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Robert D Finn, Colin J Henderson, Claire L Scott, C Roland Wolf. Unsaturated fatty acid regulation of cytochrome P450 expression via a CAR-dependent pathway. *Biochemical Journal*, 2008, 417 (1), pp.43-54. 10.1042/BJ20080740 . hal-00479009

HAL Id: hal-00479009

<https://hal.science/hal-00479009>

Submitted on 30 Apr 2010

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Unsaturated Fatty Acid Regulation of Cytochrome P450 Expression via a CAR-Dependent pathway.

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Short title: CAR-Dependent Fatty Acid Regulation of P450s.

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Key words: Constitutive androstane receptor, Cytochrome P450, Linoleic acid, P450 Oxidoreductase, Pregnane X receptor, Steatosis.

Abbreviations used: AhR – aryl hydrocarbon receptor; CAR – constitutive androstane receptor; HRN – hepatic reductase null; 3MC – 3-methylcholanthrene; MUFA – monounsaturated fatty acids; PCR – polymerase chain reaction; POR – P450 oxidoreductase; PUFA – polyunsaturated fatty acids; PXR – pregnane X receptor; SFA – saturated fatty acids.

ABSTRACT

The liver is responsible for key metabolic functions, including control of normal homeostasis in response to diet and xenobiotic metabolism/detoxification. We have previously shown that inactivation of the hepatic cytochrome P450 system through conditional deletion of P450 oxidoreductase (POR) 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 triglyceride homeostasis 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 and, to a lesser degree, PXR. To our knowledge this is the first direct evidence that P450s play a major role in controlling unsaturated fatty acid homeostasis 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.

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 manifested 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 understanding this adaptive response through the identification of transcription factors, such as the Aryl-hydrocarbon receptor (AhR), the pregnane X receptor (PXR) and the constitutive androstane receptor (CAR) 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, due 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, *et.al.* [8], have recently reported a mouse model where hepatic P450 activity has been reduced by > 95% by the conditional deletion of the P450 oxidoreductase (POR) gene. 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 hepatic reductase null (HRN) 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 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 homeostasis and liver growth and that this metabolic homeostasis 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 POR null 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 Ah receptor and administration of a single dose of 3-methylcholanthrene at 40 mg/kg results in a specific, time-dependent, hepatic deletion of POR and a phenotype identical to that observed in HRN mice [13].

In this study, we have used the new model to study the temporal biochemical changes that control lipid accumulation and P450 over-expression. We provide evidence that dietary unsaturated fatty acids play a key role in both lipid accumulation and cytochrome P450 induction. Also, using nuclear receptor/HRN double and triple knockout mice we show that P450 over-expression occurs predominantly through the activation of CAR.

EXPERIMENTAL

Reagents

All chemicals were purchased from Sigma-Aldrich (Dorset, UK), 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 previously described [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 onto 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 previously described [14, 15].

All mice were maintained under standard animal house conditions with a 12hr light/12hr dark cycle and free access to water and RM1 (control) diet (Special Diets Services, Essex, UK). 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

3-Methylcholanthrene (3MC) in corn oil was administered to *Por*^{lox/lox}/*Cre*^{CYP1A1} and *Por*^{lox/lox} mice by intraperitoneal (i.p.) injection at 40 mg/kg body weight, and animals harvested 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 old before being transferred to either a fat-deficient diet (SDS, 821459) or a fatty acid supplemented fat-deficient diet (Table S1), 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 are isocaloric and free of cholesterol. At 12 weeks of age mice were administered either a single i.p. dose of 3MC (40 mg/kg body weight) or corn oil, and harvested 14 days later.

For linoleic acid treatment, *Por*^{lox/lox}/*Cre*^{CYP1A1} 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 body weight), followed by an oral dose of linoleic acid of 65 mg/day from day 5 after 3MC administration until being harvested 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 (Bio-Rad, Herts., UK). Western blot analysis was carried out as described previously [16] using 5µg 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, His₆-Cyp3a11, His₆-Cyp7a1 - were used as standards. Immunoreactive proteins were detected using donkey anti-rabbit horseradish peroxidase IgG as a secondary antibody (DAKO, Ely, UK) and visualised by chemiluminescence (ECL+, GE Healthcare, Bucks, UK). 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 previously described [17].

RNA isolation and Real-Time Quantitative PCR analysis

Total RNA was isolated from snap-frozen liver samples using Trizol (Invitrogen, Paisley, UK). Genomic DNA was removed using RQ1 DNase (Promega, Southampton, UK), and RNA (600 ng) reverse transcribed using random hexamers and Superscript II RNase H reverse transcriptase polymerase (Invitrogen, Paisley UK). Real-Time Quantitative PCR analysis was carried out with gene-specific primers for Cyp2b10, Cyp3a11 and CAR as previously described [10] and CD36 (Mm01135198_m1), fatty acid synthase (Mm01253292_m1) and carnitine-palmitoyltransferase 1a (Mm00550438_m1) supplied by Applied Biosystems (Cheshire, UK). 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 as an internal standard.

