We provide additional evidence that, for one group of enzymes in the *Cyp3a* gene family, PXR may also play a role in defining the response to unsaturated fatty acids. The finding that feeding conditional HRN mice a fat-deficient diet prevents the induction of CYP3A11 suggests that fatty acids, as well as bile acids [14], can influence PXR function.

A possible pathway linking P450 function to the findings observed in the present study is shown in Figure 7. As described in this model, dietary unsaturated fatty acids enter the liver via fatty acid transporters, where, in wild-type mice, they are oxidized for energy purposes or detoxified by P450s. Inactivation of the P450 system as a result of *Por* deletion leads to hepatic accumulation of unsaturated fatty acids initially via increase import due to the induction of lipid transporters such as CD36 [23] and a reduction of plasma lipid levels. Hepatic lipid accumulation triggers the activation of the nuclear receptor CAR (and possibly PXR), leading to P450 induction and liver growth through the activation of other downstream growth/transcription factors [26,27]. The overexpression of P450s is aimed at re-establishing hepatic lipid homoeostasis and the prevention of toxicity via increased metabolism of unsaturated fatty acids. Inactivation of POR prevents this increased metabolism and ultimately leads to hepatic steatosis, which establishes a cyclical process that promotes further hepatic lipid accumulation and growth. The ability to control the hepatic deletion of POR conditionally using the CYP1A1–Cre system has allowed us to provide evidence in support of

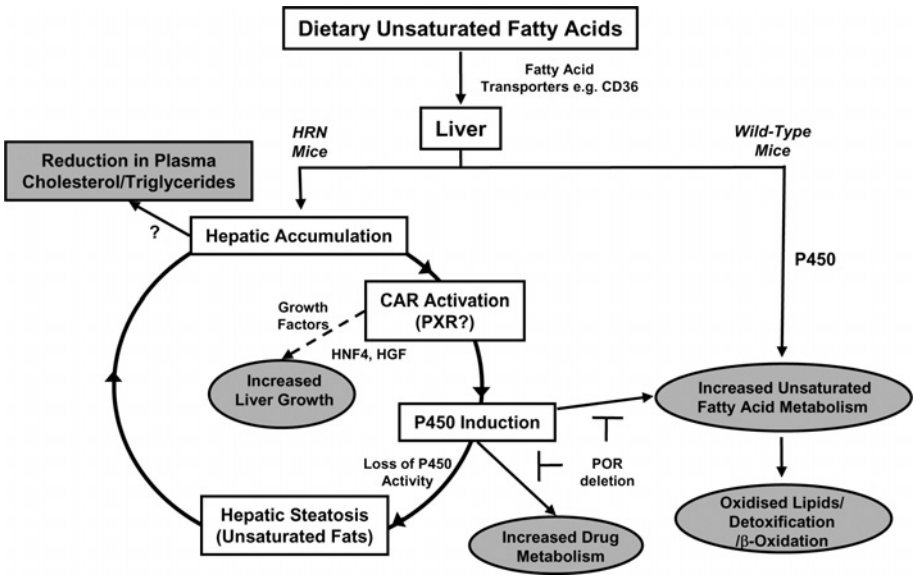

Figure 7 Schematic representation of the phenotypic changes that occur in HRN mouse models

Dietary unsaturated fatty acids enter the liver via fatty acid transporters where they are oxidized for energy purposes or detoxified by P450s. Inactivation of the P450 system as a result of *Por* deletion leads to hepatic accumulation of unsaturated fatty acids and a reduction of plasma lipid levels. Hepatic lipid accumulation triggers the activation of the nuclear receptor CAR (and possibly PXR), leading to P450 induction and liver growth. The overexpression of P450s is aimed at re-establishing hepatic lipid homoeostasis and the prevention of toxicity via increased metabolism of unsaturated fatty acids. Inactivation of POR prevents this increased metabolism and ultimately leads to hepatic steatosis. The development of steatosis establishes a cyclical process that potentiates hepatic lipid accumulation and growth. The adverse effect of P450 induction is altered drug metabolism and environmental chemical detoxification, but, in addition, the increased production of oxidized lipids could result in development of oxidative stress.

this pathway. Activation of Cre through the administration of 3MC resulted in the rapid deletion of POR, in that over 85 % of hepatocytes did not express the protein after 6 days. Although accumulation of lipid was already occurring at this time point, there appears to be a threshold at which point the CAR pathway becomes activated. This hypothesis is supported by the finding that it was only after 9–11 days that a marked change in triacylglycerol accumulation occurred which was linked directly to changes in plasma lipids, liver growth and P450 induction. Loss of P450 activity itself was not responsible for its auto-induction (although the corresponding accumulation of a metabolite produced, e.g. a bile acid precursor, which binds to CYP3A11 could explain the stabilization of this protein). Whether the inactivation of the P450 system causes the triacylglycerol accumulation and liver growth and whether their induction is to attenuate the phenotypic changes produced remains uncertain; however, it is quite clear that the accumulation of hepatic triacylglycerols activates CAR. The demonstration of cross-talk between drugs and lipids on CAR implies that there will be drug–lipid interactions associated with effects on this receptor and evidence for this is provided by the work of Li et al. [55], who showed that PUFAs could influence the induction of P450 genes by PB. Why the HRN phenotype results in the reduction in circulating triacylglycerols and cholesterol remains to be established. It is interesting to note, however, that the cholesterol 7α-hydroxylase enzyme (CYP7A1) involved in bile acid homoeostasis also appears to be affected by unsaturated fatty acid levels in a CAR-dependent manner and possibly a PXR-dependent manner, as suggested by the opposing effects on this protein's expression observed in *Car*$^{-/-}$ and *Pxr*$^{-/-}$/*Car*$^{-/-}$/HRN mice. The effect observed here on this enzyme's expression is intriguing as a previous report suggested that CAR activation represses CYP7A1 expression [56]. The contrasting results may be a consequence of the fact that fasted, as opposed to non-fasted, animals were used in the study of Miao et al. [56]. Despite this, these data suggest the existence of a new pathway for the dietary regulation of CYP7A1 by unsaturated fatty acids.

