0022-3565/07/3211-9–17$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 321:9–17, 2007

Vol. 321, No. 1
118240/3189595
Printed in U.S.A.

A Mouse Model with Liver-Specific Deletion and Global Suppression of the NADPH-Cytochrome P450 Reductase Gene: Characterization and Utility for in Vivo Studies of Cyclophosphamide Disposition

Jun Gu, Chong-Sheng Chen, Yuan Wei, Cheng Fang, Fang Xie, Kurunthachalam Kannan, Weizhu Yang, David J. Waxman, and Xinxin Ding

Wadsworth Center, New York State Department of Health, and School of Public Health, State University of New York at Albany, Albany, New York (J.G., Y.W., C.F., F.X., K.K., W.Y., X.D.); and Department of Biology, Boston University, Boston, Massachusetts (C.-S.C., D.J.W.)

Received December 6, 2006; accepted January 10, 2007

ABSTRACT

A mouse model combining liver-specific deletion with global suppression of the NADPH-cytochrome P450 reductase gene (*Cpr*) has been developed and characterized. These mice (designated "*Cpr*-low and liver-*Cpr*-null" or CL-LCN) retain the respective phenotypes of the previously reported *Cpr*-low (CL) and liver-*Cpr*-null (LCN) mouse strains, but hepatic deletion of the *Cpr* gene occurs at an earlier age in the CL-LCN mouse than in the LCN mouse. Residual hepatic microsomal CPR activities are very low in both CL-LCN and LCN mice (at 1.5 and 2.5% of wild-type levels, respectively). The utility of CL-LCN mice for in vivo drug metabolism studies was explored using the cytochrome P450 (P450) prodrug cyclophosphamide (CPA). After i.p. injection of CPA at 100 mg/kg, the $t_{1/2}$ and the area under the concentration-time curve for plasma CPA were significantly increased in mice deficient in liver CPR compared with wild-type controls, indicating a lower rate of metabolism, with the effects greater in CL-LCN mice than in LCN mice. Correspondingly, substantial decreases in C_{max}, and increases in T_{max}, and $t_{1/2}$, of 4-hydroxycyclophosphamide (4-OH-CPA) formation were observed in both LCN and CL-LCN mice relative to wild-type controls. In contrast, CPA and 4-OH-CPA pharmacokinetic parameters were essentially unchanged in CL mice, relative to wild-type controls. The slower elimination of CPA in CL-LCN mice compared with LCN mice suggests a role for extrahepatic P450 in the in vivo metabolism of CPA and demonstrates the utility of the CL-LCN model in determining the role of extrahepatic P450 enzymes in drug metabolism and chemical toxicity.

Biotransformation of drugs and other xenobiotic compounds involves a myriad of drug-metabolizing enzymes, of which the cytochrome P450 (P450) monooxygenases are important members (Porter and Coon, 1991). In vivo drug disposition is further influenced by the activities of drug transporters (Ho and Kim, 2005) and other often complex physiological factors, such as organ perfusion rates and routes of drug delivery. Thus, it is difficult to accurately predict, from the results of in vitro studies, the contributions of P450 enzymes to the metabolic activation and disposition of a given drug in vivo. In efforts to overcome this obstacle, many animal models have been developed in recent years (e.g., Gonzalez, 2003; Wu et al., 2005), including conditional NADPH-cytochrome P450 reductase (*Cpr* or *Por*) gene knockout mouse models (Gu et al., 2003; Henderson et al., 2003; Wu et al., 2003), which are useful for abolishing the activities of all microsomal P450 enzymes in an organ- or cell type-specific manner.

Initial studies using liver-specific *Cpr*-null (LCN) mice have led to interesting findings on the in vivo metabolism and toxicity of acetaminophen (Gu et al., 2005) and cyclophosphamide (CPA) (Pass et al., 2005). For acetaminophen, which causes toxicity in liver and extrahepatic tissues under overdose conditions, we found that toxicity induced in the nasal mucosa did not require hepatic CPR- and P450-catalyzed metabolic activation, whereas toxicity induced in the

This work was supported in part by the United States Public Health Service Grants CA49248 (to D.J.W.) and CA092596 and Grant ES07462 (to X.D.) from the National Institutes of Health.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.106.118240.

ABBREVIATIONS: P450, cytochrome P450; CPR, NADPH-cytochrome P450 reductase; CL, *Cpr*-low; LCN, liver-*Cpr*-null; CL-LCN, *Cpr*-low and liver-*Cpr*-null; CPA, cyclophosphamide; 4-OH-CPA, 4-hydroxycyclophosphamide; LC, liquid chromatography; MS/MS, tandem mass spectrometry; HPLC, high-performance liquid chromatography; DAPI, 4,6-diamidino-2-phenylindole; AUC, area under the curve; WT, wild type.

lung, kidney, and lateral nasal glands was at least partly dependent on liver-derived acetaminophen metabolites (Gu et al., 2005). For CPA, Pass et al. (2005) found that the loss of hepatic CPR expression caused a significant decrease, but not a total loss, of CPR- and P450-dependent CPA clearance, confirming that hepatic metabolism plays a major role in CPA disposition. However, it was not possible in those studies to determine whether the residual metabolic activity seen with acetaminophen and CPA reflects the activity of CPR/P450 in extrahepatic tissues or whether liver enzymes that are not dependent on CPR could also play a role.

A mouse model with global suppression of *Cpr* expression (designated as *Cpr*-low or CL) was recently developed and characterized in this laboratory (Wu et al., 2005). CL mice, which have decreased CPR expression in all tissues examined due to the insertion of a neo gene in the *Cpr* intron 15, are potentially useful for studying the involvement of extrahepatic tissue P450s in drug metabolism. For example, extrahepatic tissue contributions to systemic metabolism or local drug toxicity could be inferred from any differences in metabolism or toxicity between the LCN and CL mouse models. However, the utility of the CL model is limited by the presence of a relatively high residual level of hepatic CPR protein, corresponding to ~30% of wild-type levels (Wu et al., 2005). Such a residual level will, in conjunction with compensatory increases in hepatic microsomal P450 enzyme levels (Weng et al., 2005; Wu et al., 2005), result in levels of residual hepatic P450-mediated metabolic activity that are substantially higher than those in the LCN mice.

In the present study, we have developed a new mouse model (designated as "*Cpr*-low and liver-*Cpr*-null" or CL-LCN) by crossing CL mice (Wu et al., 2005) and Alb-Cre mice (Postic et al., 1999). The resulting CL-LCN mice exhibited nearly a 95% decrease in CPR protein expression in the liver (nearly 98% decrease in CPR activity levels), and greater than 80% decreases in CPR levels in extrahepatic tissues examined; thus, these characteristics overcome some of the limitations of the CL mice. The CL-LCN mice were characterized to determine whether they retained the respective biological phenotypes of the CL (Wu et al., 2005) and LCN strains (Gu et al., 2005). The time course for the onset of hepatic *Cpr* gene deletion was also determined for CL-LCN mice and was compared with that in LCN mice. The accessibility of CPR from residual CPR-expressing cells to P450s in *Cpr*-null cells in a mixed microsome preparation was also examined. The utility of the CL-LCN mice for in vivo drug metabolism studies was explored using CPA as a model compound. Plasma levels of CPA and 4-hydroxycyclophosphamide (4-OH-CPA), which is the active metabolite (Chen et al., 2004), were determined in wild-type, CL, LCN, and CL-LCN mice, following an intraperitoneal injection of CPA at 100 mg/kg. Pharmacokinetic analysis of the data obtained indicates that extrahepatic P450s contributed to the in vivo metabolism of CPA. Therefore, the CL-LCN mouse model, used together with the LCN model, is valuable for studies of the role of extrahepatic P450s in drug metabolism and chemical toxicity.

