Intrinsic Hepatic Phenotype Associated with the *Cyp1a2* Gene as Shown by cDNA Expression Microarray Analysis of the Knockout Mouse

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Several forms of cytochrome P450 (CYP) appear to metabolize principally pharmaceutical agents, as well as other dietary and plant chemicals. Other CYP forms have major roles in steroid, sterol, and bile acid metabolism. CYP1A2 expression is constitutively high in mouse liver and is well known for metabolizing several drugs and many procarcinogens to reactive intermediates that can cause toxicity or cancer. CYP1A2 is also known to carry out several endogenous functions such as uroporphyrinogen and melatonin oxidation and the 2- and 4-hydroxylations of estradiol. We have used cDNA microarray analysis of the untreated *Cyp1a2*(–/–) knockout mouse to search for changes in gene expression that might indicate important intrinsic roles for this enzyme. For 15 of the up- or downregulated genes, these increases or decreases were corroborated by reverse-transcription real-time polymerase chain reaction. Other than upregulation of the *Hprt* gene (used in the selection procedure for disrupting the *Cyp1a2* gene), we found several genes upregulated that are associated with cell-cycle regulation and lipid metabolism. Besides *Cyp1a2*, the gene exhibiting the greatest downregulation was *Igfbp1* (insulin-like growth factor binding protein-1), showing only 12% expression of that in the *Cyp1a2*(+/+) wild-type liver. Recurrent themes between both up- and downregulated genes include cell-cycle control, insulin action, lipogenesis, and fatty acid and cholesterol biosynthetic pathways. Histologically, the *Cyp1a2*(–/–) mouse exhibited an approximately 50% decrease in lipid stored in hepatocytes, and 50% increase in lipid present in interstitial fat-storing cells compared with that in the *Cyp1a2*(+/+) wild-type. These data suggest that the CYP1A2 enzyme might perform additional hepatic endogenous functions heretofore not appreciated. *Key words:* cDNA expression library; cell cycle control; cholesterol biosynthesis; CYP1A2, endogenous substrate metabolism; CYP1A2, foreign chemical metabolism; *Cyp1a2*(–/–) knockout mouse; fatty acid biosynthesis; insulin action; lipogenesis. *Environ Health Perspect* 111:855–861 (2003). doi:10.1289/txg.5925 available via *http://dx.doi.org/* [Online 20 November 2002]

During more than 2.5 billion years of evolution, it is likely that cytochrome P450 (*CYP*) genes first appeared in prokaryotes and then in early eukaryotes to carry out important roles in critical life processes; following the divergence of plants and animals some 1.8 billion years ago and especially after the radiation of innumerable phyla about 543 million years ago, animal CYP enzymes then took on the functions of metabolizing many plant products and other environmental chemicals that were consumed, inhaled, or in contact with the animal's skin (Nebert 1997; Nebert and Dieter 2000). For some CYP forms, evidence clearly indicates that their principal functions include the oxidative metabolism of endogenous molecules such as steroids, sterols, bile acids, retinoic acid, and many of the >100 eicosanoids (Nebert and Russell 2002). In addition, it is likely there are still many endogenous roles of CYP of which we remain ignorant. For instance, although CYP1A1/1A2/1B1 induction is well established in the metabolism of pro-carcinogenic polycyclic aromatic hydrocarbons and arylamines, these three enzymes are suggested to have pivotal functions in degrading the putative endogenous ligand(s) of the aryl hydrocarbon (AH) receptor and perhaps participate in apoptosis and cell-cycle regulation (Nebert et al. 2000b).

Among the few abundant CYPs that are expressed constitutively in mammalian liver, CYP1A2 carries out several known endogenous functions such as uroporphyrinogen and melatonin oxidation and the 2- and especially 4-hydroxylations of estradiol (Nebert and Russell 2002). The *Cyp1a2*(–/–) knockout mouse, however, has no apparent overt phenotype or problems with viability or fertility (Liang et al. 1996). These findings suggest that, in the absence of a foreign chemical, the expression of CYP1A2 might be redundant. In humans, CYP1A2-mediated activity varies >60-fold between individuals (Nebert 1997; Eaton et al. 1995; Nebert et al. 1996; Dorne et al. 2001), also with no overt phenotype; most of the variation does not depend on lifestyle or nutrition but is likely to be genetically determined (Le Marchand et al. 1997). It seems unlikely, however, that high constitutive CYP1A2 expression in mammalian liver continues without a particular purpose, whereas CYP1A1 expression occurs only under conditions of ligand-activation of the AH receptor (Nebert et al. 2000b). *CYP1A2* is absent in fish but present in birds and mammals, suggesting that between 380 million and 320 million years ago this gene arose from *CYP1A1* by way of a duplication event; most likely, the duplicated *CYP1A2* gene "drifted" from *CYP1A1* until it became involved in one or more critical life functions or in the metabolism of dietary components or environmental chemicals, such that the animal gained a reproductive or survival advantage (Heilmann et al. 1988; Nebert et al. 1991).

The development of mice with disruptions in specific genes allows testing of hypotheses that various CYPs might have physiologic roles not yet identified (Nebert and Duffy 1997). Many knockout mouse lines show profoundly altered phenotypes from normal, including lethality during embryogenesis if the gene participates in a nonredundant component of a metabolic pathway. In one of our laboratories we have been particularly interested in the effect of knocking out members of the *Cyp1a* family. For example, *Cyp1a1*(–/–) knockout mice are fertile and healthy and show no apparent changes in the activity or expression of other genes in the [*Ah*] gene battery, including *Cyp1a2*, following administration of AH receptor ligands (Dalton et al. 2000a). Similarly, the untreated or inducer-treated *Cyp1a2*(–/–) null mouse shows no apparent changes in expression of other genes in the [*Ah*] gene battery, including *Cyp1a1* (Liang et al. 1997). On the other hand, both *Cyp1a1*(–/–1) and *Cyp1a2*(–/–) lines show modified biochemical responses to foreign chemicals (Liang et al. 1996; Dalton et al. 2000a; Liang et al. 1997; Pineau et al. 1995; Buters et al. 1996; Peters et al. 1999;

This article was previously published in the inaugural Toxicogenomics Section of *EHP*.

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We thank B. Clothier, C. Travis, A. Andringa, and colleagues for help.

This work was funded in part by National Institutes of Health grants R01 ES06321 and P30 ES06096 (T.P.D., D.W.N.).