In summary, we have used a novel conditional HRN model to investigate the sequential development of the phenotypic changes observed in mice on deletion of POR. POR deletion and the associated inactivation of hepatic P450 function leads to accumulation of dietary fatty acids in the liver, in the form of triacylglycerols of unsaturated fatty acids, leading to organ enlargement and increased P450 expression, in the case of CYP2B, CYP2C and CYP7A1 via a CAR-dependent signalling pathway. The molecular mechanism underlying CYP3A11 expression appears to involve CAR, PXR and protein stabilization. Induction of the P450 system appears to be mediated by the accumulation of PUFAs, such as linoleic acid ($C_{18:2,n-6}$) and suggests that this may be part of an adaptive response to prevent the deleterious effects of PUFA accumulation. The finding that the major P450s that are inducible by exogenous chemicals are also regulated by changes in fatty acid homoeostasis has potentially significant implications for the variability observed in drug responses in humans.

ACKNOWLEDGEMENTS

We thank Dr Steven Kliewer for his gift of *Pxr*$^{-/-}$ and *Car*$^{-/-}$ mice and Dr Alexander Hill for carrying out the hepatic fatty acid analysis. Dianne Carrie and Catherine Hughes are thanked for excellent technical assistance with the animal work.

FUNDING

This work was funded by Cancer Research UK [grant number C463/A5661] (to C.R.W.).

REFERENCES

1 Ortiz de Montellano, P. (2005) Cytochrome P450: structure, mechanism and biochemistry, Kluwer Academic/Plenum Publishers, New York

2 Okey, A. B., Bondy, G. P., Mason, M. E., Kahl, G. F., Eisen, H. J., Guenthner, T. M. and Nebert, D. W. (1979) Regulatory gene product of the Ah locus: characterization of the cytosolic inducer–receptor complex and evidence for its nuclear translocation. J. Biol. Chem. **254**, 11636–11648

3 Kliewer, S. A., Moore, J. T., Wade, L., Staudinger, J. L., Watson, M. A., Jones, S. A., McKee, D. D., Oliver, B. B., Willson, T. M., Zetterstrom, R. H. et al. (1998) An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell **92**, 73–82

4 Lehmann, J. M., McKee, D. D., Watson, M. A., Willson, T. M., Moore, J. T. and Kliewer, S. A. (1998) The human orphan nuclear receptor PXR is activated by compounds that regulate *CYP3A4* gene expression and cause drug interactions. J. Clin. Invest. **102**, 1016–1023

5 Choi, H. S., Chung, M., Tzameli, I., Simha, D., Lee, Y. K., Seol, W. and Moore, D. D. (1997) Differential transactivation by two isoforms of the orphan nuclear hormone receptor CAR. J. Biol. Chem. **272**, 23565–23571

6 Jones, S. A., Moore, L. B., Shenk, J. L., Wisely, G. B., Hamilton, G. A., McKee, D. D., Tomkinson, N. C., LeCluyse, E. L., Lambert, M. H., Willson, T. M. et al. (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. Mol. Endocrinol. **14**, 27–39

7 Maglich, J. M., Stoltz, C. M., Goodwin, B., Hawkins-Brown, D., Moore, J. T. and Kliewer, S. A. (2002) Nuclear pregnane X receptor and constitutive androstane receptor regulate overlapping but distinct sets of genes involved in xenobiotic detoxification. Mol. Pharmacol. **62**, 638–646

8 Gu, J., Weng, Y., Zhang, Q. Y., Cui, H., Behr, M., Wu, L., Yang, W., Zhang, L. and Ding, X. (2003) Liver-specific deletion of the NADPH–cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. J. Biol. Chem. **278**, 25895–25901

9 Henderson, C. J., Otto, D. M., Carrie, D., Magnuson, M. A., McLaren, A. W., Rosewell, I. and Wolf, C. R. (2003) Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. J. Biol. Chem. **278**, 13480–13486

10 Wang, X. J., Chamberlain, M., Vassieva, O., Henderson, C. J. and Wolf, C. R. (2005) Relationship between hepatic phenotype and changes in gene expression in cytochrome P450 reductase (POR) null mice. Biochem. J. **388**, 857–867

11 Simmons, D. L., McQuiddy, P. and Kasper, C. B. (1987) Induction of the hepatic mixed-function oxidase system by synthetic glucocorticoids: transcriptional and post-transcriptional regulation. J. Biol. Chem. **262**, 326–332

12 Meehan, R. R., Forrester, L. M., Stevenson, K., Hastie, N. D., Buchmann, A., Kunz, H. W. and Wolf, C. R. (1988) Regulation of phenobarbital-inducible cytochrome P-450s in rat and mouse liver following dexamethasone administration and hypophysectomy. Biochem. J. **254**, 789–797

13 Finn, R. D., McLaren, A. W., Carrie, D., Henderson, C. J. and Wolf, C. R. (2007) Conditional deletion of cytochrome P450 oxidoreductase in the liver and gastrointestinal tract: a new model for studying the functions of the P450 system. J. Pharmacol. Exp. Ther. **322**, 40–47

14 Staudinger, J. L., Goodwin, B., Jones, S. A., Hawkins-Brown, D., MacKenzie, K. I., LaTour, A., Liu, Y., Klaassen, C. D., Brown, K. K., Reinhard, J. et al. (2001) The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc. Natl. Acad. Sci. U.S.A. **98**, 3369–3374

15 Wei, P., Zhang, J., Egan-Hafley, M., Liang, S. and Moore, D. D. (2000) The nuclear receptor CAR mediates specific xenobiotic induction of drug metabolism. Nature **407**, 920–923

16 Forrester, L. M., Henderson, C. J., Glancey, M. J., Back, D. J., Park, B. K., Ball, S. E., Kitteringham, N. R., McLaren, A. W., Miles, J. S., Skett, P. et al. (1992) Relative expression of cytochrome P450 isoenzymes in human liver and association with the metabolism of drugs and xenobiotics. Biochem. J. **281**, 359–368

