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LONGITUDINAL STUDY OF A MOUSE MODEL OF FAMILIAL PORPHYRIA CUTANEA TARDA

D.D. ARCH[1], H.A. BERGONIA[2], L. HATHAWAY[1], J. P. KUSHNER[2], J.D. PHILLIPS[2,✉] AND M.R.FRANKLIN[1]

[1]University of Utah, Department of Pharmacology and Toxicology, Salt Lake City, UT 84112
[2]University of Utah School of Medicine, Department of Internal Medicine, Salt Lake City, UT 84132
✉ John D. Phillips, 5C 330 SOM, Division of Hematology 30 N. 1900 E., Salt Lake City, UT 84132
Phone #: 801-581-6650, Fax#: 801-585-3432, Email: john.phillips@hsc.utah.edu

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Abstract – Most rodent models of porphyria cutanea tarda (PCT) share in common the administration of iron and agents that induce transcription of cytochrome P450s. Dissection of changes related to porphyrin accumulation required generation of a genetic model free from exogenous precipitants. Mice heterozygous for a null *Urod* mutation and homozygous for null *Hfe* alleles spontaneously develop major increases in hepatic and urinary porphyrins several months after weaning but the high % uroporphyrin signature of PCT is established earlier, before total hepatic and urinary porphyrins rise. Total porphyrin levels eventually plateau at higher levels in females than in males. Porphyrinogens were the dominant tetrapyrroles accumulating in hepatocytes. Hepatic Urod activity is markedly reduced but total hepatic heme content does not diminish. Microsomal heme, however, is reduced and *in vitro* metabolism of prototype substrates showed that some but not all cytochrome P450 activities are reduced. High hepatic levels of uroporphyrinogen are also associated with increased glutathione S-transferase activity and elevated mRNA of 2 transporters, *Abcc1* and *Abcc4*. This murine model of familial PCT affords the opportunity to study changes in porphyrinogen and porphyrin accumulation and transport in the absence of exogenous factors that alter P450 activity and transmembrane transporters.

Key words: Porphyria cutanea tarda, mouse model, uroporphyrinogen decarboxylase, cytochrome P450, ABC transporters.

INTRODUCTION

Uroporphyrinogen, an intermediate in the heme biosynthetic pathway is normally efficiently decarboxylated to coproporphyrinogen by uroporphyrinogen decarboxylase (UROD). In patients with porphyria cutanea tarda (PCT), the presence of hepatic siderosis is associated with a marked decrease of hepatic UROD activity due to the generation of a competitive inhibitor, namely uroporphomethene (25). This causes the accumulation of highly carboxylated porphyrinogens and/or porphyrins in the liver, which then enter plasma. Porphyrinogens and porphyrins in plasma can redistribute to the skin where they are responsible for the cutaneous manifestations of PCT and are also excreted into the urine.

We and others have generated rodent models of PCT centered around the administration of compounds such as polychlorinated biphenyls (PCBs) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) that activate a battery of genes in the liver via the arylhydrocarbon receptor. The influence of porphyrins and porphyrinogens themselves on metabolic alterations in hepatocytes has been difficult to distinguish from the effects of the compounds that precipitated the porphyric state. We previously developed a genetic murine model of familial PCT (F-PCT) by crossing porphyria-susceptible *Urod*+/- mice with mice homozygous for deletion of the hemochromatosis gene (*Hfe*) (26). The *Urod*+/-, *Hfe*-/- mouse spontaneously develops uroporphyria and permits studying the effects of porphyrinogen and porphyrin accumulation on enzymes and transporters involved in tetrapyrrole disposition without the confounding effects of exogenous factors. Here for the first time, we describe a longitudinal study of the *Urod*+/-, *Hfe*-/- mouse 8 weeks to 1 year.

Abbreviations: **Gst**, glutathione-S-transferase; **PCT**, porphyria cutanea tarda; **Urod**, uroporphyrinogen decarboxylase; **Hfe**, hemochromatosis; **PCB**, polychlorinated biphenyls

MATERIALS AND METHODS

Animals

Urod +/-, *Hfe-/-* (C57BL/6J) mice were generated as previously described (26). All procedures involving animals were approved by the University of Utah Animal Care and Use Committee and were in concordance with NIH guidelines for the humane care of laboratory animals.

