Gastrointestinal, Hepatobiliary and Pancreatic Pathology

Hepatic Gene Expression in Protoporphyic *Fech* Mice Is Associated with Cholestatic Injury but Not a Marked Depletion of the Heme Regulatory Pool

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BALB/c *Fech*^m1Pas mice have a mutated ferrochelatase gene resulting in protoporphyria that models the hepatic injury occurring sporadically in human erythropoietic protoporphyria. We used this mouse model to study the development of the injury and to compare the dysfunction of heme synthesis with hepatic gene expression of liver metabolism, oxidative stress, and cellular injury/inflammation. From an early age expression of total cytochrome P450 and many of its isoforms was significantly lower than in wild-type mice. However, despite massive accumulation of protoporphyrin in the liver, expression of the main genes controlling heme synthesis and catabolism (*Alas1* and *Hmox1*, respectively) were only modestly affected even in the presence of the cytochrome P450-inducing CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene. In contrast, in BALB/c mice exhibiting griseofulvin-induced hepatic protoporphyria with induction and destruction of cytochrome P450, both *Alas1* and *Hmox1* genes were markedly up-regulated. Other expression profiles in BALB/c *Fech*^m1Pas mice identified roles for oxidative mechanisms in liver injury while modulated gene expression of hepatocyte transport proteins and cholesterol and bile acid synthesis illustrated the development of cholestasis. Subsequent inflammation and cirrhosis were also shown by the up-regulation of cytokine, cell cycling, and procollagen genes. Thus, gene expression profiles studied in *Fech*^m1Pas mice may provide candidates for human polymorphisms that explain the sporadic hepatic consequences of erythropoietic protoporphyria. (*Am J Pathol 2005, 166:1041–1053*)

Heme is vital to the transport and utilization of oxygen in oxidative and signaling pathways. A large proportion of heme usage in the liver is accounted for by cytochrome P450 isoforms, many of which are associated with drug metabolism. In the final step of heme synthesis ferrous iron is inserted into the precursor porphyrin protoporphyrin IX by the mitochondrial enzyme ferrochelatase. The structure and regulation of ferrochelatase have been extensively studied.[1] In humans, mutations in the genes of heme synthesis may lead to clinical symptoms associated with toxic accumulation of precursor molecules.[2] These may occur in both erythroid and nonerythroid tissue especially the liver. In patients with erythropoietic protoporphyria (EPP), that have mutations of the ferrochelatase gene, leakage of protoporphyrin IX into plasma and its accumulation in tissues may lead to toxic actions in the skin because of light activation and free radical generation.[3–5] In a minority of patients there is hepatic injury, cirrhosis, and jaundice, which in extreme circumstances requires liver transplantation.[6–8] This might be because of the primary disturbance of hepatic heme synthesis or a secondary consequence of deposition in the liver of protoporphyrin formed in the erythropoietic system. How protoporphyrin in a dark environment could cause toxicity is not understood or why only some patients develop progressive liver failure.[6]

The *Fech*^m1Pas mutant BALB/c mouse contains a point mutation in the ferrochelatase gene.[9,10] The resulting enzyme exhibits less than 5% of normal ferrochelatase activity in liver and spleen and 6% of the wild-type activity when expressed as a recombinant protein. This leads to insufficiency in erythropoietic heme synthesis, hemolytic anemia, and splenic enlargement. The mice suffer from phototoxicity that can be alleviated by bone marrow transplantation and gene therapy.[11,12] Protoporphyrin accumulates in the liver and plasma of mice with associated elevated plasma transaminases and bilirubin and hyperlipidemia.[9,13,14] Progressive hepatobiliary injury

a

b
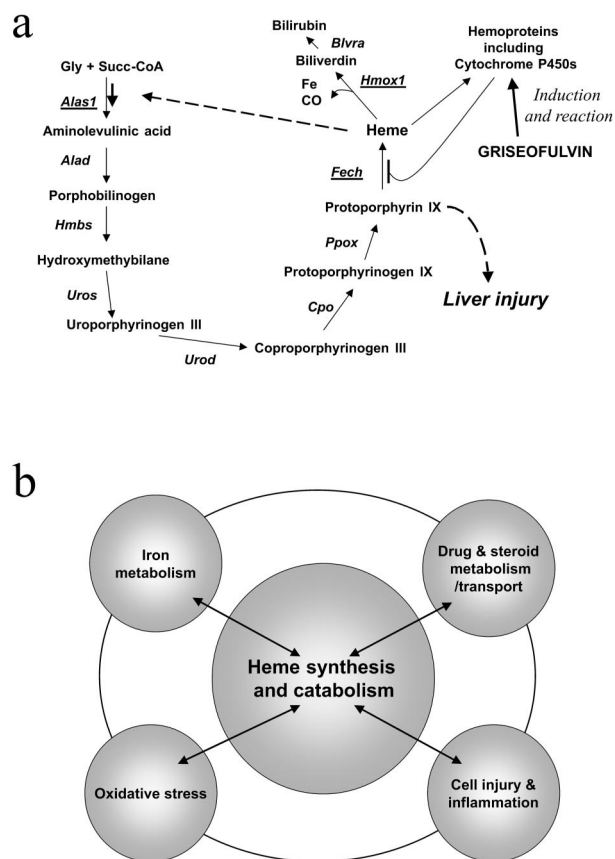
Figure 1. Hepatic heme synthesis leading to protoporphyria and possible impact with other metabolic systems. **a:** Scheme of heme metabolism showing negative feedback deregulation of ALA synthase 1 and cytochrome P450 induction and inhibition of ferrochelatase after griseofulvin treatment. **b:** Scheme of possible metabolic pathways and systems that might impact on hepatic heme metabolism.

leads to biliary fibrosis and neoplastic development[13,15] but whether the liver is suffering from heme deficiency and how the toxicity arises are not clear. A proposal has been made for the involvement of cytotoxic bile containing high concentrations of bile salts and protoporphyrin that might damage bile duct epithelium and cause biliary fibrosis.[13]

Although initiated by a ferrochelatase mutation, the pathological consequences of protoporphyria are likely to be the result of a complex interaction between metabolic systems. To understand mechanisms of liver injury and demonstrate possible candidate genes that could explain the sporadic incidence of liver injury in EPP, we

investigated hepatic gene expression in the mutant mouse using cDNA microarray analysis. We focused on expression of cytochrome P450 hemoproteins and of genes associated with bile acid and lipid metabolism, oxidative stress, and inflammation that might be affected by possible disturbance of heme synthesis and deposition of protoporphyrin (Figure 1). Results were compared to those obtained with mice exposed to the drug griseofulvin that causes protoporphyria by initiating formation in the liver of an inhibitor of ferrochelatase.[16]

