Transgenic Mice with a Hypomorphic NADPH-Cytochrome P450 Reductase Gene: Effects on Development, Reproduction, and Microsomal Cytochrome P450

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ABSTRACT

A mouse model with a hypomorphic NADPH-cytochrome P450 reductase (Cpr) gene (designated Cpr\textsuperscript{low} allele) was generated and characterized in this study. The Cpr gene in these mice was disrupted by the insertion of a neo gene in intron 15, which led to 74 to 95% decreases in CPR expression in all tissues examined, including olfactory mucosa, adrenal gland, brain, testis, ovary, lung, kidney, liver, and heart. In the liver, a pattern of pericentral distribution of CPR protein was preserved in the Cpr\textsuperscript{low} mice, despite an overall reduction in CPR expression. Genotype distribution in F2 pups indicated limited embryonic lethality associated with the Cpr\textsuperscript{low} allele, a finding that confirms the role of CPR-dependent enzymes in development. Adult male homozygotes had decreased body weight and decreased heart, lung, and kidney weights, whereas homozygous Cpr\textsuperscript{low} females, which had increased serum testosterone and progesterone and decreased copulatory activities, were infertile. Furthermore, adult Cpr\textsuperscript{low/low} mice had decreased plasma cholesterol, and some mice developed mild centrilobular hepatic lipidosis. In addition, despite apparently compensatory increases in total microsomal cytochrome P450 content in the liver and kidney, the decreases in CPR expression were accompanied by reductions in systemic clearance of pentobarbital, as well as in hepatic microsomal metabolism of acetaminophen and testosterone. These phenotypes illustrate the potential impact of a globally decreased CPR activity in human adults, and this novel knock-in mouse model provides a unique opportunity for further explorations of the in vivo roles of CPR and CPR-dependent enzymes.

The NADPH-cytochrome P450 reductase (CPR), a microsomal flavoprotein, is the obligate redox partner of all microsomal cytochrome P450 (P450) monooxygenases (Black and Coon, 1987; Strobel et al., 1995). Microsomal P450s play important roles in the biotransformation of numerous endogenous and xenobiotic compounds (Porter and Coon, 1991). CPR is also essential for heme oxygenase function (Schacter et al., 1972; Gu et al., 2003), and it functions as a redox partner for cytochrome b\textsubscript{5} (Enoch and Strittmatter, 1979; Porter, 2002), squalene monooxygenase (Ono and Bloch, 1975), 7-dehydrocholesterol reductase (Nishino and Ishibashi, 2000), and fatty acid elongase (Ilan et al., 1981).

A critical role of CPR in embryonic development was recently demonstrated in knockout mouse models with germ-line deletion of the Cpr gene (Shen et al., 2002; Otto et al., 2003). Targeted deletion of the translation start site and membrane-binding domain of CPR led to multiple embryonic defects by mid-gestation, including abnormalities in neural tube, heart, eye, and limb, or a gross retardation of development and eventually death by day 13.5 of gestation (Shen et al., 2002). Limited embryonic lethality was also observed in heterozygotes. In a second study, deletion of exons 4 to 15 of Cpr, which contain the bulk of the coding sequence, led to inhibition of vasculogenesis and hematopoiesis, as well as defects in brain and limb, and ultimately death in early to middle gestation (Otto et al., 2003).

The in vivo function of hepatic CPR in adult mice has also been examined in liver-specific Cpr\textsuperscript{-null} (liver-Cpr\textsuperscript{-null} or Alb-Cre/Cpr\textsuperscript{lox}lox) mouse models (Gu et al., 2003; Henderson et al., 2003). The liver-Cpr\textsuperscript{-null} mice were generated by mating transgenic (Alb-Cre) mice, which have liver-specific Cre recombinase expression (Postic et al., 1999) with mice having floxed Cpr (Cpr\textsuperscript{lox}) alleles (Henderson et al., 2003; Wu et al.,...
Liver-specific Cpr deletion essentially abolished liver microsomal P450 activities in homozygous adults, despite significant compensatory increases in microsomal P450 content, and it led to substantial decreases in plasma cholesterol and to the occurrence of severe hepatic lipodosis and necrosis (Gu et al., 2003; Henderson et al., 2003). The absence of hepatic Cpr was also associated with a loss of hepatic microsomal heme oxygenase activity (Gu et al., 2003) and decreases in serum triglycerides and bile production (Henderson et al., 2003). Despite these phenotypes, the homozygous liver-Cpr-null mice were normal in growth, development, and reproduction.