Liver and Urine Porphyrin Analysis

Urine samples were collected from fluid voided upon handling. Liver tissue was taken at the time of sacrifice and immediately frozen. Total porphyrin analysis of urine and liver samples was performed as previously described (7). Urine values quoted were without correction for an invariant, e.g., creatinine.

Hepatic Microsome and Cytosol Preparation

Livers were excised and the gall bladders removed. Livers were then homogenized in 0.25 M sucrose. Microsomal and cytosolic fractions were prepared by differential centrifugation (4). The protein content of both microsomes and cytosol was determined by the method of Lowry et al. (19).

Liver and microsome heme analysis

Livers were perfused in situ with ~25 ml of ice-cold saline via the portal vein prior to homogenization. The heme content of whole homogenate, and subsequently isolated microsomes was determined from the pyridine hemochromagen spectrum (35).

Hepatocyte porphyrin determination

For hepatocyte isolation, a modification of methods described by Berry and Friend (1) and Seglen (31, 32) using an *in situ* two-step EGTA/collagenase perfusion was utilized. Retrograde pefusions of livers was performed in situ via the vena cava, first with a Ca^{++}-, Mg^{++}- and indicator-free Hank's Balanced Salt Solution (HBSS; Hyclone® Laboratories, Inc., Logan UT) containing 0.2 mM EGTA (Sigma-Aldrich®, St. Louis, MO) until adequate tissue blanching occurred. This was followed by perfusion with HBSS buffer containing 100 U/ml collagenase (Type II, Worthington Biochemical Corp., Lakewood, NJ) and 2 mM $CaCl_2$ (Mallinckrodt, St. Louis, MO) until adequate tissue dissociation was apparent, usually 3-6 min. The liver (minus the gallbladder) was then macerated in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Hyclone® Laboratories, Inc., Logan UT) without phenol red or L-glutamine and with sodium pyruvate (Invitrogen™, Carlsbad, CA) supplementation. Hepatocytes were strained and washed twice. Cell yield and viability were determined by trypan blue exclusion using a hemacytometer. The cells were subjected to low-speed, iso-density Percoll (GE Healthcare Life Sciences, Piscataway, NJ) centrifugation to further eliminate non-viable cells (15). Only hepatocyte preparations with $\geq$85% viability were utilized for the porphyrin(ogen) determination assays. For this, the fluorescence (Ex 390 nm, Em 620 nm) of aliquoted cells was determined in a fluorometer (Molecular Devices, Sunnyvale, CA) before and after the addition of hydrogen peroxide (0.6% final concentration). The difference between the pre- and post-peroxide fluorescence represented the amount of porphyrinogen initially present. Fluorescence output was converted to molarity using a standard curve constructed with uroporphyrin.

Microsomal and Cytosolic assays

The microsomal cytochrome P450 concentration was quantified from the dithionite reduced ferrous heme-carbon monoxide complex using the extinction coefficient of 91 $mM^{-1}cm^{-1}$ for the 450 to 490 nm wavelength pair (23). P450 monooxygenase activities towards 7-methoxy-, 7-ethoxy- (Sigma-Aldrich®, St. Louis, MO), 7-pentoxy- and 7-benzoxyresorufin (Invitrogen™, Carlsbad, CA) and 7-ethoxytrifluoromethylcoumarin (Sigma-Aldrich®, St. Louis, MO) were determined from the rate of fluorescence increase due to the formation of the resorufin (Ex 544 nm, Em 612 nm; (27) or 7-hydroxytrifluoromethylcoumarin (Ex 409 nm, Em 550 nm) products. Bufuralol (BD Biosciences, San Jose, CA) 1'-hydroxylation and testosterone (Sigma-Aldrich®, St. Louis, MO) 6β-hydroxylation were determined by HPLC analysis of microsomal incubation extracts (5). 6β-Hydroxytestosterone was detected from the absorbance at 236 nm and 1'-hydoxybufuralol by fluorescence (Ex 285 nm, Em 310 nm). Quantification of both HPLC assays was achieved by integration of peak area and comparison with authentic metabolite standards. Microsomal NADPH-cytochrome c (P450) reductase activity was determined from the rate of reduction of cytochrome c monitored at 550 nm (21). Microsomal UDP-glucuronosyltransferase activity was determined, using detergent-activated microsomes, from the consumption of the 4-nitrophenol (4-NP) as the aglycone monitored spectrophotometrically at 417 nm (6). Cytosolic glutathione S-transferase activity was determined from the rate of formation of the 1-chloro-2,4-dinitrobenzene (CDNB) glutathione conjugate monitored at 340 nm (18).