Materials and Methods

Animals and Treatments

The BALB/c-*Fech^{m1Pas}* mouse strain (abbreviated to *Fech* mice) was originally produced by ethylnitrosourea mutagenesis and backcrossed on to BALB/c mice.[9] The mutation appears to be inherited as a single autosomal recessive gene. The strain was obtained from the Jackson Laboratories, Bar Harbor, ME, and bred according to Home Office regulations under project licenses 80/1329 and 40/2571 by homozygous mating while maintained at 21°C under reduced light to protect from skin lesions and fed RM1 diet (SDS, Witham, UK). The data shown in Tables 1 and 2 were obtained by crossing F_1 mice from mating female BALB/c mice with male homozygous *Fech^{m1Pas}* mutants to give male BALB/c mice $-/-$, $-/+$, and $+/+$ for the *Fech^{m1Pas}* mutation (wild types, heterozygotes, and homozygotes, respectively). For studies with older mice, age-matched control BALB/c mice were obtained from Harlan Ltd., Bicester, UK, or bred in-house. *Fech* mice were culled within a year as a consequence of hepatic adenomas or carcinomas. All mice analyzed were males. Mice were administered 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) by intraperitoneal injection (3 mg/kg) dissolved in corn oil (2.5 ml/kg), which was also used as a vehicle control, at 8 weeks of age and analyzed after 3 days. BALB/c mice (6 to 8 weeks old) were administered griseofulvin (Sigma, Poole, UK) (as 1% of the diet containing 2% arachis oil) for 3 weeks.[17]

Histological and Biochemical Analyses

Tissues were fixed in neutral-buffered formalin and embedded in paraffin wax. Standard histological sections (5 μm thick) were stained with hematoxylin and eosin. Col-

Table 1. Phenotypes of F_2 Mice Generated from BALB/c $\times$ *Fech^{m1Pas}* Mutation

Fech mutation	n	Body wt (g)	Liver wt (g)	Hepatic protoporphyrin (nmol/g)	Plasma *ALT* (U/L)	Plasma bilirubin Direct (μmol/L)	Total (μmol/L)
Wild type	6	26.1 ± 1.8	1.37 ± 0.23	1.3 ± 0.5	28 ± 8	0.7 ± 0.7	0.8 ± 1.2
Heterozygotes	14	26.0 ± 2.1	1.36 ± 0.21	1.1 ± 0.3	28 ± 9	1.4 ± 1.6	1.1 ± 0.8
Homozygotes	5	19.1 ± 3*	2.05 ± 0.51*	902 ± 366*	522 ± 67*	94 ± 59*	82 ± 16*

Female BALB/c mice were mated with male BALB/c *Fech^{m1Pas}* mutant mice to give a F_1 generation. From these a batch of F_2 mice was produced and the 25 males (8 weeks old) were assessed for the *Fech* mutation as described in Materials and Methods.
* Significantly different from heterozygote and homozygote mice ($P < 0.05$).

Table 2. Influence of the Homozygous *Fech^{m1Pas}* Mutation on the Hepatic Expression of Genes in Systems of Metabolism and Injury

Gene	Gene symbol	4 Weeks *Fech*/wild-type	P value	8 Weeks *Fech*/wild-type	P value
Heme synthesis/catabolism					
Aminolevulinic acid synthase 1	Alas1	1.11		1.86	0.042
Aminolevulinic acid synthase 2	Alas2	0.54	0.039	0.16	0.006
Aminolevulinate acid dehydratase	Alad	1.06		0.81	
Hydroxymethylbilane synthase	Hmbs	1.02		0.92	
Uroporphyrinogen III synthase	Uros	0.89		1.18	
Uroporphyrinogen decarboxylase	Urod	1.19		0.88	
Coproporphyrinogen oxidase	Cpo	0.96		0.71	
Protoporphyrinogen oxidase	Ppox	1.01		1.25	
Ferrochelatase	Fech	0.92		0.76	
Heme oxygenase 1	Hmox1	1.95	0.014	0.94	
Heme oxygenase 2	Hmox2	1.22		0.80	
Biliverdin reductase A	Blvra	1.36		0.60	
UDP-glucuronosyltransferase 1a6	Ugt1a6	0.57		0.70	0.035
Cytochrome P450/steroid metabolism					
Cytochrome P450 reductase	Por	1.41		0.99	
Cytochrome b5	Cyb5	0.98		0.92	
Cytochrome P450 1A2	Cyp1a2	0.53	0.026	0.35	0.025
Cytochrome P450 2A12	Cyp2a12	0.97		0.62	
Cytochrome P450 2A5*	Cyp2a5	1.3		1.65	
Cytochrome P450 2B10[†]	Cyp2b10	0.59		0.72	0.037
Cytochrome P450 2B13	Cyp2b13	0.48	0.006	0.65	
Cytochrome P450 2C29	Cyp2c29	0.22	0.028	0.18	0.006
Cytochrome P450 2D10	Cyp2d10	0.7		0.48	0.042
Cytochrome P450 2D26	Cyp2d26	1.08		0.51	0.030
Cytochrome P450 2E1	Cyp2e1	0.3	0.05	0.18	0.007
Cytochrome P450 2F2	Cyp2f2	0.25	0.011	0.09	0.009
Cytochrome P450 2J6	Cyp2j6	0.72		0.73	0.011
Cytochrome P450 3A11	Cyp3a11	1.07		1.54	0.002
Cytochrome P450 3A25	Cyp3a25	0.55		0.41	0.04
Cytochrome P450 4A10	Cyp4a10	0.45	0.006	0.17	0.012
Cytochrome P450 7A1	Cyp7a1	1.15		1.58	0.017
Cytochrome P450 7B1	Cyp7b1	0.8		0.08	0.002
Cytochrome P450 27A1	Cyp27a1	0.81		0.24	0.034
Cytochrome P450 51	Cyp51	1.55		3.07	0.006
3-OH-Me glutaryl CoA synthase 1	Hmgcs1	1.24		3.54	0.013
3-OH-Me glutaryl CoA synthase 2	Hmgcs2	ND		0.64	0.005
3-OH-Me glutaryl CoA reductase	Hmgcr	1.45		1.76	
3β-OH steroid 5-dehydrogenase	Hsd3b4	ND		0.17	0.001
11β-OH steroid dehydrogenase	Hsd11b1	0.38	0.048	0.34	0.011
Nuclear receptors					
AHR	Ahr	1.35		1.27	
HNF-1α	Tcf1	1.23		1.02	
HNF-1β	Tcf2	0.98		1.48	
HNF-4	Hnf4	1.23		1.01	
LXR	Nr1h3	1.15		0.57	
FXR	Nr1h4	1.58		0.91	
PXR/SXR	Nr1i2	0.93		0.76	
CAR	Nr1i3	0.83		0.22	0.048
RXR α	Rxra	0.9		1.08	
PPAR α	Ppara	ND		0.45	
Organic transporters					
ABC[‡] B1A (MDR1A)	Abcb1a	1.64		1.95	0.010
ABC[‡] B1B (MDR1B)	Abcb1b	1.41	0.030	1.24	
ABC[‡] B4 (phospholipid transporter) (MDR2)	Abcb4	1.58		1.37	
ABC[‡] B11 (bile acid transporter) (BSEP)	Abcb11	1.09		0.70	
ABC[‡] C2 (organic ion transporter) (MRP2)	Abcc2	0.99		0.46	
Na taurocholate cotransporter (NTCP)	Slc10a1	0.37	0.002	0.23	0.000
Organic anion transporter (OATP1)	Slco1a1	1.12		0.03	0.010
Organic anion transporter	Slco1a6	1.2		0.19	0.000
Apolipoprotein A-IV	Apoa4	1.75		5.38	0.000
Apolipoprotein B editing complex 1	Apobec1	2.51	0.000	5.56	0.020
Fatty acid-binding protein 1	Fabp1	0.35		0.36	0.000

(Table continues)

Table 2. (*Continued*)

