Targeted Disruption of the Olfactory Mucosa-Specific Cyp2g1 Gene: Impact on Acetaminophen Toxicity in the Lateral Nasal Gland, and Tissue-Selective Effects on Cyp2a5 Expression

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ABSTRACT

CYP2G1 is a cytochrome P450 monoxygenase expressed uniquely in the olfactory mucosa (OM). We have generated Cyp2g1-null mice to identify the roles of CYP2G1 in the biology and the tissue-specific toxicity of xenobiotic compounds in the nose. Homozygous Cyp2g1-null mice are viable and fertile; they show no evidence of embryonic lethality, morphological abnormality, or developmental deficits; and they seem to have normal olfactory ability. However, OM microsomes from Cyp2g1-null mice were found to have significantly lower activities than microsomes from wild-type mice in the metabolism of testosterone and progesterone (~60% decrease) and in the metabolic activation of coumarin (>70% decrease). Unexpectedly, a significant reduction in the expression of the Cyp2a5 gene was found in the liver, the lateral nasal gland (LNG), and, to a lesser extent, the kidney of adult Cyp2g1-null mice. The loss of CYP2G1 expression, and the associated decrease in the hepatic expression of CYP2A5, did not decrease systemic clearance, extent of hepatotoxicity, or OM toxicity of acetaminophen (AP). However, the LNG was protected from AP (at 400 mg/kg) toxicity in the Cyp2g1-null mice. Paradoxically, the LNG did not have detectable CYP2G1, and the decrease in LNG CYP2A5 expression in the Cyp2g1-null mice was not accompanied by decreases in microsomal AP metabolism. We hypothesize that OM CYP2G1 (through a paracrine pathway) or LNG CYP2A5 may indirectly influence resistance of the LNG to chemical toxicity, possibly by regulating gene expression in the LNG through steroid hormones or other endogenous P450 substrates and their metabolites.

The tissue-specific expression of CYP2G1 in the OM is highly conserved in mammals (for recent review, see Ding and Dahl, 2003). CYP2G1 is a major P450 enzyme in OM microsomes of rabbits and mice. In vitro metabolic assays with heterologously expressed mouse CYP2G1 or purified rabbit or bovine CYP2G1 revealed high activity with sex steroid hormones, including androstenedione, testosterone, 5a-dihydrotestosterone, progesterone, and estradiol. CYP2G1 is also active toward arachidonic acid, producing both epoxy- and hydroxyecosatrienoic acids. In addition, CYP2G1 is active in the metabolism of numerous exogenous compounds, including compounds known to cause tissue-selective toxicity in the nasal mucosa, such as coumarin, DCBN (an herbicide), and AP. The substrate specificities, combined with the tissue-selective expression pattern of this enzyme, suggest that CYP2G1 may be critical in target tissue metabolic activation and the consequent cytotoxicity of nasal toxicants and that it may have important biological functions in the olfactory chemosensory organ through its metabolic activities toward endogenous substrates. These intriguing possibilities were explored in the present study through development and characterization of a Cyp2g1-null mouse model.

Unlike many of the multigene subfamilies in the mouse Cyp2 gene family, the Cyp2g subfamily contains only a single gene, Cyp2g1. The structure of the mouse Cyp2g1 gene has been characterized; this gene contains nine exons, and the transcriptional initiation sites are located at 16 and 15 base pairs upstream of the translation start codon (Zhuo et al., http://jpet.aspetjournals.org.

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Abbreviations: OM, olfactory mucosa; DCBN, 2,6-dichlorobenzonitrile; P450, cytochrome P450; BAC, bacterial artificial chromosome; AP, acetaminophen; LNG, lateral nasal gland; PEG, polyethylene glycol; ALT, alanine aminotransferase; o-HPA, o-hydroxyphenylacetaldehyde; kb, kilobase pair; bp, base pair; CPR, NADPH-cytochrome P450 reductase; PCR, polymerase chain reaction.
2003). In this study, we have generated Cyp2g1 mouse chromosome 7, next to the Cyp2g1 gene (Wang et al., 2003). In this study, we have generated Cyp2g1-null mice by replacing exon 3 of the Cyp2g1 gene with the neo gene. The homozygous mutant mice were examined for phenotypic abnormalities, including changes in olfactory ability, the impact of Cyp2g1 gene disruption on the expression of CYP2G1, and the activities of OM microsomes toward a number of known CYP2G1 substrates. Potential compensatory increases in other P450s, and related enzymes, in the OM and liver were also examined. Furthermore, the impact of Cyp2g1 knockout on the metabolism and toxicity of AP was determined for OM and LNG (also known as Steno's gland).