Materials and Methods

Chemicals. CPA ($C_7H_{15}Cl_2N_2O_2{\cdot}H_2O$) was purchased from Sigma-Aldrich (St. Louis, MO). 4-OH-CPA was provided by ASTA Pharma (Bielefeld, Germany). Ifosfamide was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute (Bethesda, MD).

Generation and General Characterization of CL-LCN Mice. All animal use protocols were approved by the Institutional Animal Care and Use Committee of the Wadsworth Center (Albany, NY). CL-LCN mice were generated using a two-step cross-breeding scheme. In the first step, hemizygous female Alb-Cre mice (on a B6 background; The Jackson Laboratory, Bar Harbor, ME) were crossed with male CL mice ($Cpr^{low/low}$; B6/N1), yielding $Alb\text{-}Cre^{+/-}/Cpr^{low/+}$ pups. In the second step, female $Alb\text{-}Cre^{+/-}/Cpr^{low/+}$ mice were crossed again with male $Cpr^{low/low}$ mice, generating pups with four differing genotypes, including $Alb\text{-}Cre^{+/-}/Cpr^{low/low}$ (designated as CL-LCN). The number of pups in each of the four genotypes was recorded, and the information was used to detect potential in utero lethality of any given genotype. LCN mice ($Alb\text{-}Cre^{+/-}/Cpr^{lox/lox}$; B6/N5 or B6/N10), wild-type controls ($Cpr^{lox/lox}$, B6/N1), and CL mice (B6/N1) were obtained from breeding stocks maintained at the Wadsworth Center.

Growth rates were monitored by measurement of body and organ weights at various postnatal time points. Histological examination of dissected organs was performed by the Pathology Laboratory of the Wadsworth Center, as described for the LCN mice (Gu et al., 2003). For a determination of the fertility of the CL-LCN mice, female CL-LCN mice (2 to 4 months old) were mated with fertile male $Cpr^{lox/lox}$ mice. The dams were checked daily for a month for copulatory plug and pregnancy, and the number of pups born, if any, was recorded.

Animal Treatment. Two- to four-month old male mice were treated with a single i.p. injection of CPA (20 mg/ml) dissolved in saline, at a dose of 100 mg/kg. Blood samples were collected from the tail using a heparin-coated capillary at six time points following the injection (0, 5, and 25 min and 1, 2, and 4 h). For determination of CPA, 25 μl of blood was transferred to a tube containing 1 μl of heparin (1 U/μl) and 24 μl of saline. For determination of 4-OH-CPA, 25 μl of blood was transferred into a tube containing 1 μl of heparin (1 U/μl) and 25 μl of 0.5 M semicarbazide. The mixtures were centrifuged at 9000*g* for 5 min, and 25-μl aliquots of the supernatant fraction were stored at −80°C until analysis.

Determination of Plasma Levels of CPA and 4-OH-CPA. Plasma CPA concentrations were determined using liquid chromatography-tandem mass spectrometry (LC-MS and LC-MS/MS) essentially according to the method described by Pass et al. (2005). In brief, 25 μl of saline-diluted heparinized blood samples was mixed with 465 μl of Milli-Q water (Millipore Corporation, Billerica, MA) and 10 μl of ifosfamide (10 ng/μl), added as an internal standard. The samples were extracted three times with a total of 3 ml of ethyl acetate. The extracts were pooled and dried under N_2 at room temperature. The samples were redissolved in 100 μl of 50% acetonitrile in 20 mM ammonium acetate buffer, pH 5.0. CPA was analyzed using an LC/MS (Waters 2695; Waters, Milford, MA), and the results were confirmed by LC-MS/MS (API 2000; Applied Biosystems, Foster City, CA) under the same conditions (positive electrospray ionization mode, capillary temperature at 350°C, ionization voltage at 3.2 kV, collision energy at 20 V), with a Betasil C18 column (5 μm, 100 × 2.1 mm; Thermo Electron Corporation, Waltham, MA) and an isocratic mobile phase (35% acetonitrile in 20 mM ammonium acetate buffer, pH 5.0) at a flow rate of 0.3 ml/min. The selected ion-monitoring mode was used for the detection of CPA (*m/z* 140.1) and ifosfamide (*m/z* 154.1). The LC-MS/MS analysis was based on the following multiple reaction monitoring transitions: CPA 260.9 > 140.1, and ifosfamide 260.9 > 154.1. CPA was quantified using an external calibration curve prepared at five calibration points (10 ~ 1000 ng/ml).

Plasma levels of 4-OH-CPA were determined using an HPLC method described previously (Chen et al., 2005). Blood samples for determination of 4-OH-CPA were processed as described previously (Yu et al., 1999). In brief, 25 μl of plasma samples were combined

with 10 μl of 5.5% $ZnSO_4$, 10 μl of saturated barium hydroxide, and 5 μl of 0.01 M HCl. The samples were then mixed using a vortex and spun. After centrifugation, 40 μl of the supernatant fraction was combined with 20 μl of fluorescent reagent (Chen et al., 2005), and after heating at 90°C for 30 min, 20 μl of each reaction mixture was analyzed by HPLC with a Phenomenex 5-μm C18 column (150 × 2.0 mm), an isocratic mobile phase at a flow rate of 0.25 ml/min, and an online fluorescence detector. Standard curves for 4-OH-CPA were prepared using 4-OOH-CPA as described previously (Chen et al., 2005).

In Vitro Metabolism of CPA and Coumarin by Mouse Liver Microsomes. Coumarin 7-hydroxylase activity was determined according to Greenlee and Poland (1978). The reactions were carried out for 10 min at 37°C. Rates of CPA metabolism were determined via detection of 4-OH-CPA formation using an HPLC method, as described previously (Chen et al., 2004, 2005). Reaction mixtures contained 100 mM potassium phosphate buffer, pH 7.4, 0.1 mM EDTA, CPA at 0.2 or 2.0 mM, and liver microsomal protein at 0.5 mg/ml. The reactions, initiated by the addition of NADPH to 1 mM, final concentration, were carried out at 37°C for 15 min, and they were terminated by an extraction as described previously (Chen et al., 2005).

Immunohistochemical Analysis of CPR Expression in Mouse Liver. Paraffin sections of livers from 3-week-old or 3-month-old mice were processed for immunohistochemistry by a standard protocol (Chen et al., 2003). Endogenous peroxidase was blocked with 3% H_2O_2. All tissue sections were subjected to antigen-retrieval with Citra solution, pH 6.0 (Biogenex, San Ramon, CA), and they were incubated with a protein block (Dako North America, Inc., Carpinteria, CA) to prevent nonspecific binding. For detection of CPR protein, the tissue sections were incubated overnight at 25°C with a polyclonal rabbit anti-rat CPR antiserum (Stressgen Biotechnologies, Inc., San Diego, CA) at a 1:500 dilution. Antigenic sites were visualized using reagents from Biogenex, including biotinylated goat anti-rabbit secondary antibody (at 1:10 dilution) and peroxidase-conjugated streptavidin complex. Alexa Fluor 594-conjugated tyramide (Invitrogen, Carlsbad, CA) was used as peroxidase substrate. Sections were mounted with VectaShield mounting medium with DAPI (Vector Laboratories, Burlingame, CA). Fluorescent signals were detected using an Olympus BX50 microscope (Olympus, Melville, NY) and either a tetramethylrhodamine isothiocyanate filter (for Alexa-594) or a DAPI filter (for DAPI). Control sections were incubated with normal rabbit serum (Biogenex) in place of primary antibody.

