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Relationship between hepatic phenotype and changes in gene expression in cytochrome P450 reductase (POR) null mice

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Cytochrome P450 reductase is the unique electron donor for microsomal cytochrome P450s; these enzymes play a major role in the metabolism of endogenous and xenobiotic compounds. In mice with a liver-specific deletion of cytochrome P450 reductase, hepatic cytochrome P450 activity is ablated, with consequent changes in bile acid and lipid homoeostasis. In order to gain insights into the metabolic changes resulting from this phenotype, we have analysed changes in hepatic mRNA expression using microarray analysis and real-time PCR. In parallel with the perturbations in bile acid levels, changes in the expression of key enzymes involved in cholesterol and lipid homoeostasis were observed in hepatic cytochrome P450 reductase null mice. This was characterized by a reduced expression of *Cyp7b1*, and elevation of *Cyp7a1* and *Cyp8b1* expression. The levels of mRNAs for other cytochrome P450 genes, including *Cyp2b10*, *Cyp2c29*, *Cyp3a11* and *Cyp3a16*, were increased, demonstrating that endogenous factors play a role in regulating the expression of these proteins and that the increases are due, at least in part, to altered levels of transcripts. In addition, levels of mRNAs encoding genes involved in glycolysis and lipid transport were also increased; the latter may provide an explanation for the increased hepatic lipid content observed in the hepatic null mice. Serum testosterone and oestradiol levels were lowered, accompanied by significantly decreased expression of Hsd3b2 (3β-hydroxy-Δ^5-steroid dehydrogenase-2), Hsd3b5 (3β-hydroxy-Δ^5-steroid dehydrogenase-5) and Hsd11b1 (11β-hydroxysteroid dehydrogenase type 1), key enzymes in steroid hormone metabolism. These microarray data provide important insights into the control of metabolic pathways by the cytochrome system.

Key words: bile acid, cholesterol and lipid metabolism, cytochrome P450, cytochrome P450 reductase, microarray analysis.

INTRODUCTION

The cytochrome P450s constitute a superfamily of haem-containing mono-oxygenases that play a central role in the detoxification of xenobiotics, as well as in the metabolism of endogenous compounds such as steroids, fatty acids, prostaglandins and leukotrienes. In addition, cytochrome P450s also play key roles in physiological processes, such as steroidogenesis and the maintenance of bile acid and cholesterol homoeostasis [1,2]. Cholesterol homoeostasis is maintained through the co-ordinated regulation of dietary cholesterol absorption, *de novo* biosynthesis and disposal in the form of bile acids [3–5]. Bile acid biosynthesis represents the major pathway of cholesterol elimination from the body. In addition, bile acids can also directly regulate cellular signalling pathways that control cholesterol homoeostasis [6,7].

POR (NADH–cytochrome P450 reductase; NADPH:ferrihaemoprotein reductase; EC 1.6.2.4) is a 78 275 Da membrane-bound flavoprotein which transfers electrons from NADPH to cytochrome P450s located in the endoplasmic reticulum [8,9]. POR also donates electrons to haem oxygenase, resulting in the cleavage of haem to produce biliverdin, iron and carbon monoxide. This system plays a critical role in cellular haem homoeostasis [10] and the production of carbon monoxide, which may also function as a neurotransmitter [11]. In addition, POR is the electron donor for squalene mono-oxygenase, a key enzyme in sterol biosynthesis [12], and is also reported to donate electrons to 7-dehydrocholesterol reductase in this pathway [13]. POR can also transfer electrons to cytochrome b_5, which is involved in sterol and fatty acid desaturation and elongation [14,15].

We have recently described a mouse model in which hepatic POR has been deleted [16]. Gu et al. [17] have also subsequently reported a very similar mouse model. This conditional knockout of POR resulted a profound decrease in all hepatic microsomal P450 functions. In spite of this, these HRN™ (hepatic P450 reductase null) (CXR Biosciences) mice developed normally and were able to breed. As a consequence of the lower P450 levels, drug metabolism was compromised, bile acid production was markedly reduced, and hepatic lipid levels were significantly increased. Somewhat surprisingly, in parallel with these effects, circulating cholesterol and triacylglycerol levels were significantly reduced, indicating a feedback control of cholesterol uptake. In addition to these changes, a profound increase in the hepatic expression of cytochrome P450s involved in drug metabolism was observed [16].

In order to establish the regulatory pathways influenced by the hepatic P450 system, we have carried out a global analysis of hepatic mRNA expression using oligonucleotide microarray

Abbreviations used: Apo, apolipoprotein; CAR, constitutive androstane receptor; Cyp7a1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDL, high-density lipoprotein; HIF1, hypoxia-inducible factor 1; HNF4, hepatocyte nuclear factor 4; HRN, hepatic P450 reductase null; Hsd11b1, 11β-hydroxysteroid dehydrogenase type 1; Hsd3b5, 3β-hydroxy-Δ^5-steroid dehydrogenase-5; Hsd3b2, 3β-hydroxy-Δ^5-steroid dehydrogenase-2; LRH-1, liver receptor homologue-1; LPL, lipoprotein lipase; LXR, liver X receptor; Mt2, metallothionein II; MRP, multidrug-resistance-associated protein; POR, cytochrome P450 reductase; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; SCD, stearoyl-CoA desaturase; SHP-1, small heterodimer partner; SREBP, sterol regulatory element binding protein; VLACSR, very-long-chain acyl-CoA synthetase-related.

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The microarray data have been submitted to, and approved by, the GEO repository under the following accession numbers: GSE1468, GSM24728, GSM24729, GSM24730 and GSM24747.

analysis. This analysis showed that the expression of more than 268 genes was changed significantly (either > 1.5-fold increase or > 33 % decrease). These gene products participate in a variety of processes, including detoxification, bile acid and cholesterol biosynthesis, membrane transport, lipogenesis, glycolysis, lipid metabolism, and the oxidative stress response, demonstrating the central role of POR and the P450 system in controlling hepatic homoeostasis.

MATERIALS AND METHODS

Animals

HRN ($POR^{lox/lox} + Cre^{ALB}$) mice used in this study were derived as described previously [16]. Mice homozygous for loxP sites at the POR locus ($POR^{lox/lox}$) were used as controls. All mice used in the present study were male and 3 months old. Animals were allowed food and water *ad libitum*, and were kept on a standard RM1 mouse diet and a 12 h light/12 h dark cycle (light from 05:30 h to 17:30 h). Mice were always killed at the same time of day, and all procedures were carried out under the Animal (Scientific Procedures) Act (1986) in accordance with U.K. law, and following local ethical review.

Microarray analysis

Mice were killed between 10:00 and 11:00 h by a rising concentration of CO_2, and livers were removed immediately for preparation of RNA. Total RNA was isolated with a phenol/guanidine isothiocyanate reagent, TRIzol (Invitrogen), and purified further with an RNeasy Mini Kit (Qiagen Ltd) in accordance with the manufacturer's instructions. Duplicate experiments were carried out on RNA pooled from three animals of each genotype. Results from two independent experiments were exactly the same. The A_{260}/A_{280} ratio of total RNA used was typically $\geqslant 1.9$. The quality of RNA was assessed using an Agilent 2100 Bioanalyzer.