Quantitative real-time PCR determinations

For quantitative real-time PCR (qPCR), liver samples taken at the time of sacrifice were homogenized in TRIzol® Reagent (Invitrogen™, Carlsbad, CA) and total RNA extracted using the QIAGEN® RNeasy® Mini Kit (QIAGEN, Valencia, CA). Concentration and purity of the RNA extract was determined spectrophotometrically at 260 and 280 nm, respectively, (NanoDrop, Thermo Fisher Scientific, Pittsburgh, PA) and the RNA integrity verified using the Experion™ Bio-Analyzer (Bio-Rad® Laboratories, Hercules, CA). Total RNA samples with an A260:A280 value equal to or greater than 1.8, and with a 28S:18S value equal to or greater than 1.0 were considered acceptable. Total RNA was reverse-transcribed using SuperScript™ II Reverse Transcriptase reagent (Invitrogen™, Carlsbad, CA) and qPCRs of appropriate dilutions were performed on a Roche® Lightcycler® 480 (Roche Diagnostics, Indianapolis, IN). TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA) were utilized for determining gene expression of Abcb6, Abcc1 and Abcc4. SYBR Green I Master reagent (Roche Diagnostics, Indianapolis, IN) with primers constructed with *Vector* NTI® software (Invitrogen™, Carlsbad, CA) was utilized for determining gene expression of Abcg2, Abcc2 and Abcc3. *Mus musculus* proteasome subunit, beta-type 6 (NM_008946; *Psmb6*) was used as the invariant gene for normalization (29).

Gene Microarray Analysis

For microarray analysis, two groups consisting of three animals of each genotype were utilized. Total RNA extracted from liver tissue homogenized in TRIzol® reagent was analyzed for quality and quantity using a BioAnalyzer (Agilent Technologies, Foster City, CA). Total RNA was then converted to double-stranded cDNA following priming

with an oligo-dT-T7 primer. Phenol-chloroform extracted ethanol-precipitated cDNA was transcribed *in vitro* using T7 RNA polymerase in the presence of biotinylated UTP. The resultant cRNA was purified with an RNeasy® column, eluted in H_2O and quantified by UV spectrophotometry. The resultant cRNA was fragmented following the Affymetrix protocol, added to hybridization buffer and hybridized to the Affymetrix GeneChip® microarrays. Following hybridization, washing, and staining, the array was scanned using an Affymetrix GeneChip® Scanner 3000 and the raw images converted using Affymetrix software. Data were normalized using the Bioconductor Package in the R statistical environment. The GeneSifter® software package was used for data analysis and data for enzyme activities of interest were extracted and compiled manually.

Statistical Analysis

Data are presented as mean ± SEM. Statistical analyses were performed using ANOVA, followed by Fisher's protected least significant difference multiple range test. Differences were considered statistically significant at p values <0.05.

RESULTS

Urinary porphyrins

Urinary porphyrins in mature wild type mice are 1-2 µM, with the dominant component being 6-carboxyporphyrins (Table 1). Young mice (8-11 weeks old) with the *Urod+/-*, *Hfe-/-* genotype show only slightly elevated total urine porphyrin concentrations (~3 µM), but a dramatic elevation in the proportion of uroporphyrin was observed, 40-50% in males, 25% in females (Table 1). In both sexes, the major increase in total urinary porphyrins, mostly from uroporphyrin (ogen) occurs between 10.5 and 21.5 weeks. Total urine porphyrins in males reached a maximum of approximately 11 µM. In females a maximum value of approximately 20 µM was observed. In both sexes, the urinary porphyrins of older animals was dominated by uroporphyrin.

Hepatic porphyrins

Porphyrins in *Urod+/-*, *Hfe-/-* mice showed a similar pattern with age and sex to the urine porphyrin pattern. In Immature female (6 week) animals had hepatic porphyrin levels close to those of wild type animals. Hepatic porphyrin levels in mature animals reached a plateau at 16-20 weeks with higher levels present in females (150% of those in males) (Table 2).

Porphyrinogens accumulate in the liver

Illumination of liver samples from mice with experimentally induced PCT produced intense pink/orange fluorescence due to the oxidized porphyrins present. Hepatocytes isolated from collagenase-perfused livers also fluoresced.