Received 9 August 2002; accepted 20 November 2002.

Kimura et al. 1999; Shertzer et al. 2002). *Cyp1a2*(–/–) mice metabolize zoxazolamine, caffeine, and phenacetin less extensively than *Cyp1a2*(+/+) wild-type mice and are more susceptible to some aspects of toxicity of these drugs (Liang et al. 1996; Buters et al. 1996; Peters et al. 1999). On the contrary, *Cyp1a2*-null mice are unexpectedly protected from 4-aminobiphenyl-induced tumorigenesis (Kimura et al. 1999) and 4-aminobiphenyl-induced methemoglobinemia (Shertzer et al. 2002). *Cyp1a2*(–/–) mice also appear to be completely protected from hepatic uroporphyria caused by dioxin, hexachlorobenzene, and iron overload, although metabolism of these chemicals does not seem to be involved in the mechanism of their toxicity (Sinclair et al. 1998; Sinclair et al. 2000; Smith et al. 2001); in these cases, CYP1A2 appears to be functioning in a mode that does not involve production of a reactive metabolite. Other studies suggest that CYP1A2 might have a role in bilirubin metabolism (Zaccaro et al. 2001). It has been proposed that CYP1A1 metabolizes an endogenous substrate that modulates AH receptor-mediated gene expression and that, in the liver, CYP1A2 can take over this role (Nebert et al. 2000b). Since there is no constitutive expression of *Cyp1a1* in the liver, it would be interesting to investigate whether nonpathologic changes in hepatic metabolism might occur in the complete absence of constitutive CYP1A2. We have thus used cDNA microarrays to compare hepatic gene expression differences between the untreated *Cyp1a2*(–/–) knockout and the *Cyp1a2*(+/+) wild-type mouse.

Materials and Methods

The* Cyp1a2(–/–) *mouse line. Knockout mice had been generated by removing portions of exons 2 and all of exons 3–5 of the *Cyp1a2* gene; insertion of the *Hprt* minigene cassette was used for selection in embryonic stem (ES) cell cultures (Liang et al. 1996). Although the mice were originally generated from a mixture of the C57BL/6J and 129/J inbred strains, the *Cyp1a2*(–/–) genotype was subsequently backcrossed into the C57BL/6J strain to a theoretical level of >99.8%; if the mouse genome contains 40,000 genes, this would mean that the *Cyp1a2*(–/–) line in the Nebert mouse colony should have fewer than about 80 genes that might be expected to be of 129/J origin (Nebert et al. 2000a). For this reason, C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME, USA) were used as the *Cyp1a2*(+/+) controls in the present experiments. We used untreated males, approximately 10 weeks of age, in the microarray studies; females and males were compared in the histologic studies.

Mouse EST clones and preparation of cDNA. The arrays comprised 4,246 mouse expressed-sequence-tag (EST) clones (2,783 individual Genbank clusters). Two-thirds of the clones were obtained from the I.M.A.G.E. collections held at the MRC Human Gene Mapping Project (*http://www.hgmp.mrc.ac.uk/*). The remaining one-third of the EST clones were obtained from Research Genetics (RG9 set; *http://www.resgen.com*). All clones described in this article were verified by sequence analysis. cDNA from the EST was obtained via polymerase chain reaction (PCR) amplification using plasmid-specific primers. The PCR products were separated by electrophoresis on agarose gels to ensure that only a single product was obtained for each clone. The reaction products were precipitated and prepared for array, using methods described (DeRisi et al. 1997; Eisen and Brown 1999).

Printing of the arrays. Arrays were printed on poly-L-lysine-coated slides, UV-cross-linked, and blocked prior to use (DeRisi et al. 1997; Eisen and Brown 1999; Turton et al. 2001). The arrays were printed using an arrayer built essentially according to the Stanford designs (*cf. http://www.le.ac.uk/cmht/microarray_lab/Home.htm*). The center-to-center distance of the features was 210 µm, and each feature was 90–100 µm in diameter.

Labeling and hybridizations. Total RNA was prepared from mouse liver by sedimentation through CsCl. The RNA of five individual *Cyp1a2*(+/+) wild-type mice and five individual *Cyp1a2*(–/–) knockout mice were each separately labeled with both Cy3 dUTP and Cy5 dUTP. RNA labeling was carried out essentially as described (DeRisi et al. 1997; Eisen and Brown 1999; Turton et al. 2001). Priming was achieved with the oligo $dT_{(25)}$, using 4 µg of the oligo with 50 µg of total RNA. After denaturation at 70°C for 8 min, annealing was allowed to occur as the temperature fell to 42°C over 30 min. At this point, dNTPs (Pharmacia/Amersham, Bucks, UK) were added to final concentrations of 0.5 mM, with the exception of dTTP, which was at 0.2 mM. The desired Cy-labeled dUTP (Pharmacia/Amersham) was then added to a final concentration of 0.1 mM. We used the 1X first-strand buffer (Gibco/Invitrogen, Paisley, Scottland). RNAsin (20 U) was added to the reaction. Transcription was initiated by addition of 100 U of Superscript II (Gibco/Invitrogen) and allowed to proceed for 1 hr at 42°C before addition of a second 100 U of Superscript II and another 1-hr incubation at 42°C. RNA was removed from the synthesized cDNA by addition of NaOH/EDTA/sodium dodecyl sulfate (SDS) to final concentrations of 0.195 M/10 mM/0.22%, respectively, and incubated at 70°C for 10 min. The reaction was neutralized by addition of HCl and buffered to pH 7.5 by the addition of Tris–HCl. The reaction products were purified by passage through a Centri-Sep column (Princeton Separations, Inc., Adelphi, NJ, USA), dried, and resuspended in hybridization buffer. Prior to hybridization, the samples were heated to 100°C for 2 min, then at 42°C for at least 30 min.

