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<sup>low</sup> 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<sup>low</sup>/low mice, despite an overall reduction in CPR expression. Genotype distribution in F2 pups indicated limited embryonic lethality with a Cpr<sup>low</sup>/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<sup>low</sup> females, which had increased serum testosterone and progesterone and decreased copulatory activities, were infertile. Furthermore, adult Cpr<sup>low</sup>/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) monooxynogenases (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<sub>c</sub> (Enoch and Strittmatter, 1979; Porter, 2002), squalene monooxynogenase (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<sup>null</sup> (liver-Cpr-null or Alb-Cre/Cpr<sup>lox</sup>) mouse models (Gu et al., 2003; Henderson et al., 2003). The liver-Cpr-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<sup>lox</sup>) 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 lipidosis 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>flox</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<sup-null</sup> 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>flox</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>flox</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>flox</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. C57J 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-finding behavior test
was performed as described recently (Zhao et al., 2004).

For a determination of the fertility of the Cpr<sup>low</sup> mice, homozygous
Cpr<sup>low</sup> males and females were initially mixed with each other, and
the number of litters was recorded after 3 months. Subsequently,
males B6 mice were crossed with female heterozygous or homozygous
Cpr<sup>low</sup> 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 Cpr<sup>low</sup> 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
KH<sub>2</sub>PO<sub>4</sub>, 134 mM NaCl, and 8.2 mM Na<sub>2</sub>HPO<sub>4</sub>). Histological exam-
nation of various organs was performed by the Pathology Labora-
tory of the Wadsworth Center, as described recently for the liver
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-(meth-
yllnitosamino)-1-(3-pyridyl)-1-butanol (NNR) were analyzed using
radiometric high-pressure liquid chromatography with [5-<sup>3</sup>H]NNR
(Chemsyn, Lenexa, KS) as the substrate (Su et al., 2000). Metabolic
activation of acetaminophen 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 earlier (Hua et al., 1997) with the use of [1,2,6,7-<sup>3</sup>H]testos-
terone (95 Ci/mmol) (PerkinElmer Life and Analytical Sciences,
Boston, MA).

Determination of Serum Levels of Cholesterol, Testoster-
one, and Progesterone. Plasma cholesterol level was determined as described (Gu et al., 2003). For determination of serum steroids,
blood from each mouse was collected by cardiac puncture and al-
lowed to clot for 1 h at room temperature. Serum was then frozen at
−80°C until use. Enzyme immunoassay kits were used for the
determination of serum concentrations of testosterone (Neogen,
Lexington, KY) and progesterone (Assay Designs, Inc., Ann Arbor, MI).
Steroids were extracted before the assays with diethyl ether (from 100 µl of serum for each determination), according to the kit's in-
structions; extraction efficiency was ~80%.

Immunohistochemical Analysis of CPR Expression in Mouse Liver. Paraffin sections of liver from 3-week-old mice were pro-
cessed for immunohistochemistry by a standard protocol (Chen et
al., 2003). Endogenous peroxidase was blocked with 3% H<sub>2</sub>O<sub>2</sub>. All
tissue sections were subjected to antigen retrieval with the Citra
solution (pH 6.0: Biogenex, San Ramon, CA) and were incubated with a protein block (DAKO, Carpinteria, CA) to prevent nonspecific
binding. For detection of CPR protein, the tissue sections were incu-
bated overnight at 25°C with a polyclonal rabbit anti-rat CPR anti-
serum at a 1:500 dilution. Antigenic sites were visualized using reagents from Biogenex, including biotinylated goat anti-rabbit sec-
todary antibody (at 1:10 dilution), peroxidase-conjugated streptavi-
din complex, and 3-amin-9-ethylcarbazole chromogen (which pro-
duces a pink to purple-red color). For control experiments, the
sections were incubated with normal rabbit serum (Biogenex) in
place of the primary antibody.

Other Methods and Materials. Preparation of microsomal or
postmitochondrial S9 fractions and immunoblot analysis were car-
ried out essentially as described previously (Ding and Coon, 1990; Gu
et al., 1997). Protein concentration was determined by the bicincho-
ninic acid method (Pierce Chemical, Rockford, IL) with bovine serum
albumin as the standard. Microsomal P450 concentration was deter-
mined by carbon monoxide-difference spectroscopy (Omura and Sato,
1964). Microsomal CPR protein level was determined by immunoblot
(Gu et al., 2003); the intensity of the detected bands was quantified
with a densitometer. The amounts of protein loaded and the film
exposure time were adjusted to provide band intensities in the linear
range of the densitometer for immunoblot quantitation. Statistical
significance of differences between two groups in various parameters
was examined using Student's t test. Genotype distribution was
analyzed using the chi square test.