Probe labelling and hybridization procedures were conducted according to the Affymetrix Technical Manual (Affymetrix, Santa Clara, CA, U.S.A.). cDNA was synthesized from total RNA by using a Superscript ds-cDNA Synthesis Kit (Invitrogen Ltd, Paisley, U.K.) with a T7-$(dT)_{24}$ primer incorporating a T7 RNA polymerase promoter. The cRNA was prepared and biotin-labelled using *in vitro* transcription by using a BioArray High Yield RNA Transcript Labelling Kit (ENZO Biochemical). Labelled cRNA was fragmented by incubation at 94 °C for 35 min in the presence of 40 mM Tris/acetate buffer, pH 8.1, 100 mM potassium acetate and 30 mM magnesium acetate. The samples were tested by hybridization to GeneChip Test3 arrays and analysed using an Agilent Bioanalyzer. Fragmented cRNA (15 μg) was hybridized for 16 h at 45 °C to an MG_U74Av2 array (Affymetrix). After hybridization, the gene chips were washed and stained with streptavidin/phycoerythrin by using a fluidics station (Affymetrix). The chips were scanned in an Agilent G2500A scanner. Affymetrix oligonucleotide microarrays utilize multiple perfect-match and mismatch oligonucleotides to determine expression levels, so Affymetrix GCOS software was used to scan each probe set, determine the presence and the average difference value, and assess the signal intensity. Chip fluorescence was normalized by scaling total chip fluorescence intensity to a common value of 100 prior to comparison, and a normalization value was set at 1. The Affymetrix software may define a particular gene as 'absent'; however, there is still a numerical value, albeit perhaps very low, attached to the signal for the mRNA, allowing the fold change to be calculated.

Data and statistical analyses were performed with Genespring v6.1 bioinformatics algorithms (Silicon Genetics, Redwood City, CA, U.S.A.). Data sets were obtained for each mouse line from two separate pools of three animals, with each pool hybridized to a separate gene chip. Given only two data sets, an in-depth statistical analysis would not be appropriate; instead, for each gene, the ratio of the HNR value to the control value is indicated, together with a range (S.E.M.).

Real-time quantitative PCR

The same mRNA pools were used for both microarray analysis and real-time PCR. Contaminating genomic DNA was removed from 600 ng of total RNA by incubating with 1 unit of DNase (Promega UK, Chilworth, Southampton, U.K.) at 37 °C for 10 min. In each sample, single-stranded cDNA was synthesized using 100 units of Superscript II reverse transcriptase (Invitrogen) and 0.15 μg of random hexamers (Promega UK) in a 20 μl solution of 50 mM Tris/HCl, pH 8.3, 75 mM KCl and 3 mM $MgCl_2$ containing 10 mM dithiothreitol and 1 mM dNTPs. The reaction mixture was left to equilibrate at 25 °C for 10 min before synthesis was allowed to proceed at 42 °C for 50 min. Finally, the reaction was terminated by incubation at 70 °C for 10 min, and the cDNA-containing reaction mixtures were diluted to 200 μl and stored at − 20 °C until required. Matching oligonucleotide primers and fluorescent probes used for real-time PCR were designed using the software Primer Express™ (PerkinElmer Applied Biosystems), and are listed in Table 1. The primers were synthesized at the Cancer Research UK Oligonucleotide Synthesis Laboratory (Clare Hall, South Mimms, Herts., U.K.). The probes, which were labelled with a 5′ fluorescent reporter dye (6-carboxyfluorescein) and a 3′ quenching dye (6-carboxytetramethylrhodamine), were synthesized by Qiagen Ltd. (Germany). Each PCR mixture (12.5 μl) contained 2.5 μl of cDNA, 200 nM forward and reverse oligonucleotide primers and 100 nM probe in 1× (final concentration) TaqMan® Master Mix (PerkinElmer). Amplification of cDNA was performed over 41 cycles in a Prism Model 7700 Sequence Detector instrument. The first cycle was performed at 50 °C for 2 min, followed by 95 °C for 10 min. Cycles 2–41 were performed at 95 °C for 15 s, followed by 60 °C for 1 min. Reactions were monitored by measuring fluorescence at 518 nm with excitation at 494 nm. Each assay was performed in triplicate. The specificity of PCR amplifications from the various sets of oligonucleotide primers was examined routinely by agarose-gel electrophoresis. The results were analysed by using 7700 system software. The level of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as an internal standard.

Serum hormone analysis

Testosterone and oestradiol analysis was carried out at the Department of Biochemical Medicine, Ninewells Hospital, Dundee, U.K. Statistical analysis of data was performed using an unpaired *t* test in Statview 4.5 (Abacus).

RESULTS

Gene expression profile in livers from HRN mice

Hepatic mRNA levels in control and HRN mice exhibited a number of marked differences. These included genes involved in pathways of lipid/sterol metabolism (Table 2) and the stress response (Table 3), as well as cytochrome P450s (Figure 1), transporters (Table 2), secreted proteins and transcription factors (Tables 2–4 and Supplementary Tables 1–3; see http://www.BiochemJ.org/bj/388/bj3880857add.htm). Of 12488 gene sequences analysed, 143 mRNAs were found to be increased by

Table 1 Primers and probes used for real-time PCR

Primers and Taqman probes were designed using software Primer Express™ using sequence data from the NCBI database. FAM and TAMRA are reporter fluorophores.

Gene	Primer 1 (5′ → 3′)	Primer 2 (5′ → 3′)	Probe (5′-FAM/3′-TAMRA)
Ak4	gctgggtttagaccaggtcaat	tttcagtagatgtcagctttcatatgg	tgcctcaattctaggcgtcagg
AQP4	atgaaccaagatctggtcaagtttt	gacaatgccagcttcctcaca	ccgtgcagagcatatggacacct
CAR	gaggcccaggcttgcat	taccctttccaggccagtgt	ctttccccagacccccagg
Cyp2c29	ggtatctttcatcctggcttacaga	tgcatttgtactataactccagaaactg	ccctcttctctcaagacccagcaaacaca
Cyp3a11	cactggttctcatgggatcatct	tgttcaaattacaaaaacgtggttact	catccatatctcgttttctctgctaattccttttgag
Cyp7a1	caacctgccagtactagatagcatca	gtccggatattcaaggatgca	caaggaggctctgcggctctcca
Cyp27a1	cgtcctggttcccagcaa	gcccagctcggtactactgtct	tgcagaaaatgcagcctcacct
GAPDH	gtcgtggatctgacgtgcc	tgcctgcttcaccaccttct	cctggagaaacctgccaagtatgatgaca
Hal	tcatggccccggacatt	caacctcccaaaccttttgg	aggcagcacacaggttgcttctt
Hsd3b5	gcctggaacctcttgtaggtagaa	gaaatgctttggcacatgga	tcctcaggacttccacatctccc
Illr1	gaacagagccagtgtcaggaaa	actgcccctgggaacca	cccctcagcaagaaagaagagtcattg
Jun	gcatgctatcattggctcatactc	cacaccatcttctggtgtacagtct	ctcccccggcaacacacaagtc
Mt2	aatagaccatgtagaagcctagccttt	gcttttattgtcagttacatgctttatagaaa	ttgtacaaccctgactcgttctcc
PPARγ	acagagacagacatgagccttcac	ggtgggactttcctgctaataca	ccctgctccaggagatctacaagga
SHP-1	aagggcacgatcctcttcaa	ctgttgcaggtgtgcgatgt	atgtgccaggcctccgtgcc

more than 1.5-fold, which included 36 mRNAs that were increased by more than 2-fold, 12 mRNAs by more than 3-fold and 7 mRNAs with a > 10-fold increase; 125 mRNAs were decreased by greater than 33 %. In addition, 37 mRNAs were detected only in livers from HRN mice, and 14 mRNAs were present only in controls.

To validate the results of the oligonucleotide microarray, 14 genes were chosen for further analysis by real-time PCR, using GAPDH expression as a reference mRNA, since the mRNA level of GAPDH did not vary between HRN and control mice according to microarray analysis. Generally, there was good agreement between the microarray and real-time PCR analysis (Table 5), although the fold changes in mRNA levels detected by microarray analysis tended to be lower than those measured by real-time PCR. For example, the mRNA levels of Cyp3a11 and Mt2 (metallothionein II) in HRN livers were 7.7- and 13.3-fold higher respectively in HRN mice by real-time PCR (Table 5), but only 1.7- and 6.9-fold higher respectively by microarray analysis. A similar underestimate of the degree of change in mRNA expression by microarray analysis has been reported in other studies [18,19]. We arbitrarily chose 1.5-fold as the cut-off for up-regulation, and 0.67-fold for down-regulation, as this is in line with other publications reporting microarray analyses [19,20].