Other Methods. Plasma total cholesterol level was determined using the Raichem Cholesterol Reagent kit from Hemagen Diagnostics (Columbia, MD) and 10 μl of plasma per assay. Protein concentration was determined by the bicinchoninic acid method (Pierce Chemical, Rockford, IL) with bovine serum albumin as the standard. Microsomal P450 concentration was determined by CO-difference spectroscopy (Omura and Sato, 1964). CPR activity was determined using cytochrome *c* as an electron acceptor (Vermilion and Coon, 1978). Microsomal CPR protein level was determined by immunoblot (Gu et al., 2003); the intensity of the detected bands was estimated with use of a densitometer. Pharmacokinetic parameters were calculated using the WinNonlin software version 5.0.1 (Pharsight, Mountain View, CA). Statistical significance of differences between two groups was assessed for various parameters using one-way analysis of variance followed by Tukey's test (SigmaStat; SPSS Inc. Chicago, IL); for data sets that failed the variance test in analysis of variance, data were transformed into log scale before the analysis, or a nonparametric test, Kruskal-Wallis test (SigmaStat), was used, as indicated in the figure or table legends. Significance of differences in genotype distribution was analyzed using the chi-square test.

Results

General Characterization of the CL-LCN Mouse. CL mice and LCN mice both display phenotypic changes related to the loss of CPR. These include hepatomegaly and hepatic lipidosis in LCN mice, female infertility and decreases both in male body weights and in embryonic survival in CL mice, and decreases in circulating cholesterol and elevated hepatic P450 levels in both strains (Gu et al., 2005; Weng et al., 2005; Wu et al., 2005). As shown in Fig. 1, CL-LCN mice were generated through Cre-mediated recombination of floxed *Cpr* gene by crossing Alb-Cre mice with CL mice, a strategy similar to that used previously to generate the LCN mice (Gu et al., 2003). Cells that do not undergo Cre-mediated recombination are homozygous for the Cpr^{lox} allele in LCN mice or the Cpr^{low} allele in CL-LCN mice. The offspring produced by crossing $Alb\text{-}Cre^{+/-}/Cpr^{low/+}$ mice with $Cpr^{low/low}$ mice had four possible genotypes, as shown in Table 1. Analysis of the genotype distribution in a total of 259 resultant pups indicated that the CL and CL-LCN mice (both homozygous for the Cpr^{low} allele) had significantly lower numbers than did the respective heterozygotes. This outcome deviates from the expected Mendelian distribution, which predicts equal frequencies for the four groups. The breeding scheme used provides evidence for reduced survival of homozygous fetuses relative to heterozygous fetuses, but it does not allow us to compare the survival of CL-LCN fetuses or that of CL-LCN heterozygotes to the survival of wild-type fetuses.

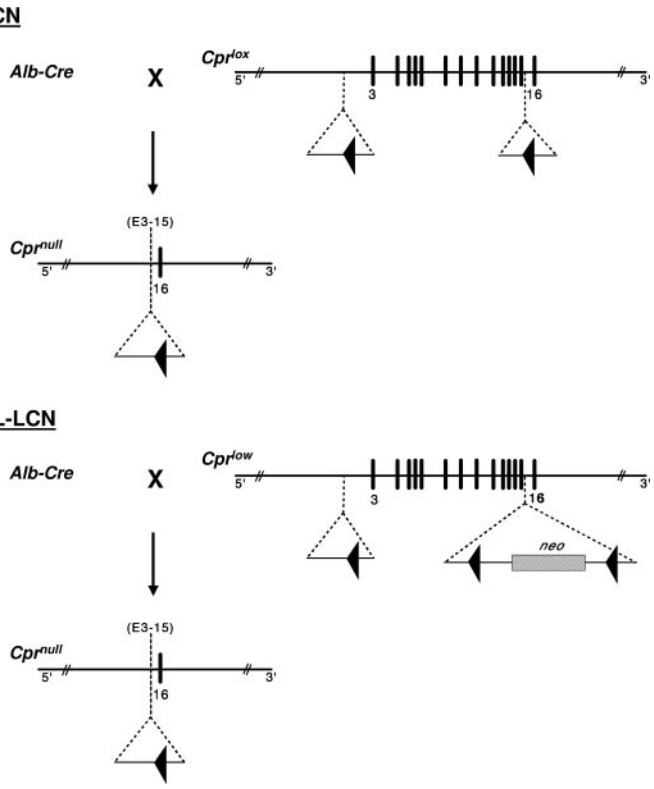

Fig. 1. Cre-mediated deletion of the *Cpr* exons 3 to 15 in hepatocytes of LCN and CL-LCN mice. Cre-mediated recombination in hepatocytes expressing the Alb-Cre transgene converts the floxed *Cpr* allele (either Cpr^{lox} or Cpr^{low}) to the Cpr^{null} allele, whereas cells that do not undergo Cre-mediated recombination are homozygous for the Cpr^{lox} (in LCN mice) or the Cpr^{low} (in CL-LCN) allele. The arrowheads represent LoxP sequences and indicate orientation. Selected exons are numbered underneath.

TABLE 1

Decreased embryonic survival of CL and CL-LCN mice

Genotype distribution was analyzed in a total of 259 pups derived from the mating of female *Alb-Cre*$^{+/-}$/*Cpr*$^{low/+}$ mice with male *Cpr*$^{low/low}$ mice. According to Mendelian distribution, there should be an equal number of pups in each genotype. Values in parentheses are percentages.

	No. of Pups in Genotype			
	Alb-Cre$^{+/-}$/Cpr$^{low/low}$ (CL-LCN)	Alb-Cre$^{+/-}$/Cpr$^{low/-}$ (Heterozygous CL-LCN)	Alb-Cre$^{-/-}$/Cpr$^{low/low}$ (CL)	Alb-Cre$^{-/-}$/Cpr$^{low/-}$ (Heterozygous CL)
Male + female	55(21.2)[a]	74 (28.6)	47(18.2)[a]	83 (32.0)
Male	31	NR	22	N.R.
Female	24	NR	25	N.R.

N.R., gender of heterozygotes was not recorded.

[a] Significantly lower than the number of corresponding heterozygous pups, assuming that the expected number of pups in each group was at least as high as in the corresponding heterozygous group ($P < 0.05$; chi-square test).

Our previous findings indicated that partial embryonic lethality occurred to the same extent (~40%) in both heterozygous and homozygous CL fetuses derived from *Cpr*$^{low/+}$ intercrosses (Wu et al., 2005). However, the genotype distribution of pups derived from the mating of female *Alb-Cre*$^{+/-}$/*Cpr*$^{low/+}$ (heterozygous CL-LCN) mice with male *Cpr*$^{low/low}$ (CL) mice (Table 1) clearly indicated a survival advantage of the heterozygotes over the homozygotes, regardless of the genotype of the Alb-Cre transgene. This survival advantage was confirmed in 124 additional fetuses resulting from the same breeding scheme, of which 21.7% pups were homozygous CL-LCN and 32.3% pups were heterozygous CL-LCN; the other 46.0% were either heterozygous or homozygous (not determined) CL pups. It remains to be established whether the presence of wild-type fetuses in the previous mating scheme (Wu et al., 2005) suppressed the survival advantage of *Cpr*$^{low/+}$ over *Cpr*$^{low/low}$ fetuses.

The CL-LCN male mice were normal in general appearance. However, the body weights of adult CL-LCN mice were significantly lower than those of wild-type controls, as shown in Table 2 for 3-month-old males. A similar extent of decrease was seen in females (data not shown). The LCN and CL mice also exhibited decreased body weights compared with wild-type mice, although the extent of decrease did not reach statistical significance. CL-LCN and LCN mice, but not CL mice, showed significant increases in liver weight compared with wild-type mice (Table 2), and, as was found previously for LCN mice (Gu et al., 2003), the CL-LCN mice displayed hepatic lipidosis (data not shown). In addition, blood total cholesterol levels were significantly lower in both CL-LCN and LCN mice (0.9 ± 0.1 and 1.3 ± 0.3 nM, respectively) than in wild-type mice (2.4 ± 0.14 nM; $P < 0.001$).