Hybridizations were conducted in humidified chambers at 42°C for approximately 16 hr. The hybridization buffer consisted of 50% deionized formamide/0.5% SDS/6× SSPE (1 M NaCl/0.33 mM NaH_2PO_4/6.6 mM EDTA, pH 7.4) and 2.5× Denhardt's/0.06 µg/µL of $polyA_{(80)}$/0.66 µg/µL human Cot 1 DNA/0.27 µg/µL yeast tRNA. The arrays were washed in 1.0× SSC (0.15 M NaCl/0.015 M Na citrate, pH 7.0), 0.03% SDS for 10 min, then washed in 0.2× SSC for 5 min, and a final wash in 0.05× SSC for 5 min at room temperature. The slides were dried by centrifugation at 800 rpm for 5 min. For each pair of *Cyp1a2*(+/+) and *Cyp1a2*(–/–) mice, the Cy3-labeled cDNA product was hybridized against the Cy5-labeled cDNA product, and vice versa—giving 10 separate hybridizations from the five pairs of animals being compared.

Analysis of fluorescence and data processing. The fluorescence of all the features on the slides was measured using the GenePix software (version 3.0.0.85; Axon Instruments, Union City, CA, USA). Feature sizes were determined using the inbuilt automated parameters in the first instance and then adjusted manually where appropriate. The fluorescence of each pixel within the feature was determined, and the median fluorescence of these pixel measurements was taken as the measure of fluorescence for the whole feature. The local background fluorescence was measured using the default GenePix parameters. The raw feature data for each channel were globally centered by reference to the median fluorescence of the whole feature set for that channel. The changes in gene expression obtained are shown as means ± standard deviations of the ratio between the Cy3 and Cy5 channels for 10 pairs of hybridizations.

Reverse-transcription real-time PCR using SYBR Green. For an alternative estimation of changes in gene expression, the relative mRNA levels of genes of interest were determined by comparison with a

mouse endogenous control gene, β-actin (*Actb*). The primers used (Table 4) were designed to cross exon-exon boundaries to eliminate the detection of any contaminating genomic DNA.

In the first step, cDNA was synthesized from total RNA. A 1,450-µL master mix was made up as follows: 200 µL PCR buffer, (catalog no. Y02028; Gibco/Invitrogen); 100 µL $MgCl_2$ (50 mM); 20 µL each of dATP, dCTP, dGTP, dTTP (all 100 mM); 19.8 µL random hexamers (catalog no. 27-2166-01; Amersham; 90 OD U/mL); dithiothreitol (Gibco 400147); and 1,030.2 µL water. For cDNA synthesis, a 40-µL reaction contained: 29 µl of the master mix; 8 µL total RNA (400 ng); 40 U RNAsin (catalog no. N211B; Promega, Southampton, UK); and 400 U SuperScript II reverse transcriptase (Gibco/Invitrogen). The mixture was heated for 10 min at 23°C, then for 30 min at 42°C, and finally for 10 min at 99°C. In the second step, 1 µL of the product of the cDNA synthesis (from 10 ng RNA), or a non-template control, was incubated with 24 µL SYBR Green PCR Master Mix (part no. 4309155; Applied Biosystems, Warrenton, UK) containing 900 nM forward primer and 300 nM reverse primer in an ABI PRISM 7700 Sequence Detection System. The thermal-cycler protocol was stage one, 50°C for 2 min; stage two, 95°C for 10 min; stage three, 40 cycles at 95°C for 15 sec and 60°C for 1 min. For every sample of *Cyp1a2*(+/+) and *Cyp1a2*(–/–) liver, the relative level of each gene examined was compared with that for β-actin. A comparison was then made for the expression of the gene between the wild-type and the knockout animal.

Histologic analysis of liver. Liver was prepared for routine light- and electron-microscopic histology and morphometry (Dalton et al. 2000b). Twenty-eight *Cyp1a2*(–/–) and 28 *Cyp1a2*(+/+) mice were used, with equal numbers of males and females (8–10 weeks of age). Phase-contrast microscopy of toluidine blue-stained 1.5-micron–thick plastic sections was used to quantify the relative amounts of parenchymal and interstitial cells and to determine the volume density of hepatocyte and of interstitial fat, glycogen pools, and necrosis. A grid of 75 intersections was visualized over the light-microscopic image using a Zeiss Photomik light-microscopy (Carl Zeiss GmbH, Vienna, Austria) and camera lucida, and the number of positive intersections lying over hepatocytes, interstitial cells, lipid, and glycogen was counted. The volume density (Vd) of each parameter was determined by dividing those values by the total number of positive intersections lying over the entire tissue.

Statistical analysis of microarray data was performed using the two-tailed paired t-test, taking the reference ratio for the population as 1.0 and comparing this with the ratio change. Means and standard-errors-of-the-mean were obtained from morphometric data, using the General Linear Model of SAS 6.1 (SAS Institute, Inc., Cary, NC, USA). A $p < 0.05$ value was regarded as statistically significant.

Results

cDNA expression microarray. To investigate potential biochemical phenotypic differences, we compared constitutive hepatic gene expression of *Cyp1a2*(–/–) mice with that of aged-matched *Cyp1a2*(+/+) mice, using cDNA microarrays. Labeling of samples with both Cy3 and Cy5 dyes (reverse-labeling) was performed to take into account any methodologic bias. Our information was thus based on 10 separate hybridizations from five mice. Although it is often customary in array work to use an arbitrary cut-off at a 2-fold change in expression, we believe that <2-fold alterations can be important in critical-life-process pathways and therefore chose to list all expressions that had changed significantly as assessed statistically at $p < 0.05$ (Tables 1, 2). Only a relatively few genes were detected that exhibited significant up- or downregulation. With the particular array used, we found a greater number of downregulated than upregulated genes in the *Cyp1a2*(–/–) mouse.

The >5-fold elevation in *Hprt* gene expression (Table 1) is likely explained by the *Hprt* minigene cassette used in the selective elimination to disrupt the *Cyp1a2*

Table 1. Hepatic genes significantly expressed to a greater extent in *Cyp1a2*(–/–) compared with that in *Cyp1a2*(+/+) mice.