The LNG is the largest of about 20 anterior nasal glands that drain by long ducts that open near the nostril in mice and rats; it has been found in most mammalian species, including man (Moe and Bojsen-Moller, 1971). The LNG is the major site for the synthesis and secretion of odorant-binding proteins, which are abundant, soluble, nasal proteins that are believed to function mainly as carriers for odorants in the nasal mucus (Snyder et al., 1988). In addition, the secretions of the LNG are believed to humidify inspired air (Snyder et al., 1988), and the large amounts of immunoglobulin A in the LNG secretions (Adams et al., 1981) may be important for the immune barrier function of the OM (Getchell and Mellert, 1991). The potential roles of the LNG in olfactory chemoreception, as well as in host defense, make it an important target for chemical toxicity. Indeed, the LNG is a known target for the toxicity of both inhaled (such as divinylbenzene-55; Morgan et al., 1997) and systemically administered (such as 4-(methylnitrosamo)-1-(3-pyridyl)-1-butanone; Belinsky et al., 1987) chemicals, and here we show for the first time that the LNG is a major target for the cytotoxicity of AP in mice.

Materials and Methods

Targeting Vector Construction. The targeting vector (Fig. 1) was prepared in a pGK-neo-tk vector (6.5 kb) provided by the Transgenic and Knockout Core Facility of the Wadsworth Center. It consists of a pBluescript backbone (Stratagene, La Jolla, CA) and, in the same orientation, a PGK-neo-bpA cassette for neo gene expression (Soriano et al., 1991) and the MC1-tk-pA cassette for thymidine kinase gene expression (Mansour et al., 1988). BAC clones containing the mouse Cyp2g1 gene have been described recently (Zhuo et al., 2001). A 7.2-kb BamHI fragment and a 2.0-kb HindIII fragment of the Cyp2g1 gene were inserted into pGK-neo-tk vector at BamHI and HindIII sites, respectively. The targeting vector containing the insert with the correct orientation was linearized with SalI and was concentrated to 1 μg/μl using a Microcon 30 spin column (Amicon, Billerica, MA).

Electroporation and Selection of ES Cells. C57 ES cells (Swiatek and Gridley, 1993) at passage 4 were trypsinized and resuspended in PBS at 2 × 10⁷ cells/ml. The resuspended cells (0.8 ml) were electroporated with 25 μg of linearized targeting vector DNA using a Bio-Rad gene pulser (500 μF, 240 V). After 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 neo gene (Stewart et al., 1992). After 24 h, the media were replaced with selection media containing, in addition to routine supplements, G418 (250 μg of active ingredient/ml; Invitrogen, Carlsbad, CA) and Ganciclovir (2 μM; Syntex, Boulder, CO). Media were changed daily. Colonies were picked individually 6 to 7 days after electroporation, dissociated in trypsin-EDTA, and plated into 48-well culture plates containing the feeder cells. The G418-resistant ES cell colonies in the 48-well dishes were trypsinized, and 50% of the cells were replica plated into new 24-well dishes to grow for DNA isolation. The remaining cells were stored at −80°C for subsequent culture and blastocyst injection.

Genotyping ES Cells and Mice. Genomic PCR was performed with DNA (500 ng for each reaction) isolated from cultured ES cells or mouse tails (Hogan et al., 1994). Primers E2U (5'-agggtcttatgtg-gcatgag-3'), NeoL (5'-caattcgttctccattg-3') and NeoL (5'-caattcgttctccattg-3'), as shown in Fig. 1, were used to amplify a 550-bp DNA fragment from the targeted allele, whereas primers E2U and E3L (5'-caattcgttcatg- gagcagag-3') were used to amplify a 1.6-kb fragment from the wild-type allele. PCR mixtures (25 μl) contained, in addition to genomic DNA and 200 nM of each primer, 1× PCR buffer (Promega, Madison, WI), 2 mM MgCl₂, 4nTPs (0.5 mM each), and 2 U of TaqDNA polymerase (Promega). The PCR program included 35 cycles of de-
at 95°C for 45 s, annealing at 59.5°C for 50 s, and extension at 72°C for 45 s. For Southern blot analysis, genomic DNA was digested overnight with 50 U of PstI (Roche Diagnostics, Indianapolis, IN) at 37°C. The fragments were separated on an agarose gel and hybridized with [α-32P]dCTP-labeled probe H, which was a 1.0-kb HindIII fragment from the 5′-flanking sequence external to the targeting vector (Fig. 1). Hybridization was performed in QuikHyb hybridization solution (Stratagene), with salmon sperm DNA, at 58°C for 4 h. Fragments of 6.5 and 5.5 kb were expected to represent the wild-type and targeted alleles, respectively.