The availability of Cpr<sup>lox</sup> mice makes it possible to generate additional mouse models with selective deletion of the Cpr gene in a given tissue or cell type. These tissue-specific Cpr-null mouse models will be valuable in the determination of tissue-specific functions of Cpr and Cpr-dependent enzymes. However, since the potential influences of genetic and environmental factors on human CPR activity are unlikely to occur in a tissue-specific fashion, it is desirable to also have an animal model with a global suppression of CPR expression.

In the present study, a mouse model (designated CPR-low mouse) with a hypomorphic Cpr (Cpr<sup>low</sup>) allele was generated. The Cpr gene in these mice was disrupted by the insertion of neo in intron 15, leading to a global substantial decrease in CPR expression. Embryonic stem cells containing the Cpr<sup>low</sup> allele were generated in a recent study in which the neo gene was used as a marker for positive selection in the first step of our successful effort to obtain the Cpr<sup>low</sup> mice; neo was subsequently removed by Cre recombinase-mediated recombination (Wu et al., 2003). This CPR-low mouse model was characterized with respect to the levels and patterns of residual CPR expression and to identify any phenotypes in development, growth, and reproduction. The effects of the decreased CPR expression on P450 expression and on P450-mediated metabolism of endogenous and xenobiotic compounds were also examined. The findings from our initial characterization of this novel knock-in mouse model identify the potential impact of a globally decreased CPR activity in human adults.

**Materials and Methods**

**Targeting Vector.** A 129/SvJ mouse bacterial artificial chromosome (BAC) genomic library was screened by Genome Systems (St. Louis, MO), and one positive Cpr clone (19020) was identified. A 6.5-kb BamHI fragment (containing Cpr exons 3 to 15), a 2.1-kb EcoRI fragment (containing the 3′-end of intron 2), and a 2.0-kb BamHI-SphI fragment (containing exon 16 and 3′-flanking sequence) were isolated and subcloned in pUC18 (New England Biolabs, Beverly, MA). The targeting vector (Fig. 1), which has been described recently (Wu et al., 2003), was linearized with Sall and was concentrated to 1 mg/ml using a Microcon 30 spin column (Millipore Corporation, Bedford, MA).

**Electroporation and Selection of Embryonic Stem Cells.** Electroporation and selection of embryonic stem cells, as well as blastocyst injection, were performed in the Transgenic and Knockout Core Facility of the Wadsworth Center. CJ7 embryonic stem cells (Swiatek and Gridley, 1993) at passage 4 were used for electroporation. Following electroporation, cells were cultured on tissue culture plates containing mitomycin C-treated primary embryonic fibroblast feeder layers prepared from a transgenic mouse line that expresses the neomycin-resistance gene (neo) (Stewart et al., 1992). After 24 h, the media was replaced with selection media containing, in addition to routine supplements, G418 (250 μg of active ingredient per milliliter; Invitrogen, Carlsbad, CA) and ganciclovir (2 μM; Syntex, Boulder, CO). Individual G418- and ganciclovir-resistant embryonic stem cell clones were screened using polymerase chain reaction (PCR) and Southern blot analysis (Wu et al., 2003). Integrity of the loxP sites was confirmed by sequencing of the PCR products.

**Blastocyst Injection and Animal Breeding.** Embryonic stem cells from two positive clones were trypsinized, centrifuged, and resuspended in injection media (Hogan et al., 1994). Embryonic stem cells were injected into the blastocysts from C57BL/6J (B6) female mice. The blastocysts were implanted into the uterus of a pseudopregnant B6CBA F1 mouse to generate offspring. The male chimeras were bred with wild-type B6 female mice to obtain germline transmission F1 mice that are heterozygous for the mutant allele. In panel D, genomic DNA was digested with EcoRI and was analyzed on Southern blots with the Int P probe. The approximate positions of the 8-kb and 12-kb fragments in a 1-kb DNA ladder are indicated.
heart, liver, kidney, adrenal glands, testes, ovary, brain, and olfac-
tory mucosa, were collected (at 1 wk, 1 month, and 3 months) and
weighed prior to storage at −80°C. The food-feeding behavior test
was performed as described recently (Zhuo et al., 2004).