Results

Insertion of neo in Intron 15 Led to Global Suppression
of CPR Expression. As an intermediate step in the generation of a floxed Cpr allele, a loxP site was inserted into intron 2, and a neo gene flanked by two loxP sites was introduced into intron 15 of the mouse Cpr gene (Wu 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 identified
using the primers and probes shown in Fig. 1A. Two positive embryonic stem cell clones, 135 and 142, were
rejected 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-
logous 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 fractions
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
five selected organs (Table 1) indicated that the extent of
decrease in the Cpr<sup>low</sup> mice, compared with the B6 mice,
ranged from 74 to 95% 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 microsco-
mal 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
(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 $Cpr^{low/low}$ 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/$Cpr^{lox/lox}$ 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/$Cpr^{lox/lox}$ 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 $Cpr^{low/low}$ mice were compared with those of B6 and 129/Sv mice at 1 and 3 months of age. Although the $Cpr^{low/low}$ mice were normal in general appear-
ance, the body weights of males were significantly lower (10–15%) than those of B6 and 129/Sv males at 1 and 3 months of age (Table 2). The body weights of the Cpr<sub>low/low</sub> 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 Cpr<sub>low/low</sub> 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 Cpr<sub>low/low</sub> mice apparently had normal olfactory ability, as demonstrated in a food-finding behavioral test. Thus, 3-month-old male Cpr<sub>low/low</sub> 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 lipodisosis in two of the three female homozygotes. In further studies, mild centrilobular hepatic lipodisosis 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 Cpr<sub>low/low</sub> 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 Cpr<sub>low/low</sub> mice, we bred the male 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, 8 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 Cpr<sub>low/low</sub> mice are fertile, whereas the female Cpr<sub>low/low</sub> mice have severely impaired reproductive ability. However, histopathologic examination of 3-month-old female homozygotes revealed no abnormalities.

### Table 2

Decreased body weight in homozygous Cpr<sub>low</sub> mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Body Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1-Month-Old</td>
</tr>
<tr>
<td>B6</td>
<td>19.4 ± 1.6</td>
</tr>
<tr>
<td>129/Sv</td>
<td>18.6 ± 1.5</td>
</tr>
<tr>
<td>Cpr&lt;sub&gt;low/low&lt;/sub&gt;</td>
<td>16.6 ± 2.1</td>
</tr>
</tbody>
</table>

* Significantly different from B6 (P < 0.05).

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

*Numbers in parentheses indicate percentage of total number of pups.*

### Table 3

Decreased organ weights in homozygous male Cpr<sub>low</sub> mice

<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>mg</td>
<td>158 ± 23</td>
<td>179 ± 12</td>
<td>432 ± 14</td>
</tr>
<tr>
<td>B6</td>
<td></td>
<td>164 ± 13</td>
<td>167 ± 14</td>
<td>469 ± 49</td>
</tr>
<tr>
<td>129/Sv</td>
<td></td>
<td>114 ± 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>138 ± 7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>345 ± 24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cpr&lt;sub&gt;low/low&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
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</table>

<sup>a</sup> Significantly different from B6 and 129/Sv strains (P < 0.05).
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 decrease in the Cprlow/low mice 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 Cprlow/low mice than in the parental wild-type strains at 3 months of age (Table 7) for both male and female mice. This finding is consistent with the increased plasma testosterone levels in the Cprlow/low mice, although hepatic clearance is not the only pathway by which serum testosterone levels are regulated. Similar decreases in microsomal P450 activity were also observed for the metabolic activation of acetaminophen. For this drug, a strain difference in the rates of liver microsomal metabolism was found in females, but not in males (Table 7). Furthermore, consistent with the decreases in hepatic P450-mediated metabolic activity, the Cprlow/low mice showed a significantly decreased ability to clear pentobarbital (Table 8). The reduced CPR expression in the

### Table 5
Decreased reproductive ability in female homozygous Cprlow mice

<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 x female Cprlow/+</td>
<td>10</td>
<td>8</td>
<td>8</td>
<td>61</td>
</tr>
<tr>
<td>Male B6 x female Cprlow/low (experiment 1)</td>
<td>9</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Male B6 x 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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Testosterone ng/ml</th>
<th>Progesterone ng/ml</th>
</tr>
</thead>
<tbody>
<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)</td>
<td>12.0 ± 4.0 (n = 19)</td>
</tr>
</tbody>
</table>