**Blastocyst Injection and Animal Breeding.** Recombinant ES cells were trypsinized, centrifuged, and resuspended in injection media (Hogan et al., 1994). Ten to 15 ES cells were injected into the blastocoele cavity of 3.5 day postcoitum blastocysts from C57BL/6J (B6) female mice. The blastocysts were implanted into the uteri of pseudopregnant B6/CBA F1 mice to generate offspring. The male chimeras were bred with wild-type B6 female mice to obtain germ-line transmission F1 mice that are heterozygous for the targeted allele. The F2 homozygotes were obtained by intercrossing the F1 heterozygotes.

**RNA Blot Analysis.** Total RNA was isolated from mouse tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. For RNA blot analysis, hybridization was carried out overnight, at 58°C, in QuikHyb hybridization solution (Stratagene), with an [α-32P]dCTP-labeled full-length mouse Cyp2g1 cDNA (Hua et al., 1997) or a Cyp2a5 cDNA (Su et al., 1998).

**RNA-PCR.** Qualitative PCR detection of CYP1A2, CYP2A5 (Zhang et al., 2003), and CYP2B1 (Gu et al., 1999) transcripts was performed as described previously; the expected products are 359, 318, and 489 bp, respectively. First-strand cDNA was synthesized by reverse transcription from 1 μl of total RNA with use of Moloney Murine Leukemia virus reverse transcriptase (Promega) and a dT(18) primer (Applied Biosystems, Foster City, CA). Real-time PCR was carried out in a LightCycler instrument (Roche Diagnostics), with the PCR mixtures containing 2 μl of FastStart DNA Master SYBR Green I (Roche Diagnostics), 2 mM MgCl2, 0.4 μM of each primer, and 1 μl of a reverse transcription mixture, in a total volume of 20 μl. For quantitation of CYP2A5 mRNA, a pair of CYP2A5-specific primers was used (forward primer: 5′-cgcacagcaggtatcagcgtgc-3′; reverse primer: 5′-cttacctctcagcaggtc3′; reverse primer: 5′-ctcctgggaatcgc-3′), which do not amplify CYP2A4 or CYP2A12. The PCR program included 50 cycles of denaturation at 95°C for 2 s, annealing at 60°C for 5 s, and extension at 72°C for 25 s. CYP2F2 (forward primer: 5′-gtagatacatctgactgtgcacag-3′; reverse primer: 5′-cagctctactacaagtctgtg-3′) for 5′ s, annealing at 62°C, and extension for 30 s) and β-actin (forward primer: 5′-accagatgttttggac-3′; reverse primer: 5′-tttgctacaactcagtgc-3′ (Fiorenza and Mangia, 1998); denaturation for 5 s, and extension for 20 s) were determined under similar conditions. Different amounts of CYP2A5 (Su et al., 1998), CYP2F2 (generated with PCR according to the sequence of Ritter et al., 1991), and mouse β-actin (generated by PCR) cDNAs were used as standards for quantitation. Detection of fluorescence in each reaction was carried out at 3°C below the melting temperature of the corresponding PCR product. The specificity of the PCR products was confirmed by melting-curve analysis and by sequencing. Quantification and melting temperature analysis were performed using the LightCycler data analysis software.

**Genotyping Cyp2a5 in Cyp2g1−/− Mice.** A 1.1-kb genomic fragment was amplified using forward (5′-ctcttatatactatggcagc-3′) and reverse primers (5′-gcagcagactgctc-3′). The PCR products, which contain a polymorphic marker that distinguishes the Cyp2a5 locus of 129/Sv strain (C) from that of the B6 strain (T), were analyzed by DNA sequencing to determine the strain from which the Cyp2a5 gene in the knockout mice was derived.