When intact cells were treated with hydrogen peroxide, fluorescence increased dramatically. The difference between pre- and post-peroxide fluorescence represented the amount of porphyrinogen initially present. Using this assay most (~70%) of the accumulated hepatic tetrapyrroles were present as the reduced porphyrinogens (Table 3).

Hepatic heme content

These experiments utilized saline perfused livers from female mice which contained higher porphyrin (ogen) levels than males. Total hepatic heme levels were not statistically different from wild type controls (Table 4). Heme levels in the endoplasmic reticulum fraction (microsomes), however, were significantly decreased and there was a general decrease in the level of microsomal P450 (Table 4). The decrease in microsomal heme (0.2 nmoles/mg protein) matched the decrease in total cytochrome P450. The non-P450 microsomal heme, mostly cytochrome b5, was unaltered. To determine if these results represented a global decline in all the P450s present in mouse microsomes, a slate of monooxygenase activities (many of which purported to be selective or semi-selective for subfamilies of P450), were examined in additional animals (Table 5). In this larger group of animals, total cytochrome P450 concentration was depressed by 56%. There was no shift in the absorbance maximum of the carbon monoxide (CO) complex, suggesting no selective depression of Cyp1a versus other subfamilies.

There was no significant decrease in testosterone 6β-hydroxylation and pentoxyresorufin O-dealkylation, two activities that are generally ascribed to Cyp3a and Cyp2b subfamilies respectively (Table 5). There was a significant decrease in benzoxyresorufin O-dealkylase activity, an activity generally ascribed to Cyp2bs. The lack of change for pentoxyresorufin activity would implicate differences in Cyp2bs contributing to these two reactions. Bufuralol 1'-hydroxylation is ascribed to the CYP2D family in humans and this activity showed a decrease of ~50%. Methoxy- and ethoxyresorufin O-dealkylase activities are attributed to human CYP1A subfamilies and these showed 33 and 43% decreases, respectively. The selective effect on some P450 activities and not others, and the absence of depletion of total hepatic heme (Table 4) suggest that porphyrin accumulation and/or iron

Table 1. Progression of uroporphyria in *Urod+/-, Hfe-/-* mice.

	Urine Porphyrins*	**% 8COOH**	**% 7COOH**	**% 6COOH**	**% 5COOH**	**% 4COOH**
MALE**						
Wild type						
28 (4)	1.2 ± 0.06	7.9 ± 0.7	6.3 ± 0.5	77.3 ± 0.9	1.6 ± 0.1	7.1 ± 0.4
Urod+/-, Hfe-/-						
10.5 (4)	3.05 ± 0.78	49.7 ± 8.2	9.6 ± 1.3	35.5 ± 5.8	1.5 ± 0.2	3.8 ± 0.9
21.5 (9)	7.19 ± 0.64	63.4 ± 2.9	10.9 ± 1.7	16.0 ± 1.6	2.7 ± 0.2	6.9 ± 1.0
23.5 (9)	8.22 ± 0.96	67.9 ± 1.4	9.3 ± 0.5	13.2 ± 1.0	2.8 ± 0.2	6.8 ± 0.6
30 (10)	8.62 ± 1.01	73.7 ± 3.2	7.5 ± 1.0	10.9 ± 1.2	2.1 ± 0.2	5.9 ± 1.1
35 (9)	11.22 ± 1.71	72.6 ± 2.1	7.6 ± 0.6	12.6 ± 1.3	2.2 ± 0.3	5.0 ± 0.6
41 (7)	10.80 ± 0.73	74.5 ± 1.7	7.5 ± 0.7	11.5 ± 1.0	1.9 ± 0.2	4.6 ± 0.5
FEMALE**						
Wild type						
19 (2)	1.85 ± 0.07	4.1 ± 0.5	2.6 ± 0.6	67.9 ± 5.8	1.7 ± 0.1	24.0 ± 5.8
Urod+/-, Hfe-/-						
10.5 (2)	2.84 ± 0.41	25.3 ± 8.2	9.6 ± 0.9	48.3 ± 13.1	1.7 ± 0.2	15.3 ± 4.0
21.5 (2)	19.96 ± 2.81	71.3 ± 0.3	8.2 ± 0.3	9.0 ± 0.5	2.3 ± 0.2	9.4 ± 0.2
23.5 (4)	19.25 ± 5.00	77.7 ± 1.7	6.1 ± 0.1	7.4 ± 0.9	1.7 ± 0.1	7.1 ± 0.7

* Porphyrins measured in µM. ** Age in weeks, (n) number of animals/group.