Gene	Gene symbol	4 Weeks *Fech*/wild-type	*P* value	8 Weeks *Fech*/wild-type	*P* value
Iron metabolism					
Transferrin	Trf	1.38		1.69	0.043
Transferrin receptor	Trfr	0.77		0.89	
Transferrin receptor 2	Trfr2	0.77		0.67	0.040
HFE	Hfe	1.21		1.01	
Ferritin light chain 1	Ftl1	1.09		1.62	0.000
Ferritin heavy chain	Fth	1.59		1.52	0.009
Aconitase 1	Aco1	1.11		0.95	
ABC[‡] B7	Abcb7	1.14		1.10	
Ferroportin	Slc40a1	1.18		1.37	
NRAMP1	Slc11a1	1.43		3.21	0.037
β2-microglobulin	B2m	0.97		1.45	0.011
Hepcidin	Hamp	1.49		1.20	
Hephaestin	Heph	1.53	0.001	2.74	0.003
Ceruloplasmin	Cp	2.19		3.53	0.001
Hemopexin	Hpxn	2.45		4.54	0.003
Oxidative stress					
Jun oncogene	Jun	1.16		3.38	0.016
Nuclear factor E2-derived 2, like 2	Nfe2l2	1.63	0.006	3.92	0.003
Metallothionein 1	Mt1	1.19		2.52	0.008
Glutathione synthetase	Gss	1.55	0.053	4.05	0.005
Glutathione peroxidase 4	Gpx4	2.78	0.025	3.85	0.000
Glutathione S-transferase α2	Gsta2	1.79		3.58	0.000
Glutathione S-transferase μ1	Gstm1	2.25		2.42	0.002
Glutathione S-transferase μ5	Gstm5	1.83		1.97	0.011
Fumarylacetoacetate hydrolase	Fah	0.83		0.57	0.037
Catalase	Cat	0.61		0.50	0.052
Superoxide dismutase 2	Sod2	0.87		0.43	0.032
Glutamine synthase	Glul	0.93		0.30	0.013
Carbonic anhydrase 3	Car3	0.17	0.033	0.36	0.017
Uncoupling protein 2	Ucp2	2.34	0.005	3.92	
Inflammation and wound healing					
TGF-β induced	Tgfbi	2.02	0.013	2.58	0.003
Transglutaminase 2	Tgm2	2.84	0.009	4.64	0.002
Osteopontin	Spp1	6.28	0.005	10.82	0.000
Osteonectin	Sparc	1.89	0.008	2.77	0.012
Procollagen, type III, α1	Col3a1	4.1	0.012	5.82	0.000
Procollagen, type IV, α1	Col4a1	2.49	0.036	5.85	0.038
Procollagen, type VI, α3	Col6a3	1.97		2.94	0.008
Annexin A2	Anxa2	4.1	0.013	10.17	0.007
Annexin A5	Anxa5	4.18	0.001	6.63	0.000
Biglycan	Bgn	1.8	0.017	4.12	0.001
Chemokine ligand 2	Ccl2	2.83	0.003	7.94	0.011
Cyclin D1	Ccnd1	1.61		3.08	0.005
CD14 antigen	Cd14	2.82	0.002	3.87	0.003
Lymphocyte antigen 6 complex	Ly6 days	6.9	0.000	10.33	0.005
Lymphocyte antigen 86	Ly86	2.29	0.001	7.69	0.009

Results are the mean of three to four mice per group. Changes in expression are given as normal ratios relative to comparison with the wild-type mice. Degrees of significance (*P* values) were calculated from the original log$_2$ data that were then converted to the normal values for ease of appreciation. Other genes in the groups were present on the array but have been omitted from the table for the sake of brevity or because the abundance of mRNA was too low for reliable quantitation. Symbols are from Genebank data base (*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db = unigene*).

  * Also would probably detect *Cyp2a4* (cytochrome P450 2A4).
  † Described as *Cyp2b20* in Unigene but categorized as *Cyp2b10* by Nelson and co-workers.[57]
  ‡ ABC is ATP-binding cassette protein super family.
  ND, not determined.

lagen was demonstrated with van Gieson's stain. Tissue for electron microscopy was processed and examined as previously reported.[18] Plasma alanine aminotransferase (ALT) and bilirubin levels were estimated with Sigma kits. Protoporphyrin levels were determined by fluorescence spectroscopy.[19] RNA and microsomes were obtained as reported in previous publications.[17,20] Total cytochrome P450 was assessed as described.[21]

Immunoblotting

Sodium dodecyl sulfate electrophoresis and Western blotting of microsomal proteins were performed as reported previously[20] using chemiluminescence detection and primary antibodies from the following sources: heme oxygenase (HMOX1) with a rabbit antibody anti-rat HMOX1 peptide (Stressgen, Victoria, Canada), rat HMOX1 was a

positive control; CYP1A2 with a goat antibody against rat enzyme (Gentest, Woburn, MA); CYP2A5 with a rabbit antibody against human CYP2A6 peptide (Chemicon, Temecula, CA) that recognizes the mouse ortholog[22] with human CYP2A6 (Gentest) as a positive control; CYP2B10 with rabbit anti-human CYP2B6 (Chemicon). Rat microsomes expressing human CYP2B6 (74.4% homology with mouse CYP2B10) gave a single band but with a lower molecular weight. CYP2E1 and cytochrome P450 reductase were detected using rabbit antibodies (Chemicon) to the human forms and compared with human and rat proteins, respectively, from Gentest. CYP3A11 was detected with a rabbit antibody against rat CYP3A2 that may also recognize CYP2C29 (Gentest). Quantitation was achieved by densitometry and changes expressed relative to wild-type mice.[20]

Determination of Fech Genotype

The mutated ferrochelatase gene in BALB/c *Fech^m1Pas* mice carries a T→A transversion at nucleotide 293 leading to a methionine to lysine substitution at position 98.[10] To confirm the mutation, cDNA clones encoding the appropriate region of the ferrochelatase gene were isolated by amplification of cDNA from total liver RNA from wild-type and *Fech^m1Pas* BALB/c mice using touchdown polymerase chain reaction (PCR) with primers 5′-GGTA-CATCATGCCAAGACCA-3′ and 5′-ACGACCTACTCAAT-AGGGGA-3′. A fragment of ~300 bp was observed by 2.5% agarose gel electrophoresis for both strains. After amplification by PCR, purification and sequencing the T→A transversion were confirmed occurring at nucleotide 137 of the product from the *Fech^m1Pas* mouse. The *Fech* heterozygotes and *Fech* homozygotes have been distinguished previously by phenotypic characteristics or dot blotting of cDNA fragments followed by hybridization with allele-specific oligonucleotides;[9,10,13] neither method has been entirely satisfactory. Instead therefore, the wild-type 300-bp fragment was digested with the restriction enzyme *Pag1*, to two fragments of 158 and 145 bp. The *Pag1* site is not present in the *Fech^m1Pas* mutation and thus wild-type, heterozygous, and homozygous *Fech* mice were genotyped at the termination of experiments using liver cDNA and this procedure (Figure 2).