Blood Chemistry

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

Histopathology

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

Lipid Isolation and Analysis

A small piece of liver was homogenised in phosphate-buffered saline containing 0.1% Triton X-100 and hepatic triglyceride and cholesterol levels determined using assay kits (Thermo Trace, Eastleigh, UK) on a Cobas Fara II centrifugal analyser (Roche, Herts., UK) [18].

Detailed fatty acid analysis of the various diets was carried out by Special Diet Services, Essex, UK (Tables S2 and S3).

Plasmid Construction

The plasmids pCMX Gal4-hPXR-LBD and p4*4xGal4-UAS-luciferase were a generous 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 the following: 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 738bp PCR product was cloned as an *EcoRI/BamHI* fragment into pCMX Gal4-hPXR-LBD replacing the hPXR-LBD 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, Paisley, UK. HepG2 cells were cultured in DMEM containing 10% heat-inactivated foetal bovine serum, 1X non essential amino acids, 100U/ml penicillin, 100µg/ml streptomycin and 2mM L-glutamine at 37°C and 5% CO₂.

HepG2 cells were transiently transfected with pCMX Gal4-mCAR-LBD/p4*4xGal4-UAS-luciferase according to the method of Vignati *et.al.* [19], with the following modifications – Eugene6 (Roche, Herts., UK) was used instead of Lipofectamine Plus (Invitrogen, Paisley, UK), assays were carried out in serum-containing media and 0.2µg of the

plasmid pRL-TK was included to allow normalisation of luciferase reporter gene activity. Cells were treated with 0.25-0.5 μ M 1,4-bis[2-3,5-dichloropyridyloxy]-benzene (TCOPBOP) and 1mM phenobarbital (PB) as positive controls and DMSO as a further control. Linoleic acid, complexed to fatty-acid free bovine serum albumin at a molar ratio of 2:1, was tested at concentrations ranging from 0-12.5 μ M, with the addition of 20 μ M α -tocopheryl succinate to prevent lipid peroxidation. Luciferase reporter activity was determined using the Dual-Luciferase Reporter Assay System (Promega, Southampton, UK).

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RESULTS

Temporal analysis of the phenotypic changes observed in the Hepatic POR null 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 to 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.

Six 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 B(i)), increasing to approximately 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 (data not shown). Treatment with 3MC resulted in the anticipated induction of Cyp1a1 as a consequence of the activation of the Ah receptor. By day 7 – 9 Cyp1a1 levels, presumably due to the metabolism and elimination of the 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, similar to Cyp1a1, however return of POR is dependent on cell turnover, which in the case of hepatocytes is several weeks. In *Por^{lox/lox}/Cre^{CYP1A1}* mice, no return of POR is observed 60 days post 3MC treatment (data 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 day 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 POR null mice, after which time they remained slightly higher in POR nulls (data 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 the induction of Cyp1a1 (data not shown).

Associated with the time-dependent reduction in POR were changes in liver size and non-fasting plasma total cholesterol and triglycerides levels (Figures 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 triglyceride 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 triglycerides 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 localised 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 localisation 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 triglycerides 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 triglyceride content were closely correlated with changes in both liver/body weight ratio and the reduction in plasma triglycerides (Figures 2C and D). This suggests that the level of hepatic triglycerides determines the circulating triglyceride levels and that they are related to liver enlargement.

Changes in hepatic lipid homeostasis 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 both at the protein and mRNA levels [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 (Figure S1A(i) and (ii)) 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 (Figure S1A(iii)). In addition, Cyp2b10 mRNA levels were correlated with hepatic triglyceride concentration (Figure S1A(iv)). Similar analysis for Cyp3a11 indicated that the increased protein expression occurred more rapidly than Cyp2b10, steadily increasing from day two, 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 nulls this did not correlate with the level of Cyp3a11 protein, indicating a more complex mechanism of regulation of this gene (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 triglyceride levels was observed (Figure S1B(iv)). Thus differences observed in the induction kinetics of Cyp2b10 and Cyp3a11, both at the protein and mRNA levels, suggests that the mechanisms regulating their over-expression 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 the lipid transporter CD36, fatty-acid synthase (FAS) and carnitine-palmitoyltransferase 1a (Cpt1a) were determined in 3MC-treated *Por^{lox/lox}/Cre^{CYP1A1}* mice compared to *Por^{lox/lox}* mice (Figure S2). No significant changes in the expression levels of these genes were observed, except for Cpt1a on day 28 which showed a minor 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 earlier than 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 hepatic POR null mice