For a determination of the fertility of the Cprloxlox mice, homozygous
Cprloxlox males and females were initially mixed with each other, and
the number of litters was recorded after 3 months. Subsequently,
males and females were crossed with female heterozygous or homozygous
Cprloxlox mice. The number of copulatory plugs was recorded daily in
the morning for 1 month, and the number of litters and pups was also
counted until the end of 2 months. The colonies were maintained by
crossing homozygous male with heterozygous females.

The pentobarbital clearance test was performed essentially as
described by Tsuji and coworkers (Tsuji et al., 1996). Male, −4
months old, B6, 129/Sv, and homozygous Cprlox mice were treated
with a single i.p. dose of pentobarbital (60 mg/kg; Sigma-Aldrich, St.
Louis, MO) in phosphate-buffered saline (2.7 mM KCl, 1.5 mM
KH2PO4, 134 mM NaCl, and 8.2 mM Na2HPO4). Histological exam-
ination of various organs was performed by the Pathology Labora-
tory of the Wadsworth Center, as described recently for the liver of
Cpr-null mice (Gu et al., 2003).

Determination of Microsomal P450 Activities. All assays
were carried out in duplicate. The rates of product formation were
corrected for zero time blanks that were quenched prior to the
addition of NADPH. Reactions were carried out for lengths of time that
permitted determination of initial rates. Metabolites of 4-(methyl-
nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) were analyzed using
radiometric high-pressure liquid chromatography with [5-3H]NNK
(Chemsyn, Lenexa, KS) as the substrate (Su et al., 2000). Metabolic
activation of acetonaminophen was assayed by a determination of the
rates of formation of glutathione-acetaminophen adduct (Gu et al.,
1998). Metabolism of testosterone was assayed essentially as de-
scribed previously (Ding and Coon, 1990; Gu et al., 2003). The structure and restriction map of the recombinant
Cpr allele, with the neo gene insert, are shown in Fig. 1A.

Homologous recombinant embryonic stem cell clones were
identifying the primers and probes shown in Fig. 1A. Two positive embryonic stem cell clones, 135 and 142,
were used for injection into the blastocoele cavities of B6 embryos.
Nine male chimeras, two from 142 (2856 and 2857) and seven
from 135 (2880–2886) were identified, seven of which were
subsequently able to transmit the targeted Cpr allele to F1
progeny upon breeding with wild-type B6 female mice.

The presence in the mutant mice of the loxP site and the
floxed neo gene in introns 2 and 15, respectively, was con-
formed by PCR and Southern blot analysis, as shown in Fig.
1, panels B–D. For PCR analysis of the intron-15 insertion
(Fig. 1B), the primer R1 is external to the target sequence,
and it therefore only detects the alleles derived from a ho-
mologous recombination, but not alleles with random-inte-
grated targeting construct. From the analysis of the loxP site
in intron 2 (Fig. 1C), we determined that the relative levels of
PCR products corresponding to the wild-type and recombin-
ant alleles were about the same in heterozygotes, indicating
that no nonspecific integration of the targeting construct was
occurring. This was confirmed by Southern blot data, which
indicated a single band in EcoRI-digested genomic DNA from
homozygous mutant mice and two bands of equal intensity
(and with the expected sizes) in heterozygotes, detected by
the Int P probe (Fig. 1D) or the 3P probe (not shown).

The insertion of neo led to dramatic decreases in CPR
expression in all tissues examined, including olfactory mu-
cosa, adrenal gland, brain, testis, ovary, lung, kidney, liver,
and heart (Fig. 2 and Table 1). As shown in Fig. 2, the levels of
CPR protein in microsomes or postmitochondrial S9 frac-
tions from mice homozygous for the targeted Cpr allele were
much lower than the levels from the two parental wild-type
strains (B6 and 129/Sv) at 7, 30, and 90 days after birth.
There was no significant difference between B6 and 129/Sv
mice in the level of CPR protein in any of these organs.

Quantitative immunoblot analysis of CPR protein levels in
determined mutations in five selected organs (Table 1) indicated that the extent of
decline in the Cprloxlox mice, compared with the B6 mice, was
85% with the greatest reductions found in
female brain at all three ages and in male and female lung at
90 days after birth. The extent of decrease in liver microsomal
CPR level in heterozygotes was about one-half of that in
the homozygotes (data not shown). Similar deficiencies in
CPR expression were found in five mouse lines examined

Results

Insertion of neo in Intron 15 Led to Global Suppression
of CPR Expression. As an intermediate step in the

"..."
(2857, 2880, 2882, 2883, and 2886); therefore, only two lines (2882 and 2857) were used for further studies. Reduced CPR expression was also confirmed in 8-month-old homozygotes (data not shown).