Expression of cytochrome P450s in livers of HRN mice

A total of 55 probe sets specific for mouse cytochrome P450s, representing 47 mouse cytochrome P450 genes, are present on the MG_U74Av2 array. Of these, 13 cytochrome P450s could not be detected in either control or HRN mice – Cyp1a1, Cyp1b1, Cyp2b9, Cyp2b13, Cyp2b19, Cyp2j6, Cyp2s1, Cyp4b1, Cyp11a, Cyp17a1, Cyp19a1, Cyp24a1 and Cyp27b1. Altoghether, 31 cytochrome P450s were detected in control livers and 34 in HRN livers (Figure 1). Cyp2e1 mRNA was expressed abundantly in mouse liver (Figure 1A), although the level was not affected by hepatic deletion of POR. Nine hepatic Cyp mRNAs were increased by 1.5–3.7-fold in livers from HRN mice – Cyp2a4, Cyp2c29, Cyp3a11, Cyp3a16, Cyp4a10, Cyp4a14, Cyp7a1, Cyp8b1 and Cyp26a1. The most significant increase (more than 30-fold) was in Cyp2b10. However, it should be noted that the basal level of expression of this gene in controls was very low (Figure 1B). The expression of certain P450 mRNAs was decreased in HRN livers, the most significant effect being on the expression of Cyp7b1, which was reduced by 50 % (Figure 1B). Four P450s showed a 30–40 % reduction in mRNA expression – Cyp2c70, Cyp2d26, Cyp2f2 and Cyp4v3. Microarray analysis revealed that there was no difference in the sum for the signal intensity of 34 Cyp transcripts in the liver between HNR and control mice.

The transcripts of three Cyp genes, *Cyp2b10*, *Cyp3a16* and *Cyp2c29*, which were essentially undetectable in control livers, were markedly induced in livers from HRN mice (Figure 1). Cyp3a11, which catalyses the side-chain hydroxylation of bile acid intermediates, displayed a 1.7-fold increase by microarray analysis and a 7.7-fold increase by real-time PCR. In addition, Cyp2a4 mRNA was up-regulated 2.6-fold in the liver of HRN mice, as assessed by microarray analysis.

It has been established that one major group of P450 inducers, typified by phenobarbital, consists of a host of structurally diverse chemicals that induce a subset of P450 genes within the *Cyp2a*, *2b*, *2c* and *3a* subfamilies, with the *Cyp2b* genes being most affected. It has also been shown that the CAR (constitutive androstane receptor) mediates this induction process, at least in part [21,22]. In addition, it has been demonstrated that Cyp2b10 and Cyp3a are regulated by the orphan nuclear PXR (pregnane X receptor), which is activated by a broad spectrum of xenobiotics [23–25]. Results from the microarray analysis showed that the levels of mRNAs encoding CAR and PXR did not change significantly in livers from HRN mice (both values being approx. 80 % of control) (Supplementary Table 2; http://www.BiochemJ.org/bj/388/bj3880857add.htm). This suggests that the elevation in Cyp2b10 and Cyp3a expression was not due to changes in CAR or PXR levels, although the cellular localization of these transcription factors is critical to their function

The expression of the mRNA encoding Cyp26a1, which catalyses the hydroxylation of retinoic acid, was increased 2-fold in livers from HRN mice (Figure 1B). This could be explained if retinoic acid, which is an inducer of Cyp26a1, accumulated in the HRN liver due to a decrease in its catabolism by Cyp26a1. Interestingly, other retinoic acid-regulated genes, such as those encoding Raet1c (retinoic acid early inducible protein) and Asns (asparagine synthetase), were also induced (4- and 2-fold respectively) in HRN liver (Table 2). However, the expression of the transcription factor RXRα (retinoid X receptor α), which can activate these genes, was not affected significantly (Supplementary Table 2; http://www.BiochemJ.org/bj/388/bj3880857add.htm).

Table 2 Effects of POR deletion on the expression of genes involved in lipid/sterol metabolism and carbohydrate metabolism

Pooled liver RNA from three male 3-month-old mice of the same genotype was used for microarray analysis. The experiments were carried out twice, and the results shown are the averages of the two experiments. Up-regulated genes had an mRNA level > 1.5 times greater than that of the control. Genes classified as down-regulated had an mRNA level < 0.67 of control. Only genes whose 'average difference' is > 34 are shown. The average difference is an indication of hybridization intensity to perfect-match oligonucleotide probe sets compared with mismatch oligonucleotide probe sets, with 34 being the threshold chosen for reliable expression. GenBank® accession numbers are shown. *Genes present in livers of HRN mice but absent from controls; †genes absent from HRN liver but present in controls.