In view of the finding that changes in hepatic fatty acid homeostasis correlated with the increases in P450 expression, detailed fatty acid analysis was carried out on livers from HRN and *Por^{lox/lox}* (control) mice (Tables S4 and S5). Changes in all fatty acid classes occurred; however, the most significant changes were in monounsaturated and polyunsaturated fatty acids (Table S5), with C16:1, C18:1 and C20:3 increasing by 6.78, 14.06 and 6.19-fold respectively (Table S4). These data are in close agreement with the report of Weng, *et.al.*, [23] who, for their hepatic POR null model, reported additional fold changes of 7.3 and 14.9 for C18:3 and C20:1 unsaturated fatty acids respectively. Together these findings suggest that

the accumulated hepatic triglycerides 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 prior to 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 (data 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 triglyceride levels were measured on deletion of POR in *Por^{lox/lox}/Cre^{CYP1A1}* mice fed a fat-deficient diet (Figure S3). These data indicate that hepatic triglyceride accumulation in POR null mice originates from the diet and that as a consequence of the inactivation of the P450 system an inability to maintain hepatic lipid homeostasis 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 Table S1. *Por^{lox/lox}/Cre^{CYP1A1}* and *Por^{lox/lox}* were maintained on the supplemented diets and analysed 14 days following 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 approximately 88% unsaturated fatty acids) (Figure 3B, Table S2). The 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 (data 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 (Figures 3B(ii) and (iii)). Analysis of plasma lipid levels revealed some minor decreases in both total cholesterol and triglyceride 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-fold ($p = 0.013$) decrease was observed, respectively (Figures S3A and B). Hepatic lipid accumulation was observed for all diets, except 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 (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 (Tables S2 and S3), identified C18:2, linoleic acid, an n-6 PUFA, 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 mRNA levels for Cyp2b10 and Cyp3a11 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 to *Por^{lox/lox}/Cre^{CYP1A1}* mice maintained on a control diet and appeared concentrated around the blood vessels, a pattern similar to that observed at early time points following 3MC administration (Figure 1C), however in this case 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 hepatic POR null 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 triglyceride homeostasis [27]. In order to investigate whether these nuclear receptors mediate the P450 over-expression in the HRN mice, we crossed the HRN animals with mice null at either the *Car* or *Pxr* locus alone or in combination, to generate *Car^{-/-}/HRN*, *Pxr^{-/-}/HRN* and *Pxr^{-/-}/Car^{-/-}/HRN* genotypes.

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^{-/-}* null mice exhibited slight increases in Cyp3a11 protein [14], and also Cyp2b and Cyp2c proteins (Figure 5A(i)); Cyp3a11 mRNA was also elevated relative to 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 hepatic POR null animals, however a reduction in both Cyp2b10 and Cyp3a11 mRNA levels was observed, but this was not significant compared to HRN mice (Figure 5A(ii)). No changes in plasma and hepatic lipid levels were observed in *Pxr^{-/-}/HRN* mice compared to HRN mice (Table S6 and Figure 5D), however, in *Pxr^{-/-}* mice non-fasting plasma total cholesterol and triglyceride levels were reduced, the former significantly, in comparison to wild-type mice (Table S6).

In relation to HRN mice null for CAR, profound changes in the HRN phenotype were observed. The elevated expression of P450s Cyp2b, Cyp7a and Cyp2c was markedly attenuated, however, Cyp3a11 expression appeared 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 triglyceride levels observed in the HRN mice were however not reversed on the CAR null background (Table S6), however a significant reduction in hepatic lipid content for *Car^{-/-}/HRN* mice compared to 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, due 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 nulls, the expression of Cyp2b and Cyp2c proteins was profoundly reduced, whereas in contrast Cyp7a expression levels were only slightly affected (Figure 5C). In mice null 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 level (Figure 5C(ii)). A slight decrease in

Cyp3a11 mRNA was observed in *Pxr*^{-/-}/*Car*^{-/-}/HRN mice, although this was not significantly different from 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 triglycerides was also observed, this was not significantly different to the HRN value (Table S6). Hepatic lipid content was reduced in *Pxr*^{-/-}/*Car*^{-/-}/HRN mice compared to HRN mice, similar to levels observed in *Car*^{-/-}/HRN mice but still elevated compared to controls (Figure 5D); however this reduction did result in a change in liver size (Table S6).

Direct activation of the nuclear receptor CAR by linoleic acid in Hepatic POR null mice

In order to establish whether PUFAs could activate CAR directly we carried out transactivation assays using a Gal4-CAR-LBD reporter assay in HepG2 cells [32]. In these experiments, both TCPOBOP and linoleic acid could activate CAR directly (Figure 6). Phenobarbital, 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, peroxisome proliferator-activated receptor α (PPAR α) can effect CAR expression [33, 34] and that the effects in the POR null mice may be indirect, we measured CAR mRNA expression in *Por*^{lox/lox}/*Cre*^{CYP11A1} mice. Expression levels of CAR were found to be unchanged (Figure S2D).

DISCUSSION

The liver is the major organ controlling metabolic homeostasis 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 this 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 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 phenobarbital [38].

The metabolic dialogue between the pathways of detoxification and metabolic homeostasis are manifested in the findings that when the P450 system is inactivated profound changes in lipid homeostasis 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 triglycerides. 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 triglyceride accumulation, where as 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 are also reflected in the finding that the over-expression of these proteins is at least in part mediated by different transcription factors (see below). We have also demonstrated that hepatic steatosis *per se* 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.