RNA Extraction, Labeling, and cDNA Gene Arrays

The mouse cDNA microarray consisted of ~4000 candidate-expressed sequence tag clones obtained from Research Genetics, now Invitrogen, Carlsbad, CA and the IMAGE collection held originally at MRC Human Geneome Mapping Project. These clones represented many genes encompassing heme, iron, and drug metabolism; liver function; oxidative stress; apoptosis; cell cycling; inflammation; and wound-healing responses. Total RNA was extracted from liver tissues using Tri-Reagent (Sigma, UK). The RNA labeling, hybridization, analysis of fluorescence, and data processing were performed as previously de-
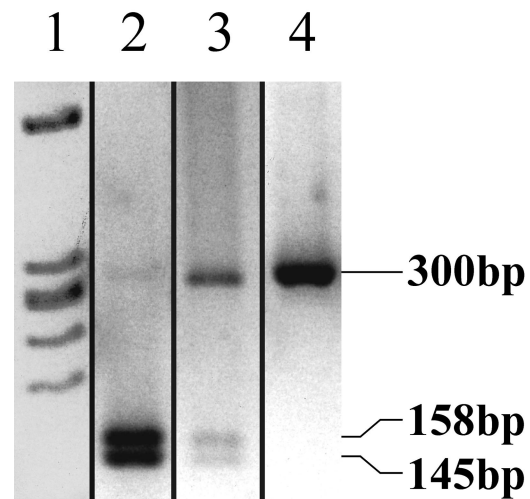
Figure 2. Genotyping of *Fech^m1Pas* mutation by cleavage of cDNA fragment of ferrochelatase. A 300-bp product amplified by PCR from ferrochelatase cDNA of wild-type or mutant mice was digested with *Pag1* restriction enzyme. **Lane 1**, molecular weight markers; **lane 2**, homozygous wild-type BALB/c (−/−); **lane 3**, heterozygote; **lane 4**, homozygous mutant.

scribed.[17,23,24] The cDNAs of four mice were compared against cDNAs from four wild-type mice as appropriate for *Fech* or TCPOBOP studies. Clones for all genes reported were verified by sequence analysis.[24] Data has been deposited via MIAMExpress in ArrayExpress at the European Bioinformatics Institute, Hinxton, UK (http://www.ebi.ac.uk/arrayexpress). ArrayExpress is a public repository for microarray data in accordance with Microarray Gene Expression Data Society's MIAME recommendations. The accession numbers are as follows: experiment accession numbers, E-MEXP-189 and E-MEXP-194; array design accession number, A-MEXP-113 and A-MEXP-116.

Statistical Analyses

The microarray data were normalized using a program developed at the MRC Toxicology Unit, version NorTTNov2003.exe using the LOWESS option.[25] Statistical analysis of the data were performed using the two-tailed paired *t*-test on the logarithm (base 2) of the ratio of the data. Statistical significance of phenotype and Western blotting data were performed using two-tailed Student's *t*-test.

Real-Time Reverse Transcriptase (RT)-PCR

mRNA expressions from *Alas1* and *Hmox1* genes were determined by real-time RT-PCR with primers designed to cross exon-exon boundaries to avoid interference from genomic DNA. Primers were designed using Primer Express Software v2.0, Applied Biosystems. Primers were as follows: *Hmox1* forward primer, 5′-CACTTCGTCAG-AGGCCTGCTA-3′ and reverse primer, 5′-GTCTGGGAT-GAGCTAGTGCTGAT-3′; *Alas1* forward primer, 5′-TCT-TCCGCAAGGCCAGTCT-3′ and reverse primer, 5′-TG-GGCTTGAGCAGCCTCTT-3′. Results were normalized against β2-microglobulin (β2m). *B2m* forward primer, 5′-

CATACGCCTGCAGAGTTAAGCA-3′; reverse primer, 5′-GATCACATGTCTCGATCCCAGTAG-3′.

cDNA was synthesized from total RNA using random hexamers and SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA) as per the manufacturer's protocols. Product cDNAs (from 10 ng RNA), or a nontemplate control, were incubated with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) containing 900 nmol/L forward primer and 300 nmol/L reverse primer in an ABI Prism 7700 sequence detection system. The thermal-cycler protocol was: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles at 95°C for 15 seconds, and 60°C for 1 minute.

Results

Phenotypic Characteristics of Fech Mutation

Male wild-type, heterozygous, and homozygous *Fech* mice bred as a F_2 cross from BALB/c and BALB/c *Fech^{m1Pas}* parent strains at 8 weeks of age representing early adulthood mice were phenotyped for hepatic porphyria and plasma levels of bilirubin and ALT (Table 1). Only the homozygous *Fech* mice showed hepatic porphyria and elevated plasma levels of ALT and bilirubin. All homozygous *Fech* mice had enlarged livers and spleens. Livers contained moderate amounts of protoporphyrin as fine droplets of red-brown pigment in hepatocytes as well as variable-sized deposits in bile canaliculi, bile ducts, and interstitial cells of both hepatic portal tracts and parenchyma.[9,13] Portal tracts also showed varying degrees of inflammation, fibrosis, bile duct, and oval cell proliferation. The enlarged spleens showed active hematopoiesis in the zones of red pulp. Only 1 heterozygote of 14 exhibited slight hepatic portal tract proliferation, the remainder were identical to the wild-type group.

Histological findings in 4-week-old homozygous *Fech* mice (1 week after weaning) were qualitatively similar to those that were 8 weeks old, but there was obviously less pigment within hepatic parenchyma and less florid proliferative alterations and collagen around portal tracts (data not shown). In comparison, in older *Fech* mice at 13 weeks and 26 weeks although protoporphyrin continued to be widely distributed, showing red birefringence under polarized light,[5] it tended to aggregate into larger deposits around portal tracts with greater inflammation, fibrosis, and proliferation of bile ducts and oval cells than seen at 8 weeks (Figure 3). Cytological changes in hepatocytes became marked with foci of chronic inflammation. By electron microscopy most hepatocytes appeared normal with only a few signs of lipid accumulation. The marked distension and inflammation of portal regions was accompanied by ingress of numerous macrophages containing lipofuscin and crystalline needles of protoporphyrin that were rarely seen in Kupffer cells. Small amounts of densely staining material were seen in the lumen of bile canaliculi and between hepatocytes (Figure 3). By 32 to 52 weeks livers became so enlarged that they accounted for up to 25% of body weight with large eosinophilic and clear cell foci, adenomas, and adenocarcinomas as reported by Libbrecht and colleagues.[15]

### Impact of Fech Mutation on Gene Expression

The expression of a wide range of genes was investigated by cDNA array analysis focusing on metabolic systems that we hypothesized might show interaction (Figure 1). As with histological assessment, few significant changes were detected in heterozygous mice carrying one copy of the *Fech* mutation and are not discussed further. A summary of the main changes in 4-week-old *Fech* homozygous mice compared with young adults (8 weeks of age) is shown in Table 2. Most changes were greater at 8 weeks than 4 weeks.

Heme and Hemoprotein Metabolism

Few consistent changes in the regulation of genes of the nonerythroid pathway of heme synthesis were detected in homozygous *Fech* mice. Thus despite the fact that hepatic ferrochelatase activity is <5% of normal in the livers of homozygous *Fech* mice,[9] this did not lead to a regulatory hepatic heme pool sufficiently lowered to cause transcriptional activation of the *Alas1* gene or increased stability mRNA.[26,27] Curiously, down-regulation of *Alas2*, which is associated with erythroid heme synthesis, was observed. The mutated *Fech* gene was not up-regulated to compensate for the low enzyme activity. There was no evidence that heme catabolism was stimulated because expression of heme oxygenase1 (*Hmox1*) was not markedly elevated.