17 Smith, G. C., Tew, D. G. and Wolf, C. R. (1994) Dissection of NADPH–cytochrome P450 oxidoreductase into distinct functional domains. Proc. Natl. Acad. Sci. U.S.A. **91**, 8710–8714

18 Yoshinari, K., Takagi, S., Yoshimasa, T., Sugatani, J. and Miwa, M. (2006) Hepatic CYP3A expression is attenuated in obese mice fed a high-fat diet. Pharm. Res. **23**, 1188–1200

19 Vignati, L. A., Bogni, A., Grossi, P. and Monshouwer, M. (2004) A human and mouse pregnane X receptor reporter gene assay in combination with cytotoxicity measurements as a tool to evaluate species-specific CYP3A induction. Toxicology **199**, 23–33

20 Li-Masters, T. and Morgan, E. T. (2001) Effects of bacterial lipopolysaccharide on phenobarbital-induced CYP2B expression in mice. Drug Metab. Dispos. **29**, 252–257

21 Wolf, C. R., Moll, E., Friedberg, T., Oesch, F., Buchmann, A., Kuhlmann, W. D. and Kunz, H. W. (1984) Characterization, localization and regulation of a novel phenobarbital-inducible form of cytochrome P450, compared with three further P450-isoenzymes, NADPH P450-reductase, glutathione transferases and microsomal epoxide hydrolase. Carcinogenesis **5**, 993–1001

22 Oinonen, T. and Lindros, K. O. (1998) Zonation of hepatic cytochrome P-450 expression and regulation. Biochem. J. **329**, 17–35

23 Weng, Y., Dirusso, C. C., Reilly, A. A., Black, P. N. and Ding, X. (2005) Hepatic gene expression changes in mouse models with liver-specific deletion or global suppression of the NADPH–cytochrome P450 reductase gene: mechanistic implications for the regulation of microsomal cytochrome P450 and the fatty liver phenotype. J. Biol. Chem. **280**, 31686–31698

24 Honkakoski, P. and Negishi, M. (2000) Regulation of cytochrome P450 (CYP) genes by nuclear receptors. Biochem. J. **347**, 321–337

25 Xie, W., Barwick, J. L., Simon, C. M., Pierce, A. M., Safe, S., Blumberg, B., Guzelian, P. S. and Evans, R. M. (2000) Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. Genes Dev. **14**, 3014–3023

26 Huang, W., Zhang, J., Washington, M., Liu, J., Parant, J. M., Lozano, G. and Moore, D. D. (2005) Xenobiotic stress induces hepatomegaly and liver tumors via the nuclear receptor constitutive androstane receptor. Mol. Endocrinol. **19**, 1646–1653

27 Zhou, J., Zhai, Y., Mu, Y., Gong, H., Uppal, H., Toma, D., Ren, S., Evans, R. M. and Xie, W. (2006) A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. J. Biol. Chem. **281**, 15013–15020

28 Honkakoski, P., Zelko, I., Sueyoshi, T. and Negishi, M. (1998) The nuclear orphan receptor CAR-retinoid X receptor heterodimer activates the phenobarbital-responsive enhancer module of the *CYP2B* gene. Mol. Cell. Biol. **18**, 5652–5658

29 Zhang, J., Huang, W., Qatanani, M., Evans, R. M. and Moore, D. D. (2004) The constitutive androstane receptor and pregnane X receptor function coordinately to prevent bile acid-induced hepatotoxicity. J. Biol. Chem. **279**, 49517–49522

30 Xie, W., Yeuh, M. F., Radominska-Pandya, A., Saini, S. P., Negishi, Y., Bottroff, B. S., Cabrera, G. Y., Tukey, R. H. and Evans, R. M. (2003) Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. Proc. Natl. Acad. Sci. U.S.A. **100**, 4150–4155

31 Pascussi, J. M., Gerbal-Chaloin, S., Duret, C., Daujat-Chavanieu, M., Vilarem, M. J. and Maurel, P. (2008) The tangle of nuclear receptors that controls xenobiotic metabolism and transport: crosstalk and consequences. Annu. Rev. Pharmacol. Toxicol. **10**, 1–32

32 Honkakoski, P., Jaaskelainen, I., Kortelahti, M. and Urtti, A. (2001) A novel drug-regulated gene expression system based on the nuclear receptor constitutive androstane receptor (CAR). Pharm. Res. **18**, 146–150

33 Wieneke, N., Hirsch-Ernst, K. I., Kuna, M., Kersten, S. and Puschel, G. P. (2007) PPARα-dependent induction of the energy homeostasis-regulating nuclear receptor NR1i3 (CAR) in rat hepatocytes: potential role in starvation adaptation. FEBS Lett. **581**, 5617–5626

34 Guo, D., Sarkar, J., Suino-Powell, K., Xu, Y., Matsumoto, K., Jia, Y., Yu, S., Khare, S., Haldar, K., Rao, M. S. et al. (2007) Induction of nuclear translocation of constitutive androstane receptor by peroxisome proliferator-activated receptor α synthetic ligands in mouse liver. J. Biol. Chem. **282**, 36766–36776

35 Kato, J., Ikemoto, A. and Mizutani, T. (2003) The effect of dietary fatty acids on the expression levels and activities of hepatic drug metabolizing enzymes. J. Health Sci. **49**, 105–114

36 Yoshinari, K., Takagi, S., Sugatani, J. and Miwa, M. (2006) Changes in the expression of cytochromes P450 and nuclear receptors in the liver of genetically diabetic *db*/*db* mice. Biol. Pharm. Bull. **29**, 1634–1638

37 Khemawoot, P., Yokogawa, K., Shimada, T. and Miyamoto, K. (2007) Obesity-induced increase of CYP2E1 activity and its effect on disposition kinetics of chlorzoxazone in Zucker rats. Biochem. Pharmacol. **73**, 155–162