Cytochromes P450 of the CYP2B family are the major enzymes induced in the liver by barbiturate drugs such as phenobarbital [42] and have the capacity to metabolise fatty acids such as arachidonic acid [43]. Interestingly, Fulco and co-workers demonstrated over 20 years ago that a fatty acid monooxygenase involved in fatty acid oxidation in *Bacillus megaterium* is highly inducible by phenobarbital [44, 45]. In fact studies on this pathway demonstrated that its induction provided 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 presented here does 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 homeostasis and liver growth, including PPAR α , CAR, PXR, liver X receptor (LXR) and hepatocyte nuclear factor 4 α (HNF4 α) [26, 27, 47-49]. For example, it has been demonstrated that PPAR α mediates liver growth induced by perturbations in fatty acid homeostasis as a consequence of peroxisome proliferation and that PXR plays a role in controlling hepatic triglyceride homeostasis [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 homeostasis 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 AMP kinase [52], interaction with the forkhead transcription factor FoxO1 [53] and activation during fasting [54]. It has also been postulated that CAR function can be modulated by the fatty acid sensitive transcription factor PPAR α [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 homeostasis and endogenous P450 expression has not been previously reported. 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 POR nulls 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 here is shown in Figure 7. As described in this model dietary unsaturated fatty acids enter the liver via fatty acids transporters, where in wild type mice they are oxidised 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 over-expression of P450s is aimed at re-establishing hepatic lipid homeostasis 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 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 triglyceride accumulation occurred which was directly linked to changes in plasma lipids, liver growth and P450 induction. Loss of P450 activity *per se* 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 stabilisation of this protein). Whether the inactivation of the P450 system causes the triglyceride 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 triglycerides activates CAR. The demonstration of cross talk between drugs and lipids on CAR implies 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.* who showed that polyunsaturated fatty acids could influence the induction of P450 genes by phenobarbital [55]. Why the HRN phenotype results in the reduction in circulating triglycerides and cholesterol remains to be established. It is interesting to note, however, that the cholesterol 7 α -hydroxylase enzyme (*Cyp7a1*) involved in bile acid homeostasis 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 Miao's study. 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 hepatic POR null 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 triglycerides 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 stabilisation. Induction of the P450 system appears to be mediated by the accumulation of PUFAs, such as linoleic acid (C18:2n-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 inducible by exogenous chemicals are also regulated by changes in fatty acid homeostasis has potentially significant implications for the variability observed in drug responses in man.

Acknowledgements: The authors would like to thank Dr. Steven Kliewer for his gift of *Pxr* and *Car* null 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. This work was funded by Cancer Research UK programme grant to C.R.W.

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FIGURE LEGENDS

Figure 1. Time-course of the phenotypic changes in conditional hepatic P450 oxidoreductase null mice.

Por^{lox/lox}/*Cre*^{CYP1A1} and *Por*^{lox/lox} mice were treated with a single dose of 3MC (40 mg/kg) following which 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 to body weight ratios, ■ = *Por*^{lox/lox}/*Cre*^{CYP1A1} and ■ = *Por*^{lox/lox}, (iii) Non-fasting plasma total cholesterol and (iv) Non-fasting plasma triglyceride levels. Values represent mean ± S.E.M. of three animals per time point and in the case of POR activity and plasma lipid levels are expressed as a % compared to *Por*^{lox/lox} mice at the same time point. $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$, compared to *Por*^{lox/lox} mice. (C) Hepatic lipid content was visualised by Oil red O staining of snap-frozen sections. Photomicrographs were taken at 10x magnification, bright field, and are representative of the results from at least three mice per group.

Figure 2. Hepatic lipid accumulation and its relationship with organ weight and plasma lipid levels in conditional hepatic P450 oxidoreductase null mice.

Por^{lox/lox}/*Cre*^{CYP1A1} and *Por*^{lox/lox} mice were treated with a single dose of 3MC (40 mg/kg), and harvested on the indicated days. (A) Hepatic triglyceride and (B) cholesterol concentrations, and the correlation of hepatic triglyceride concentration with (C) liver/body weight ratio and (D) non-fasting plasma triglyceride levels. Values represent the mean ± S.E.M. of three animals per time point. $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$, compared to lipid level on day one.

Figure 3. Development of phenotypic changes in conditional hepatic P450 oxidoreductase null mice on restricted fatty acid diets.

Por^{lox/lox}/*Cre*^{CYP1A1} mice were maintained on a control diet until 8 weeks old, and then either placed on a fat-deficient or fatty acid supplemented diet, or continued on the control diet, for the following 6 weeks. At 12 weeks old, they were treated with a single dose of 3MC (40 mg/kg) and harvested 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 and hepatic mRNA levels of (ii) Cyp2b10 and (iii) Cyp3a11 expressed as a fold change compared to *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 and hepatic mRNA levels of (ii) Cyp2b10 and (iii) Cyp3a11 expressed as a fold change compared to *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 10x magnification, bright field, and are representative of the results from at least three mice per group. For mRNA analysis, all values represent the mean ± S.E.M., $n = 3$. $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$, compared to *Por*^{lox/lox} mice. ■ = *Por*^{lox/lox}/*Cre*^{CYP1A1} and ■ = *Por*^{lox/lox}. SFA = plus saturated fatty acids, MUFA = plus monounsaturated fatty acids, PUFA = plus polyunsaturated fatty acids and SunFl = plus 1% sunflower oil.