Cytochrome P450 isoforms account for a considerable proportion of the total liver hemoprotein content. Many of the genes for major constitutively expressed isoforms such as CYP1A2, CYP2C29, and CYP2E1, were down-regulated in *Fech* mice even at 4 weeks of age (Table 2). However, the decreased expression of cytochrome P450 genes was not uniform. *Cyp3a11*, the isoform that is involved in the hydroxylation of bile acids was significantly induced by 8 weeks. The genes for cytochrome P450 reductase and cytochrome b5 were unaffected. The *Cyp3a11* gene is a target gene of the pregnane X receptor (PXR) as are a number of other genes involved in liver metabolism whose expression may be mediated in part by bile acid ligands for example *Abcb1b* (MDR1B).

Pertinent to the established disturbance of bile acid excretion in *Fech* mice was the effect on the expression of the genes for CYP7 sterol 7α-hydroxylases in bile acid synthesis. CYP7A1 enzyme catalyzes the rate-limiting initial 7α-hydroxylation step in metabolism of cholesterol in the neutral pathway with PXR, LXR, and FXR receptors implicated in the regulation of the gene.[28] A small significant increase in expression of *Cyp7a1* was observed in *Fech* mice at 8 weeks. In contrast, the *Cyp7b1* gene that codes for a CYP isoform with oxysterol 7α-hydroxylase activity that is not regulated by these receptors was >90% down-regulated in 8-week-old mice. CYP7B1 enzyme acts in the acidic pathway of bile acid synthesis that is initiated by 27-hydroxylation of cholesterol.[29] Ex-
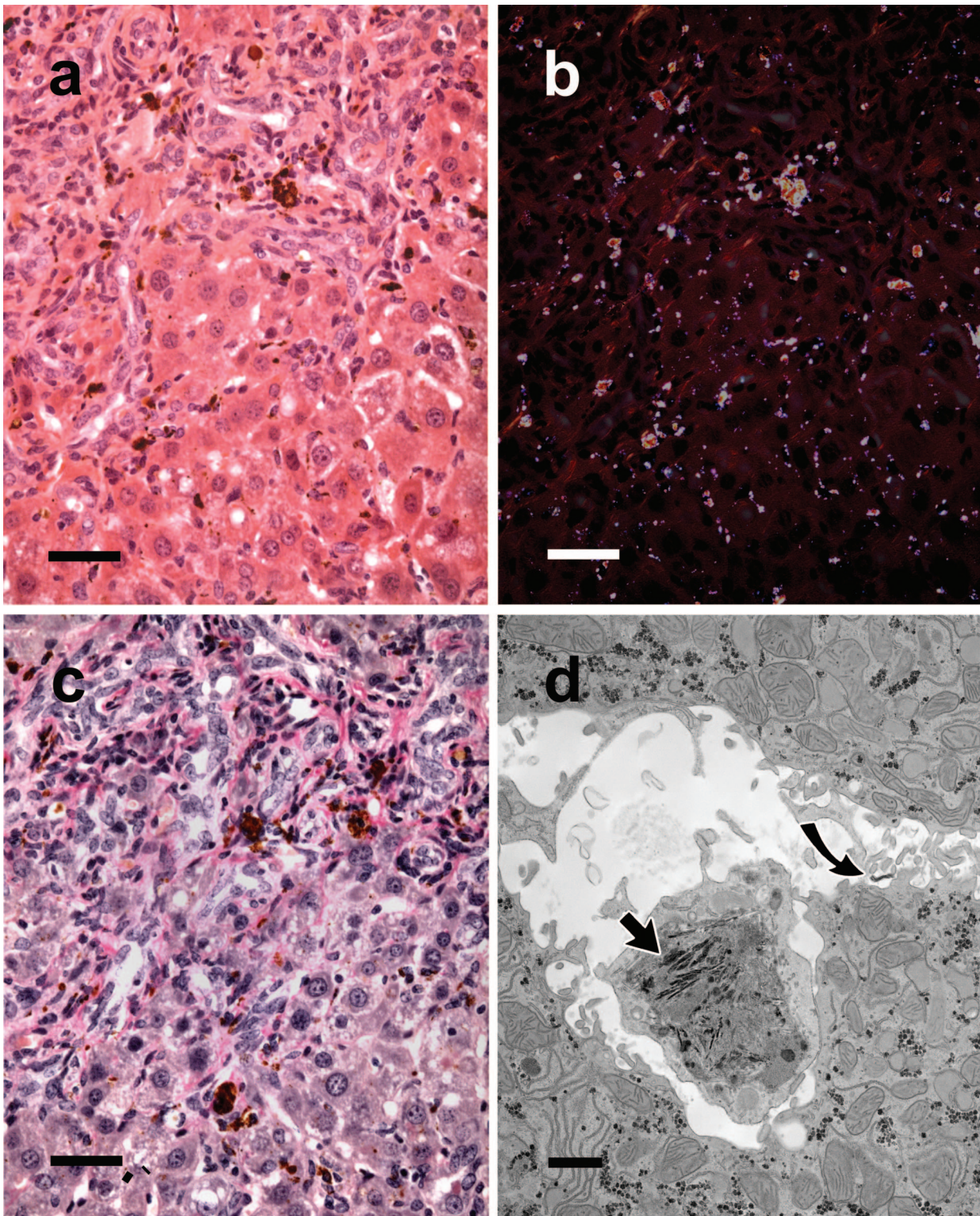
Figure 3. Microscopic examination of 13-week-old homozygous male mouse *Fech* liver. **a:** Shows a portal zone from liver with bile duct proliferation and a scattering of red-brown pigment mainly located in and around portal tracts as well as in interstitial cells of the hepatic parenchyma. **b:** Shows the same microscopic field under crossed polarizing filters revealing the wide distribution of the pigment. H&E stain. **c:** Same zone as in **a** and **b** stained for collagen. Pink collagen bands can be seen spreading from the portal zones into the hepatic parenchyma as shown by van Gieson's stain. **d:** Electron microscopy showing a macrophage containing needle-shaped crystals (**arrowhead**) in the dilated space between two hepatocytes exhibiting normal ultrastructure. Traces of a densely staining material are also evident (**curved arrow**) in the extracellular space. Scale bars: 50 μm (**a–c**); 1 μm (**d**).

pression of the *Cyp27a1* gene also became depressed (Table 2). Additionally, the mRNA level from *Cyp51*, a controlling gene of cholesterol synthesis, became significantly increased (Table 2) compatible with the elevated biliary excretion of cholesterol and total bile acids in these mice.[13]

Expression of Transport Proteins

The proposal that disruption of bile acid metabolism contributed to the mechanism of liver injury was strengthened by expression of organic anion transport proteins concerned with influx and efflux from hepatocytes and chondriocytes and consistent with bile acid-mediated genes in cholestasis.[30,31] Basolateral bile salt uptake genes *Slc10a1* (sodium taurocholate transporter or NTCP) and especially *Slco1a1* and *Slcola6* (organic anion transporters) became markedly down-regulated in *Fech* mice. In contrast, the genes *Abcb11* and *Abcc2* (BSEP and MRP2, respectively) associated with canalicular bile salt and bilirubin efflux showed only a statistically nonsignificant decrease. The *Abcb1a* gene (MDR1A) was up-regulated and the *Abcb4* (MDR2) gene unchanged, the products of which are associated with phospholipid transport and cholestasis.[32] Genes for CYP4A10 and fatty acid-binding protein are potentially co-ordinately expressed with *Abcb4* via the nuclear receptor PPARα, but were both significantly down-regulated (Table 2). Transcript profiles of apolipoprotein A- and apolipoprotein B-editing complex genes (Table 2) were markedly elevated in the livers of *Fech* mice but other apolipoprotein clones on our arrays showed no significant changes from wild-type mice (data not shown).