Cellular distribution of the reduction in hepatic CPR expression was examined by immunohistochemical analysis of liver tissue sections from 3-week-old mice. As shown in Fig. 3, CPR protein was detected primarily in centrilobular hepatocytes in control mice with normal CPR expression (panel A). In the livers of the Cprlow/low mice, there was a general reduction in staining intensity, but the pattern of staining was not changed; this is consistent with a reduction of CPR expression in all cells (panel B). This result contrasts markedly with the staining pattern in the liver of an Alb-Cre/Cprlox/lox double transgenic mouse, with Cre-mediated liver-specific deletion of the Cpr gene (Gu et al., 2003); in these mice, no staining could be detected in association with a particular lobular zone, but intense staining was seen in randomly distributed small clusters of cells (panel C). These cells probably represent hepatocytes in which Cre protein levels are not yet sufficient to cause deletion of the floxed Cpr gene. It had been shown previously that the liver microsomal CPR level in 3-week-old Alb-Cre/Cprlox/lox mice was about 18% of that in wild-type mice (Gu et al., 2003). The high intensity of CPR staining in these cells, in regions normally expressing CPR at only low levels, suggests that CPR expression is up-regulated in cells that still have a functional Cpr gene.

**Impact of Reduced CPR Levels on Growth and Fertility.** Growth rates of the Cprlow/low mice were compared with those of B6 and 129/Sv mice at 1 and 3 months of age. Although the Cprlow/low mice were normal in general appear-
ance, the body weights of males were significantly lower (10–15%) than those of B6 and 129/Sv at 1 and 3 months of age (Table 2). The body weights of the Cprlow/low females were significantly lower than those of B6 females at both time points (about 10%), but they were not significantly different from those of 129/Sv females. The heart, lung, and kidney weights were significantly decreased in the Cprlow/low males at 3 months of age (about 20–30%) compared with B6 and 129/Sv males (Table 3). The weights of other organs examined (adrenal gland, liver, brain, testis, and ovary) were not affected at 3 months of age, nor were the weights of any of the examined organs at 1 week or 1 month of age in either males or females (not shown). In addition, the Cprlow/low mice apparently had normal olfactory ability, as demonstrated in a food-finding behavioral test. Thus, 3-month-old male Cprlow/low mice took 45 ± 23 s (n = 10) to find a buried food pellet, a time not significantly different from that taken by B6 mice (52 ± 29 s; n = 10; P = 0.58) and only marginally different from that taken by the 129/Sv mice (69 ± 27 s; n = 10; P = 0.042) in the same experiment. Both homozygotes and heterozygotes appeared to have a normal life span; >90% of those retained for an aging experiment lived longer than 24 months.

Initial histopathologic examination of tissues from three male and three female homozygotes (about 3 months old) revealed no abnormalities in any of the major organ systems, except for a mild centrilobular hepatic lipidosis in two of the three female homozygotes. In further studies, mild centrilobular hepatic lipidosis was detected in three of four 4-month-old homozygotes and in two of eleven 2- to 3-month-old homozygotes examined (data not shown).

Breeding statistics suggested the occurrence of embryonic lethality for heterozygotes and homozygotes (Table 4). Among 207 F2 pups obtained from intercrosses of F1 heterozygotes, the numbers of homozygous and heterozygous offspring were significantly lower than the numbers predicted according to Mendelian distribution. It appears that about 40% each of the heterozygous and homozygous fetuses died in the uterus. The decreased embryonic survival affected both males and females.

Initial efforts to establish F2 Cprlow/low breeding pairs were unsuccessful, a fact which suggested possible deficiencies in reproductive ability in the homozygotes. To determine the fertility of the male and female Cprlow/low mice, we bred the homozygotes with B6 females and the female homozygotes with B6 males in preliminary studies. The results (not shown) indicated that, whereas the male homozygotes were fertile, the female homozygotes were not. Therefore, the mutant mouse colonies were maintained by breeding the male homozygotes with female heterozygotes; 12 of 14 such breeding pairs were fertile, averaging six pups per litter.