Figure 4. Induction of hepatic P450 expression in conditional hepatic P450 oxidoreductase null mice by linoleic acid.

Por^{lox/lox}/*Cre*^{CYP1A1} mice were maintained on a control diet until 8 weeks old, and then placed on a fat-deficient diet for the following 6 weeks. At 12 weeks old, they were treated with a

single dose of 3MC (40 mg/kg) and 5 days later administered linoleic acid (65 mg/day) for 9 days before being harvested. (A) POR and P450 expression levels were determined by Western blotting. (B) Hepatic mRNA levels of Cyp2b10 and Cyp3a11 expressed as a fold change compared to *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 10x magnification, bright field, and are representative of the results from at least three mice per group. For RNA analysis, all values represent the mean \pm S.E.M. of $n = 3$. $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$, compared to *Por*^{lox/lox}/*Cre*^{CYP1A1} mice maintained on a fat-deficient diet. ■ = Cyp2b10 and ■ = Cyp3a11.

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 mRNA levels of Cyp2b10 and Cyp3a11 expressed as a fold change compared to *Por*^{lox/lox} mice. (B) POR and P450 expression levels in *Car*^{-/-}/HRN mice; (i) protein expression determined by Western blotting and (ii) hepatic mRNA levels of Cyp2b10 and Cyp3a11 expressed as a fold change compared to *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 mRNA levels of Cyp2b10 and Cyp3a11 expressed as a fold change compared to *Por*^{lox/lox} mice. (D) Hepatic lipid content determined by Oil red O staining of snap-frozen liver sections. Photomicrographs were taken at 10x magnification, bright field, and are representative of the results from at least three mice per group. For mRNA analysis, all values represent the mean \pm S.E.M. of $n = 3$. $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$, compared to *Por*^{lox/lox} mice. ■ = Cyp2b10 and ■ = Cyp3a11.

Figure 6. Activation of the nuclear receptor CAR by linoleic acid.

HepG2 cells were transfected with pCMX Gal4-mCAR-LBD and p4*4xGal4-UAS4-luciferase as outlined in the Experimental procedures. Transfected cells were subsequently treated with linoleic acid at a concentration ranging from 0 – 12.5 μ M and luciferase activity analysed 24hr later. Transfected cells were treated with 0.25 – 0.5 μ M TCPOBOP or 1mM PB as positive and negative controls. All values represent the mean \pm S.E.M. of $n = 6$ determinations and are expressed as a fold change compared cells treated with either DMSO in the case of TCPOBOP and PB or the 0 μ M sample in the case of linoleic acid. $p \leq 0.05 = *$, $p \leq 0.01 = **$, $p \leq 0.001 = ***$.

Figure 7. A schematic representation of the phenotypic changes that occur in hepatic P450 oxidoreductase null mouse models.

Dietary unsaturated fatty acids enter the liver via fatty acids transporters where they are oxidised 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 over-expression of P450s is aimed at re-establishing hepatic lipid homeostasis 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 oxidised lipids could result in development of oxidative stress.