Oxidative Stress and Iron Metabolism

The hypothesis that oxidative stress might play a role in *Fech* liver injury (Figure 1) was supported by the array data. In particular, expression of genes for Jun, antioxidant transcription factor NFE2, glutathione peroxidase 4, glutathione transferases, glutathione synthetase, and metallothionine 1 were increased and other genes decreased as occurs in oxidative stress (Table 2). On the other hand, although heme oxygenase 1 (*Hmox1* gene) can be regulated by the anti-oxidant transcription factor NFE2, it showed little increase in *Fech* mice.

Modest effects on the hepatic expression of iron metabolism genes were detected (Table 2). In particular, synthesis of the plasma heme transporter hemopexin and ceruloplasmin, haephaestin, and NRAMP1, believed to be involved in plasma iron regulation, were significantly induced, possibly as a response to hemolysis and inflammation.

Liver Injury and Inflammation

Whatever the precise mechanism of liver dysfunction as a consequence of protoporphyrin deposition, up-regulation of many genes associated with cytokines and collagens and with lymphocytes and macrophage infil-
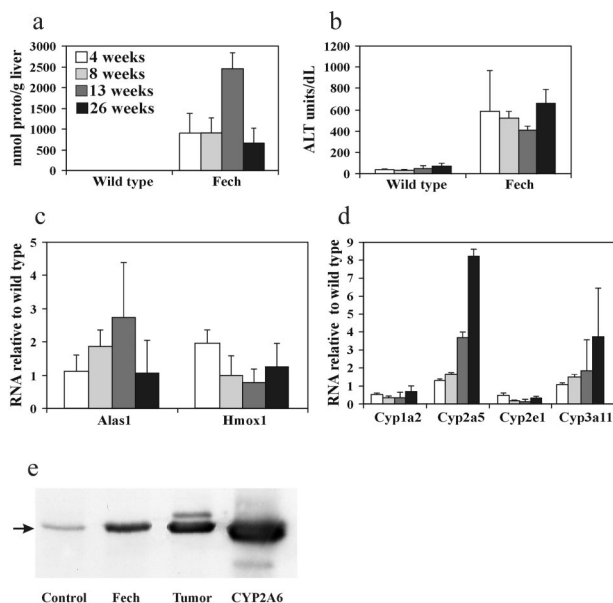

Figure 4. Phenotypic and gene expression changes in male *Fech* mice with increased age from 4 to 26 weeks compared to wild-type mice. **a:** Hepatic protoporphyrin. **b:** Plasma ALT. **c:** Comparison of *Alas1* and *Hmox1* gene expressions. **d:** Comparison of *Cyp1a2*, *Cy2a5*, *Cyp2e1*, and *Cyp3a11* gene expressions in *Fech* mice relative to wild-type mice. Values represent means ± of three to five mice. *Cyp1a2* and *Cy2e1* in *Fech* were significantly ($P < 0.05$) less than wild type at all ages. **e:** Detection of CYP2A5 protein by Western blot in liver from wild-type and *Fech* mice after 26 weeks and an example of hepatic adenoma (taken from a 42-week-old mouse) using antibody against human CYP2A6 to detect the mouse ortholog.[22] Mean increased level for *Fech* liver was 3.2 of wild-type ($n = 3$).

tration (such as *Tgm2*, *Spp1*, *Col4a1*, *Anxa5*, *Cd14*, and *Ly6d*) was consistent with observations of subsequent bile tract inflammation and proliferation (Table 2).

Modulation of Expression with Age

Previous biochemical changes in *Fech* mice have been examined in mice of an age range of 3 to 6 months.[9,13,14] Here hepatic protoporphyrin and plasma ALT levels were compared with gene expression in mice from 4 weeks (1 week after weaning) up to 26 weeks of age. Porphyrin and ALT levels were already markedly increased by 4 weeks although protoporphyrin levels were maximum at 13 weeks whereas ALT levels were sustained throughout the period (Figure 4, a and b). Despite porphyria only a small increase in *Alas1* expression was detected at even 13 weeks and little in *Hmox1* at any time (Figure 4c). In contrast, genes for major cytochrome P450 isoforms, such *Cyp2e1*, *Cyp1a2*, and *Cyp2c29* were markedly down-regulated from the earliest time and throughout the studies. Two exceptions were expressions of *Cyp3a11* and *Cyp2a5* that increased steadily in older mice (Figure 4d). The latter has been associated with porphyria, liver injury, and hepatic tumors.[33–35] Western blotting confirmed the marked presence of CYP2A5 protein in livers of 26-week mice and hepatic adenoma tissue (Figure 4e). Changes in gene expressions associated with hepatocyte bile acid up-take, lymphocyte infiltration, remodeling, cirrhosis, and proliferation in older mice were mainly significantly less or absent in the 4-week-old mice com-
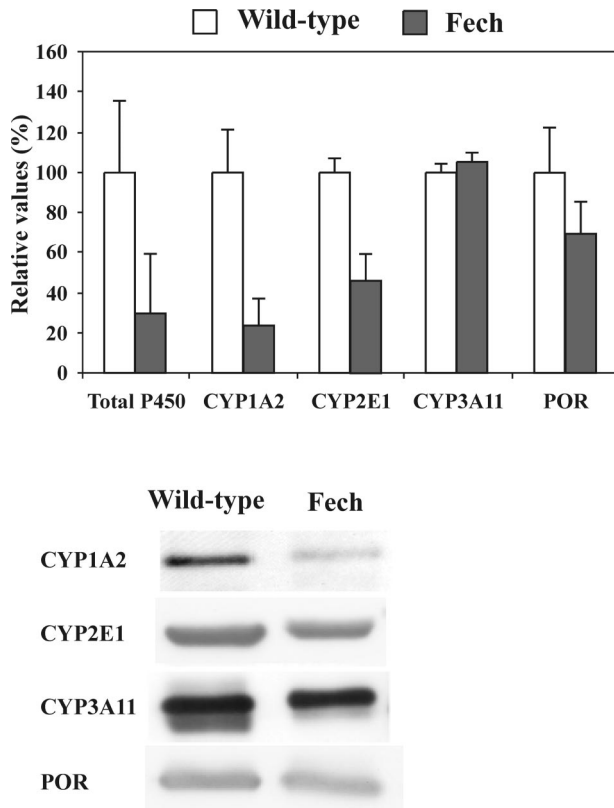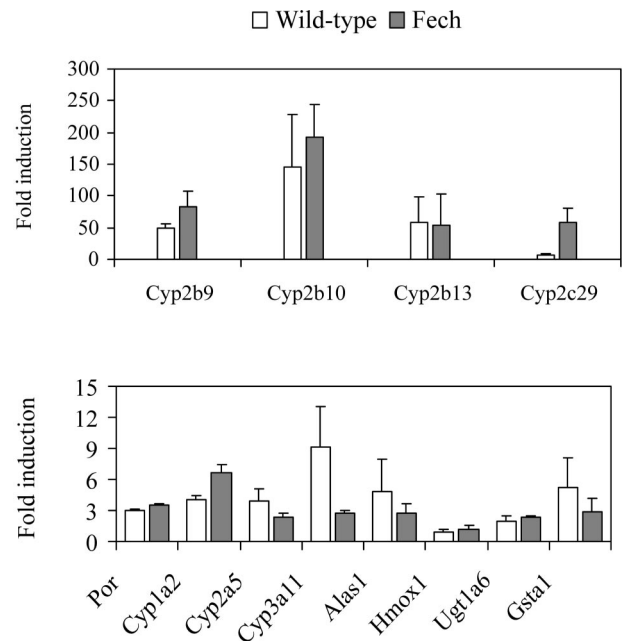
Figure 5. Comparison of levels of total cytochrome P450 and some specific isoforms in liver microsomes from 6-week-old *Fech* and wild-type BALB/c mice as assessed by Western blotting. Antibodies, sources, and standards (not shown) were as described in Materials and Methods. Anti-CYP3A11 may also detect CYP2C29. Total cytochrome P450, CYP1A2, and CYP2E1 were significantly ($P < 0.05$) different between the strains. CYP3A11 and cytochrome P450 reductase (POR) were not different.