To further investigate the extent and mechanisms of the female infertility, we paired 10 wild-type B6 males with 10 heterozygous females and another nine wild-type B6 males with nine homozygous females. As shown in Table 5, of the 10 heterozygous females had copulatory plugs (which appeared between 2 and 10 days after pairing), whereas only four of the nine homozygous females had copulatory plugs (which appeared between 13 and 20 days after pairing). The eight heterozygous females with plugs all became pregnant and produced a total of 61 pups, whereas the four homozygous females with plugs did not produce any pups. In another experiment (Table 5, experiment 2), the females in 5 of 12 breeding pairs of wild-type B6 males and homozygous females had copulatory plugs (which appeared between 3 and 22 days after pairing); three of the five females became pregnant and produced a total of eight pups. Thus, the male Cprlow/low mice are fertile, whereas the female Cprlow/low mice have severely impaired reproductive ability. However, histopathologic examination of 3-month-old female homozygotes showed no abnormalities except for a mild centrilobular hepatic lipidosis in two of the three female homozygotes.

TABLE 2
Decreased body weight in homozygous Cprlow mice

Results are shown as the mean ± S.D. with the number of animals examined shown in parentheses.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Body Weight (g)</th>
<th>1-Month-Old</th>
<th>3-Month-Old</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>19.4 ± 1.6 (n = 27)</td>
<td>28.1 ± 2.1 (n = 21)</td>
<td>16.0 ± 1.3 (n = 13)</td>
</tr>
<tr>
<td>129/Sv</td>
<td>18.6 ± 1.5 (n = 17)</td>
<td>28.4 ± 2.2 (n = 20)</td>
<td>15.3 ± 2.2 (n = 14)</td>
</tr>
<tr>
<td>Cprlow/low</td>
<td>16.6 ± 2.1 (n = 17)</td>
<td>25.3 ± 2.9 (n = 25)</td>
<td>14.6 ± 1.2 (n = 14)</td>
</tr>
</tbody>
</table>

a Significantly different from B6 (P < 0.05).

b Significantly different from 129/Sv (P < 0.05).

c Significant difference compared with B6 (P < 0.05).

d Significant difference compared with 129/Sv (P < 0.05).

TABLE 3
Decreased organ weights in homozygous male Cprlow mice

The values presented are means ± S.D., n = 3. The organs shown were obtained from 3-month-old male mice. No difference was observed among the three strains in 3-month-old females or in either gender in 1-week-old or 1-month-old mice for these three organs.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Organ Weight</th>
<th>Heart</th>
<th>Lung</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>mg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>158 ± 23</td>
<td>179 ± 12</td>
<td>432 ± 14</td>
<td></td>
</tr>
<tr>
<td>129/Sv</td>
<td>164 ± 13</td>
<td>167 ± 14</td>
<td>469 ± 49</td>
<td></td>
</tr>
<tr>
<td>Cprlow/low</td>
<td>114 ± 10a</td>
<td>138 ± 7a</td>
<td>345 ± 24a</td>
<td></td>
</tr>
</tbody>
</table>

a Significantly different from B6 and 129/Sv strains (P < 0.05).

TABLE 4
Decreased embryonic survival of Cprlow mice

Genotype distribution was analyzed in a total of 207 F2 pups derived from intercrosses of F1 Cprlow/low mice. Numbers in parentheses indicate percentage of total number of pups.

<table>
<thead>
<tr>
<th>Gender</th>
<th>Number of F2 Pups in Each Genotype from F1 Cprlow/low Intercrosses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Homozygous</td>
</tr>
<tr>
<td>Male</td>
<td>21</td>
</tr>
<tr>
<td>Female</td>
<td>21</td>
</tr>
<tr>
<td>Total (male + female)</td>
<td>42b (20%)</td>
</tr>
<tr>
<td>Expectedc</td>
<td>74</td>
</tr>
</tbody>
</table>

a Sex of wild-type mice was not recorded.

b Significantly lower than expected value; P < 0.05.

c The number of pups expected in each genotype was calculated by assuming Mendelian distribution for the wild-type littermates.
gotes indicated that the ovaries have normal corpora lutea and follicles (data not shown).

**Impact of Reduced CPR Levels on Circulating Hormone Levels.** The female reproductive deficiency, despite a lack of structural abnormality in the reproductive organs, suggested disturbance in sex hormone levels in the female homozygotes. As shown in Table 6, the levels of serum progesterone and testosterone were both significantly elevated in female Cprlow/low mice. The extent of the increases (2.3- to 3.8-fold for testosterone and 3.8- to 6.7-fold for progesterone) was much greater in value than the strain difference seen between B6 and 129/Sv mice in the circulating levels of these hormones.