### Table 7
In vitro metabolism of acetaminophen and testosterone in liver microsomes from Cprlow/low and control mice

<table>
<thead>
<tr>
<th>Gender</th>
<th>Strain</th>
<th>Acetaminophen pmol product/min/mg microsomal protein</th>
<th>Testosterone pmol product/min/mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>B6</td>
<td>270 ± 20</td>
<td>770 ± 170</td>
</tr>
<tr>
<td></td>
<td>129/Sv</td>
<td>330 ± 20</td>
<td>880 ± 120</td>
</tr>
<tr>
<td></td>
<td>Cprlow/low</td>
<td>170 ± 20*</td>
<td>360 ± 10</td>
</tr>
<tr>
<td>Female</td>
<td>B6</td>
<td>380 ± 20</td>
<td>690 ± 150</td>
</tr>
<tr>
<td></td>
<td>129/Sv</td>
<td>620 ± 60</td>
<td>870 ± 10</td>
</tr>
<tr>
<td></td>
<td>Cprlow/low</td>
<td>220 ± 20*</td>
<td>340 ± 50</td>
</tr>
</tbody>
</table>

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

**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

### Table 8
Decreased pentobarbital clearance in homozygous Cprlow mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Onset of Sleep (min)</th>
<th>Sleep Duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6 (n = 4)</td>
<td>4.5 ± 0.9</td>
<td>50 ± 6</td>
</tr>
<tr>
<td>129/Sv (n = 5)</td>
<td>6.4 ± 2.0</td>
<td>65 ± 10</td>
</tr>
<tr>
<td>Cprlow/low (n = 5)</td>
<td>4.5 ± 1.4</td>
<td>203 ± 23*</td>
</tr>
</tbody>
</table>

*Significantly different from the two control groups; P < 0.01.
TABLE 9

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

P450 was determined by carbon monoxide-difference spectroscopy. Microsomes were prepared from individual mice at the age of 3 months. Values reported are means ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Liver P450 Cont</th>
<th>Kidney P450 Cont</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (nmol P450/mg microsomal protein)</td>
<td>Female (nmol P450/mg microsomal protein)</td>
</tr>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>B6</td>
<td>0.56 ± 0.12</td>
<td>0.41 ± 0.19</td>
</tr>
<tr>
<td>129/Sv</td>
<td>0.70 ± 0.06</td>
<td>0.61 ± 0.11</td>
</tr>
<tr>
<td>Cpr&lt;sup&gt;lox&lt;/sup&gt;low</td>
<td>1.21 ± 0.18&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.94 ± 0.13&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> P < 0.01, compared with either B6 or 129/Sv (same gender).
<sup>b</sup> P < 0.05, compared with either B6 or 129/Sv (same gender).

allele in conditional gene-targeting experiments (Nagy, 2000), as was done in our Cpr<sup>b<sup>b</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/lox</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/lox</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-null mice. Notably, the limited embryonic lethality in the Cpr<sup>lox/lox</sup> and Cpr<sup>lox/lox</sup> mice was unlikely to be due to nonspecific mutations, since the Cpr<sup>lox</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-null 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-null mice. In the liver-Cpr-null 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-null 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-null 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-null 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-null mice, as was the occurrence of hepatic lipidosis and necrosis (Gu et al., 2003; Henderson 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-null 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-null 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. It is likely that the drastic decrease in cholesterol biosynthesis in liver-Cpr-null mice has resulted in a reduced biosynthesis of sex-steroid hormones, thus circumventing the normal accumulation of reproductive hormones that is seen in the CPR-low mice.

Significant increases in total P450 content were found in the kidneys, as well as in the liver, of the Cpr<sup>lox/lox</sup> mice, a result that contrasts with the tissue-specific P450 induction in the liver of the liver-Cpr-null mice (Gu et al., 2003) and suggests that the increases in P450 expression seen in both mouse models occurred in response to intracellular signals—rather than systemic signals—upon a loss or a substantial decrease in CPR expression. The effects of CPR loss on P450 expression in other extrahepatic tissues of the Cpr<sup>lox/lox</sup> mice are currently under investigation, including the specific P450s affected and potential tissue differences. We anticipate that the Cpr<sup>lox/lox</sup> mouse model will be valuable for the identification of the mechanistic aspects of regulation of microsomal P450 expression in various extrahepatic tissues, such as the involvement of endogenous P450 substrates or metabolites in the induction process.

The apparently compensatory increases in P450 expression in the Cpr<sup>lox/lox</sup> mouse may have reduced the impact of
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 low 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 Cpr^low/low mice also differ from the germine 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 Cpr^low/low 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 (Shephard et al., 1992). 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), ellipticine (Guenthner et al., 1980), and cadmium (Trakshel et al., 1986).

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.

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