Table 2. Hepatic porphyrins in wild type and *Urod+/-, Hfe-/-* mice as a function of age.

Gender &	**Age**	**Liver porphyrins**		
Genotype	**(weeks)**	**(nmol/g liver)**	**% 8COOH**	**% 7COOH**
MALE				
Wild type	11	0.14 ± 0.05 (4)	100 ± 0	0 ± 0
	28	0.10 ± 0.02 (4)	100 ± 0	0 ± 0
Urod+/-, Hfe-/-	16	298.9 ± 75.4 (2)	91 ± 1	9 ± 1
	38	289.3 ± 16.5 (3)	85 ± 1	14 ± 1
FEMALE				
Wild type	8	0.10 ± 0.00 (2)	100 ± 0	0 ± 0
	10	0.37 ± 0.16 (6)	nd	nd
	16.5	0.58 ± 0.18 (6)	98 ± 2	2 ± 2
Urod+/-, Hfe-/-	5.5	0.63 ± 0.13 (4)	54 ± 5	46 ± 5
	20.5	454.8 ± 56.0 (5)	89 ± 3	11 ± 1
	59	406.0 ± 58.6 (6)	85 ± 2	15 ± 2

Table 3. Isolated hepatocyte porphyrin and porphyrinogen levels in *Urod+/-, Hfe-/-* mice.

Gender		**Pre-peroxide**	**Post-peroxide**	**%**
age in weeks (n)	**% viability***	**Porphyrins ****	**Porphyrins ****	**porphyrinogen**
Male, 29.7 ± 3.3 (6)	90.5 ± 1.8	5.1 ± 0.3	22.5 ± 7.6	68.7± 6.1
Female, 30.7 ± 7.5 (8)	90.3 ± 2.5	10.2 ± 3.7	36.9 ± 14.1	68.3 ± 4.8

*Cell suspension at 10^5 viable cells/ml. ** Concentration of porphyrins in nM.

Table 4. Hepatic heme and cytochrome P450 levels, and NADPH cytochrome c reductase activity in female *Urod+/-*, *Hfe-/-* and wild type-mice.

Parameter	Wild type*	*Urod+/-, Hfe-/-**	Change**
NADPH cytochrome c reductase (nmol/mg protein/min)	123 ± 6	94 ± 3	-29***
Liver heme (nmol/g wet tissue)	76 ± 13	104 ± 7	+28
Microsomal heme (nmole/mg protein)	1.14 ± 0.17	0.92 ± 0.055	-0.22***
Microsomal P450 (nmol/mg protein)	0.64 ± 0.03	0.44 ± 0.018	-0.20***
Microsomal non-P450 heme (nmol/mg protein)	0.50 ± 0.04	0.48 ± 0.04	-0.02

* (n) = 3 animals per group.
** *Urod+/-, Hfe-/-* vs. wild type.
*** Statistically significant, p<0.05.

accumulation selectively affects either rates of synthesis or degradation of specific P450s.

Transferases and transporters

To determine if porphyrin accumulation selectively affected only heme-containing enzyme activities in the liver, the activity and/or transcriptional regulation of NADPH cytochrome c reductase, two transferases and six transmembrane transporters of tetrapyrroles and other compounds were examined (Table 6 & Figure 1). There was no effect on the microsomally-located UDP-glucuronosyltransferase activity, a slight (24%) but statistically significant decline in NADPH cytochrome c reductase activity and a major increase (81%) in glutathione S-transferase activity located in the cytoplasm. The increase in glutathione S-transferase activity and the absence of a change in glucuronosyltransferase activity was mirrored in the gene array where mRNAs of multiple *Gst*s were markedly elevated (Figure 1, lines 1-6) while the mRNA of *Ugt1a6* (Figure 1, line 7) showed no change. Effects on mRNA expression of the ABC transporters, examined by qPCR, were highly variable (Table 6). Three, *Abcc6*, *Abcg2* and *Abcc3*, were not altered, two, *Abcc1* and *Abcc4* were increased (~70%) and one, *Abcc2*, was depressed (~50%).