Similar to previous findings with the liver-Cpr-null mice (Gu et al., 2003; Henderson et al., 2003), the level of circulating cholesterol was decreased in the Cprlow/low mice, but the extent of the decreases (2.3- to 3.8-fold for cholesterol) was not as great as in mice with liver-specific deletion of the Cpr gene. Thus, plasma total cholesterol levels (in millimolars) in 4-month-old male Cprlow/low mice (2.1 ± 0.6; mean ± S.D.; n = 5) were about 49% lower (p < 0.01) than in the 129/Sv mice (4.1 ± 0.8; n = 5) and 25% lower (p = 0.07) than in the B6 mice (2.8 ± 0.4; n = 5); note, however, that the latter difference did not reach statistical significance.

**Impact of Reduced CPR Levels on Microsomal P450 Activities and in Vivo Pentobarbital Clearance.** Liver microsomal testosterone hydroxylase activities (per milligrams of microsomal protein) were 50 to 60% lower in the microsomal testosterone hydroxylase activities (per milligrams of microsomal protein). The values presented are means ± S.E.M.

**TABLE 5**
Decreased reproductive ability in female homozygous Cprlow mice
Male B6 mice (2- to 5-month-old) were mixed with female heterozygous (Cprlow/+: 9- to 12-month-old) or homozygous (Cprlow/low; 10- to 14-month-old in experiment 1 and 2- to 10-month-old in experiment 2) Cprlow mice. Mice were observed daily for 2 months. The appearance of copulatory plugs was recorded for 1 month. The number of litters and the total number of live pups produced in the 2 months after mixing the breeding pairs were also recorded.

<table>
<thead>
<tr>
<th>Mating Group</th>
<th>No. of Pairs</th>
<th>No. of Females with Plug</th>
<th>No. of Litters</th>
<th>Total No. of Pups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male B6 × female Cprlow/+</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>61</td>
</tr>
<tr>
<td>Male B6 × female Cprlow/low (experiment 1)</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Male B6 × female Cprlow/low (experiment 2)</td>
<td>12</td>
<td>5</td>
<td>3</td>
<td>8</td>
</tr>
</tbody>
</table>

**TABLE 6**
Altered levels of circulating testosterone and progesterone in homozygous female Cprlow mice
Blood samples were collected from 2- to 3-month-old female mice for determination of testosterone and progesterone. The values presented are means ± S.E.M.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Testosterone</th>
<th>Progesterone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg/ml</td>
<td>ng/ml</td>
</tr>
<tr>
<td>B6</td>
<td>83 ± 14 (n = 20)</td>
<td>3.2 ± 1.0 (n = 20)</td>
</tr>
<tr>
<td>129/Sv</td>
<td>130 ± 20 (n = 19)</td>
<td>1.8 ± 0.3 (n = 23)</td>
</tr>
<tr>
<td>Cprlow/low</td>
<td>300 ± 50 (n = 20)a</td>
<td>12.0 ± 4.0 (n = 19)b</td>
</tr>
</tbody>
</table>

a P < 0.001 vs. B6, B6 < 0.01 vs. 129/Sv.
b P < 0.05 vs. B6, P < 0.01 vs. 129/Sv.

**TABLE 7**
In vitro metabolism of acetaminophen and testosterone in liver microsomes from Cprlow/mice and control mice
Hepatic microsomes were prepared from individual, 3-month-old, B6, 129/Sv, and Cprlow/low mice. The rates of formation of glutathione-acetaminophen from acetaminophen were determined with 0.5 mM acetaminophen and 1.0 mg/ml microsomal protein, whereas the rates of formation of total testosterone metabolites were determined with 0.01 mM testosterone and 0.15 mg/ml microsomal protein. The values reported are means ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Gender</th>
<th>Strain</th>
<th>Rate of Metabolism</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acetaminophen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pmol product/min/mg microsomal protein</td>
</tr>
</tbody>
</table>

**TABLE 8**
Decreased pentobarbital clearance in homozygous Cprlow mice
Adult male mice received pentobarbital (10 mg/ml in phosphate-buffered saline) i.p. at a dose of 60 mg/kg. The lengths of time between drug administration and the loss and the subsequent recovery of righting reflex were recorded. Values presented are means ± S.E.M.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Onset of Sleep</th>
<th>Sleep Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>4.5 ± 0.9</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>129/Sv</td>
<td>6.4 ± 2.0</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>Cprlow/low</td>
<td>4.5 ± 1.4</td>
<td>203 ± 23*</td>
</tr>
</tbody>
</table>

*Significantly different from the two control groups; P < 0.01.