CL, but not LCN, female mice were previously found to be infertile (Gu et al., 2003; Wu et al., 2005). For determination of the fertility of female CL-LCN mice, five female CL-LCN mice (2 to 4 months old) were mated with fertile male wild-type (*Cpr*$^{lox/lox}$) mice. Two female heterozygous CL-LCN mice (*Alb-cre*$^{+/-}$/*Cpr*$^{low/+}$) were included as controls. Within 1 month after the breeding pairs had been set up, the two control females each produced a litter of live pups, whereas none of the five homozygous CL-LCN females displayed a copulatory plug or produced a litter. Therefore, female CL-LCN mice are demonstrably infertile. The fertility of male CL-LCN mice was not determined.

TABLE 2

Decreased body weight and increased liver weight in CL-LCN mice

Values are means ± S.E. for four 3-month-old male mice in each group.

Strain	Body Wt	Liver Wt
	g	
Wild type	29.3 ± 0.8	1.54 ± 0.11
LCN	26.4 ± 1.2	2.38 ± 0.12[a]
CL	26.6 ± 0.6	1.20 ± 0.06
CL-LCN	25.2 ± 0.5[a]	2.39 ± 0.16[a]

[a] Significantly different from wild type; $P < 0.05$ (Tukey's test).

Early Onset of Hepatic *Cpr* Gene Deletion in CL-LCN Mice. The extent and time course of *Cpr* deletion was examined by immunoblot analysis of mouse microsomal CPR protein in liver, lung, and kidney tissue samples at three different ages (Fig. 2). As observed previously (Gu et al., 2003; Wu et al., 2005), LCN mice had substantial, residual *Cpr* expression at 1 and 3 weeks, but not at 3 months of age, reflecting the postnatal onset of the Alb-Cre transgene expression (Postic et al., 1999). Furthermore, at 3 months of age, hepatic microsomal CPR protein level was much higher in CL than in LCN mice. However, in CL-LCN mice, hepatic CPR was barely detectable at 3 weeks of age, in contrast to the relatively high signal intensity seen in LCN and CL mice. Semiquantitative immunoblot analysis (data not shown) indicated that the levels of CPR protein were ~20, 6, and 1% of wild-type level in CL-LCN mice at 1 week, 3 weeks, and 3 months of age, respectively, compared with ~40, 40, and 3%, respectively, in LCN mice. CPR levels in extrahepatic tissues were decreased to similar extents in the CL and CL-LCN mice, as shown in Fig. 2 for lung and kidney, whereas CPR levels in the LCN mouse lung and kidney were unchanged compared with wild-type, consistent with the liver specificity of the deletion.

The immunoblot data seemed to indicate an earlier onset of *Cpr* gene deletion in CL-LCN mice than in LCN mice. However, before Cre-mediated recombination, all cells have the

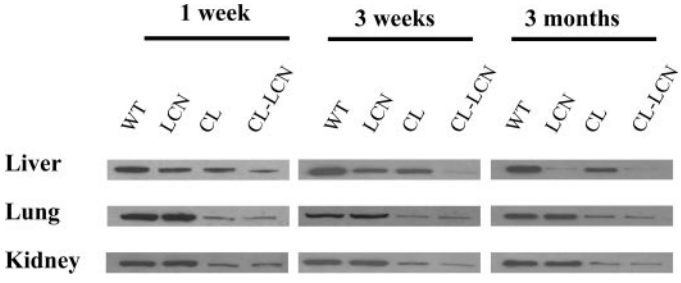

Fig. 2. Differing time course of the Cre-mediated hepatic *Cpr* deletion in LCN and in CL-LCN mice. Immunoblot analysis was performed with microsomal preparations from wild-type (WT), LCN, CL, and CL-LCN mice at 1 week, 3 weeks, and 3 months of age. Tissues, pooled from three to four mice in each group, were obtained from males. CPR protein was detected with a polyclonal antibody to rat CPR. Microsomal proteins were analyzed at 5 μg per lane for all tissues.

$Cpr^{low/low}$ genotype in the CL-LCN liver, whereas all cells have the $Cpr^{lox/lox}$ genotype in the LCN liver. As a result, cells not undergoing Cre-mediated recombination will have lower CPR levels in CL-LCN than in LCN livers. Thus, the lower CPR levels seen in CL-LCN than in LCN livers might reflect only the CL status, rather than an earlier *Cpr* deletion. To clarify whether *Cpr* deletion occurred earlier in CL-LCN than in LCN livers, we performed immunohistochemical analysis. At 3 weeks of age (Fig. 3, left), CPR protein expression in wild-type liver exhibited a typical zonal distribution pattern, with the strongest CPR staining intensity observed in hepatocytes surrounding the central vein in hepatic lobules. The zonal pattern of CPR staining was absent in LCN liver, as expected; however, although the majority of cells were negative for CPR staining at 3 weeks of age, there were still many intensely stained hepatocytes, which were randomly distributed throughout the section. In CL liver, an overall decrease in the intensity of CPR immunostaining was observed, but the zonal staining pattern was preserved. In contrast to either the LCN liver or the CL liver, the CL-LCN liver had only a few hepatocytes that were intensely stained.

At 3 months of age (Fig. 3, right), both wild-type mice and CL mice displayed patterns of CPR expression similar to those seen at three weeks of age. However, in both LCN and CL-LCN livers, intensely stained cells were no longer detected, indicating completion of Cre-mediated *Cpr* deletion in essentially all of the hepatocytes. Thus, although it remains to be confirmed that Cre expression was turned on earlier in the CL-LCN liver than in the LCN liver, the differences in the number of CPR-positive cells (Fig. 3) between CL-LCN and LCN livers at 3 weeks of age strongly suggest an earlier developmental onset of Cre-mediated *Cpr* deletion in the CL-LCN mice.

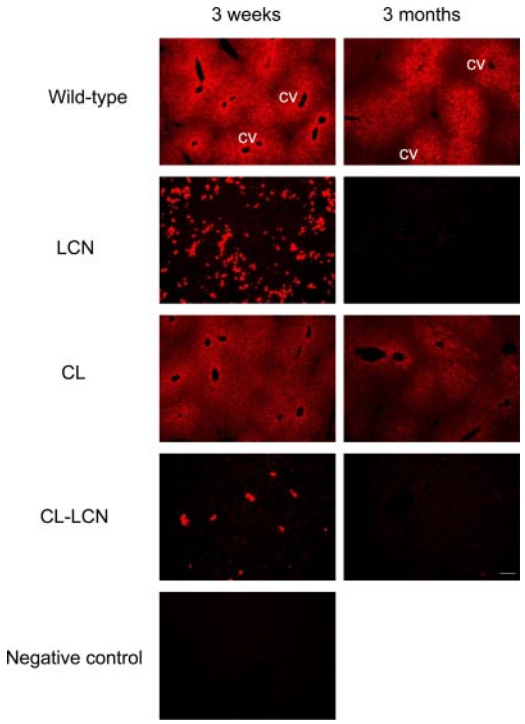

Fig. 3. Immunohistochemical analysis of hepatic CPR expression in wild-type, LCN, CL, and CL-LCN mice. Paraffin sections (4 μm) of livers from 3-week-old and 3-month-old male mice were stained with a rabbit anti-rat CPR antibody. Alexa Fluor 594-conjugated tyramide was used as the peroxidase substrate. Fluorescent signals were detected with a tetramethylrhodamine isothiocyanate filter (for Alexa-594). No signal was detected for any of the four strains when the primary antibody was replaced by a normal rabbit serum (negative control). Scale bar, 100 μm. CV, central vein.

Hepatic CPR Activity, P450 Level, and in Vitro Microsomal CPA Metabolism. The completion of hepatic *Cpr* gene deletion in the CL-LCN and LCN mice at 3 months of age was further confirmed through measurement of liver microsomal CPR activities, using cytochrome *c* as the substrate (Table 3). Residual hepatic CPR activities, presumably derived from CPR expressed in nonhepatocytes, was significantly lower in CL-LCN mice than in LCN mice, at 1.5 and 2.5% of wild-type levels, respectively. The difference in CPR activity probably reflects the *Cpr*-low status of the residual CPR-positive cells in the CL-LCN liver. The decrease in microsomal CPR activity was moderate (~55%) in CL liver. Total hepatic microsomal P450 content was significantly increased in the CL-LCN, LCN, and CL mice (6-, 5-, and 2-fold increases, respectively, compared with wild-type mice) (Table 3).