38 Xiong, H., Yoshinari, K., Brouwer, K. L. and Negishi, M. (2002) Role of constitutive androstane receptor in the *in vivo* induction of Mrp3 and CYP2B1/2 by phenobarbital. Drug Metab. Dispos. **30**, 918–923

39 Posti, K., Leinonen, S., Tetri, S., Kottari, S., Viitala, P., Pelkonen, O. and Raunio, H. (1999) Modulation of murine phenobarbital-inducible CYP2A5, CYP2B10 and CYP1A enzymes by inhibitors of protein kinases and phosphatases. Eur. J. Biochem. **264**, 19–26

40 Oesch-Bartlomowicz, B. and Oesch, F. (2003) Cytochrome-P450 phosphorylation as a functional switch. Arch. Biochem. Biophys. **409**, 228–234

41 Zangar, R. C., Kimzey, A. L., Okita, J. R., Wunschel, D. S., Edwards, R. J., Kim, H. and Okita, R. T. (2002) Cytochrome P450 3A conjugation to ubiquitin in a process distinct from classical ubiquitination pathway. Mol. Pharmacol. **61**, 892–904

42 Honkakoski, P. and Negishi, M. (1998) Regulatory DNA elements of phenobarbital-responsive cytochrome P450 CYP2B genes. J. Biochem. Mol. Toxicol. **12**, 3–9

43 Keeney, D. S., Skinner, C., Wei, S., Friedberg, T. and Waterman, M. R. (1998) A keratinocyte-specific epoxygenase, CYP2B12, metabolizes arachidonic acid with unusual selectivity, producing a single major epoxyeicosatrienoic acid. J. Biol. Chem. **273**, 9279–9284

44 Ruettinger, R. T. and Fulco, A. J. (1981) Epoxidation of unsaturated fatty acids by a soluble cytochrome P-450-dependent system from *Bacillus megaterium*. J. Biol. Chem. **256**, 5728–5734

45 Narhi, L. O. and Fulco, A. J. (1982) Phenobarbital induction of a soluble cytochrome P-450-dependent fatty acid monooxygenase in *Bacillus megaterium*. J. Biol. Chem. **257**, 2147–2150
46 Palmer, C. N., Axen, E., Hughes, V. and Wolf, C. R. (1998) The repressor protein, Bm3R1, mediates an adaptive response to toxic fatty acids in *Bacillus megaterium*. J. Biol. Chem. **273**, 18109–18116
47 Li, Y., Nara, T. Y. and Nakamura, M. T. (2005) Peroxisome proliferator-activated receptor α is required for feedback regulation of highly unsaturated fatty acid synthesis. J. Lipid Res. **46**, 2432–2440
48 Hayhurst, G. P., Lee, Y. H., Lambert, G., Ward, J. M. and Gonzalez, F. J. (2001) Hepatocyte nuclear factor 4α (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. Mol. Cell. Biol. **21**, 1393–1403
49 Laffitte, B. A., Chao, L. C., Li, J., Walczak, R., Hummasti, S., Joseph, S. B., Castrillo, A., Wilpitz, D. C., Mangelsdorf, D. J., Collins, J. L. et al. (2003) Activation of liver X receptor improves glucose tolerance through coordinate regulation of glucose metabolism in liver and adipose tissue. Proc. Natl. Acad. Sci. U.S.A. **100**, 5419–5424
50 McCarthy, T. C., Pollak, P. T., Hanniman, E. A. and Sinal, C. J. (2004) Disruption of hepatic lipid homeostasis in mice after amiodarone treatment is associated with peroxisome proliferator-activated receptor-α target gene activation. J. Pharmacol. Exp. Ther. **311**, 864–873
51 Yang, Q., Ito, S. and Gonzalez, F. J. (2007) Hepatocyte-restricted constitutive activation of PPARα induces hepatoproliferation but not hepatocarcinogenesis. Carcinogenesis **28**, 1171–1177
52 Rencurel, F., Stenhouse, A., Hawley, S. A., Friedberg, T., Hardie, D. G., Sutherland, C. and Wolf, C. R. (2005) AMP-activated protein kinase mediates phenobarbital induction of CYP2B gene expression in hepatocytes and a newly derived human hepatoma cell line. J. Biol. Chem. **280**, 4367–4373
53 Kodama, S., Koike, C., Negishi, M. and Yamamoto, Y. (2004) Nuclear receptors CAR and PXR cross talk with FOXO1 to regulate genes that encode drug-metabolizing and gluconeogenic enzymes. Mol. Cell. Biol. **24**, 7931–7940
54 Maglich, J. M., Watson, J., McMillen, P. J., Goodwin, B., Willson, T. M. and Moore, J. T. (2004) The nuclear receptor CAR is a regulator of thyroid hormone metabolism during caloric restriction. J. Biol. Chem. **279**, 19832–19838
55 Li, C. C., Lii, C. K., Liu, K. L., Yang, J. J. and Chen, H. W. (2006) $n-6$ and $n-3$ polyunsaturated fatty acids down-regulate cytochrome P-450 2B1 gene expression induced by phenobarbital in primary rat hepatocytes. J. Nutr. Biochem. **17**, 707–715
56 Miao, J., Fang, S., Bae, Y. and Kemper, J. K. (2006) Functional inhibitory cross-talk between constitutive androstane receptor and hepatic nuclear factor-4 in hepatic lipid/glucose metabolism is mediated by competition for binding to the DR1 motif and to the common coactivators, GRIP-1 and PGC-1α. J. Biol. Chem. **281**, 14537–14546

Received 11 April 2008/1 September 2008; accepted 9 September 2008
Published as BJ Immediate Publication 9 September 2008, doi:10.1042/BJ20080740

Biochem. J. (2009) **417**, 43–54 (Printed in Great Britain) doi:10.1042/BJ20080740

online data

SUPPLEMENTARY ONLINE DATA

Unsaturated fatty acid regulation of cytochrome P450 expression via a CAR-dependent pathway

Robert D. FINN, Colin J. HENDERSON, Claire L. SCOTT and C. Roland WOLF[1]

Cancer Research UK Molecular Pharmacology Unit, Biomedical Research Centre, Level 5, Ninewells Hospital and Medical School, Dundee DD1 9SY, U.K.