Description[a]	Symbol[a]	Accession no.[a]	Mean[b]	SD
Hypoxanthine guanine phosphoribosyltransferase	*Hprt*[b]	BF148132	**5.38**	1.80
Growth arrest and DNA-damage-inducible 45 gamma	*Gadd45g*	AI662702	**3.16**	1.06
Cytochrome P450, 2a4[c]	*Cyp2a4*	AI118260	**1.94**	0.50
Major urinary protein-1	*Mup1*	AI225954	**1.89**	1.06
RIKEN cDNA 0610010A22 gene	0610010A22Rik	AA198519	**1.85**	0.52
Cytochrome P450, steroid inducible 3a11	*Cyp3a11*	AI255364	**1.67**	0.75
E4F transcription factor-1	*E4f1*	AA270628	**1.65**	0.76
Cytochrome P450, 7b1	*Cyp7b1*	AI287063	**1.64**	0.89
Glutathione *S*-transferase, pi 2	*Gstp2*	AA152555	**1.59**	0.46
Ferritin light chain-1	*Ftl1*	AA059749	**1.61**	0.70
NADPH-P450 (cytochrome) oxidoreductase	*Por*	BI144049	**1.53**	0.38

[a]From GenBank database (*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene*). [b]Ratio of expression in the *Cyp1a2*(–/–) compared with that in the *Cyp1a2*(+/+) mouse. Results are means of five mice per line and significantly different at $p < 0.05$. [b]This may arise, in part or in total, from the HAT-selective minigene used for the selection of the transfected ES cells. [c]Also probably recognizing CYP2A5 cDNA and any other mouse CYP2A cDNA.

Table 2. Hepatic genes significantly expressed to a lower degree in *Cyp1a2*(–/–) compared with that in *Cyp1a2*(+/+) mice.

Description[a]	Symbol[a]	Accession no.[a]	Mean[b]	SD
Cytochrome P450, 1a2, aromatic compound inducible	*Cyp1a2*	AA242360	**<0.01**	<0.01
Insulin-like growth factor binding protein-1	*Igfbp1*	W10866	**0.12**	0.08
G0/G1 switch gene-2	*G0s2*	NM_008059	**0.20**	0.09
Fatty acid synthase	*Fasn*	BF531259	**0.27**	0.15
Cytochrome P450, 4a14	*Cyp4a14*	AI893426	**0.28**	0.26
Stearoyl-coenzyme A desaturase-1	*Scd1*	AA269438	**0.33**	0.17
3-Hydroxy-3-methylglutaryl–coenzyme A reductase	*Hmgcr*	W18347	**0.36**	0.25
Glucokinase	*Gk*	AI194797	**0.38**	0.18
Fatty acid-binding protein 2, intestinal	*Fabp2*	AA596309	**0.40**	0.09
Similar to tyrosine aminotransferase		AA066836	**0.41**	0.22
Cytochrome P450, 4a10	*Cyp4a10*	AB018421	**0.42**	0.15
UDP-glucose ceramide glucosyltransferase	*Ugcg*	AA116263	**0.45**	0.28
Protein tyrosine phosphatase, nonreceptor type 16	*Ptpn16*	AI528560	**0.45**	0.37
Glutamyl aminopeptidase	*Enpep*	AA170635	**0.45**	0.33
Phosphoenolpyruvate carboxykinase-1, cytosolic	*Pck1*	AA063800	**0.47**	0.34
Pre-B-cell leukemia transcription factor 1	*Pbx1*	W85267	**0.48**	0.39
Apolipoprotein A-IV	*Apoa4*	AI385459	**0.54**	0.15
Serum amyloid P-component	*Sap*	AI157330	**0.57**	0.27
Pyruvate dehydrogenase E1alpha subunit	*Pdha1*	AW106750	**0.58**	0.19
Farnesyl diphosphate synthetase	*Fdps*	BE986882	**0.58**	0.25
Glutamate dehydrogenase	*Glud*	W09330	**0.61**	0.37

[a]From GenBank database (*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene*). [b]Ratio of expression in the *Cyp1a2*(–/–) compared with that in the *Cyp1a2*(+/+) mouse. Results are means of five mice per line and significantly different at $p < 0.05$.

(Liang et al. 1996), or any other gene (van der Lugt et al. 1991), in ES cells. The *Gadd45g* gene, the *Sprlc1* gene, and the *Cyp2a4/2a5* gene(s) were upregulated 3.2-, 2.4- and 1.9-fold, respectively. In addition to the marked decrease in *Cyp1a2* gene expression (Table 2), as expected, several other genes were downregulated by 2.5-fold or more. These included the *Igfbp1, G0s2, Fasn, Cyp4a14, Scd1, Hmgcr, Gk* and *Fabp2* genes. Recurrent functional themes among both these up- and downregulated genes include cell-cycle control, insulin action, lipogenesis, and fatty acid and cholesterol biosynthetic pathways.

Besides *Cyp1a2*, there were at least three other mouse *Cyp* genes upregulated (Table 1) and two others downregulated (Table 2). We also documented in our microarray that the expression of at least nine other mouse *Cyp* genes was not significantly altered (Table 3). Expression of genes encoding the AH receptor and both forms of heme oxygenase was also not significantly changed (Table 3).

Reverse-transcription real-time PCR. To prove the accuracy of the cDNA microarray, we chose 15 of the genes that had exhibited significant differences in expression between the genotypes (in Tables 1, 2) for further analysis by reverse-transcription real-time PCR, and we used *Actb* expression as a reference "housekeeping" gene. For the 6 upregulated and the 9 downregulated genes investigated (Table 4), there was good agreement with those changes that had been observed using the cDNA arrays. The data in Table 4 thus confirm the robustness of the cDNA expression microarray approach.

Histology and morphometry. Conducting histology on mouse liver several years ago, it had independently been noted in one of our laboratories that the lipid-containing cells differ between *Cyp1a2*(–/–) and *Cyp1a2*(+/+) mice (Figure 1). Morphometric analysis of 28 *Cyp1a2*(–/–) and 28 *Cyp1a2*(+/+) mouse livers corroborated that these lipid-storage differences are consistent and statistically significant (Table 5). The Vd of hepatic glycogen pools was slightly but not significantly greater in the *Cyp1a2*(–/–) mouse. The increase in *Cyp1a2*(–/–) interstitial fat accounted for a small increase in the Vd of interstitium overall, which had a p value of 0.089. Total lipid stores were significantly (p = 0.035) decreased in the *Cyp1a2*(–/–) liver compared with that in *Cyp1a2*(+/+) liver. This decrease principally reflected a Vd of hepatocyte-containing lipid droplets in *Cyp1a2*(–/–) liver that was approximately 55% of that seen in *Cyp1a2*(+/+) liver. On the other hand, there was a 45% increase in the Vd of lipid found within the *Cyp1a2*(–/–) interstitial fat-storing cells compared with that in *Cyp1a2*(+/+) liver. Gender influenced the response, with females showing a greater increase than males in fat stored in interstitial cells; in retrospect, therefore, it might have been worthwhile in the microarray experiments to include comparisons between males and females.