Affymetrix ID	HRN/control	Range	Accession no.	Common name	Description
Nuclear receptors and transcription factors					
97926_s_at	1.64	0.09	U10374	*Pparg*	PPARγ
160894_at	1.63	0.25	X61800	*Cebpd*	C/EBP (CCAAT/enhancer binding protein) δ
101889_s_at	0.63	0.04	U53228	*Rora*	Retinoic acid receptor-related orphan receptor α
103052_r_at	0.61	0.13	X76653	*Nr2f2*; *COUP-TF*	Nuclear receptor subfamily 2, group F, member 2
93264_at	0.50	0.06	AI843895	*Srebf1*	SREBP-1
97123_at	0.38	0.28	L76567	*Nr0b2*; *SHP*; *SHP-1*†	Small heterodimer partner
Lipid/sterol metabolism					
95758_at	3.71	0.62	M26270	*Scd2**	SCD (Δ^9-desaturase 2)
100078_at	3.04	0.21	M64248	*Apoa4*	ApoA-IV
104342_i_at	2.51	0.47	AI845798	*Pla2g12*	Phospholipase A_2
160083_at	2.35	0.48	M63335	*Lpl*	LPL gene
100569_at	1.94	0.60	M14044	*Anxa2*	Annexin A2
101173_at	1.81	0.38	Z50024	*Pctp*	Phosphatidylcholine transfer protein
104524_at	1.80	0.09	AI842825	*Gltp**	Glycolipid transfer protein
97560_at	1.60	0.20	AF037437	*Psap*; *SGP-1*	Mouse prosaposin gene, promoter and exon 1, partial cds
160737_at	1.58	0.31	AW060927	*Lss*	Lanosterol synthase
160770_at	1.57	0.30	AW049778	*Mvd**	Mevalonate (diphospho) decarboxylase
104285_at	1.56	0.34	M62766	*Hmgcr*	3-Hydroxy-3-methylglutaryl-CoA reductase
93038_f_at	1.55	0.07	M69260	*Anxa1*; *Lpc1*	Annexin A1
95633_r_at	1.54	0.23	AW122653	*Mvk*	Mevalonate kinase
160808_at	0.66	0.06	AI854287	*Prkab1*	Protein kinase, AMP-activated, β1 non-catalytic subunit
97867_at	0.48	0.11	X83202	*Hsd11b1*	Hydroxysteroid 11β-dehydrogenase 1
101659_at	0.47	0.04	M75886	*Hsd3b2*	Hydroxysteroid dehydrogenase-2, $\Delta\langle 5\rangle$-3-beta
99521_at	0.33	0.02	AB020239	*Ak4*	Adenylate kinase
94795_at	0.26	0.49	L41519	*Hsd3b5*	Hydroxysteroid dehydrogenase
Transport					
102200_at	1.97	0.14	AF018952	*AQP8*	Aquaporin 8
103953_at	1.94	0.21	U91538	*Sec22l1*	SEC22 vesicle trafficking protein-like 1 (*S. cerevisiae*)
103646_at	1.77	0.04	X85983	*Crat*	Carnitine acetyltransferase
93867_at	1.71	0.07	AJ001166	*Abcd4*	ATP-binding cassette, subfamily D (ALD), member 4
103394_at	1.70	0.06	U72680	*Fxyd5**	FXYD domain-containing ion transport regulator 5
103035_at	1.50	0.28	U60020	*Tap1*	Transporter 1, ATP-binding cassette, subfamily B (MDR/TAP)
92612_at	0.65	0.12	AJ223959	*Slc27a5*, *VLACSR*	Solute carrier family 27 (fatty acid transporter), member 5
92790_at	0.64	0.03	D55720	*Kpna2*	Karyopherin (importin) α2
97430_at	0.62	0.02	AF080469	*G6pt1*	Glucose-6-phosphatase; transport protein 1
94463_at	0.62	0.03	X78874	*Clcn3*	Chloride channel 3
102863_at	0.59	0.00	AI550530	*9130423L19Rik*	RIKEN cdna 9130423L19 gene
94563_at	0.58	0.08	AJ011080	*Afm*	Afamin
97472_at	0.57	0.01	AJ006341	*Slc25a17*	Solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein), member 17
96665_at	0.55	0.06	AW211618	*Vps26*	Vacuolar protein sorting 26 (yeast)
160417_at	0.54	0.03	U86090	*Kif5b*	Kinesin family member 5B
103818_at	0.53	0.04	AJ012754	*Slc7a7*	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 7
102704_at	0.22	0.08	U88623	*AQP4*†	Aquaporin 4
Metabolism					
92688_at	1.97	0.15	X57199	*Acp2*	Acid phosphatase 2, lysosomal
99559_at	1.81	0.06	U14390	*Aldh3a2*	Aldehyde dehydrogenase family 3, subfamily A2
92848_at	1.81	0.06	X64837	*Oat*	Ornithine aminotransferase
92796_at	1.69	0.33	J02980	*Akp2*	Alkaline phosphatase 2, liver
96110_at	1.56	0.15	U31966	*Cbr1*	Carbonyl reductase 1
103299_at	1.56	0.13	AW123773	*AI132321**	RIKEN library clone:A430098J23
94778_at	1.50	0.18	U96401	*Aldh1a7*	Aldehyde dehydrogenase family 1, subfamily A7
104616_g_at	0.65	0.00	M96265	*Galt*	Galactose-1-phosphate uridyltransferase
92768_s_at	0.61	0.08	M15268	*Alas2*	Aminolaevulinic acid synthase 2, erythroid
102773_at	0.51	0.09	X61397	*Car8*	Carbonic anhydrase-like sequence 1
Glycolysis					
103297_at	1.88	0.10	X98848	*Pfkfb1*	6-Phosphofructo-2-kinase/fructose-2,6-biphosphatase 1
104234_at	1.57	0.23	AW125120	*Mrps25**	Mitochondrial ribosomal protein S25
102651_at	1.56	0.21	L41631	*Gck*; *Gk*; *GLK*	Putative; mouse glucokinase gene, complete cds
96066_s_at	1.50	0.12	X97047	*Pkm2*; *Pk3*	M2-type pyruvate kinase.
Retinoic acid-inducible					
102649_s_at	3.93	0.57	D64162	*Raet1c*	Retinoic acid early inducible protein 1γ
95133_at	2.34	0.17	U38940	*Asns**	Asparagine synthetase

Table 3 Effects of POR deletion on mRNAs encoding proteins involved in the oxidative stress response and pro-cancer

Microarray and data analysis were carried out as described in the legend to Table 2. *Genes present in livers of HRN mice but absent from controls.

Affymetrix ID	HRN/control	Range	Accession no.	Common name	Description
Transcription factors					
100130_at	3.82	0.2	X12761	*Jun**	Jun oncogene
98579_at	3.6	1.81	M28845	*Egr1*; *egr**	Zinc finger protein (krox-24) gene, exon 2
104155_f_at	2.75	0.16	U19118	*Atf3*	Leucine zipper protein; transcription factor Irg-21 mRNA
98083_at	2.03	0.33	AW049031	*Copeb*	Kruppel-like factor
103990_at	1.55	0.25	X14897	*Fosb**	Fosb protein (aa 1–338)
100307_at	0.62	0.02	AA002843	*Rtp801*	HIF-1 responsive RTP801
92647_at	0.61	0.02	U35141	*Rbbp4*	Retinoblastoma binding protein 4
93728_at	0.52	0.00	X62940	*Tgfb1i4*	Transforming growth factor β1-induced transcript 4
99603_g_at	0.44	0.17	AF064088	*Tieg1*	Tgfb-inducible early growth response 1
Stress response					
97682_r_at	10.97	1.06	J03953	*Gstm3*	Glutathione S-transferase, Mu3
101561_at	6.95	0.33	K02236	*Mt2*	Metallothionein 2
93573_at	4.75	0.1	V00835	*Mt1*	Metallothionein 1
93009_at	3.35	1.06	J04696	*Gstm2*	Glutathione S-transferase, Mu2
100946_at	2.71	1.3	AF109906	*Hspa1b*	Heat shock protein 1b
93875_at	2.07	0.45	M12571	*Hspa1a**	Heat shock protein 1a
96679_at	1.81	0.28	AW120711	*Dnajb9*	Dnaj (Hsp40) homologue, subfamily b, member 9
94897_at	1.7	0.01	D87896	*Gpx4*	Glutathione peroxidase 4
93853_at	1.55	0.1	AA763918	*2010306G19Rik*	Riken cDNA 2010306g19 gene
98111_at	1.52	0.04	L40406	*Hsp105*	Heat shock protein 105
96085_at	0.67	0.06	L06047	*Gsta4*	Glutathione S-transferase, Alpha 4
Oxidative stress-related metabolic functions					
95693_at	2.04	0.26	U51167	*Idh2*	Isocitrate dehydrogenases, mitochondrial
103597_at	0.4	0.08	X63349	*Dct*	Dopachrome tautomerase
92833_at	0.32	0.06	L07645	*Hal*	Histidine ammonia lyase
Genotoxicity/repair					
104230_at	2.41	0.1	AI225445	*Dclre1a*	Dclre1a DNA-crosslink repair gene snm1
96146_at	2.33	0.24	D83745	*Btg3*	B-cell translocation gene 3 matrix
160590_r_at	2.19	0.26	AI853323	*1810030M08Rik**	1810030M08Rik
102331_at	2.18	0.07	AW120586	*2010004M01Rik**	2010004M01Rik

Hepatic POR deficiency results in altered expression of cytochrome P450s involved in bile acid biosynthesis

The degradation of cholesterol occurs principally by conversion into bile acids in the liver. This process involves both neutral and acidic pathways of bile acid synthesis [26]. The rate-limiting enzyme in the neutral bile acid synthesis pathway is Cyp7a1 (cholesterol 7α-hydroxylase), and the expression of the *Cyp7a1* gene was elevated 3.5-fold in livers from HRN mice as assessed by microarray analysis, and 5.1-fold as assessed by real-time PCR. In addition, the mRNA encoding an additional gene in the neutral pathway, *Cyp8b1* (encoding sterol 12α-hydroxylase), was also increased 2.7-fold (Figure 1). Interestingly, the mRNA expression of FXR (farnesoid X receptor), LXRα (liver X receptor α; NR1H3), HNF4α (hepatocyte nuclear factor 4α) and LRH-1 (liver receptor homologue-1), which regulate the enzymes involved in bile acid homoeostasis, was not significantly affected (Supplementary Table 2; http://www.BiochemJ.org/bj/388/bj3880857add.htm). In contrast, the mRNA level of SHP-1 (small heterodimer partner), which is a repressor of LXR, HNF4α and LRH-1 function, was reduced by more than 60 % in HRN livers (Table 2), suggesting a role for this protein in the increased Cyp7a1 and Cyp8b1 transcription.