Figure 6. Induction of expressions of hepatic cytochrome P450 2B and 5-aminolevulinate synthase 1 genes in BALB/c and *Fech* mice after exposure to the CAR ligand TCPOBOP. Mice (8 weeks old) were administered TCPOBOP (3 mg/kg) and after 3 days hepatic gene expression was determined by cDNA array analysis. No induction of heme oxygenase 1 (*Hmox1*) was detected. Full names of genes corresponding to gene symbols can be found in Table 2.

pared with 8 weeks (Table 2) and continued to be greatly altered throughout the study period consistent with the more severe pathology.

Further Studies of Heme Supply

That *Alas1* and *Hmox1* were not greatly up-regulated suggested that the supply of heme in the protoporphyric *Fech* mutant mouse was sufficient to obviate major regulatory changes in *Alas1* transcription or RNA stability[27] (Figure 1) as a consequence of early down-regulation of major cytochrome P450 isoforms. The total hepatic cytochrome P450 level of 6-week-old *Fech* mice was ~30% of that in age-matched controls (Figure 5). Western blotting confirmed significantly lower protein levels of the major hepatic isoforms CYP1A2 and CY2E1 in *Fech* mice compared with wild type. That this was not a simple sequela of general microsomal damage after liver injury was illustrated by the lack of significant effects on expression of *Cyp3a11* and cytochrome P450 reductase (Table 2 and Figure 5).

Induction of *Cyp2b* and *Alas1* genes by phenobarbital-type drugs can be effected through the CAR/PXR transcription factors independent of heme status.[36,37] After administration of TCPOBOP, a potent CAR agonist and cytochrome P450 inducer, *Cyp2b* isoforms were mark-

edly up-regulated in control and *Fech* mice (Figure 6). *Alas1* was modestly increased (but not *Hmox1*) in both strains demonstrating that in *Fech* mice the potential for up-regulation of the *Alas1* gene by this mechanism is present. The induction was lower than in the wild type possibly as a consequence of reduced CAR expression (Table 2).

Comparison of Fech and Griseofulvin Porphyric Mice

The drug griseofulvin causes protoporphyria in mice by formation of an inhibitor of ferrochelatase in the liver after cytochrome P450 induction (Figure 1).[16] When griseofulvin-treated BALB/c mice with porphyria were compared with *Fech* mice the hepatic levels of protoporphyrin (Figure 7a) and the intra- and intercellular distributions were similar[17] as were the depressed ferrochelatase activities (B. Clothier and A.G. Smith, unpublished data). However, comparison of array data from 8-week *Fech* mice (Table 2) with that generated from griseofulvin-treated BALB/c mice[17] indicated that in these two types of protoporphyria *Alas1* and *Hmox1* showed different responses. Further analysis using real-time RT-PCR demonstrated that expressions of *Alas1* and *Hmox1* in the livers of *Fech* mice were not significantly changed whereas both genes were massively induced in the livers of mice treated with griseofulvin (Figure 7, b and c). Western blotting confirmed that the level of HMOX1 protein was greatly increased after drug treatment. In addition, the level of cytochrome P450 protein of the phenobarbital-inducible
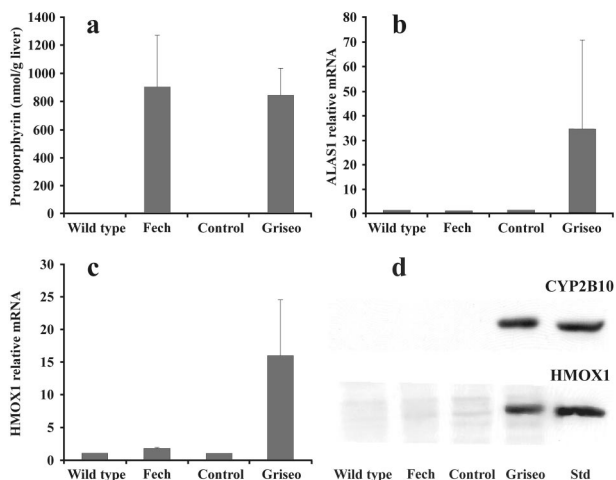
Figure 7. Comparison of hepatic 5-aminolevulinate synthase 1 and heme oxygenase 1 expression, using real-time RT-PCR, in protoporphyric homozygous 8-week-old *Fech* mice and BALB/c mice treated with griseofulvin for 3 weeks. **a:** Hepatic protoporphyrin levels. **b:** RT-PCR of *Alas1* expression. **c:** RT-PCR of *Hmox1* expression. Expression of β2-microblobulin was used as a reference. **d:** Western blotting of proteins for HMOX1 and CYP2B10 using rabbit anti-rat HMOX1 and rabbit anti-human CYP2B6 polyclonal antibodies. HMOX1 mean value was 0.96-fold of wild type for *Fech* mice and 6.6-fold of controls for griseofulvin-treated mice ($n = 3$). CYP2B10 mean value was1.2-fold of wild type for *Fech* mice and 224-fold of controls for griseofulvin-treated mice ($n = 3$). Rat protein was used as a positive control for HMOX1 and microsomes from TCPOBOP-treated mice as a standard for CYP2B isoforms, particularly CYPB10. Rat cell microsomes expressing human CYP2B6 (CYP2B10 in the mouse is one of the orthologs of this human gene[57] with a 74% protein homology) gave a single band but with a lower molecular weight (data not shown).

type CYP2B10, was highly elevated (Figure 7) in line with gene array analysis in our previous study.[17] As in other studies[38] and the *Fech* mouse (Table 2) no marked compensatory increase in ferrochelase RNA was detected (data not shown).