Figure 1

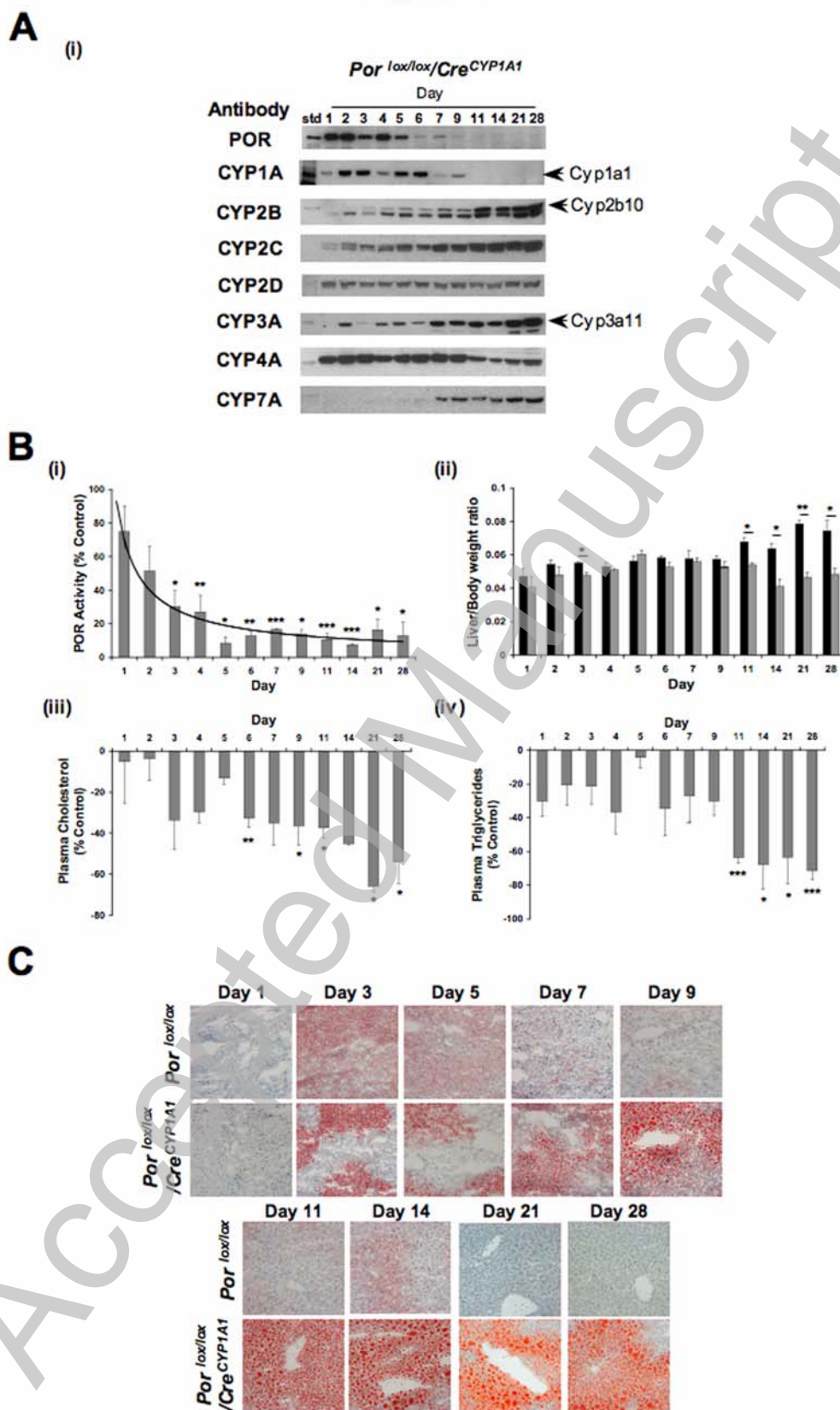


Figure 2

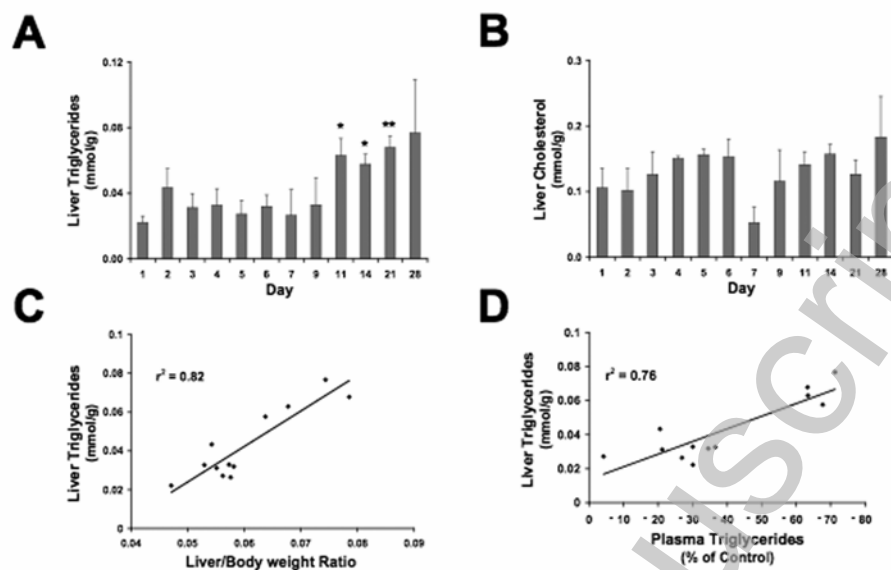