**Immunoblot Analysis and Quantitation.** The relative levels of P450 and other biotransformation enzymes were determined by immuno blot, using the following antibodies: goat antibodies against rat CYP1A1/2, CYP2B1, CYP2C6, CYP3A2, and CPR (BD Gentest, Woburn, MA); rabbit anti-human CYP2E1 (Oxford Biomedical Research, Oxford, MI); rabbit anti-rat CYP2J4 (Zhang et al., 1998); rabbit anti-CYP2A5 (Gu et al., 1998); and rabbit anti-rat microsomal epoxide hydrolase (a gift from Dr. Charles Kasper, University of Wisconsin, Madison, WI). Microsomal preparation and immunoblot analysis were carried out essentially as described previously (Ding and Coon, 1990). Protein concentrations were determined by the bichinonic acid method (Bio-Rad) with bovine serum albumin as the standard. The intensity of the detected bands was quantified with a densitometer (Personal Densitometer SI and ImageQuant version 5.0 software (Amersham Biosciences Inc., Piscataway, NJ).

**Determination of Catalytic Activity.** Assays contained 50 mM potassium phosphate buffer, pH 7.4 (unless otherwise indicated); reactions were initiated by the addition of NADPH to a final concentration of 1 mM and carried out at 37°C. Metabolism of testosterone and progesterone was assayed essentially as described previously (Hua et al., 1997), with [1,2,6,7-3H]testosterone (95 Ci/mmol) and [1,2,6,7-3H]progesterone (104 Ci/mmol) (PerkinElmer Life Sciences, Boston, MA). Reaction mixtures contained 10 or 5 μM substrate and 0.1 mg/ml microsomal protein. Reactions were carried out for 10 min (for testosterone) or 30 min (for progesterone). DCBN-protein adduct formation was assayed as described previously (Ding et al., 1996). Reaction mixtures contained OM microsomes (0.1 mg of protein/ml) and 2.6-[ring-14C]DCBN (16.7 Ci/ml; Sigma-Aldrich, St. Louis, MO) at a concentration of 3 or 30 μM. The reactions were carried out for 30 min. Coumarin 7-hydroxylase activities were assayed using a fluorometric method, with coumarin at 0.5 mM, or by radiometric high-performance liquid chromatography, as described previously (Zhao et al., 1999). For radiometric analysis of coumarin metabolites, the reaction mixtures contained 0.2 to 0.3 mg/ml microsomal protein and 20 or 72 μM [4-14C]-coumarin (14.5 μCi/ml; Sigma-Aldrich); reactions were carried out for 5 to 10 min. Metabolic activation of AP was assayed by determining the rates of formation of AP glutathione adduct (Gu et al., 1998). The reaction mixtures contained 50 mM potassium phosphate, pH 7.6, 0.05 to 0.5 mM AP, 10 mM reduced glutathione, 1 mM NADPH, and 0.3 to 1.0 mg/ml S9 fraction or microsomal proteins. For assays involving LNG, an NADPH-generating system (5 mM glucose 6-phosphate, 3 mM MgCl2, 1 mM NADP+, and 1.5 U of glucose-6-phosphate dehydrogenase) was used in place of NADPH.

**Animal Treatments.** Animals were normally maintained at 22°C with a 12-h light/12-h off light cycle and were allowed free access to water and a standard laboratory diet. For determination of circadian effects on CYP2A5 expression, animals were kept in the light-off cycle for 4 days and were sacrificed at either 9:00 AM or 9:00 PM, eastern standard time, of the 5th day, using a protocol described by Lavery et al. (1999). The food-feeding behavior test was performed according to Harding et al. (1978), using 2-month-old female mice. Animals were deprived of food overnight and were tested the next morning.

For determination of AP clearance and toxicity, animals were given a single i.p. injection (at 9:00 to 10:00 AM after overnight fasting) of AP (50 mg/ml in warm saline (Genter et al., 1998) or in 25% (w/v) PEG (Sigma-Aldrich) (Nishimura et al., 1999)), at a dose of 200, 400, 600 mg/kg. Animals in the control groups received the corresponding vehicle only. Blood samples (15 μl each) were collected in heparin-coated capillaries by tail bleeding, at 15 min and 1, 2, 6, and 12 h after the injection, and were mixed with an equal volume of saline. The samples were spun at 800g for 5 min at 4°C; the supernatant fraction was mixed with one-half volume of 1 M perchloric acid and spun again at 2000g for 5 min to remove precipitated proteins. Aliquots of the final supernatant were analyzed for AP concentration on a high-performance liquid chromatograph according to the method described previously (Gu et al., 1998).
blood samples were collected by cardiac puncture. ALT levels were determined using a kit from Sigma-Aldrich (DG159-UV), with 10 to 50 μl of plasma. The liver tissue was collected and stored frozen at −80°C before analysis for the content of nonprotein thiols, which was determined according to Tonge et al. (1998). Glutathione was used as a standard. The nasal tissues were fixed in Bouin’s fixative or formaldehyde for histological examination, as described previously (Gu et al., 1997). The nasal cavity was sectioned at three levels (corresponding to levels 3, 5, and 6 in Young, 1986), embedded in paraffin, and stained with hematoxylin and eosin.