In the acidic bile acid synthetic pathway, Cyp27a1 converts cholesterol into both 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid. The probe set for Cyp27a1 is not present on the array. However, real-time PCR analysis showed that the expression of Cyp27a1 mRNA was not affected by the hepatic deletion of POR (Table 5). In addition, the expression of Cyp7b1, an additional protein involved in the acidic pathway, was repressed by 50 %.

Cholesterol biosynthetic genes are activated in livers from HRN mice

The mRNA levels of three enzymes involved in cholesterol biosynthesis were increased significantly in the HRN liver (Table 2; see also Figure 3). These were 3-hydroxy-3-methylglutaryl-CoA reductase, which is involved in the rate-limiting step of cholesterol biosynthesis (1.6-fold), mevalonate kinase (1.5-fold) and mevalonate decarboxylase (1.6-fold). The transcript levels of squalene epoxidase, 7-dehydrocholesterol reductase and Cyp51, enzymes which require the function of POR, were not significantly affected in the HRN liver [Supplementary Table 3 (http://www.BiochemJ.org/bj/388/bj3880857add.htm); see also Figure 3].

Hepatic POR deficiency results in altered expression of transporters involved in cholesterol homoeostasis

The expression of VLACSR (very-long chain acyl-CoA synthetase-related; Slc27a5), which is highly expressed in mouse liver and a candidate enzyme for bile acid conjugation [27,28], was decreased by 35 % in livers from HNR mice (Table 2). mRNAs encoding several ABC (ATP-binding cassette) transporters did not vary significantly. These included those for Abcd1, Abcb4, Abcg2, Abcc6, Abcd3, Abca2 and Tap2. The expression of Slc10a1 (or NTCP), which is a sodium/bile acid co-transporter and is responsible for the uptake of bile acids,

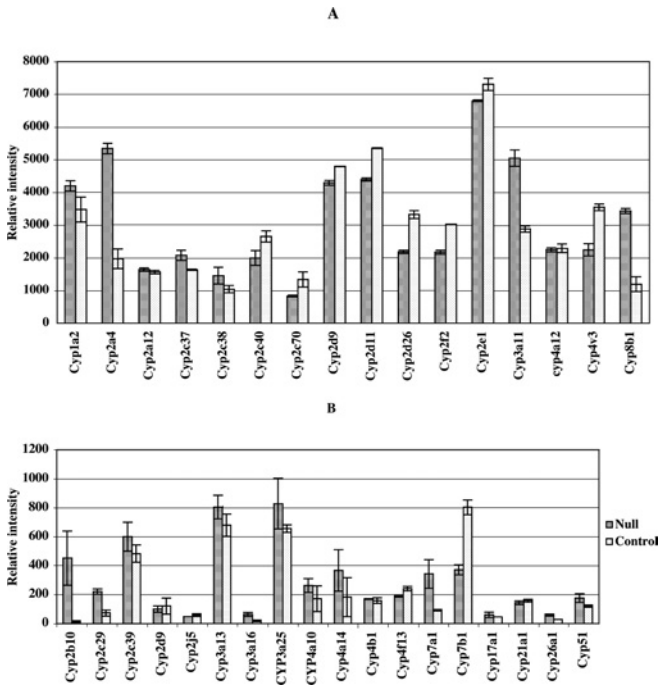

Figure 1 Hepatic cytochrome P450 mRNA levels in HRN and control mice

The Affymetrix MG_U74Av2 array was used for hybridization, and Affymetrix GCOS software was used to scan and analyse the relative abundance of the average difference of intensities of each probe sets. Pooled liver RNA from three male 3-month-old mice of the same genotype was used for microarray analysis. The experiments were carried out twice, and the results shown are averages for the two experiments. (**A**) Sixteen P450 mRNAs were detected at a high level (> 1000 relative units). (**B**) Eighteen P450 mRNAs were detected at a low level (< 1000 relative units).

was not changed significantly (Supplementary Table 3; http://www.BiochemJ.org/bj/388/bj3880857add.htm).

Interestingly, the expression of two genes involved in the formation of water channels was altered in livers from HRN mice. The expression of *AQP8*, the gene encoding aquaporin 8, which facilitates osmotic water transport during canalicular bile secretion [29], increased 2-fold (Table 2). In contrast, the AQP4 mRNA level was reduced dramatically by over 80 % when examined by either microarray or real-time PCR analysis (Tables 2 and 5). Little is known about the mechanism of the regulation of *AQP4* and *AQP8* gene expression in mouse liver.

Altered expression of genes associated with lipid metabolism in livers from HRN mice

LPL (lipoprotein lipase) plays a central role in the hydrolysis of triacylglycerols carried by very-low-density lipoprotein and chylomicrons. In the liver, LPL is also believed to promote uptake of HDL (high-density lipoprotein)-cholesterol, and thereby facilitate reverse cholesterol transport [30,31]. LPL mRNA was increased 2-fold in livers from HRN mice. ApoAIV (apolipoprotein AIV), which is involved in lipid transport and is able to influence the activity of LPL [32], was up-regulated 3-fold. In addition, the expression of the gene encoding phospholipase A_2, an enzyme that hydrolyses phospholipids, was increased 2.5-fold (Table 2). These results indicate that the uptake of fatty acids and HDL-associated cholesteryl esters into the liver is increased in HRN mice. Other genes associated with lipid metabolism remained unaffected by hepatic POR deficiency. These included *ApoAI*, *ApoAII*, *ApoE*, *Apo5*, *Apof* and *Apobec1*. Other transcripts that also did not appear to change included those for the scavenger receptor-BI and the low-density lipoprotein receptor (Supplementary Table 3; http://www.BiochemJ.org/bj/388/bj3880857add.htm).

Cytochrome P450s of the Cyp4a subfamily have been associated with fatty acid degradation in situations of hyperlipidaemia. These enzymes catalyse the ω-1 oxidation of fatty acids. Cyp4a14 and Cyp4a10 mRNAs were increased by 1.9- and 1.5-fold respectively in the HRN liver (Figure 1B). Also, the level of the mRNA encoding the transcription factor PPARγ (peroxisome proliferator-activated receptor γ) was increased 2.6-fold (real-time PCR) and 1.6-fold (microarray analysis) (Table 2). PPARs, particularly PPARα, control the regulation of *Cyp4a* genes. However, the expression of genes involved in mitochondrial or peroxisomal fatty acid β-oxidation was not changed significantly (Supplementary Table 3).

Alterations in mRNAs involved in lipogenesis

In relation to lipogenesis, the expression of the transcription factor SREBP-1 (sterol regulatory element binding protein-1; Srebf1) was decreased by 50 % in the livers of HRN mice. It is well established that SREBP-1c, which is the main SREBP-1 isoform expressed in the liver, plays a critical role in regulating the expression of hepatic lipogenic genes [3–5]. In contrast, the expression of the majority of the genes in the lipogenic pathway, e.g. those encoding acetyl-CoA carboxylase, fatty acid synthase and SCD1 (stearoyl-CoA desaturase 1), was not changed significantly in HRN livers (Supplementary Table 3). In contrast, the isoform SCD2, which was not detected in control liver, was induced (3.7-fold) in HRN liver (Table 2), suggesting that SCD1 and SCD2 are regulated by different mechanisms.

Alterations in mRNAs involved in glycolysis

mRNAs for glucokinase (hexokinase IV), which phosphorylates glucose, and pyruvate kinase were increased 1.7-fold and 1.6-fold respectively in HRN livers (Table 2). In addition, the mRNA encoding the bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphophatase, which was recently identified as a binding partner of glucokinase [33,34], was up-regulated 1.9-fold. These data indicate that there was some alteration in glucose homoeostasis, although serum glucose levels were not significantly different between control and HRN mice (C. J. Henderson, unpublished work).