EXPERIMENTAL

Lipid isolation and analysis

For detailed fatty acid analysis, livers from 12-week-old HRN male mice were collected and lipid-extracted using a modified version of the Folch method [1]. Briefly, internal standard triheptadecanoin was added to a weighed amount of wet mouse liver and homogenized in chloroform/methanol (1:1, v/v). An equal volume of saline was added, before being centrifuged, and the organic layer was removed and dried under vacuum. The lipid residue was transesterified with 5 % sulfuric acid in methanol, and the methyl fatty acids were extracted into heptane. Separation was achieved by injection into an SGE BPX-70 capillary column in a Unicam series 610 gas chromatograph with temperature programming. Data were collected with a Phillips PU6030 data capture unit and processed using Unicam 4880 data handling software. Fatty acid quantification was by internal standardization with sample concentrations calculated by comparing the sample fatty acid/internal standard ratio against the fatty acid/internal standard ratio of known amounts of a commercial fatty acid standard within a known linear range of standard ratios. The same amount of internal standard was added to both standard and sample.

Table S1 Fatty acids added to a fat-deficient base diet

Values are expressed as the amount of fatty acid (in g) in 100 g of food (w/w). The control diet is included for comparison purposes. A fourth supplemented diet was used consisting of a fat-deficient base diet plus 1 % sunflower oil. None of the diets contained cholesterol and were all isocaloric. MUFA, mono-unsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid. NA, not applicable.

Fatty acid	Control diet	SFA diet	MUFA diet	PUFA diet
Lauric acid ($C_{12:0}$)	0.02	0.02	NA	NA
Myristic acid ($C_{14:0}$)	0.14	0.14	NA	NA
Palmitic acid ($C_{16:0}$)	0.31	0.31	NA	NA
Stearic acid ($C_{18:0}$)	0.04	0.04	NA	NA
Myristoleic acid ($C_{14:1}$)	0.02	NA	0.02	NA
Palmitoleic acid ($C_{16:1}$)	0.09	NA	0.09	NA
Oleic acid ($C_{18:1}$)	0.77	NA	0.77	NA
Linoleic acid ($C_{18:2,n-6}$)	0.69	NA	NA	0.69
Linolenic acid ($C_{18:3,n-3}$)	0.06	NA	NA	0.06
Arachidonic acid ($C_{20:4,n-6}$)	0.13	NA	NA	0.13
Total fatty acids	2.27	0.51	0.88	0.88

Table S2 Fatty acid analysis of the diets used

Values are expressed as a percentage of total fatty acid content on a fresh weight basis. MUFA, mono-unsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Fatty acid group	Control diet	SFA diet	MUFA diet	PUFA diet	Sunflower oil diet
Saturated fatty acids	19.3	96.5–97.9	1.9–3.4	29.2–30.2	12.4–13.3
Mono-unsaturated fatty acids	18.3	1.7–2.1	95.1–95.5	11.8	24.8–25.1
Polyunsaturated fatty acids	62.2	1.0–2.0	2.1–3.2	16.0–16.3	61.9–62.9
Other unknown fatty acids	0.2	0.9	1.29	41.7–43.0	0.9

Table S3 Fatty acid analysis of the diets used

Values are expressed on a w/w basis in the diet. MUFA, mono-unsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

Fatty acid group	Control diet	SFA diet	MUFA diet	PUFA diet	Sunflower oil diet
Lauric acid ($C_{12:0}$)	< 0.003	0.016	0.001	0.004	0.001
Myristic acid ($C_{14:0}$)	0.006	0.099	0.002	0.012	0.003
Myristoleic acid ($C_{14:1,n-5}$)	< 0.003	< 0.001	0.012	0.003	< 0.001
Pentadecanoic acid ($C_{15:0}$)	0.003	0.001	0.001	0.1	0.001
Palmitic acid ($C_{16:0}$)	0.48	0.32	0.009	0.05	0.067
Palmitoleic acid ($C_{16:1,n-7}$)	0.01	< 0.001	0.07	0.003	0.001
Stearic acid ($C_{18:0}$)	0.05	0.05	0.001	0.017	0.039
Oleic acid ($C_{18:1,n-9}$)	0.47	0.007	0.74	0.039	0.237
Vaccenic acid ($C_{18:1,n-7}$)	0.03	< 0.001	0.01	0.002	0.006
Linoleic acid ($C_{18:2,n-6}$)	1.63	0.002	0.017	0.106	0.616
Linolenic acid ($C_{18:3,n-3}$)	0.17	< 0.001	< 0.001	0.002	0.001
Eicosanoic acid ($C_{20:0}$)	0.01	< 0.001	0.001	0.001	0.002
Gondoic acid ($C_{20:1,n-9}$)	0.02	< 0.001	< 0.001	0.004	0.002
Eicosadienoic acid ($C_{20:2,n-6}$)	0.003	< 0.001	< 0.001	0.001	< 0.001
Dihomo-γ-linolenic acid ($C_{20:3,n-6}$)	< 0.003	< 0.001	< 0.001	< 0.001	< 0.001
Eicosatrienoic acid ($C_{20:3,n-3}$)	< 0.003	0.001	0.001	0.008	0.001
Arachidonic acid ($C_{20:4,n-6}$)	< 0.003	< 0.001	< 0.001	0.001	< 0.001
Eicosatetraenoic acid ($C_{20:4,n-3}$)	< 0.003	< 0.001	< 0.001	0.001	< 0.001
Timnodonic acid ($C_{20:5,n-3}$)	< 0.003	< 0.001	< 0.001	0.002	0.001
Behenic acid ($C_{22:0}$)	0.01	< 0.001	< 0.001	0.01	0.007
Cetoleic acid ($C_{22:1,n-11}$)	0.003	< 0.001	< 0.001	0.023	0.002
Erucic acid ($C_{22:1,n-9}$)	0.003	< 0.001	< 0.001	0.024	< 0.001
Adrenic acid ($C_{22:4,n-6}$)	0.003	0.001	0.001	0.004	0.001
Osbond acid ($C_{22:5,n-6}$)	0.003	< 0.001	< 0.001	0.002	< 0.001
Clupanodonic acid ($C_{22:5,n-3}$)	< 0.003	< 0.001	< 0.001	0.003	< 0.001
Docosahexaenoic acid ($C_{22:6,n-3}$)	< 0.003	< 0.001	< 0.001	0.012	< 0.001
Lignoceric acid ($C_{24:0}$)	0.01	< 0.001	< 0.001	0.005	0.002
Nervonic acid ($C_{24:1,n-9}$)	0.003	< 0.001	< 0.001	0.007	< 0.001
Unknown C_{4-10} acids	< 0.003	0.002	0.003	0.11	0.001
Unknown C_{10-15} acids	< 0.003	0.002	0.002	0.041	0.001
Unknown C_{15-20} acids	0.003	0.001	0.006	0.072	0.004
Unknown C_{20-25} acids	0.01	< 0.001	0.001	0.157	0.003