The impact of decreased *Cpr* expression on hepatic drug metabolism was examined with the P450 prodrug CPA used as a test compound. Rates of P450-dependent, microsomal CPA 4-hydroxylation are expected to be decreased in CL-LCN mice, as was reported for the LCN mice (Pass et al., 2005). However, it was not clear whether the small difference in residual CPR levels between the LCN and CL-LCN mice would lead to significant differences in residual CPA hydroxylase activity. As shown in Table 3, the rates of microsomal CPA 4-hydroxylation were decreased to ~15 to 20% of wild-type rates in both LCN and CL-LCN mice, and there was no significant difference between the two groups. The same results were obtained when activity was assayed at either of two CPA concentrations (0.2 or 2.0 mM CPA). In contrast to the decreases in activity seen in the CL-LCN and LCN mice, the rates of microsomal CPA 4-hydroxylation were apparently higher in CL mice than in wild-type mice, although the difference did not reach statistical significance (Table 3). This rate increase probably reflects 1) the induction in CL mice of hepatic P450s (Table 3), particularly CYP2B enzymes (Weng et al., 2005), which are active catalysts of CPA 4-hydroxylation (Chen et al., 2004) and 2) the occurrence of only a moderate decrease in hepatic CPR activity (Table 3).

Impact of *Cpr* Status on CPA Disposition and 4-OH-CPA Formation in Vivo. Following an i.p. injection of CPA at 100 mg/kg, plasma CPA levels were significantly higher in LCN and CL-LCN mice than in wild-type controls (Fig. 4, top). The $t_{1/2}$ and AUC for plasma CPA levels were significantly higher in CL-LCN mice than in LCN mice (Table 4), indicating that the Cpr^{low} allele confers further decreases in the rate of CPA metabolism, compared with the decrease conferred by the liver-Cpr^{null} allele alone. Substantial decreases in the C_{max} value and increases in the T_{max} and $t_{1/2}$ values of 4-OH-CPA formation were observed in both LCN and CL-LCN mice compared with the wild-type controls (Fig. 4, bottom; Table 5), consistent with the importance of hepatic CPR and, thus, hepatic P450 enzymes in the conversion of CPA to 4-OH-CPA. However, no significant differences in the 4-OH-CPA C_{max} and AUC values were apparent between the LCN and CL-LCN mice. Finally, plasma profiles of CPA and 4-OH-CPA were essentially unchanged in the CL mice compared with wild-type controls (Fig. 4; Tables 4 and 5).

TABLE 3

Hepatic microsomal CPR activity, P450 content, and rates of CPA 4-hydroxylation

Liver microsomes were prepared from individual 3-month-old male mice. CPR activity was determined using cytochrome *c* as the electron acceptor. P450 content was determined spectrally. Rates of 4-OH-CPA formation from CPA were determined as described under *Materials and Methods*. Reaction mixtures contained 0.2 or 2.0 mM CPA. The values presented are means ± S.E. (n = 4 mice/group). Values in parentheses are percentages. Data were log-transformed before statistical analysis using Tukey's test.

Strain	CPR Activity	P450 Content	Rate of 4-OH-CPA Formation	
			0.2 mM CPA	2.0 mM CPA
	nmol cytochrome c reduced/min/mg protein	*nmol/mg protein*	*nmol/min/mg protein*	
Wild type	155 ± 7 (100)	0.48 ± 0.05 (100)	0.60 ± 0.13	2.14 ± 0.32
LCN	4.0 ± 0.5[a] (2.5)	2.48 ± 0.18[a] (516)	0.12 ± 0.02[b]	0.42 ± 0.06[b]
CL	68 ± 6[a] (45)	0.98 ± 0.14 (204)	0.77 ± 0.08	2.50 ± 0.17
CL-LCN	2.0 ± 0.2[a,c] (1.5)	2.95 ± 0.11[a] (615)	0.10 ± 0.02[b]	0.31 ± 0.03[b]

[a] $P < 0.01$, compared with wild type.
[b] $P < 0.05$, compared with wild type.
[c] $P < 0.05$, compared with LCN.

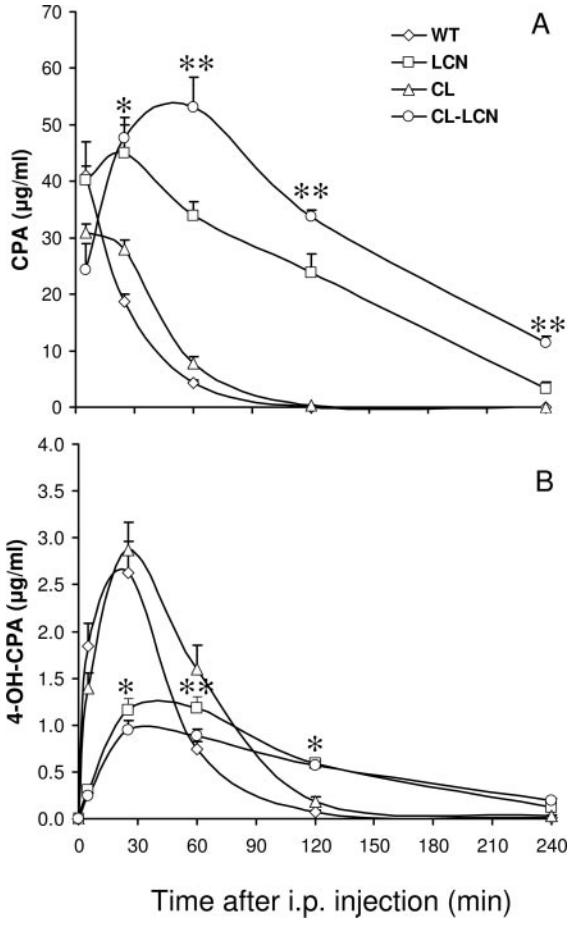

Fig. 4. Plasma CPA and 4-OH-CPA levels. Two- to 4-month old, male, WT, LCN, CL, and CL-LCN mice were given a single i.p. injection of CPA in saline, at a dose of 100 mg/kg. Blood samples were collected from individual animals, at various time points after injection, for determination of plasma concentrations of CPA (n = 4) and 4-OH-CPA (n = 11 for wild-type group and n = 7 for the other groups). Values presented are means ± S.E. Data from three sets of experiments were combined for analysis. Kruskal-Wallis test was used for statistical analysis. CL-LCN is significantly different from wild-type (*, $P < 0.01$); CL-LCN is significantly different from LCN (**, $P < 0.05$).

Accessibility of CPR to P450s in Microsomal Preparations. Our present finding that hepatic microsomes from LCN and CL-LCN mice, which contain only 1.5 to 2.5% residual CPR activity, retain 15 to 20% residual CPA 4-hydroxylase activity was unexpected. This high residual P450 activity cannot be explained solely by strong overexpression of CYP2B enzymes active in CPA metabolism, since hepatocytes with strong CYP2B overexpression no longer express Cpr. We suspected that the overexpressed CYP2B enzymes, located on microsomal vesicles derived from *Cpr*-null hepatocytes, were able to gain access to CPR that was associated with microsomal vesicles derived from the nonparenchymal cells that express CPR, resulting in further increases in P450 activity. In the LCN liver, the residual CPR-expressing cells have the $Cpr^{lox/lox}$ genotype, whereas in the CL-LCN liver, the residual CPR-expressing cells have the $Cpr^{low/low}$ genotype (Fig. 1). We hypothesized that, in liver microsomes prepared from LCN and CL-LCN mice, CPR derived from $Cpr^{lox/lox}$ and $Cpr^{low/low}$ cells supports electron transfer to P450s expressed in *Cpr*-null cells, artificially increasing the microsomal P450 activity that is measured in vitro. This access to CPR may involve electron transfer between individual microsomal vesicles of differing cellular origin, or it may occur by fusion of microsomal vesicles derived from different cells at the time of liver homogenization and microsome preparation.