The expression of genes involved in hormone metabolism is altered in the livers of HRN mice

Hsd3b5 (3β-hydroxy-Δ^5-steroid dehydrogenase-5), a male-specific enzyme found only in liver and which is involved in the inactivation of steroid hormones, is abundantly expressed in the livers of control mice, but expression was reduced by 74 % in HRN livers (Table 2). In addition, expression of Hsd3b2 (3β-hydroxy-Δ^5-steroid dehydrogenase-2), which catalyses the rate-limiting step in the conversion of dihydroepiandrosterone into sex steroids, was reduced by 53 % in HRN liver. Together, these data suggest alterations in steroid hormone homoeostasis in HRN mice. Indeed, analysis of testosterone and oestradiol in the serum of HRN and control mice showed that, in the former, levels of these hormones were significantly lowered in both males and females (Figure 2). Hsd11b1 (11β-hydroxysteroid dehydrogenase type 1) is an enzyme that converts cortisone into the active glucocorticoid, cortisol. Microarray analysis showed that

Table 4 Effects of POR deletion on mRNAs encoding proteins involved in cytoskeleton organization, immune response, protein amino acid phosphorylation, and signal transduction

Microarray and data analysis was carried out as described in the legend to Table 2. *Genes present in livers of HRN mice but absent from controls; †genes absent from HRN liver but present in controls. MAPK, mitogen-activated protein kinase.

Affymetrix ID	HRN/control	Range	Accession no.	Common name	Description
Transcription factors					
93425_at	0.61	0.02	AF028725	*Irf5*	Interferon regulatory factor 5
95081_at	0.61	0.27	AI840176	*P38ip*†	Transcription factor (p38-interacting protein)
104701_at	0.60	0.07	Y07836	*Bhlhb2*	Basic helix–loop–helix-domain-containing, class B2
92658_at	0.43	0.13	AF010405	*Foxq1*; *Hfh-1L*; *HNF-3*	HNF3/fork head homologue-1 like; member of HNF3/forkhead transcription factor family
Cytoskeleton organization and biogenesis					
94288_at	1.97	0.39	J03482	*Hist1h1c*	Histone 1, h1c
101009_at	1.76	0.05	X15662	*Krt2-8*	Cytokeratin endo A; mouse gene for cytokeratin endo A (no 8) 5′ end
98129_at	1.73	0.09	AI852553	*Tmsb10*	Thymosin β10
98477_s_at	1.70	0.17	L40632	*Ank3*	Ankyrin 3, epithelial
160462_f_at	1.69	0.40	AW050256	*Tubb3*	Tubulin β3
94270_at	1.61	0.26	M22832	*Krt1-18*	Mouse cytokeratin (endob) gene, complete cds
92608_at	1.59	0.02	D88793	*Csrp1*	Cysteine-rich protein 1
98409_at	0.61	0.01	L00923	Myo1b	Myosin IB
160417_at	0.54	0.03	U86090	*Kif5b*	Kinesin family member 5B
92280_at	0.47	0.04	AA867778	*Actn1*	Actinin α1
Matrix					
94305_at	3.48	1.12	U03419	*Col1a1**	α1 Type I collagen preproprotein
103675_at	2.95	0.35	Y17793	*Robo1**	Roundabout homologue 1 (*Drosophila*)
98331_at	2.32	0.48	X52046	*Col3a1**	α3 subunit; *COL3A1* gene for collagen αI
96708_at	2.05	0.22	AW120643	*P24b*	P24b
95706_at	1.99	0.27	X16834	*Lgals3*	Lectin, galactose binding, soluble 3
92558_at	1.77	0.30	M84487	*Vcam1*; *CD106*;	Vascular cell adhesion molecule 1
92877_at	1.58	0.06	L19932	*Tgfbi*	Transforming growth factor β-induced
97160_at	1.55	0.11	X04017	*Sparc*	Secreted acidic cysteine-rich glycoprotein
96752_at	1.52	0.05	M90551	*Icam1*; *CD54*;	Intercellular adhesion molecule 1, exons 6 and 7
99476_at	1.51	0.09	AJ131395	*Col14a1*	Procollagen, type XIV, α1
93353_at	1.49	0.21	AF013262	*Lum*; *Ldc*	Lumican
92835_at	0.59	0.03	AI840501	*Cml1*	Camello-like 1
104480_at	0.52	0.01	AI152659	*Dsg2*	Desmoglein 2
160137_at	0.41	0.01	AW260308	*B3gnt1*†	β-1,3-*N*-Acetylglucosaminyltransferase bgnt-1
Cytolysis					
100611_at	2.05	0.30	M21050	*Lyzs*; *Lys*; *Lzm*; *Lzp*	Precursor; mouse lysozyme M gene, exon 4
101753_s_at	1.69	0.20	X51547	*Lzp-s*	P lysozyme, structural
103458_at	0.66	0.00	M35525	*Hc*	Haemolytic complement
104424_at	0.58	0.06	X05475	*C9*	Complement component 9
Immune response					
100328_s_at	1.92	0.16	U96684	*Pira3*	Paired-Ig-like receptor A3
98088_at	1.75	0.16	X13333	*Cd14**	CD14 antigen
94061_at	1.60	0.06	M13018	*Crip1**	Cysteine-rich intestinal protein
94224_s_at	1.51	0.11	M74123	*Ifi205*; *D3*; *D3cdna*	Interferon-activated gene 205
103693_at	0.59	0.06	AA288979	*C130027E04Rik*	RIKEN cDNA C130027E04 gene
104189_at	0.54	0.02	AI122079	*C630032O20Rik*	RIKEN cDNA C630032O20 gene
Protein amino acid phosphorylation					
92473_at	1.76	0.05	Y11092	*Mknk2*	MAPK-interacting serine/threonine kinase 2
100994_at	0.56	0.06	AF011908	*Aatk*†	Apoptosis-associated tyrosine kinase
Proteolysis and peptidolysis					
102316_at	1.68	0.00	Y10656	*Capn5**	Calpain 5
95608_at	1.68	0.20	AI851255	*Ctsb*	Cathepsin B
103588_at	1.59	0.16	AF006196	*Adam15*	A disintegrin and metalloproteinase domain 15 (metargidin)
101019_at	0.61	0.01	U74683	*Ctsc*	Cathepsin C
Signal transduction					
92539_at	1.96	0.01	M16465	*S100a10*	S100 calcium binding protein A10 (calpactin)
93212_at	1.89	0.63	Z97207	*Hspc121*	Butyrate-induced transcript 1
101841_at	0.60	0.04	AW049716	*Egfr*	Epidermal growth factor receptor
94353_at	0.52	0.03	U75530	*Eif4ebp2*	Eukaryotic translation initiation factor 4E binding protein 2
100494_at	0.50	0.08	M30641	*Fgf1*	Fibroblast growth factor 1
160607_at	0.49	0.12	AW123707	*Pard3*†	Par-3 (partitioning defective 3) homologue (*C. elegans*)
161121_f_at	0.46	0.11	AV007820	*S100a13*	S100 calcium binding protein A13
93914_at	0.39	0.01	M20658	*Il1r1*	Interleukin 1 receptor, type I
Peroxisome organization and biogenesis					
103660_at	1.72	0.14	AF093669	*Pex11a*	Peroxisomal biogenesis factor 11a
101844_at	0.64	0.00	U94700	*Pipox*	Pipecolic acid oxidase
Ubiquitin-dependent protein catabolism					
96695_at	1.52	0.09	AF089812	*Ube2a*	Ubiquitin-conjugating enzyme E2A, RAD6 homologue (*S. cerevisiae*)
93069_at	0.61	0.03	U62483	*Ube2d2*	Ubiquitin-conjugating enzyme E2D 2
104046_at	0.58	0.06	AI854141	*Arih1*	Ariadne ubiquitin-conjugating enzyme E2 binding protein homologue 1 (*Drosophila*)

Table 5 Changes in gene expression in the livers of adult HRN compared with control mice as analysed by real-time PCR

The ratios of expression in the livers of HRN compared with control mice, relative to that of GAPDH, are shown. Results are means and range of two experiments. Each experiment was done in triplicate; the variance between the triplicates was $< 5\%$. NA, data not available.