[1] To whom correspondence should be addressed (email c.r.wolf@dundee.ac.uk).

Table S4 Fatty acid analysis of total hepatic lipids of control ($Por^{lox/lox}$) and HRN mice

Results are means ± S.E.M. ($n = 3$ livers). Fold change is expressed as the ratio of HRN mice to control mice.

	Fatty acid concentration (μM)			
Fatty acid	Control	HRN	*P*	Fold change
Myristic acid ($C_{14:0}$)	1.3 ± 0.2	3.5 ± 1.4	0.202	2.7
Palmitic acid ($C_{16:0}$)	24.5 ± 1.0	86.7 ± 2.9	< 0.001	3.5
Palmitoleic acid ($C_{16:1}$)	2.5 ± 0.01	17.1 ± 3.8	0.019	6.8
Stearic acid ($C_{18:0}$)	10.3 ± 0.2	25.2 ± 2.6	0.005	2.5
Oleic acid ($C_{18:1}$)	15.2 ± 0.5	213.3 ± 18.1	< 0.001	14.1
Linoleic acid ($C_{18:2}$)	12.8 ± 0.5	38.8 ± 10.1	0.063	3.0
Eicosatrienoic acid ($C_{20:3}$)	1.2 ± 0.1	7.5 ± 0.4	< 0.001	6.2
Arachidonic acid ($C_{20:4}$)	10.1 ± 0.6	18.3 ± 1.1	0.003	1.8
Docosahexaenoic acid ($C_{22:6}$)	4.9 ± 0.2	11.1 ± 0.8	0.002	2.3

Table S5 Fatty acid analysis of total hepatic lipids of control ($Por^{lox/lox}$) and HRN mice

Results are means ± S.E.M. ($n = 3$ livers). Fold change is expressed as the ratio of HRN mice to control mice.

	Fatty acid concentration (μM)			
Fatty acid group	Control	HRN	*P*	Fold change
Total	82.7 ± 1.1	421.4 ± 32.4	< 0.001	5.1
$C_{16:0}/C_{18:0}$ ratio	2.4 ± 0.1	3.5 ± 0.3	0.024	1.5
Saturated	36.1 ± 1.1	115.4 ± 5.7	< 0.001	3.2
Mono-unsaturated	17.7 ± 0.5	230.4 ± 20.8	< 0.001	13.0
Polyunsaturated	28.9 ± 0.6	75.7 ± 11.9	0.018	2.6
$n-3$	4.9 ± 0.2	11.1 ± 0.8	0.002	2.3
$n-6$	24.1 ± 0.5	64.6 ± 11.2	0.022	2.7
Triacylglycerol (mM)	0.03 ± 0.0004	0.14 ± 0.01	< 0.001	5.1

Table S6 The induction of other phenotypes in HRN mice on nuclear receptor-null backgrounds

$Pxr^{-/-}$/HRN, $Car^{-/-}$/HRN and $Pxr^{-/-}/Car^{-/-}$/HRN mice were generated as outlined in the Experimental section of the main text and were analysed at 12 weeks of age for non-fasting plasma lipid concentrations and liver/body weight ratio as detailed in the Experimental section of the main text. Results are means ± S.E.M. (n = 3). *$P \leqslant 0.05$; **$P \leqslant 0.01$; ***$P \leqslant 0.001$ compared with $Por^{lox/lox}$ (control) mice. †$P \leqslant 0.05$ compared with HRN mice.

Genotype	Plasma cholesterol	Plasma triacylglycerols	Liver/body weight ratio
Control ($Por^{lox/lox}$)	3.73 ± 0.24	1.09 ± 0.27	0.051 ± 0.002
HRN	1.15 ± 0.10***	0.29 ± 0.09*	0.075 ± 0.004**
$Pxr^{-/-}$	2.31 ± 0.16*	0.41 ± 0.13	0.062 ± 0.001*
$Car^{-/-}$	2.90 ± 0.05	0.70 ± 0.16	0.045 ± 0.002
$Pxr^{-/-}/Car^{-/-}$	2.60 ± 0.49	0.95 ± 0.01	0.051 ± 0.002
$Pxr^{-/-}$/HRN	1.03 ± 0.32**	0.14 ± 0.01*	0.091 ± 0.002***
$Car^{-/-}$/HRN	1.35 ± 0.15**	0.28 ± 0.04*	0.073 ± 0.007*
$Pxr^{-/-}/Car^{-/-}$/HRN	2.63 ± 0.28†	0.61 ± 0.12	0.073 ± 0.004**