To test this hypothesis, livers of equal wet weight from wild-type and LCN mice were homogenized together, and microsomes were prepared to mimic microsome preparation from tissues containing mixed cell populations that differ in their P450 and CPR content. As shown in Table 6, the rates of P450-catalyzed coumarin 7-hydroxylation (Lindberg and Negishi, 1989) in microsomes prepared from pooled wild-type and LCN mouse livers were about twice as high (125% of wild-type activity) as the averaged microsomal metabolic rates of the respective individual strains (60% of wild-type activity). The apparent increase in the LCN liver microsomal P450 activity was ~7.5-fold (75% compared with 10% of wild-type activity; see Table 6, footnote *d*) if we assume that the activity of the P450 enzymes from the wild-type livers was not decreased by the higher levels of P450s in the LCN liver through competition for available CPR. Thus, P450s expressed in LCN liver could interact with CPR from wild-type liver when livers from the two strains were homogenized together, leading to higher P450 activity. As a control, we added purified, exogenous CPR to both wild-type and LCN liver microsomal preparations, and measured the coumarin 7-hydroxylase activities in the resultant "CPR-fortified" microsomes. This addition of exogenous CPR had minimal effects on the P450 activity of wild-type liver microsomes (i.e., increase to 107% of wild-type activity), indicating that the level of CPR was already saturating. In contrast, the same

TABLE 4

Pharmacokinetic parameters for CPA clearance

Plasma CPA levels (from Fig. 4) were used to calculate pharmacokinetic parameters, including AUC, T_{max}, C_{max}, $t_{1/2}$, and clearance. Values are means ± S.E. (n = 4 for each strain). Tukey's test was used for statistical analysis.

Strain	T_{max}	C_{max}	$t_{1/2}$	AUC_{INF}	Clearance
	min	*μg/ml*	*min*	*μg · min/ml*	*ml/min*
Wild type	5.0 ± 0.0	41.1 ± 1.8	16.8 ± 1.2	1200 ± 50	2.00 ± 0.08
LCN	15.0 ± 5.8	51.9 ± 2.5[a]	62.5 ± 8.3[b,c]	6030 ± 670[a,d]	0.41 ± 0.05[a,d]
CL	10.0 ± 5.0	32.1 ± 1.0[e]	15.7 ± 1.0	1540 ± 65[e]	1.57 ± 0.06[e]
CL-LCN	51.3 ± 8.8[a,d,e]	54.1 ± 5.2[a,d]	167 ± 39[b,c,f]	10,800 ± 735[a,d,f]	0.23 ± 0.01[a,d,e]

[a] $P < 0.01$, compared with CL.
[b] $P < 0.05$, compared with wild type.
[c] $P < 0.05$, compared with CL.
[d] $P < 0.01$, compared with wild type.
[e] $P < 0.01$, compared with LCN.
[f] $P < 0.05$, compared with LCN.

TABLE 5

Pharmacokinetic parameters for 4-OH-CPA formation

Plasma 4-OH-CPA levels (see Fig. 4) were used to calculate pharmacokinetic parameters. Values are mean ± S.E. (n = 11 for wild-type group and n = 7 for the other groups). Tukey's test was used for statistical analysis.

Strain	T_{max}	C_{max}	$t_{1/2}$	AUC_{INF}
	min	*μg/ml*	*min*	*μg · min/ml*
Wild type	23.2 ± 1.8	2.6 ± 0.3	28.1 ± 3.0	139 ± 15
LCN	45.0 ± 7.1[a]	1.2 ± 0.1[a]	70.4 ± 6.1[a]	168 ± 11
CL	25.0 ± 0.0	2.9 ± 0.3	31.9 ± 2.7	191 ± 27
CL-LCN	48.6 ± 13.4[b]	1.0 ± 0.1[a]	96.7 ± 11.9[a]	163 ± 7

[a] $P < 0.01$, compared with wild type.
[b] $P < 0.05$, compared with wild type.

TABLE 6

Accessibility of CPR to P450s in microsomal preparations

Coumarin 7-hydroxylase activity was determined in reactions containing 0.5 mg/ml total microsomal protein, 0.1 mM coumarin, and 1.0 mM NADPH. All microsomes were prepared from livers of 2-month-old male mice, either WT or LCN. Where indicated, purified rat CPR was added to liver microsomes at 0.2 nmol CPR/mg microsomal protein; these "CPR-fortified" microsomes (samples marked "+ CPR") were kept on ice for ~15 min before the addition of the other components of the reaction mixtures. The values presented are averages of two determinations, with variations less than 10% of the mean.

Sample	Coumarin 7-Hydroxylation Activity (%WT)
WT	100[a]
LCN	20
WT/2 + LCN/2	50 + 10 = 60[b]
WT/LCN mix[c]	125 (50 + 75)[d]
WT + CPR	107
LCN + CPR	686
+CPR only	0

[a] The wild-type rate was 170 pmol/min/mg.
[b] The activity shown, 60, was calculated by summing one half of the activity observed for each component of a putative (1:1) WT/LCN mix and assuming that there are no interactions between the activity contributed by WT microsomes (50) and that contributed by LCN microsomes (10).
[c] Microsomes prepared from mixed livers in equal proportions (wet weight) from the two strains.
[d] Numbers in parentheses indicate the apparent contributions from the WT microsomes (50) and LCN microsomes (75), respectively, assuming that the activity of P450s from WT mice was not decreased by the presence of the P450s from the LCN mice.

CPR addition increased P450 activity more than 30-fold (from 20 to 686%) in the LCN microsomes. Thus, in LCN liver microsomes, the P450 enzymes, which are present at elevated levels in response to the loss of CPR (Gu et al., 2003), are intact, and their functions can be restored in the presence of additional CPR. Together, these findings support the hypothesis that, in LCN and CL-LCN liver microsomes, CPR present in $Cpr^{low/low}$ and $Cpr^{lox/lox}$ cells becomes accessible to P450s expressed in *Cpr*-null cells, artificially increasing the in vitro microsomal P450 activity compared with that which is manifest in intact liver in vivo.

Discussion

The mechanisms of tissue-selective toxicity are poorly understood for most xenobiotic compounds. Extrahepatic P450 enzymes are thought to play important roles in the in situ metabolic activation and disposition of drugs and other xenobiotic compounds. However, until recently, few studies directly assessed the contributions of various organs to P450-dependent drug metabolism in vivo; the paucity of such studies was mainly the result of the lack of suitable animal models or experimental approaches that permit organ-selective modulation of P450 expression or function. The recently developed LCN mouse model (Gu et al., 2003) and the similar hepatic cytochrome P450 reductase null mouse model (Henderson et al., 2003) have made it possible to investigate the roles of hepatic P450 enzymes in the in vivo disposition and toxicity of a number of xenobiotic compounds, such as acetaminophen (Henderson et al., 2003; Gu et al., 2005) and CPA (Pass et al., 2005). However, the utility of the liver-specific Cpr-null mouse model to study the role of extrahepatic P450s in drug metabolism and toxicity is limited. Thus, although drug toxicity observed in extrahepatic tissues of LCN mice, such as acetaminophen toxicity in nasal mucosa and lung (Gu et al., 2005), can be taken to indicate that hepatic P450-dependent metabolic activation is not responsible, what cannot be determined using this mouse model is whether the observed toxicity reflects metabolic activation by extrahepatic P450s or by non-P450 enzymes expressed in the liver or elsewhere. Likewise, although a decrease in the rates of in vivo metabolism for a given drug, such as CPA (Pass et al., 2005), in mice with liver-specific *Cpr* knockout can be taken to indicate the involvement of hepatic P450 in systemic drug metabolism, what cannot be determined using this mouse model is whether the residual CPA metabolic activity in these mice is mediated by extrahepatic P450s or by non-P450 enzymes expressed in the liver. Alternative mouse models are required, in which CPR is either absent or down-regulated in both liver and in extrahepatic tissues, if we are to distinguish between these possibilities. The CL-LCN mouse described here is such a model.