**Discussion**

The neo gene is frequently used as a selection marker in gene-targeting experiments (Soriano et al., 1991). The insertion of neo may affect expression of the target gene; therefore, it is usually desirable to remove the neo cassette from the floxed
allelic in conditional gene-targeting experiments (Nagy, 2000), as was done in our Cpr<sup>+</sup> mouse model (Wu et al., 2003). In the present study, embryonic stem cells with the floxed neo cassette inserted in the last intron of Cpr were used for the generation of knock-in mice. The neo cassette was in the reverse orientation to Cpr, a situation that is likely to cause greater compromise of the expression of the target gene than if neo was inserted in the same orientation as the target gene (Jacks et al., 1994; Nagy, 2000). The varying extents of CPR loss in different organs of our Cpr<sup>lox/lox</sup> mice may be due either to tissue differences in the extent of feedback induction of CPR expression or to differences in the strength of the Cpr promoter.

Germline deletion of mouse Cpr is known to cause embryonic death (Shen et al., 2002; Otto et al., 2003). The limited embryonic lethality observed for the Cpr<sup>lox/lox</sup> and Cpr<sup>lox/neo</sup> mice seems to reflect the fact that CPR expression is not completely lost in these mice, but it is not yet clear why there was not a greater lethality for the Cpr<sup>lox/lox</sup> fetuses than for the Cpr<sup>lox/neo</sup> fetuses. Our observation, that even a <50% reduction in CPR level can hinder embryonic development, confirms the earlier report by Shen and coworkers (Shen et al., 2002) in which a limited embryonic lethality was observed in heterozygous germline Cpr<sup>-null</sup> mice. Notably, the limited embryonic lethality in the Cpr<sup>lox/lox</sup> and Cpr<sup>lox/neo</sup> mice was unlikely to be due to nonspecific mutations, since the Cpr<sup>lox/neo</sup> mouse that was derived from the Cpr<sup>lox/lox</sup> embryonic stem cells does not show any embryonic lethality (Wu et al., 2003). The impact of the decreased CPR expression on development was also evidenced as a reduction in body weight and in heart, lung, and kidney weights in male Cpr<sup>lox/lox</sup> mice. Although the three organs did not show any gross structural abnormalities, it remains to be determined whether their physiological functions are fully intact.

Female Cpr<sup>lox/lox</sup> mice have severely impaired reproductive ability, but males and heterozygous females are fertile. Impairment of reproductive ability was also observed following targeted deletion of Cyp19 (aromatase) in both males and females (Fisher et al., 1998; Honda et al., 1998; Robertson et al., 1999). However, unlike the phenotypes in the Cyp19-null mice, the infertility in the female Cpr<sup>lox/lox</sup> mice was not associated with any overt structural alterations in the reproductive organs. The precise mechanism of female infertility in Cpr<sup>lox/lox</sup> mice has not been identified, but the sterility appears to be at least partly due to a disruption of steroid hormone homeostasis; this disruption includes increases in the levels of circulating progesterone and testosterone and probably other hormonal changes that have yet to be characterized. The apparent decrease in copulatory activity in these females additionally suggests an involvement of neuroendocrine mechanisms.

Major differences exist between the Cpr<sup>lox/lox</sup> mice and the liver-specific Cpr<sup>null</sup> mice (Gu et al., 2003; Henderson et al., 2003). The reduction in CPR expression is global in the Cpr<sup>lox/lox</sup> mice, whereas the loss of CPR expression was limited to hepatocytes in the liver-Cpr<sup>null</sup> mice. In the Cpr<sup>lox/lox</sup> mice, the residual amount of CPR protein may still provide limited electron transfer for the functions of those CPR partners that are involved in essential biological pathways. On the other hand, in the conditional Cpr<sup>null</sup> models, a cell will either have or not have a functional Cpr gene; thus, all CPR-dependent pathways will be affected in cells that have undergone Cre-mediated Cpr gene deletion. Furthermore, the drastic decrease in CPR expression appears to occur relatively early in development (at least as early as 1 week after birth) in the Cpr<sup>lox/lox</sup> mice, whereas in the liver-Cpr<sup>null</sup> mice, loss of CPR was not yet complete at 3 weeks of age (Gu et al., 2003). Moreover, the pattern of pericentral distribution of CPR protein was preserved in the Cpr<sup>lox/lox</sup> mice, despite an overall reduction in CPR expression, whereas this zonal distribution pattern was abolished in the liver-Cpr<sup>null</sup> mice. These differences will likely affect the outcome of experiments designed to study the in vivo role of CPR-dependent enzymes using these mouse models.