Effects of sodium orthovanadate on porphyrin transport

Sodium orthovanadate, an inhibitor of a number of ATPases, is a potent inhibitor of ABC transporters (10, 24, 37). We suspected that ABC transporters mediated the efflux of porphyrins across the cellular membrane, based on the physiochemical properties of uroporphyrin and the finding that ABC transporters are involved in the translocation of other tetrapyrroles (13, 16, 17). A 1 mM concentration of sodium orthovanadate was incubated with isolated hepatocytes with and without electroporation, and the amount of uroporphyrin effluxed into the media evaluated hourly over 4 hours. No loss of cell viability was observed nor was there a significant decrease in porphyrin efflux (data not shown).

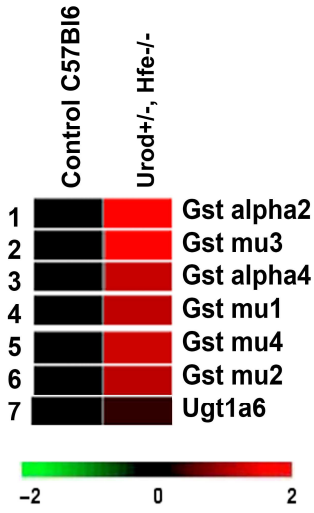

Figure 1. Expression of selected genes in wild type and *Urod+/-*, *Hfe-/-* mice. Hepatic mRNA expression from an equal pool of 3 animals per group, were hybridized to an Affymetrix 430 2.0 mouse array. Selected family members from the glutathione-S-transferases and glucuronosyltransferases are presented. The $\log_2$ scale below indicates relative expression levels.

Localization of porphyrins in hepatocytes

We utilized confocal microscopy to determine if compartmentalization of porphyrins occurred in isolated porphyric hepatocytes. Our results demonstrate that in the *Urod+/-*, *Hfe-/-* mouse model, porphyrins localize to the lysosome (Figure 2).

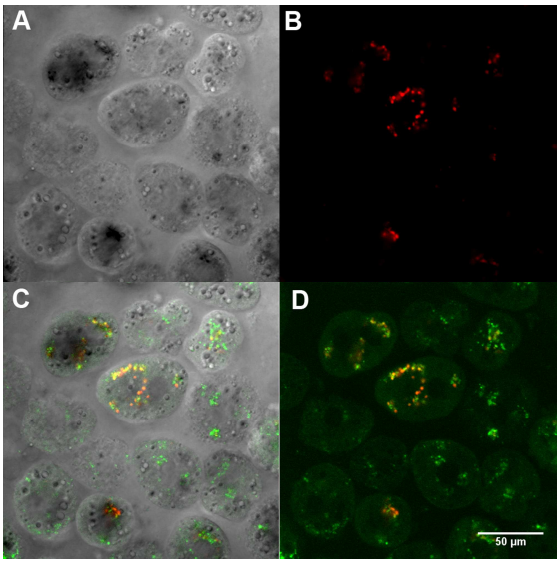

Figure 2. Localization of porphyrins to lysosomes. Isolated hepatocytes were imaged using a FV1000-XY Confocal Olympus IX81 Microscope. Panel A: transmitted light; Panel B: confocal image of oxidized porphyrins seen by exciting at 400 nM and capturing at 600 nM; Panel C: LysoTracker green overlay showing co-localization of porphyrins (yellow); Panel D: same as panel C on a dark field. White scale bar is equivalent to 50 microns.

DISCUSSION

The *Urod+/-*, *Hfe-/-* mouse, like humans with penetrant F-PCT, accumulate hepatic porphyrins. Iron is necessary as the *Urod+/-*, *Hfe+/+* has no phenotype (26). Total urine porphyrin concentration is minimally elevated in young *Urod+/-*, *Hfe-/-* mice but there is a clear shift from wild type in the pattern of accumulation favoring highly carboxylated porphyrins, indicating a further reduction in Urod activity. Once urine porphyrins have plateaued, by week 20-30, changes in the levels of hepatic porphyrins have also reached a plateau. It is not known if the pattern of urine porphyrins in humans with mutant alleles of *UROD* also changes before a clinical phenotype is evident but this might provide a method for screening asymptomatic family members. The mechanism responsible for enhanced phenotypic expression in *Urod+/-*, *Hfe-/-* female mice is not known but administration of medicinal estrogens to both men and women has been recognized as a risk factor for expression of PCT (2). In contrast, medicinal androgens have not been reported as a risk factor.