Figure 3

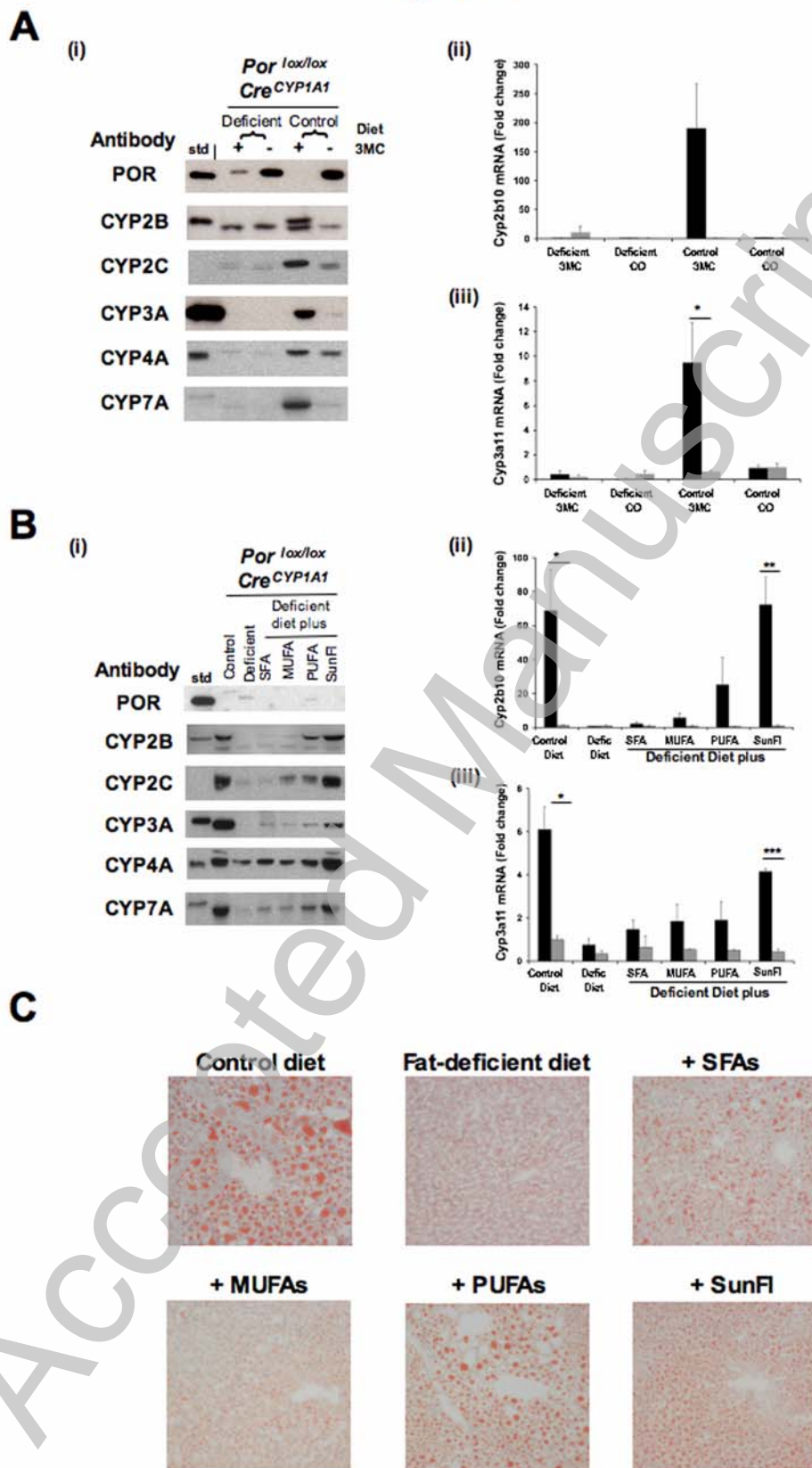


Figure 4

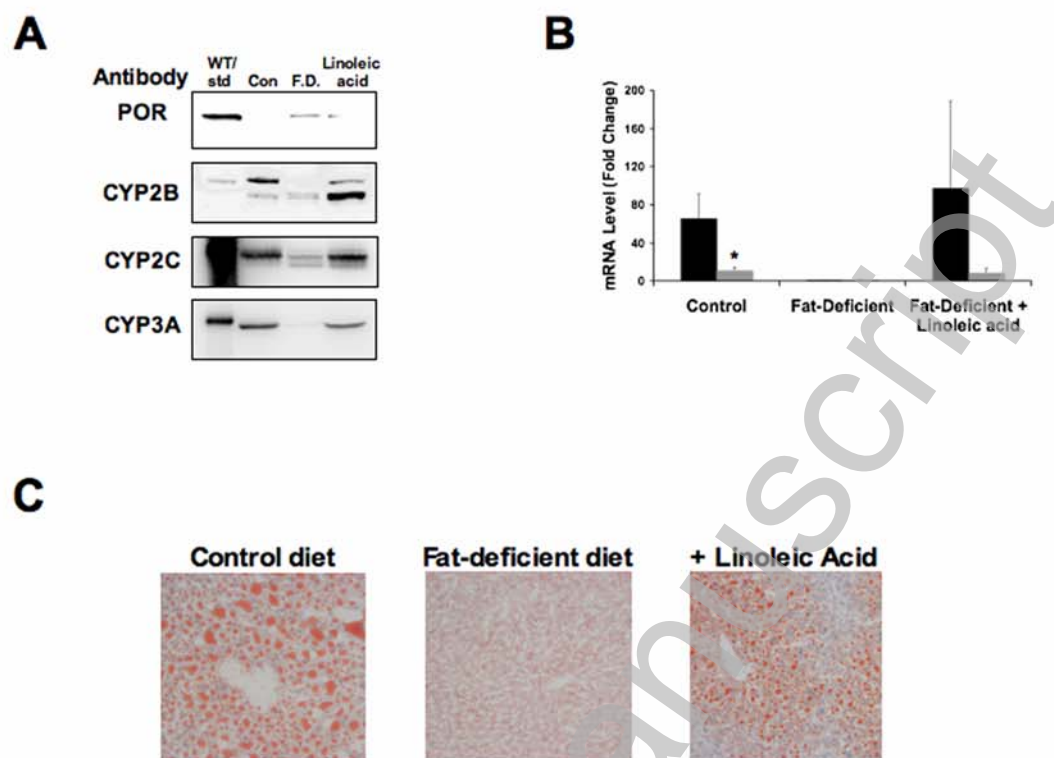


Figure 5