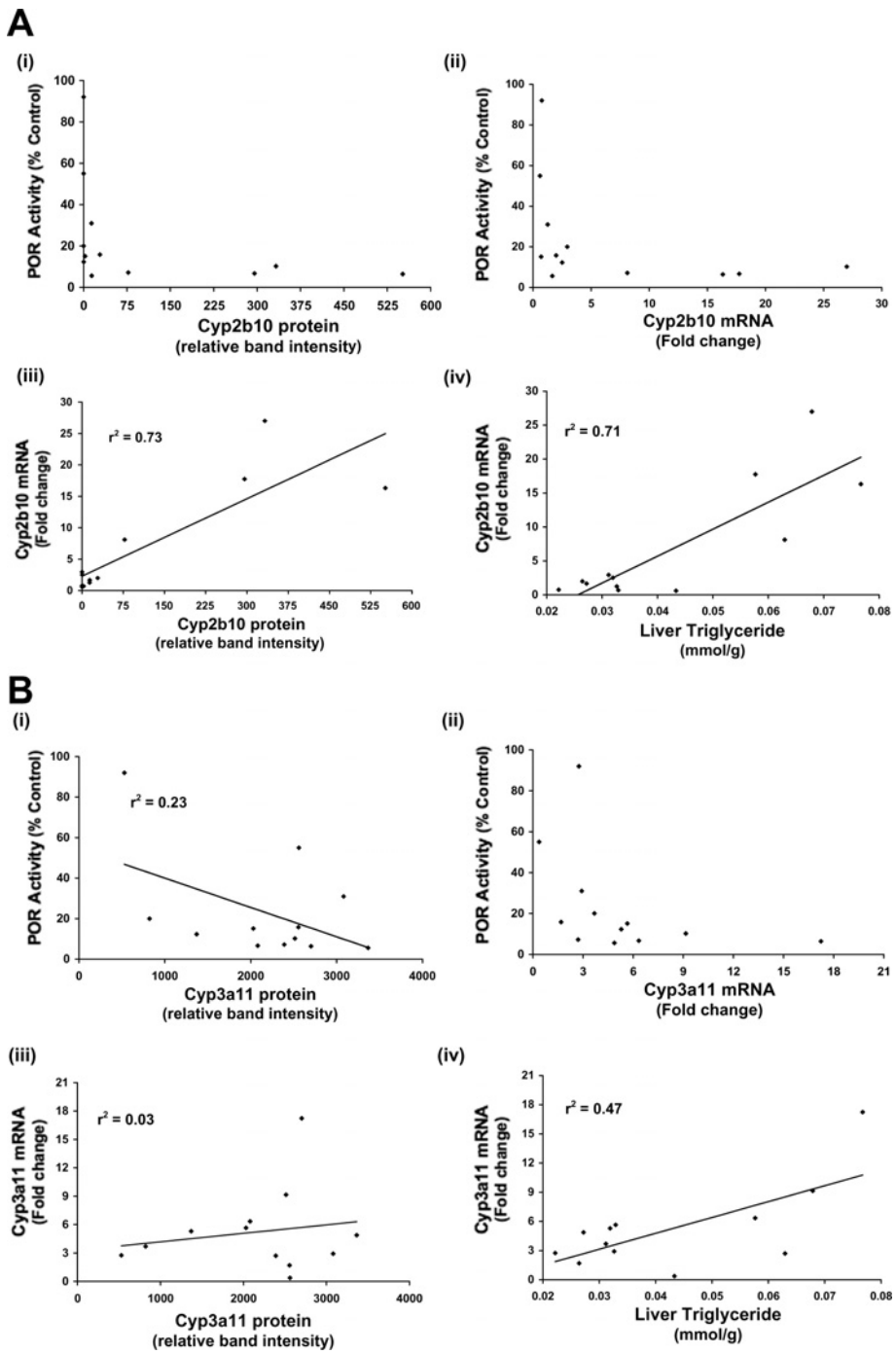

Figure S1 Relationship of CYP2B10 and CYP3A11 expression with loss of POR activity and hepatic lipid accumulation

Por$^{lox/lox}$/*Cre*^{CYP1A1} and *Por*$^{lox/lox}$ mice were treated with a single dose of 3MC (40 mg/kg of body weight), and harvested over a 28 day period. Hepatic triacylglycerol levels, mRNA and protein levels of CYP2B10 and CYP3A11, and POR activity were determined as detailed in the Experimental section of the main text. (**A**) Relationship between POR activity and (i) CYP2B10 protein, (ii) *Cyp2b10* mRNA content. Correlation of *Cyp2b10* mRNA with (iii) CYP2B10 protein and (iv) hepatic triacylglycerol (triglyceride) concentration. (**B**) Relationship between POR activity and (i) CYP3A11 protein and (ii) *Cyp3a11* mRNA content. Correlation of *Cyp3a11* mRNA with (iii) CYP3A11 protein and (iv) hepatic triacylglycerol concentration. mRNA expression and POR activity are expressed compared with 3MC-treated *Por*$^{lox/lox}$ mice. Results are means for three animals per experimental time point.