There were no significant differences in hepatocyte necrosis, inflammatory infiltrate, binucleated hepatocytes, or number of apoptotic hepatocytes that could be attributed to the loss of CYP1A2. The mitotic index in *Cyp1a2*(–/–) liver was slightly elevated but not statistically significant compared with that in *Cyp1a2*(+/+) liver (p = 0.16).

Table 3. Hepatic *Cyp* and other possibly relevant genes whose expression is not significantly different between the *Cyp1a2*(–/–) and *Cyp1a2*(+/+) mice.

Description[a]	Symbol[a]	Accession no.[a]	Mean[b]	SD
Cytochrome P450, 17	*Cyp17*	AA097768	**0.80**	0.19
Cytochrome P450, 2a12	*Cyp2a12*	AA255330	**1.00**	0.16
Cytochrome P450, 2b10, phenobarbital-inducible, type b	*Cyp2b10*	AI196037	**1.02**	0.20
Cytochrome P450, 2c29	*Cyp2c29*	AI529126	**1.10**	0.37
Cytochrome P450, 2d9	*Cyp2d9*	AA986388	**0.94**	0.15
Cytochrome P450, 2e1, ethanol-inducible	*Cyp2e1*	AA717630	**0.92**	0.43
Cytochrome P450, 2f2	*Cyp2f2*	AA242452	**1.17**	0.31
Cytochrome P450, 2j6	*Cyp2j6*	AI323934	**0.72**	0.21
Cytochrome P450, 3a25	*Cyp3a25-pending*	AA061713	**1.15**	0.37
Aryl-hydrocarbon receptor	*Ahr*	AA274836	**1.19**	0.37
Heme oxygenase (decycling)-1	*Hmox1*	W08092	**1.30**	0.16
Heme oxygenase (decycling)-2	*Hmox2*	AI428016	**1.00**	0.23

[a]From GenBank database (*http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene*). [b]Ratio of expression in the *Cyp1a2*(–/–) compared with that in the *Cyp1a2*(+/+) mouse. Results are means of five mice per line and not significantly different ($p > 0.05$).

Discussion

The data in this study strongly suggest that mouse hepatic CYP1A2 is involved in a number of previously unrecognized endogenous functions. These findings are in contrast to popular opinion that CYP1A2 exists solely for the metabolism of pharmaceuticals and other environmental chemicals.

Table 4. Real-time PCR changes in expression of some hepatic genes in *Cyp1a2*(–/–) compared with that in *Cyp1a2*(+/+) mice.

Expression	Mean[a]	SD	Forward primer	Reverse primer
Upregulation				
Gadd45g	**4.26**	2.57	GACCGCTGGCGTCTACGA	GCACGCAAAAGGTCACATTGT
Cyp2a4/2a5	**2.40**	0.97	TCGAGGAGCGCATCCAA	GACTGTTCGGCTAAGGTAGAAGGT
Mup1	**1.72**	0.58	GGGAAACCTTCCAGCTGATG	GGATTCCATGCTCCTCACATAGT
Cyp3a11	**1.36**	0.55	TTAAGAATGTGCTAGTGAAGGAATGTTT	CATCCTTAGATATTGAGATAGCTTTACTCATTA
Cyp7b1	**1.42**	0.54	GCTCTCGGCCCTGTTCCT	GGGCCATGCCAAGATAAGG
Por	**1.21**	0.34	GCTGCAGGCCCGCTACTA	CGCAGATGTGCACGGAGTT
Downregulation				
Igfbp1	**0.09**	0.05	CCATCAGCACCTATAGCAGCAT	ATTTGTAGATTTCATCTCCTGCTTTCT
G0s2	**0.13**	0.03	AACGCCAAAGCCAGTCTGA	CCTTGGCCAGAGGGATCA
Fasn	**0.21**	0.10	CATTGGTGGTGTGGACATGGT	GACCGCTTGGGTAATCCATAGAG
Cyp4a14	**0.11**	0.12	CAAGACCCTCCAGCATTTCC	GCTCCCCGAGAGACACTGTAA
Scd1	**0.24**	0.10	CAACACCATGGCGTTCCA	GGTGGGCGCGGTGAT
Hmgcr	**0.37**	0.25	CGAGGAAAGACTGTGGTTTGTG	CGTCAACCATAGCTTCCGTAGTT
Gk	**0.33**	0.05	GCACACGTGGTGCTTTTGAG	GCCTTCGGTCCCCAGAGT
Fabp2	**0.43**	0.13	CCTAGAGACACACACAGCTGAGATC	CCAAGCTTCCTCTTCATCACATT
Pck1	**0.53**	0.27	TGTCGGAAGAGGACTTTGAGAAA	TGCTGAATGGGATGACATACATG

[a]Ratio of expression in the *Cyp1a2*(–/–) compared with that in the *Cyp1a2*(+/+) mouse, relative to that of *Actb* (forward primer, GATTACTGCTCTGGCTCCTAGCA; reverse primer, GCCACCGATCCACACAGAGT). Results are means ± SD of three to four *Cyp1a2*(–/–) and three to four *Cyp1a2*(+/+) mice.

Cell cycle control. The >3-fold upregulation of the *Gadd45g* gene in *Cyp1a2*(–/–) mice suggests there is some cellular stress (Fornace et al. 1989), perhaps in genomic stability, or alterations in cell cycle control that might occur in the absence of hepatic CYP1A2 constitutive expression; GADD45γ is also associated with apoptosis (Hollander et al. 2001), though this was not reflected in an increase in apoptotic hepatocytes. This marked increase in *Gadd45g* expression could be related to disruption of the *Cyp1a2* gene directly, or to some secondary response caused by the complete absence of CYP1A2 activity. It might also be pertinent that there is a 5-fold downregulation of the G0/G1 switch gene-2 (*G0s2*) (Table 2) and a 65% increase in the E4F1 transcription factor (*E4f1*) gene (Table 1), both involved in cell-cycle regulation. Likely reasons for CYP1A2 involvement in oxidative stress, cell cycle control, and apoptosis have been recently reviewed (Nebert et al. 2000b). Histologically, however, the mitotic index in the *Cyp1a2*(–/–) mice is only slightly elevated (not statistically significant), and the percent binucleated hepatocytes (which reflects hepatocyte hypertrophy and karyokinesis without cytokinesis) in *Cyp1a2*(–/–) is almost identical to that in the *Cyp1a2* (+/+) wild type.