Decreases in plasma cholesterol were previously observed in the liver-Cpr<sup>null</sup> mice, as was the occurrence of hepatic lipidosis and necrosis (Gu et al., 2003). However, in accord with the partial loss of CPR expression, the Cpr<sup>lox/lox</sup> mice had much lower extents of decrease in circulating cholesterol levels than did the liver-Cpr<sup>null</sup> mice. Similarly, hepatic lipidosis was only mild and focal in the Cpr<sup>lox/lox</sup> mice and was only seen in some animals, whereas necrosis was never detected. On the other hand, the liver-Cpr<sup>null</sup> mice, unlike the CPR-low mice, had normal fertility (Gu et al., 2003) despite a near-complete loss of liver microsomal P450 activities for steroid-hormone metabolism.

Mice with a Hypomorphic Cpr Gene

### TABLE 9
Increased liver and kidney microsomal P450 content in homozygous Cpr<sup>lox/lox</sup> mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Liver P450 Content</th>
<th>Kidney P450 Content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>B6</td>
<td>129/Sv</td>
<td>Cpr&lt;sup&gt;lox/lox&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>0.56 ± 0.12</td>
<td>0.41 ± 0.19</td>
</tr>
<tr>
<td></td>
<td>0.70 ± 0.06</td>
<td>0.61 ± 0.11</td>
</tr>
</tbody>
</table>

**: 0.05, compared with either Cprlow/low or Cprlow/low mice (same gender).**

**P < 0.01, compared with either B6 or 129/Sv mouse (same gender).**

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**Mice with a Hypomorphic Cpr Gene**
the decreased CPR levels on microsomal P450-dependent activities. However, the striking zonal expression pattern of CPR expression in the liver suggests that induction of P450 in cells with very low expression of CPR (e.g., interlobular hepatocytes) would not significantly increase intracellular P450 activity. Nonetheless, the increase in total microsomal P450 level will serve to increase in vitro P450 activity when cells with lower or high CPR levels are mixed by homogenization, leading to underestimation of the impact of the partial CPR loss on P450-mediated activities in vivo.

The Cprlow mice also differ from the germline Cpr-null mice, which are embryonic lethal (Shen et al., 2002; Otto et al., 2003), in that their CPR expression is globally reduced, but not abolished. The residual amount of CPR expression in Cprlow mice appears to be sufficient for most essential biological functions in adult mice, thus providing a unique animal model in which to study the potential impact of a reduction in CPR activity on the biotransformation of various endogenous and xenobiotic compounds in human adults. In this regard, although a <3-fold variation in the level of CPR protein was found in human liver microsomes in a previous study (Yamano et al., 1989), the impact of such relatively small variations in the mean CPR level on the in vivo function of microsomal P450s and other CPR-dependent enzymes will be more significant when we consider the zonal distribution of CPR protein, particularly for those enzymes that have lower affinities for CPR (Trakshel et al., 1986; Backes and Kelley, 2003). Genetic polymorphisms of the human CPR have not been thoroughly characterized, although variant forms have been known for some time (Black SD and Coon MJ, 1987). However, several functionally significant single-nucleotide polymorphisms were identified in a recent study in which CPR mutations were associated with disordered steroidogenesis (Flück et al., 2004). In addition, it is known that CPR activity can be inhibited by a variety of drugs and other chemical agents, such as cyclophosphamide (Marinello et al., 1981), which can inhibit NADPH-cytochrome P450 reductase activity, and cyclophosphamide (Marinello et al., 1981) is known to inhibit NADPH-cytochrome P450 reductase. Nonetheless, the increase in total microsomal P450 activity can be inhibited by a variety of drugs and other chemical agents, such as cyclophosphamide (Marinello et al., 1981).

In summary, we have generated and characterized a mouse model that has globally decreased CPR expression. A >70% loss in CPR expression led to limited embryonic lethality. Adult CPR-low mice, although they appear to be affected to a lesser extent than are developing fetuses by the decreased CPR expression, show impaired reproductive ability (in the females) and significant decreases in microsomal P450-mediated metabolism of endogenous and xenobiotic compounds. Further characterization of this novel knock-in mouse model will improve our understanding of the in vivo roles of CPR and CPR-dependent enzymes and the potential involvement of these enzymes in human diseases.

Acknowledgments

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