Measurements of the ratio of porphyrinogen to porphyrin accumulating in the livers of humans with PCT or in animal models of PCT have not been widely reported, due to the difficulties in isolating and quantifying reduced porphyrinogens. Using a freshly isolated hepatocyte cell system we demonstrated that most of the "total tetrapyrrole" in the porphyric hepatocyte existed as the reduced porphyrinogen (Table 3). Maintaining the reduced porphyrinogens presents a significant cellular challenge. The mRNA levels. The levels of many glutathione S-transferase (*Gst*) genes were increased (Figure 1) as were many genes in the oxidoreductase pathway, likely reflecting a response to the increased oxidative challenge of both the porphyrinogens and the excess iron. This increase in *Gst* expression is supported by a significant increase in enzyme activity (Table 6).

The role of specific cytochrome P450s in the development of PCT in humans and mice has been investigated since early rodent models mimicking hexachlorobenzene exposure (30). There is a clear role for Cyp1A2 in mice (8), but this has not been shown in humans (2). The *Urod+/-*, *Hfe-/-* genetic mouse model of PCT, as with other high iron models, made it possible to examine changes in heme and P450s without the confounding problem of transcriptional activation of the Ah-receptor battery of genes (8). First, and most apparent, is the demonstration of a slightly increased total liver heme concentration (27%), with reduced microsomal heme concentration (24%) (Table 4), along with a corresponding reduction in functional protein for cytochrome P450 activities (5-55%) (Table 5). A loss of activity resulting from mechanism based inactivation of the P450s is unlikely based on (a) no compound was administered to this genetic model for the precipitation of PCT to cause such an inactivation (22), and (b) there is no loss of free heme (20). Our results suggest that incorporation of the heme moiety into the P450 apoprotein may be inhibited by the excess of porphyrin(ogens). The mechanism of heme incorporation into cytochrome P450s has not yet been fully defined.

The role of ABC type transporters in the metabolism and compartmentalization of porphyrin(ogen)s and heme is beginning to be unraveled. Recently Abcb6, Abcg2 have been

Table 5. Hepatic cytochrome P450 activities in *Urod+/-*, *Hfe-/-* and wild-type mice.

Cytochrome P450 parameter*	**Wild type ****	***Urod+/-, Hfe-/-****	**Change**
P450 concentration (nmol/mg)	0.549 ± 0.056 (4)	0.244 ± 0.022 (5)	-56%***
P450-CO absorbance max. (nm)	450.1 ± 0.2 (4)	449.9 ± 0.4 (6)	-0.2 nm
Testosterone 6ß-hydroxylation	2837 ± 125 (5)	2564 ± 117 (6)	-10%
Pentoxyresorufin O-dealkylation	2.29 ± 0.11 (4)	2.18 ± 0.44 (6)	-5%
Benzoxyresorufin O-dealkylation	33.8 ± 3.3 (4)	15.3 ± 3.3 (6)	-55%***
Bufuralol 1'-hydroxylation	73.8 ± 7.1 (4)	37.6 ± 4.1 (5)	-49%***
Methoxyresorufin O-demethylation	78.5 ± 1.1 (5)	52.3 ± 6.5 (6)	-33%***
Ethoxyresorufin O-deethylation	32.7 ± 3.3 (5)	21.8 ± 3.3 (6)	-43%***
7-EFC- O-dealkylation****	296.7 ± 32.5 (5)	143.6 ± 19.7 (6)	-52%***

* oxidation activities are in pmol/mg microsomal protein/min.
** number (n) = number of animals per group.
*** Statistically significant $p<0.05$.
**** 7-ethoxy-4-trifluoromethylcoumarin O-deethylation

Table 6. Hepatic phase II enzyme activities and ABC transporter mRNA expression in female *Urod+/-*, *Hfe-/-* and wild-type mice.