Insulin action, lipogenesis, and fatty acid and cholesterol biosynthetic pathways. Other than *Cyp1a2*, the gene exhibiting apparently the greatest downregulation was *Igfbp1* (insulin-like growth factor binding protein-1), which showed only 12% expression of that in the *Cyp1a2*(+/+) wild-type mouse (Table 2). Again, this may be linked to an overall downregulation of cell proliferation or alterations in lipid metabolism. The *Igfbp1* promoter shares common insulin regulatory response elements with the phosphenolpyruvate carboxykinase (*Pck1*) gene, which catalyzes the rate-limiting and committed step in gluconeogenesis, although different mechanisms are used by insulin to inhibit expression of these two genes (Yeagley et al. 2001). *Pck1* was downregulated more than 2-fold in the *Cyp1a2*(–/–) mouse (Table 2). Interestingly, glucokinase, another gene associated with insulin action, was also downregulated. In hepatocytes, insulin stimulates a rapid increase in transcription of the gene coding for sterol response-binding protein-1c, which has been implicated in the expression of lipogenic genes; moreover, cholesterol synthesis genes in hepatocytes include fatty acid synthase (*Fasn*), steroyl-coenzyme A desaturase (*Scd1*), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*Hmgcr*), glucokinase (*Gk*), and farnesyl diphosphate synthase (*Fdps*) (Yeagley et al. 2001; Joseph et al. 2002). The expression of these five genes was significantly decreased in *Cyp1a2*(–/–) mouse liver (Table 2). The 1.6-fold increase in *Cyp7b1* (Table 1), and the downregulation of fatty acid-binding protein-2 (*Fabp2*) and apolipoprotein A-IV (*Apoa4*) (Table 2), provide further evidence of the possible involvement of CYP1A2 in fatty acid and cholesterol pathways. This possible perturbation of fatty acid and cholesterol pathways might be reflected in the histologic assessment (Table 5) in which the *Cyp1a2*(–/–) mouse liver has less volume density of lipid within its hepatocytes, concomitant with increases in lipid in the fat-storing cells in its interstitium compared with that in the *Cyp1a2*(+/+) wild-type liver.

Cyp gene expression. As expected, the signal for *Cyp1a2* gene expression was markedly decreased by >99% in the *Cyp1a2*(–/–) compared with that in the *Cyp1a2*(+/+) mouse (Table 2). Since the *Cyp1a2* gene is constitutively quite highly expressed in wild-type mice, it was of interest to see whether there might be compensatory expression of any other CYP enzymes. The upregulation of *Cyp3a11* (Table 1) and the downregulation of *Cyp4a10* and *Cyp4a14* (Table 2) might be related to alterations in the arachidonic acid cascade or other physiologic homeostasis (Nebert and Dieter 2000; Nebert and Russell 2002) in the absence of CYP1A2. The ESTs for each of the two *Cyp4a* genes would have detected either cDNA and, thus, these two could not be properly distinguished—although differences in degree of upregulation were seen.

The greatest difference (1.94-fold upregulation) was observed for *Cyp2a4* (Table 1). Although this gene encodes steroid 15α-hydroxylase in the synthesis of testosterone and estradiol, both CYP2A4 and CYP2A5 have been shown to be modulated by circadian rhythm (Lavery et al. 1999; Akhtar et al. 2002). CYP2A5 has coumarin 7-hydroxylase activity and metabolically activates many chemicals such as nitrosamines and aflatoxin that are known hepatic carcinogens in mice (Negishi et al. 1989; Camus-Randon et al. 1996). CYP2A5 induction differs from most because it seems to occur by a variety of agents, including not only drugs and chemicals such as pyrazole, phenobarbital and cobalt but also viral and parasitic inflammation and in hepatic neoplasia (Camus-Randon et al. 1996; Wastl et al. 1998). This often occurs under circumstances in which other CYP isoforms are decreased. Thus, it would seem that hepatic *Cyp2a5*

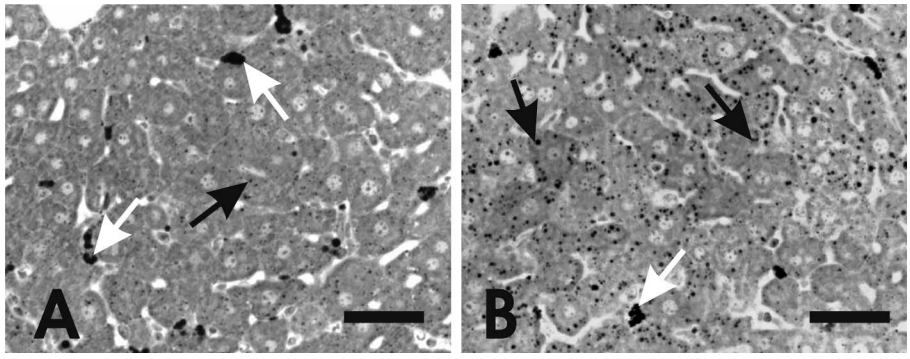

Figure 1. Histology of mouse liver. Plastic sections (1-μ thick) of tissue were stained with toluidine blue to show lipid droplets. (*A*) In the untreated *Cyp1a2*(–/–) knockout mouse, the number and size of tiny lipid droplets in hepatocytes (black arrow) are greatly decreased, whereas the amount of large lipid droplets in interstitial fat-storing cells (white arrows) is increased. (*B*) In the untreated *Cyp1a2*(+/+) wild-type mouse, small osmiophilic lipid droplets (black arrows) are common in the hepatocytes, whereas larger droplets occur occasionally in fat-storing cells of the interstitium (white arrow). Each bar is 25 μm.

Table 5. Intrahepatocyte versus interstitial fat in the liver of *Cyp1a2*(–/–) and *Cyp1a2*(+/+) mice.