Discussion

Demonstration of Cholestatic Gene Expression

To our knowledge this is the first example of gene array analysis applied to a model of human porphyrias that is purely genetically derived. The development of cholestasis and liver failure in human EPP is not an automatic progression, although liver disease may develop in patients before minor clinical manifestations.[5] The precise mechanism of uptake of protoporphyrin into the liver is not understood if, as is likely, a major part originates from the hemopoietic system. Study of the *Fech* mouse should increase our understanding. After plasma transport on albumin and hemopexin,[39] uptake of protoporphyrin to the liver is thought to depend on affinity for fatty acid-binding protein in hepatocytes,[39] the expression of which is markedly down-regulated in *Fech* (Table 2) and griseofulvin-treated mice.[40] This may reflect attempts by hepatocytes to limit protoporphyrin uptake. Similarly, genes of basolateral transport proteins (*Slc10a1* and *Slco1a1*) for import of anions and bile acids from the blood into the hepatocytes became markedly down-regulated. There is evidence that export of protoporphyrin

from cells is mediated by ABC protein MDR2 (ABCB4)[3] and expression of this was increased in our studies (Table 2). Other evidence implicates ABCG2.[41] These patterns of transport protein expressions are characteristic of cholestatic experimental models with down-regulation of hepatocyte systems associated with uptake from portal blood flow, for bile acids mediated by HNF-1α and HNF-4α,[31] and less reduction or even up-regulation of transport systems associated with export.[30,42,43] How protoporphyrin causes this effect and whether it is purely physical or a result of interference in a specific signaling pathway remains to be elucidated. The marked rise in apolipoprotein IV-A expression may be an adaptive response to the elevated plasma porphyrin levels and has been reported for protoporphyric drugs.[44] It may also be linked to the rise in cholesterol synthesis. A manifestation of the homozygous *Fech* mutation mouse is hyperlipidemia and atherosclerotic lesions.[14]

Consistent with previous work[13] we found no major disturbances of the neutral pathway of bile acid synthesis from cholesterol in *Fech* mice; in fact there was a small increase in *Cyp7a1* expression. In contrast, the *Cyp7b1* gene became markedly down-regulated after 8 weeks. CYP7A1 and CYP7B1 proteins have identical sterol 7α-hydroxylase activity, but the latter acts in the acidic pathway of bile acid metabolism after 27-hydroxylation of cholesterol. In humans, a mutation of the equivalent gene is associated with accumulation of toxic sterols.[45] Down-regulation of the preceding gene of this path, *Cyp27a1* (Table 2) might be a protective mechanism, but null *Cyp27a1* ($-/-$) mice also show significant down-regulation of *Cyp7b1* and disturbance of lipid metabolism.[46] Similar changes occur after rat bile duct ligation.[47] In *Fech* mice, total bile acid excretion and bile acid levels in plasma are greatly increased.[13] Thus part of the toxic response in the protoporphyric livers is probably a consequence of disturbed bile acid homeostasis with toxic bile acids and oxysterols perhaps acting in combination with protoporphyrin.[13] If oxidized porphyrins besides protoporphyrin are formed in *Fech* livers they might contribute to the toxicity. The up-regulation of *Cyp3a11* is probably an adaptive response in cholestasis to detoxify bile salts by 6β-hydroxylation to less toxic products such as β-muricholic acid, the excretion of which by *Fech* mice rises significantly.[13,48]

The expression data also demonstrate that oxidative processes[49] could contribute to cell injury in *Fech* livers but it is not possible to conclude whether this would be a consequence of cholestasis or is initiated by protoporphyrin. The net result is inflammation with expression of genes to be expected from invading cells as well as injured hepatocytes (Table 2). The marked up-regulation of hemopexin, ceruloplasmin, NRAMP1, and haephaestin are probably part of such mechanisms to minimize heme-catalyzed free radical production because of intravascular hemolysis that leads to splenomegaly and liver inflammation.[50]

Potential for Individual Variation

One reason for the sporadic incidence of liver injury in EPP might be because of individual differences in the

mechanism of protoporphyrin uptake and export since most EPP patients have normal protoporphyrin levels and liver function.[5] Preliminary data suggest that the severity of the effects of the *Fech^m1Pas* mutation on liver injury may depend on the genetic background of the mice.[5] The present studies demonstrate a number of metabolic systems associated with protoporphyria, bile acid transport, and metabolism as well as subsequent fibrosis[51] in which genetic polymorphisms (for instance genes responsible for hepatocyte protoporphyrin import or export) might have phenotypic influence in the development of cholestasis and liver injury in different *Fech* mice and some EPP patients.

Response of Heme Metabolism

The results of this study also demonstrate that although highly porphyric, heme supply seemed sufficient in the *Fech* mouse liver to require only modest up-regulation of *Alas1* or other enzymes of the heme synthetic pathway. This might be explained by reduced heme usage for cytochrome P450 as an early response in this mutant. Decreased *Cyp1a2*, *Cyp2e1*, and *Cyp2c29* occurred before response of other genes such as *Slco1a1* and *Slco1a6* (Table 2). In addition, induction of heme oxygenase that would exacerbate heme shortage was not observed. Only modest elevation of *Alas1* in both BALB/c and the *Fech* mutant in response to the phenobarbital type cytochrome P450 inducer TCPOBOP occurred, despite large increases in *Cyp2b* expression, supporting the view that basal heme enzyme levels might be sufficient to cope with general cell function and could be up-regulated by drugs inducing cytochrome P450. In contrast, mice heterozygous for the null *Hmbs* gene (hydroxymethylbilane synthase or porphobilinogen deaminase) in heme synthesis exhibit super induction of *Alas1* expression in response to phenobarbital.[22] The difference may reflect relative activities of hydroxymethylbilane synthase and ferrochelatase in some circumstances of heme synthesis.[52] That the liver in *Fech* mice does not show marked *Alas1* and *Hmox1* induction but contains large quantities of protoporphyrin is also consistent with a significant proportion of the hepatic porphyrin originating from an erythropoietic source as is likely for EPP patients.[5,11] The detection and down-regulation of *Alas2*, the erythroid-isoform of aminolevulinate synthase, in hepatic tissue is not simple to explain but may reflect an inability of the gene in a particular cell type to be up-regulated in these mice.[53]

Comparison with Griseofulvin Protoporphyria

The present finding demonstrates that heme metabolism and catabolism in *Fech* mice differ from that seen when protoporphyria in mice is induced by griseofulvin. Hepatic ferrochelatase activities would be expected to be decreased by >90% in both *Fech* mice and those treated with griseofulvin for 3 weeks.[9,54,55] Despite equivalent levels of hepatic protoporphyrin and cholestatic injury in the two models there was a marked stimulation of cyto-

chrome P450 isoforms, *Hmox1* and *Alas1* expression after drug treatment that was not seen in the mutant mouse (Figure 7).[17,38] This induction of *Alas1* and *Hmox1* was much greater than the modest change observed with the phenobarbital-like CAR agonist TCPOBOP. Griseofulvin not only induces cytochrome P450 but also stimulates turnover of its heme moiety in a process that induces *Hmox1*.[56] Thus with griseofulvin, protoporphyria is a hepatic consequence of not only ferrochelatase inhibition but the induction and destruction of cytochrome P450 with accompanying *Alas1* derepression.[16] Furthermore in the longer term, differences between *Fech*- and griseofulvin-treated hepatic tissue may reflect adaptation of cells throughout their lifetime in the mutant compared to the more acute exposure of mice to the drug.

Summary

In conclusion, hepatic gene expression in protoporphyric *Fech^m1Pas* mice is consistent with deposition of protoporphyrin causing complex gene expressions associated with cholestasis but not with a marked depletion of the heme regulatory pool as observed with the protoporphyric drug griseofulvin.

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