Germline *Cpr*-knockout mice die during gestation (Shen et al., 2002; Otto et al., 2003), which renders impossible any use of the whole-body *Cpr*-null approach to study the functions of microsomal P450 enzymes in adults. The CL mouse model (Wu et al., 2005), which has a decrease in CPR expression in essentially all tissues, offers unique advantages, because adult animals have no debilitating biological phenotypes,

other than female infertility. However, the residual CPR activity in CL mouse livers is substantial, nearly 45% of wild-type level in hepatic microsomes. The high residual CPR activity, coupled with increased expression of many P450 genes in CL mouse liver (Weng et al., 2005), could result in unchanged rates or even a net increase in the rates of hepatic drug metabolism compared with rates in wild-type controls. The fact that hepatic P450 levels in CL mice differ from those in wild-type and LCN mice makes it difficult to distinguish, through comparisons to these two latter mouse strains, the contributions of extrahepatic P450s to in vivo xenobiotic metabolism and toxicity. This deficiency of the CL model is overcome in the CL-LCN mouse model, which has a CPR activity in the liver that is only 1.5% of wild-type CPR activity and that is accompanied by an ~80% decrease in CPR levels in all extrahepatic tissues examined. Thus, by comparing the pharmacokinetics of CPA and 4-OH-CPA in CL-LCN mice and in LCN mice, we were able to obtain evidence that extrahepatic P450 enzymes contribute to the metabolic activation and systemic clearance of CPA, a prodrug that requires P450 metabolism to exert its antitumor effects (Sladek, 1988; Fleming, 1997). It is noteworthy that these extrahepatic contributions could come from tissues other than liver as well as from nonparenchymal cells in the liver, several of which express xenobiotic-metabolizing P450 enzymes (Steinberg et al., 1987).

As was found previously in hepatic *Cpr* null mice (Pass et al., 2005), the rate of in vivo CPA metabolism was significantly decreased in LCN mice compared with wild-type controls. However, significantly greater decreases in CPA clearance were observed in CL-LCN mice. This difference indicates that extrahepatic tissues contribute significantly to CPA metabolism in vivo. In contrast, we did not observe a significant difference between these two mouse strains in the pharmacokinetic parameters for the CPA metabolite 4-OH-CPA. This difference in impact of extrahepatic CPR on the pharmacokinetics of CPA versus the pharmacokinetics of 4-OH-CPA can be explained by the fact that serum levels of 4-OH-CPA are affected by two offsetting changes in the CL-LCN mouse: additional decreases in the rate of 4-OH-CPA formation due to the Cpr^{low} allele and further increases in serum CPA levels, both of which are expected to result from the significant decrease in extrahepatic CPR activity. The absence of a change in AUC (4-OH-CPA) in LCN and CL-LCN mice compared with wild-type mice indicates that alternative pathways of metabolism and elimination are not activated when the rate of CPA clearance is substantially reduced [5- to 9-fold increase in AUC (CPA); Table 4] as a consequence of CPR deficiency. A similar conclusion was reached in the rat model when hepatic CPR was suppressed by the antithyroid drug methimazole (Huang et al., 2000).

It is important to note that the actual contribution of extrahepatic P450-dependent activity to systemic CPA clearance in wild-type mice could be lower than the apparent contribution determined here in CL-LCN mice, since an intact hepatic CPR/P450 system in the wild-type mice will likely reduce the amounts of CPA reaching extrahepatic tissues. Nevertheless, our findings demonstrate that extrahepatic P450s have significant capacity to metabolize CPA and that they can play a significant role in CPA clearance when hepatic P450 activity is absent or suppressed, as occurs during liver transplant, or as a consequence of pathological changes such as liver inflammation (Morgan, 2001).

The CL-LCN mice described here were generated by cross-breeding Alb-Cre mice (Postic et al., 1999) with CL mice. In CL mice, the *Cpr* locus contains a loxP site in intron 2 and a floxed neo gene in intron 15; the presence of the neo gene is thought to lead to the suppression in *Cpr* expression (Wu et al., 2005). In CL-LCN mice, the liver-specific expression of Cre leads to deletion of *Cpr* exons 3 to 15 in hepatocytes, thus converting both Cpr^{low} alleles to Cpr^{null} alleles in these cells (Fig. 1). Few CPR-expressing hepatocytes were detected in the CL-LCN livers, indicating that partial recombination of the Cpr^{low} allele resulting in deletion of only the floxed neo gene in intron 15, while sparing the *Cpr* exons (thus converting the Cpr^{low} allele to a Cpr^{lox} allele), did not occur to any significant extent. However, residual CPR-expressing cells in the liver, such as endothelial cells, would have maintained the $Cpr^{low/low}$ genotype. As a comparison, the LCN mouse was made by cross-breeding Alb-Cre and $Cpr^{lox/lox}$, the latter having two loxP sites, one each in introns 2 and 15 (Wu et al., 2003). Cre-mediated recombination converts the two Cpr^{lox} alleles to Cpr^{null} alleles, and residual CPR-expressing cells would have a $Cpr^{lox/lox}$ genotype. Consistent with the differing genotypes of the CPR-expressing cells (nonhepatocytes) in the liver, the residual CPR activity in adult LCN liver (2.5% of wild-type level) was significantly higher than that in adult CL-LCN liver (1.5% of wild-type level).

The marginal change in CPA clearance in CL mice, relative to wild-type mice, may be explained by the increases in hepatic P450s in CL mice (Weng et al., 2005; Wu et al., 2005), coupled with a modest decrease in hepatic CPR levels, and, presumably, a decrease in extrahepatic contribution to systemic CPA clearance. In this regard, CPA is a preferred substrate for CYP2B enzymes (Clarke and Waxman, 1989; Chen et al., 2004), which are highly induced in the liver upon deletion (Gu et al., 2003; Henderson et al., 2003) or down-regulation (Weng et al., 2005) of *Cpr*.

The onset of hepatic *Cpr* gene deletion was found to occur much earlier in CL-LCN mice than in LCN mice, a feature that makes CL-LCN mice valuable for the study of P450-dependent drug metabolism and chemical toxicity in young as well as in adult animals. Possible mechanisms include earlier activation of the Alb-Cre transgene in the CL-LCN mice. The Cre recombinase is driven by the rat albumin promoter in Alb-Cre mice (Postic et al., 1999). It is known that expression of the albumin gene is influenced by hormones, such as insulin, glucagon, and glucocorticoids (Flaim et al., 1985; Kimball et al., 1995), and that the mouse albumin gene promoter contains binding sites for nuclear proteins, such as CCAAT/enhancer-binding protein, that mediate inducible gene expression (Maire et al., 1989). A comprehensive analysis of the hormonal changes in the two CPR-deficient mouse strains has not been performed, although we know that both strains display decreased plasma cholesterol levels. Nonetheless, the infertility of female CL-LCN mice, but not female LCN mice, suggests potential differences in steroidogenic activities in the two strains. Such differences, which would be predicted from the unique loss of CPR in extrahepatic tissues of the CL-LCN mice, may underlie the two strains' differing developmental onsets of Cre-mediated *Cpr* deletion.