Gene name	RT-PCR Ratio (HRN/control)	RT-PCR Range	Ratio by microarray analysis (HRN/control)
Up-regulated genes			
Cyp2c29	6.41	1.66	2.86
Cyp3a11	7.70	0.21	1.66
Cyp7a1	5.05	0.52	3.53
JUN	3.56	0.58	3.82
Mt2	13.32	0.19	6.95
PPARγ	2.59	0.49	1.64
Down-regulated genes			
Hal	0.29	0.06	0.32
Ak4	0.26	0.07	0.33
AQP4	0.12	0.02	0.22
Hsd3b5	0.17	0.11	0.26
Illr1	0.35	0.06	0.39
SHP-1	0.33	0.17	0.38
Genes not changed significantly			
CAR	0.89	0.35	0.82
Cyp27a1	0.92	0.09	NA

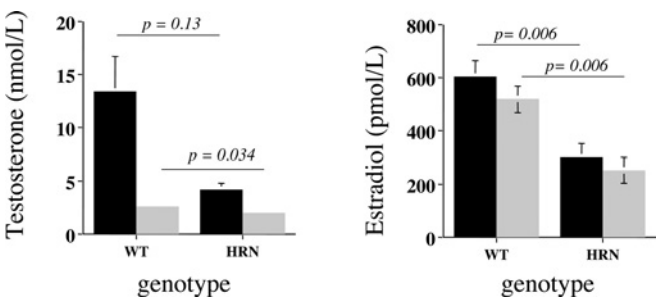

Figure 2 Serum testosterone and oestradiol levels in HRN and control mice

Testosterone and oestradiol levels were measured in the serum of adult male (black bars) and female (light grey bars) HRN and control mice as described in the Materials and methods section; $n = 6$–20.

Hsd11b1 mRNA was decreased by 50% in the livers of HRN mice. The marked reduction in Hsd11b1 mRNA could result in lower local glucocorticoid concentrations in the liver of HRN mice.

Changes in stress response genes in livers of HRN mice

It has been established that the transcription factors Jun, Fosb, Egr1 and Atf3 are immediate early genes implicated in the response of cells to both metabolic changes and a variety of stressful stimuli. Studies have shown that the induction of these genes is associated with cell growth as well as the cellular stress response. mRNAs encoding Jun, Fosb, Egr1 and Atf3 were not detectable in the livers of control mice (Table 3), but were markedly induced in HRN livers. A group of genes involved in antioxidant defence exhibited elevated mRNA levels in HRN liver; these included those encoding metallothionein I, metallothionein II, heat shock proteins (Hsp1a, Hsp1b, Hsp105, and Hsp40), glutathione S-transferase Mu2 and glutathione S-transferase Mu3 (Table 3). In addition, mRNAs encoding several genes involved in the repair of genotoxic damage were induced in HRN livers (Table 3). These results may indicate that the metabolic changes that have occurred in the livers of HRN mice have induced a range of stress responses. Although there is some minor evidence of cellular damage, examination of liver pathology in HRN mice revealed no sign of inflammation, little indication of fibrosis and no evidence of collapse in architecture, with pericentral–periportal spacing similar to that in control liver. The micro- and macrovesicular fatty change observed in HRN liver occurs mostly in the mid-zone region. Gu et al. [17], who also generated a liver-specific POR-null mouse model, reported that the protein level of haem oxygenase-1 was induced 9-fold by hepatic deletion of POR. In contrast, our microarray analysis showed that the haem oxygenase-1 mRNA level was increased only slightly (1.4-fold) (shown in Supplementary Table 3; http://www.BiochemJ.org/bj/388/bj3880857add.htm), and real-time PCR indicated a 2-fold increase (X. J. Wang, unpublished work).

DISCUSSION

In the present study we have used oligonucleotide microarrays and real-time PCR to analyse alterations in hepatic gene expression in mice lacking POR in the liver. Liver-specific deletion of the *POR* gene resulted in significant changes in hepatic gene expression. Several key findings have emerged from these studies, including profound changes in the expression of genes involved in cholesterol and lipid homoeostasis, as well as genes associated with oxidative stress response.

Thirty-four forms of cytochrome P450 were detected in livers from control and/or HRN mice. The total hepatic P450 mRNA levels encoded by these genes was the same in the two groups of animals. In our previous study, we showed that HRN mice exhibited a 5-fold increase in total cytochrome P450 protein content [16]. The reason for this apparent inconsistency between total P450 protein and mRNA levels in HRN mouse livers is not clear, and would suggest that post-transcriptional events, such as translation efficiency and/or protein turnover, are altered in HRN mice. This could help to identify important new pathways of cytochrome P450 protein regulation.

Several hepatic cytochrome P450 mRNAs were increased in livers from HRN mice, including Cyp2b10, Cyp3a11, Cyp3a16, Cyp2a4 and Cyp2c29. This did not appear to be due to increased levels of the transcription factors CAR and PXR, which control expression of these genes, as the expression of both transcription factors did not vary significantly between control and HRN mice. However, it is possible that CAR and/or PXR are activated by endogenous compounds whose level is altered in HRN mice. It is clear from other studies involving the mechanism of action of PXR and CAR that the absolute level of expression of these transcription factors is less important in determining their function than their location (i.e. nuclear) and activation status [35]. A further possible explanation is based on the decrease in the expression of the transcriptional repressor SHP-1 (Table 2). This transcription factor has been shown to interact with many members of the nuclear receptor superfamily, and, in addition to FXR, LRH-1 can modulate the functions of CAR, PXR, LXR, HNF4α and RXR, and inhibit the activity of its binding partners [23]. Whether PXR or CAR is involved in the transcriptional changes remains to be determined; however, the expression of several other genes regulated by the transcription factors was not changed in HRN mice. It is also possible that other, as yet unknown, nuclear receptors or transcription factors might mediate the up-regulation of P450s independently of CAR or PXR.

The absence of hepatic POR, and thus hepatic P450 function, appears to result in few compensatory changes in the expression

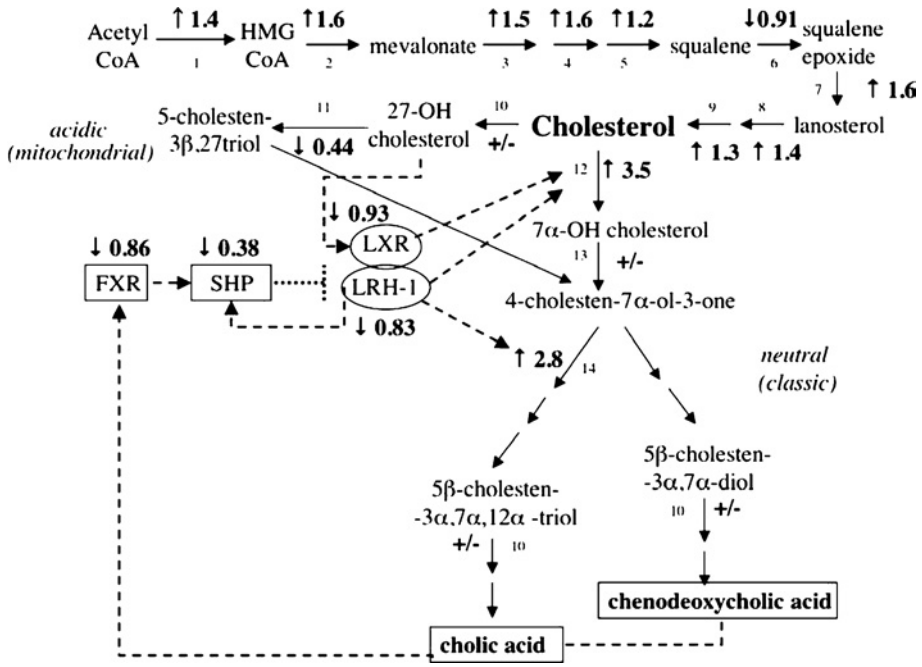

Figure 3 Changes in gene expression in the cholesterol and bile acid biosynthetic pathway