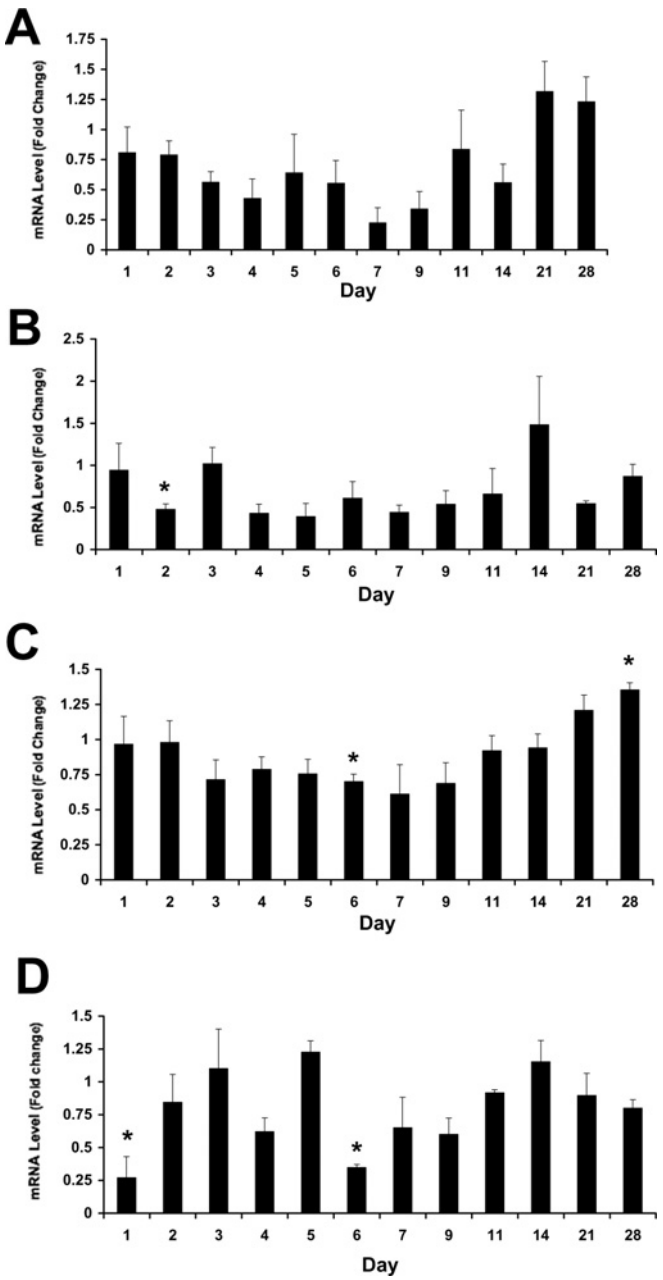

Figure S2 Gene expression changes on loss of POR

$Por^{lox/lox}/Cre^{CYP1A1}$ and $Por^{lox/lox}$ mice treated with a single dose of 3MC (40 mg/kg of body weight) were analysed for changes in the expression levels of various genes involved in controlling hepatic lipid levels. mRNA expression of the fatty-acid transporter CD36 (**A**), FAS (**B**), CPT1a (**C**) and CAR (**D**) as a function of time post 3MC administration. Results are means $\pm$ S.E.M. ($n = 3$) and are expressed as a fold change compared with $Por^{lox/lox}$ mice at the same time point. $^{*}P \leqslant 0.05$.

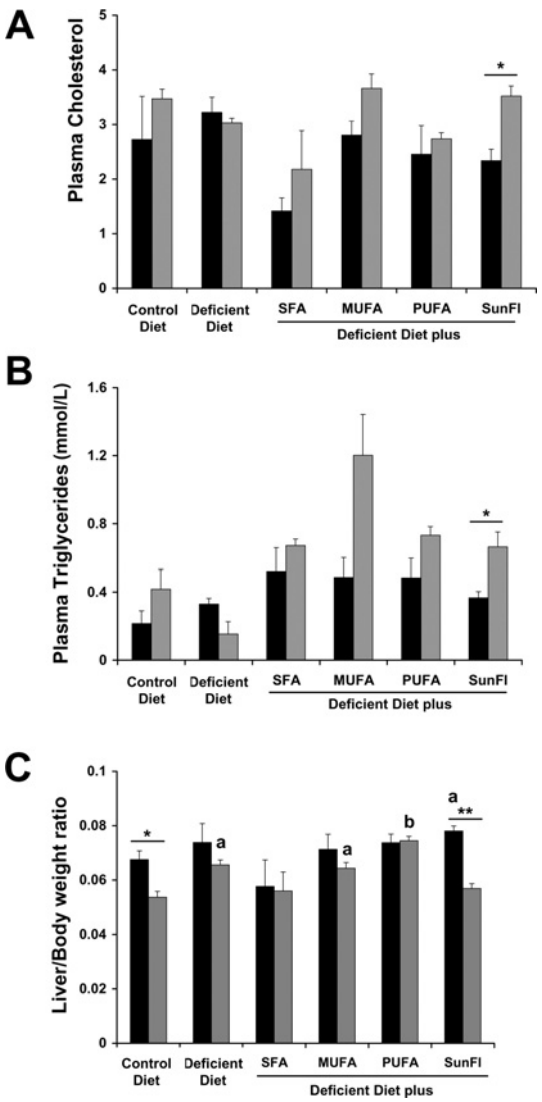

Figure S3 Development of phenotypic changes in conditional HRN mice on restricted fatty acid diets

$Por^{lox/lox}/Cre^{CYP1A1}$ and $Por^{lox/lox}$ mice were maintained on a control, fat-deficient or fatty-acid-supplemented diet until 12 weeks of age as outlined in the Experimental section of the main text, then treated with a single dose of 3MC (40 mg/kg of body weight) and killed 14 days later. Plasma lipid concentrations and liver/body weight ratios were determined as detailed in the Experimental section of the main text. (**A**) Non-fasting total plasma cholesterol. (**B**) Non-fasting plasma triacylglycerol (triglyceride) levels. (**C**) Liver/body weight ratio. Black bars: $Por^{lox/lox}/Cre^{CYP1A1}$. Grey bars: $Por^{lox/lox}$. Results are means $\pm$ S.E.M. ($n = 3$). $^{*}P \leqslant 0.05$; $^{**}P \leqslant 0.01$ compared with the same experimental conditions for $Por^{lox/lox}$ mice. $^{a}P \leqslant 0.05$; $^{b}P \leqslant 0.01$ compared with mice of the same genotype on a control diet. SFA, saturated fatty acids; MUFA, mono-unsaturated fatty acids; PUFA, polyunsaturated fatty acids; SunFl, 1 % sunflower oil.

REFERENCE

1 Folch, J., Lees, M. and Sloane Stanley, G. H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**, 497–509

Received 11 April 2008/1 September 2008; accepted 9 September 2008
Published as BJ Immediate Publication 9 September 2008, doi:10.1042/BJ20080740