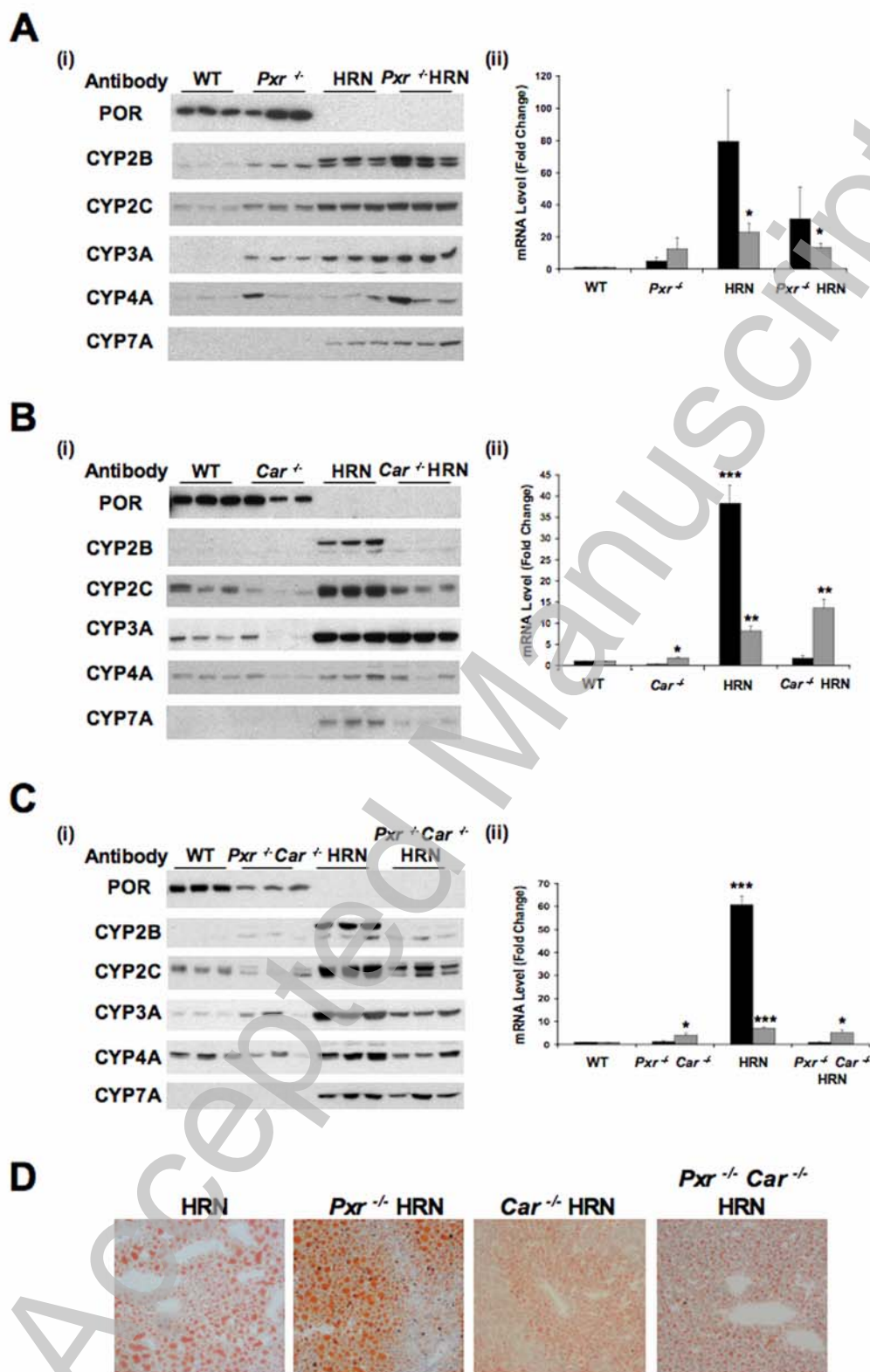


Figure 6

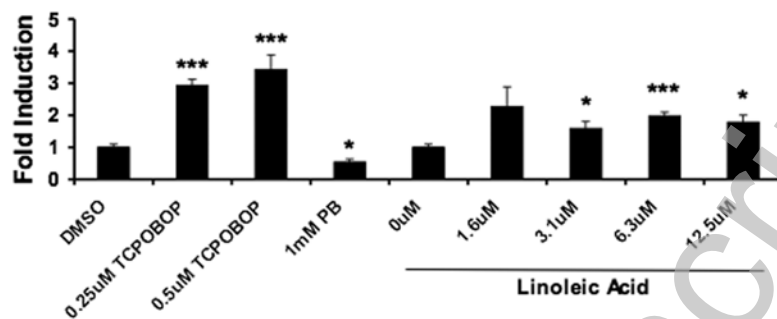


Figure 7