Numbers represent fold increase (↑) or decrease (↓) in mRNA expression as derived from microarray analysis (Cyp27a1 expression was measured by real-time PCR). +/− denotes unchanged expression. Only those genes represented on the Affymetrix chip are shown. Broken and dotted lines represent positive and negative regulation respectively. 1, 3-Hydroxy-3-methylglutaryl-CoA synthase; 2, 3-hydroxy-3-methylglutaryl-CoA reductase; 3, mevalonate kinase; 4, diphosphomevalonate decarboxylase; 5, squalene synthase; 6, squalene epoxidase; 7, lanosterol synthase; 8, lanosterol demethylase (Cyp51); 9, 7-dehydrocholesterol reductase; 10, cholesterol 27α-hydroxylase (Cyp27a1); 11, oxysterol 7α-hydroxylase (Cyp7b1); 12, cholesterol 7α-hydroxylase (Cyp7a1); 13, 3β-hydroxysteroid oxidoreductase (HSD3B7); 14, sterol 12α-hydroxylase (Cyp8b1).

of genes encoding other Phase I and Phase II drug-metabolizing enzymes. Among the glutathione S-transferases, only Gstm2 and Gstm3 showed significant induction (Table 3 and Supplementary Table 2; http://www.BiochemJ.org/bj/388/bj3880857add.htm), while the single UDP-glucuronyltransferase (UGT2b5) and the two sulphotransferases (Sult1a1 and Sult1b1) represented on the Affymetrix chip were not induced significantly (Supplementary Table 2).

The deletion of hepatic POR results in profound changes in bile acid production, which are shown here in the expression of genes involved in controlling bile acid homoeostasis. The expression of Cyp7a1 is activated by the orphan receptor LRH-1 and regulated further through a feed-forward activation by oxysterols, mediated by LXRα [36,37]. Feedback repression of Cyp7a1 is mediated by binding of bile acids to FXR, leading to increased transcription of the gene encoding SHP-1 [38,39]. This heterodimerizes with LRH-1, and leads to promoter-specific repression of Cyp7a1 [40–42]. The regulation of the expression of the *Cyp8b1* gene is controlled by a similar mechanism. The reduction of bile acid production could explain the increases in Cyp7a1 and Cyp8b1 mRNAs. Our data suggest that when bile acid synthesis is decreased in the liver, de-repression, as a consequence of a decrease in SHP-1, is an important endogenous mechanism of cytochrome P450 induction. In contrast with the phenotype observed in Cyp7a1-deficient mice [43,44], deletion of hepatic POR, and thus hepatic P450 function, does not lead to an induction of the acidic (alternative) bile acid biosynthetic pathway, but rather to up-regulation of key enzymes in the neutral pathway. The absence of hepatic POR means that, despite the increased expression of Cyp7a1 and Cyp8b1, bile acid production is profoundly decreased in HRN mice, due to an inability of these enzymes to function ([16]; C. J. Henderson, unpublished work). A summary of the changes in expression of genes involved in cholesterol and bile acid biosynthesis is shown in Figure 3.

Of the MRP (multidrug-resistance-associated protein) transporters represented on the Affymetrix chip, the expression of both MRP2 (Abcc2), which has been implicated in the biliary transport of anionic conjugates, and MRP6 (Abcc6) was unchanged. However, levels of MRP3 (Abcc3) were elevated almost 1.5-fold (Supplementary Table 3; http://www.BiochemJ.org/bj/388/bj3880857add.htm); it has been suggested that this protein is involved in the biliary excretion of organic anions and is up-regulated in the cholestatic liver, via a mechanism possibly involving FXR and LRH-1 (http://nutrigene.4t.com/humanabc.htm).

We reported previously that the cholesterol level in the serum of HRN mice was reduced [16]. However, the cholesterol level in the liver of HRN mice was essentially unchanged relative to controls (K. Fon Tacer, D. Rozman and C. J. Henderson, unpublished work). As hepatocytes in the HRN liver are unable to either synthesize cholesterol *de novo* or process cholesterol to bile acids easily, this suggests that cholesterol homoeostasis in the HRN liver is tightly controlled, possibly at the expense of serum cholesterol. The overall reduction in hepatic cholesterol biosynthesis and catabolism implies that cholesterol/fatty acid transport may be altered. Changes in these pathways were indeed observed in the HRN liver, characterized by an increased expression of LPL and Apoa4, and a decreased expression of VLACSR. Other perturbations in fatty acid homoeostasis in HRN livers were the changes observed in SCD expression. SCD is a microsomal enzyme required for the biosynthesis of oleate and palmitoleate, which are the major monounsaturated fatty acids of membrane phospholipids, triacylglycerols and cholesteryl esters. Two well-characterized isoforms of SCD, SCD1 and SCD2, exist in the mouse. Most mouse tissues express SCD1 and SCD2, although

the liver expresses mainly the SCD1 isoform [45]. Microarray analysis revealed that the expression of *Scd1* was unchanged in HRN liver. In contrast, the mRNA level for *Scd2* was elevated 3.7-fold. It has been shown that the SCD2 enzyme is expressed at higher levels in the livers of mice overexpressing the truncated nuclear form of SREBP-1a [46]; in this case the livers were massively enlarged, engorged with triacylglycerols and cholesterol, and contained increased levels of unmonosaturated fatty acids. The physiological significance of increased SCD2 in the liver of HRN mice is not clear. However, the increased expression of SCD2 and phospholipase A_2 may affect the fatty acid composition and also contribute to lipid accumulation.

Increased hepatic fatty acid accumulation is often correlated with activation of PPARα and peroxisome proliferation [47,48]. In the present studies there was no evidence for the induction of peroxisomal mRNAs, and the mRNA level of PPARα was not changed. However, induction of PPARγ was observed. The LPL gene is an established target of PPARγ in several tissues, including macrophages, liver and adipocytes [49]. In addition, it has also been shown that PPARγ induces the expression of Apoa4 and represses Hsd11b1. Studies have shown that PPARγ can be activated by a range of fatty acids, as well as prostaglandins [50]. Collectively, these results suggest that PPARγ might play a central role in altering the expression of genes related to lipid, cholesterol and glucose homoeostasis in the liver of HRN mice.

A cluster of antioxidant defence genes was activated in the liver of HRN mice, providing evidence that hepatic deletion of POR results in an altered redox state. It is at present unclear what has triggered these changes. However, the transcription factors Hsf1 (heat shock transcription factor 1), Mtf (metal response element binding transcription factor), HIF1 (hypoxia-inducible factor 1) and NFκB (nuclear factor κB), which mediate these responses, remained unchanged at the mRNA level (Supplementary Table 2; http://www.BiochemJ.org/bj/388/bj3880857add.htm). One explanation could be the changes in glucose and lipid metabolism. The reason for the overexpression of these genes remains to be elucidated.

In conclusion, the present study provides potential molecular mechanisms for the phenotypic changes observed in HRN mice. Further studies to evaluate the pathways affected as a consequence of POR deficiency are currently in progress.

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