In summary, we have developed a new mouse model of

CPR deficiency that can aid in investigations of the role of extrahepatic P450 enzymes in drug metabolism and chemical toxicity, both in neonatal and in adult mice. Using this new model in combination with the previously developed LCN model, we have identified a role for extrahepatic P450 enzymes in the clearance of CPA, an anticancer prodrug. Additional mouse models with selective *Cpr*-knockout in individual extrahepatic tissues, such as the brain and the lung, are currently being developed in one of our laboratories and should help to better define the specific tissues and cells of origin of reactive intermediates that contribute to target tissue toxicity.

Acknowledgments

We gratefully acknowledge the use of the services of the Pathology Laboratory and the Biochemistry and Molecular Genetics Core Facilities of the Wadsworth Center. We thank Drs. Laurence Kaminsky and Adriana Verschoor for reading the manuscript and Dr. Stewart Sell for advice on liver histopathology.

References

Chen CS, Jounaidi Y, and Waxman DJ (2005) Enantioselective metabolism and cytotoxicity of *R*-ifosfamide and *S*-ifosfamide by tumor cell-expressed cytochromes P450. *Drug Metab Dispos* **33:**1261–1267.

Chen CS, Lin JT, Goss KA, He YA, Halpert JR, and Waxman DJ (2004) Activation of the anticancer prodrugs cyclophosphamide and ifosfamide: identification of cytochrome P450 2B enzymes and site-specific mutants with improved enzyme kinetics. *Mol Pharmacol* **65:**1278–1285.

Chen Y, Liu YQ, Su T, Ren X, Shi L, Liu D, Gu J, Zhang Q-Y, and Ding X (2003) Immunoblot analysis and immunohistochemical characterization of CYP2A expression in human olfactory mucosa. *Biochem Pharmacol* **66:**1245–1251.

Clarke L and Waxman DJ (1989) Oxidative metabolism of cyclophosphamide: identification of the hepatic monooxygenase catalysts of drug activation. *Cancer Res* **49:**2344–2350.

Flaim KE, Hutson SM, Lloyd CE, Taylor JM, Shiman R, and Jefferson LS (1985) Direct effect of insulin on albumin gene expression in primary cultures of rat hepatocytes. *Am J Physiol* **249:**E447–E453.

Fleming RA (1997) An overview of cyclophosphamide and ifosfamide pharmacology. *Pharmacotherapy* **17:**146S–154S.

Gonzalez FJ (2003) Role of gene knockout and transgenic mice in the study of xenobiotic metabolism. *Drug Metab Rev* **35:**319–335.

Greenlee WF and Poland A (1978) An improved assay of 7-ethoxycoumarin *O*-deethylase activity: induction of hepatic enzyme activity in C57BL/6J and DBA/2J mice by phenobarbital, 3-methylcholanthrene and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *J Pharmacol Exp Ther* **205:**596–605.

Gu J, Cui H, Behr M, Zhang L, Zhang Q-Y, Yang W, Hinson JA, and Ding X (2005) In vivo mechanisms of tissue-selective drug toxicity: effects of liver-specific knockout of the NADPH-cytochrome P450 Reductase gene on acetaminophen toxicity in kidney, lung, and nasal mucosa. *Mol Pharmacol* **67:**623–630.

Gu J, Weng Y, Zhang Q-Y, Cui H, Behr M, Wu L, Yang W, Zhang L, and Ding X (2003) Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. *J Biol Chem* **278:**25895–25901.

Henderson CJ, Otto DME, Carrie D, Magnuson MA, McLaren AW, Rosewell I, and Wolf CR (2003) Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. *J Biol Chem* **278:**13480–13486.

Ho RH and Kim RB (2005) Transporters and drug therapy: implications for drug disposition and disease. *Clin Pharmacol Ther* **78:**260–277.

Huang Z, Raychowdhury MK, and Waxman DJ (2000) Impact of liver P450 reductase suppression on cyclophosphamide activation, pharmacokinetics and antitumoral activity in a cytochrome P450-based cancer gene therapy model. *Cancer Gene Ther* **7:**1034–1042.

Kimball SR, Horetsky RL, and Jefferson LS (1995) Hormonal regulation of albumin gene expression in primary cultures of rat hepatocytes. *Am J Physiol* **268:**E6–E14.

Lindberg RL and Negishi M (1989) Alteration of mouse cytochrome P450coh substrate specificity by mutation of a single amino-acid residue. *Nature (Lond)* **339:**632–634.

Maire P, Wuarin J, and Schibler U (1989) The role of cis-acting promoter elements in tissue-specific albumin gene expression. *Science (Wash DC)* **244:**343–346.

Morgan ET (2001) Regulation of cytochrome P450 by inflammatory mediators: why and how? *Drug Metab Dispos* **29:**207–212.

Omura T and Sato R (1964) The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J Biol Chem* **239:**2370–2378.

Otto DM, Henderson CJ, Carrie D, Davey M, Gundersen TE, Blomhoff R, Adams RH, Tickle C, and Wolf CR (2003) Identification of novel roles of the cytochrome P450 system in early embryogenesis: effects on vasculogenesis and retinoic acid homeostasis. *Mol Cell Biol* **23:**6103–6116.

Pass GJ, Carrie D, Boylan M, Lorimore S, Wright E, Houston B, Henderson CJ, and Wolf CR (2005) Role of hepatic cytochrome P450s in the pharmacokinetics and toxicity of cyclophosphamide: studies with the hepatic cytochrome P450 reductase null mouse. *Cancer Res* **65:**4211–4217.

Porter TD and Coon MJ (1991) Cytochrome P-450. Multiplicity of isoforms, substrates, and catalytic and regulatory mechanisms. *J Biol Chem* **266:**13469–13472.

Postic C, Shiota M, Niswender KD, Jetton TL, Chen Y, Moates JM, Shelton KD, Lindner J, Cherrington AD, and Magnuson MA (1999) Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using cre recombinase. *J Biol Chem* **274:**305–315.

Shen AL, O'Leary KA, and Kasper CB (2002) Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J Biol Chem* **277:**6536–6541.

Sladek NE (1988) Metabolism of oxazaphosphorines. *Pharmacol Ther* **37:**301–355.

Steinberg P, Lafranconi WM, Wolf CR, Waxman DJ, Oesch F, and Friedberg T (1987) Xenobiotic metabolizing enzymes are not restricted to parenchymal cells in rat liver. *Mol Pharmacol* **32:**463–470.

Vermilion JL and Coon MJ (1978) Purified liver microsomal NADPH-cytochrome P-450 reductase. Spectral characterization of oxidation-reduction states. *J Biol Chem* **253:**2694–2704.

Weng Y, DiRusso CC, Reilly AA, Black PN, and Ding X (2005) Hepatic gene expression changes in mouse models with liver-specific deletion or global suppression of the NADPH-cytochrome P450 reductase gene. Mechanistic implications for the regulation of microsomal cytochrome P450 and the fatty liver phenotype. *J Biol Chem* **280:**31686–31698.

Wu L, Gu J, Cui H, Zhang Q-Y, Behr M, Fang C, Weng Y, Kluetzman K, Swiatek PJ, Yang W, et al. (2005) Transgenic Mice with a hypomorphic NADPH-cytochrome P450 reductase gene: effects on development, reproduction, and microsomal cytochrome P450. *J Pharmacol Exp Ther* **312:**35–43.

Wu L, Gu J, Weng Y, Kluetzman K, Swiatek P, Behr M, Zhang Q-Y, Zhuo X, Xie Q, and Ding X (2003) Conditional knockout of the mouse NADPH-cytochrome P450 reductase gene. *Genesis* **36:**177–181.

Yu LJ, Drewes P, Gustafsson K, Brain EG, Hecht JE, and Waxman DJ (1999) In vivo modulation of alternative pathways of P-450-catalyzed cyclophosphamide metabolism: impact on pharmacokinetics and antitumor activity. *J Pharmacol Exp Ther* **288:**928–937.

Address correspondence to: Dr. Xinxin Ding, Wadsworth Center, New York State Department of Health, Empire State Plaza, Box 509, Albany, NY 12201-0509. E-mail: xding@wadsworth.org