Phase II enzyme activities:	**Wild type***	***Urod+/-, Hfe-/-****	**Change****
UDP-glucuronosyltransferase [4-NP] (nmol/mg microsomal protein/min)	1.30 ± 0.10 (4)	1.49 ± 0.09	+14%
Glutathione S-transferase [CDNB] (nmol/mg cytosolic protein/min)	2575 ± 75 (6)	4658 ± 258 (6)	+81%**
Phase III transporters:***			
Abcb6 (coproporphyrin transport)	0.804 ± 0.127	0.720 ± 0.078	-10%
Abcg2 (Bcrp) (protoporphyrin IX transport****)	0.826 ± 0.088	0.670 ± 0.044	-19%
Abcc1 (Mrp1)	1.538 ± 0.166	2.608 ± 0.308	+70%**
Abcc2 (Mrp2) (bilirubin transport)	2.706 ± 0.167	1.370 ± 0.073	-49%**
Abcc3 (Mrp3)	1.272 ± 0.125	1.252 ± 0.122	-2%
Abcc4 (Mrp4)	1.032 ± 0.061	1.732 ± 0.119	+68%**

*(n) = 5 unless otherwise indicated in parentheses.
** *Urod+/-*, *Hfe-/-* vs. wild type; changes are statistically significant, $p<0.05$.
***mRNA expression is normalized to the mRNA expression of *Psmb6*.

shown to transport tetrapyrroles (13, 16, 17, 28). Additionally Mrp2 and ABC-me have been shown to transport tetrapyrroles (11, 33). Phase III transporters measured in the Urod+/-, Hfe-/- animals showed both increases and decreases. It is not clear why there was a significant increase in expression of Abcc1 and Abcc4 expression as neither of these transporters has been shown to transport porphyrins and there have been no transporters that have been directly shown to transport porphyrinogens. Our results with suspensions of porphyric hepatocytes indicate that two transmembrane transport steps occur.

First, porphyrins are transported from the cell interior to the medium. This must occur *in vivo* as porphyrins are present in plasma and reach the kidney for excretion. Sodium orthovanadate, an inhibitor of ABC transporter activity, had no effect on cellular export of porphyrins suggesting that a transporter of another class might mediate this transport step. Feline leukemia virus subgroup C receptor (FLVCR), a member of the major facilitator superfamily, has been demonstrated to transport both heme and protoporphyrin from cytoplasm to plasma (14), raising the possibility that other symporters or antiporters might mediate transmembrane transport of uroporphyrin. Our finding that porphyrins localize to lysosomes raises the possibility that cellular export of porphyrins might not be mediated by a transmembrane transporter. Lysosomal contents can exit cells by fusion of lysosomes with the plasma membrane or by recycling of lysosomal membranes (3, 9).

Second, porphyrinogens or porphyrins are transported from cytosol to the lysosomal

compartment. Detection of porphyrins in lysosomes has been previously reported in a hexachlorobenzene-induced model of PCT in rats (36) but our data are the first to demonstrate lysosomal localization in the absence of an exogenous compound. Yeast models of porphyria created by deletion of *HEM12* the yeast gene encoding Urod, demonstrated localization of uroporphyrin in the vacuole, the yeast equivalent of the lysosome (38). Deletion of HEM15 the yeast gene encoding ferrochelatase led to accumulation of protoporphyrin in the the cytosol but not in the vacuole (38). These findings suggest the presence of a transmembrane transporter selective for highly anionic porphyrins.

Our data demonstrate that the first sign of abnormal porphyrin biosynthesis in the *Urod+/-*, *Hfe-/-* mouse occurs at 8 to 10 weeks of age, a point at which iron accumulation in the liver due to the *Hfe-/-* genotype becomes significant (7). In spite of the marked reduction of Urod activity (7), total hepatic heme synthesis remains the same as wild type but P450 heme is reduced. These data indicate, as have other models, that induction of P450 activity is not required for development of the porphyric phenotype. Although the presence of porphyrinogens in porphyric liver has been shown previously (34), amounting to 21-42% in the hexachlorobenzene rat PCT model, our peroxide treatment of hepatocytes isolated from *Urod+/-*, *Hfe-/-* porphyric mice demonstrates that it can be much higher, and that uroporphyrinogen is the dominant compound present in the liver. Since porphyrinogens never appear in the isolated hepatocyte medium, our data suggest that oxidation to uroporphyrin is a prerequisite fortransport across the cell membrane. Oxidation may also be required for transport across the lysosomal membrane. The nature of the transporters involved remains unknown but they may not be members of the ABC transporter family. The efflux of porphyrins rather than porphyrinogens leaves for further investigation the origin of porphyrinogens present in the urine (12).

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