Volume density (%)	*Cyp1a2*(–/–)	*Cyp1a2*(+/+)	*p*- value
Interstitium	11.0 ± 0.54	9.52 ± 0.47	0.089
Glycogen in hepatocytes	6.59 ± 1.0	5.37 ± 0.85	NS
Lipid in the whole liver	7.84 ± 1.08	12.1 ± 1.63	0.035
Hepatocyte lipid (in hepatocytes only)	6.38 ± 1.09	11.7 ± 1.72	0.012
Hepatocyte lipid in whole liver	5.72 ± 0.93	10.7 ± 1.58	0.009
Interstitial lipid (in interstitium cells only)	25.6 ± 2.29	17.6 ± 2.09	0.02
Interstitial lipid in whole liver	2.19 ± 0.23	1.35 ± 0.15	0.004

NS, not significant. The morphometric data from 28 *Cyp1a2*(–/–) and 28 *Cyp1a2*(+/+) mice, studied in our laboratory for more than 12 months from a number of different experiments, were combined to assess the intrahepatocyte versus interstitial lipid stores.

induction might be associated with a subtle form of liver injury, although nothing was seen histologically. Moreover, mouse Cyp2a5 has been found to possibly be involved in perturbation of the cell cycle and apoptosis (Pelkonen 2002).

There was no significant change in the expression of heme oxygenase-1 (inducible form) (Table 3), however, which is often associated with CYP heme turnover and oxidative stress. In addition, there was no detectable elevation in the transcription of *Alas1*, coding for 5-aminolevulinate synthase, which can also occur in situations of heme insufficiency (unpublished data). The expression of nine other CYPs was detected but was not significantly different between *Cyp1a2*(–/–) and *Cyp1a2*(+/+) mice (Table 3). Interestingly, this includes the *Cyp2e1* gene, encoding an enzyme that is constitutively quite highly expressed in mouse liver and known to be induced by ethanol and involved in small-molecule intermediary metabolism, diabetes mellitus and ketosis, as well as in the metabolism of drugs and dietary components.

Hprt *gene expression.* The unexpected observation that the *Hprt* gene is elevated more than 5-fold (Table 1) can most likely be explained by use of the *Hprt* minigene cassette in the selective elimination of the *Cyp1a2* gene in ES cells (Liang et al. 1996). In all probability, *Hprt* expression is being driven by the nearby *Cyp1a2* 5′-flanking regulatory region and promoter. We are unable to conclude with certainty that none of the changes observed (Tables 1, 2) are the downstream consequence of this striking elevation in *Hprt* expression.

Conclusions

In summary, absence of the mouse *Cyp1a2* gene appears to lead to changes in expression of some genes related to cell growth as well as to a downregulation of genes in energy production mediated by insulin. These endogenous functions would appear to be separate from any activity involving the metabolism of environmental chemicals. It is possible that this *Cyp1a2* gene knockout reflects a general disturbed metabolic activity and an intrinsic function (Nebert and Dieter 2000) of CYP1A2 in liver and energy metabolism, which results in the decreased ability of lipid to accumulate within the hepatocyte cytoplasm. It is also not possible at this stage to deduce if there is any phenotypic consequence of *Hprt* overexpression resulting from the use of this gene in developing the knockout mouse line. It will be easy enough in the near future, however, to disrupt this *Hprt* selection gene and re-examine the *Cyp1a2*(–/–) knockout mouse in the absence of *Hprt*.

References

Akhtar RA, Reddy AB, Maywood ES, Clayton JD, King VM, Smith AG, et al. 2002. Circadian cycling of the mouse liver transcriptome, as revealed by cDNA microarray, is driven by the suprachiasmatic nucleus. Curr Biol 12:540–550.

Buters JT, Tang BK, Pineau T, Gelboin HV, Kimura S, Gonzalez FJ. 1996. Role of CYP1A2 in caffeine pharmacokinetics and metabolism: studies using mice deficient in CYP1A2. Pharmacogenetics 6:291–296.

Camus-Randon AM, Raffalli F, Bereziat JC, McGregor D, Konstandi M, Lang MA. 1996. Liver injury and expression of cytochromes P450: evidence that regulation of CYP2A5 is different from that of other major xenobiotic-metabolizing CYP enzymes. Toxicol Appl Pharmacol 138:140–148.

Dalton TP, Dieter MZ, Matlib RS, Childs NL, Shertzer HG, Genter MB, et al. 2000a. Targeted knockout of *Cyp1a1* gene does not alter hepatic constitutive expression of other genes in the mouse [*Ah*] battery. Biochem Biophys Res Commun 267:184–189.

Dalton TP, Miller ML, Wu X, Menon A, Cianciolo E, McKinnon RA, et al. 2000b. Refining the mouse chromosomal location of *Cdm*, the major gene associated with susceptibility to cadmium-induced testicular necrosis. Pharmacogenetics 10:141–151.

DeRisi JL, Iyer VR, Brown PO. 1997. Exploring the metabolic and genetic control of gene expression on a genomic scale. Science 278:680–686.

Dorne JL, Walton K, Renwick AG. 2001. Uncertainty factors for chemical risk assessment. Human variability in the pharmacokinetics of CYP1A2 probe substrates. Food Chem Toxicol 39:681–696.

Eaton DL, Gallagher EP, Bammler TK, Kunze KL. 1995. Role of cytochrome P450 1A2 in chemical carcinogenesis: implications for human variability in expression and enzyme activity. Pharmacogenetics 5:259–274.

Eisen MB, Brown PO. 1999. DNA arrays for analysis of gene expression. Meth Enzymol 303:179–205.

Fornace AJ Jr, Nebert DW, Hollander MC, Luethy JD, Papathanasiou M, Fargnoli J, et al. 1989. Mammalian genes coordinately regulated by growth arrest signals and DNA-damaging agents. Mol Cell Biol 9:4196–4203.

Heilmann LJ, Sheen YY, Bigelow SW, Nebert DW. 1988. The trout P450 IA1. cDNA and deduced protein sequence, expression in liver, and evolutionary significance. DNA 7:379–387.

Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Haines DC, et al. 2001. Dimethylbenzanthracene carcinogenesis in *Gadd45a*-null mice is associated with decreased DNA repair and increased mutation frequency. Cancer Res 61:2487–2491.

Joseph SB, Laffitte BA, Patel PH, Watson MA, Matsukuma KE, Walczak R, et al. 2002. Direct and indirect mechanisms for regulation of fatty acid synthase gene expression by liver X receptors. J Biol Chem 277:11019–11025.

Kimura S, Kawabe M, Ward JM, Morishima H, Kadlubar FF, Hammons GJ, et al. 1999. CYP1A2 is not the primary enzyme responsible for 4-aminobiphenyl-induced hepatocarcinogenesis in mice Carcinogenesis 20:1825–1830.

Lavery DJ, Lopez-Molina L, Margueron R, Fleury-Olela F, Conquet F, et al. 1999. Circadian expression of the steroid 15α-hydroxylase (*Cyp2a4*) and coumarin 7-hydroxylase (*Cyp2a5*) genes in mouse liver is regulated by the PAR leucine-zipper transcription factor DBP. Mol Cell Biol 19:6488–6499.

Le Marchand L, Franke AA, Custer L, Wilkens LR, Cooney RV. 1997. Lifestyle and nutritional correlates of cytochrome CYP1A2 activity: inverse associations with plasma lutein and α-tocopherol. Pharmacogenetics 7:11–19.

Liang HC, Li H, McKinnon RA, Duffy JJ, Potter SS, Puga A, et al. 1996. *Cyp1a2*(–/–) null mutant mice develop normally but show deficient drug metabolism. Proc Natl Acad Sci U S A 93:1671–1676.

Liang HC, McKinnon RA, Nebert DW. 1997. Sensitivity of CYP1A1 mRNA inducibility by dioxin is the same in *Cyp1a2*(+/+) wild-type and *Cyp1a2*(–/–) null mutant mice. Biochem Pharmacol 54:1127–1131.

Nebert DW. 1997. Polymorphisms in drug-metabolizing enzymes: what is their clinical significance and why do they exist? Am J Hum Genet 60:265–271.

Nebert DW, Dalton TP, Stuart GW, Carvan MJ III. 2000a. "Gene-swap knock-in" cassette in mice to study allelic differences in human genes. Ann NY Acad Sci 919:148–170.

Nebert DW, Dieter MZ. 2000. The evolution of drug metabolism. Pharmacology 61:124–135.

Nebert DW, Duffy JJ. 1997. How knockout mouse lines will be used to study the role of drug-metabolizing enzymes and their receptors during reproduction and development, and in environmental toxicity, cancer, and oxidative stress. Biochem Pharmacol 53:249–254.

Nebert DW, McKinnon RA, Puga A. 1996. Human drug-metabolizing enzyme polymorphisms: effects on risk of toxicity and cancer. DNA Cell Biol 15:273–280.

Nebert DW, Nelson DR, Coon MJ, Estabrook RW, Feyereisen R, Fujii-Kuriyama Y, et al. 1991. The P450 superfamily: update on new sequences, gene mapping, and recommended nomenclature. DNA Cell Biol 10:1–14.

Nebert DW, Roe AL, Dieter MZ, Solis WA, Yang Y, Dalton TP. 2000b. Role of the aromatic hydrocarbon receptor and [*Ah*] gene battery in the oxidative stress response, cell cycle control, and apoptosis. Biochem Pharmacol 59:65–85.

Nebert DW, Russell DW. 2002. Clinical importance of the cytochromes P450. Lancet 360:1155–1162.

Negishi M, Lindberg R, Burkhart B, Ichikawa T, Honkakoski P, Lang M. 1989. Mouse steroid 15α-hydroxylase gene family: identification of type II P-450(15α) as coumarin 7-hydroxylase. Biochemistry 28:4169–4172.

Pelkonen O. 2002. Cross-talk between signal transduction systems and cytochrome P450 regulation. Pharmacologist 44(2) (suppl 1):A142.

Peters JM, Morishima H, Ward JM, Coakley CJ, Kimura S, Gonzalez FJ. 1999. Role of CYP1A2 in the toxicity of long-term phenacetin feeding in mice. Toxicol Sci 50:82–89.

Pineau T, Fernandez-Salguero P, Lee SS, McPhail T, Ward JM, Gonzalez FJ. 1995. Neonatal lethality associated with respiratory distress in mice lacking cytochrome P450 1A2. Proc Natl Acad Sci U S A 92:5134–5138.

Shertzer HG, Dalton TP, Talaska G, Nebert DW. 2002. Decrease in 4-aminobiphenyl-induced methemoglobinemia in *Cyp1a2*(–/–) knockout mice. Toxicol Appl Pharmacol 181:32–37.

Sinclair PR, Gorman N, Dalton T, Walton HS, Bement WJ, Sinclair JF, et al. 1998. Uroporphyria produced in mice by iron and 5-aminolaevulinic acid does not occur in *Cyp1a2*(–/–) null mutant mice. Biochem J 330:149–153.

Sinclair PR, Gorman N, Walton HS, Bement WJ, Dalton TP, Sinclair JF, et al. 2000. CYP1A2 is essential in murine uroporphyria caused by hexachlorobenzene and iron. Toxicol Appl Pharmacol 162:60–67.

Smith AG, Clothier B, Carthew P, Childs NL, Sinclair PR, Nebert DW, et al. 2001. Protection of the *Cyp1a2*(–/–) null mouse against uroporphyria and hepatic injury following exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol Appl Pharmacol 173:89–98.

Turton NJ, Judah DJ, Riley J, Davies R, Lipson D, Styles JA, et al. 2001. Gene expression and amplification in breast carcinoma cells with intrinsic and acquired doxorubicin resistance. Oncogene 20:1300–1306.

van der Lugt N, Maandag ER, te Riele H, Laird PW, Berns A. 1991. A *pgk::hprt* fusion as a selectable marker for targeting of genes in mouse embryonic stem cells: disruption of the T-cell receptor δ-chain-encoding gene. Gene 105:263–267.

Wastl UM, Rossmanith W, Lang MA, Camus-Randon AM, Grasl-Kraupp B, Bursch W, et al. 1998. Expression of cytochrome P450 2A5 in preneoplastic and neoplastic mouse liver lesions. Mol Carcinog 22:229–234.

Yeagley D, Guo S, Unterman T, Quinn PG. 2001. Gene- and activation-specific mechanisms for insulin inhibition of basal and glucocorticoid-induced insulin-like growth factor binding protein-1 and phosphoenolpyruvate carboxykinase transcription. Roles of forkhead and insulin response sequences. J Biol Chem 276:33705–33710.

Zaccaro C, Sweitzer S, Pipino S, Gorman N, Sinclair PR, Sinclair JF, et al. 2001. Role of cytochrome P450 1A2 in bilirubin degradation. studies in *Cyp1a2*(–/–) mutant mice. Biochem Pharmacol 61:843–849.