**Dissection and Fractionation of LNG.** The mouse head was trimmed to remove the eyes, skin, and lower jaw. The nose was cut, perpendicular to the hard palate and the nasal septum, at the second palatal ridge (Young et al., 1986). The LNG, which surrounds the maxillary turbinate, was identified on both sides of the cut under a dissection microscope and extracted with a fine-tipped surgical forceps. The wet weight of dissected LNG was about 5 to 6 mg/mouse.

**Generation and General Characterization of Cyp2g1-Null Mice.** The structures of the wild-type Cyp2g1 allele, the targeting construct, and the targeted Cyp2g1-null allele are shown in Fig. 1, A to C. A Cyp2g1 genomic clone isolated from the 129/Sv strain was used to construct the targeting vector. The strategy used for targeted disruption of the mouse Cyp2g1 gene by homologous recombination in ES cells was to replace its exon 3, which presumably encodes parts of the substrate-binding site in enzymes of the CYP2 family, with a neomycin-resistance gene. After electroporation, ES cell colonies that survived the double selection with G418 and ganciclovir were genotyped. Analyses of genomic DNA from ES cells with the probe H confirmed that six out of 144 colonies examined (efficiency, 4.2%) were homologous recombinants; the 5.5-kb and 6.5-kb diagnostic fragments on Southern blots corresponding to the targeted allele and the wild-type allele, respectively. ES cells from a homologous recombinant cell line, designated 1D4, were injected into the blastocoeal cavity of B6 embryos, from which 16 chimeric males were generated. When bred with wild-type B6 females, seven chimeras exhibited germine transmission. Homozygous mutant mice (Cyp2g1−/−) were produced by crossbreeding the heterozygous littermates (Cyp2g1+/−). Homozygotes, heterozygotes, and wild-type littermates were identified by the presence of the characteristic bands on Southern blots (Fig. 1D).

**Results**

The impact of the targeted mutation on Cyp2g1 expression in the OM was examined by Northern blot analysis, with a full-length mouse CYP2G1 cDNA probe (Hua et al., 1997). As shown in Fig. 1E, two transcripts were found in the wild-type littermate (+/+), with one of higher mobility being the major transcript. Abundance of the CYP2G1 transcripts was much lower in the heterozygote (+/−), whereas no signal was detected in the homozygous mutant mouse (−/−). The absence of any positive signal in the Cyp2g1−/− mouse also indicated that a transcript from the targeted Cyp2g1 allele was either not generated or not properly processed.

Mice homozygous for the disrupted allele were indistinguishable from their wild-type and heterozygous littermates in growth rate and reproductive ability. No deviation from Mendelian distribution was observed, suggesting that CYP2G1 is not important for embryonic development. No histological abnormalities were detected in the OM, brain, heart, lung, kidney, liver, small intestine, and testis of the Cyp2g1-null mice (data not shown). The Cyp2g1−null mice apparently had normal olfactory ability, as demonstrated in a food-finding behavior test; the Cyp2g1−null mice took 66 ± 23 s (n = 8) to find a buried food pellet, which was not significantly different from the time taken by B6 (96 ± 63 s; n = 8) or 129/Sv (67 ± 32 s; n = 8) mice in the same experiment.

The expression of several other P450 enzymes, including CYP1A2, 2B, 2C, 2E1, 2J, and 3A, as well as CPR and microsomal epoxide hydrolase, was examined by immunoblot analysis of OM microsomes from Cyp2g1−/− mice and the parental wild-type strains as controls. As shown in Fig. 2, expression levels were similar for each of these enzymes in OM microsomes of male and female B6, 129/Sv, and Cyp2g1−/− mice. Thus, disruption of the Cyp2g1 gene did not cause compensatory increases in the expression of these enzymes in the OM.

**Effects of Cyp2g1 Mutation on Cyp2a5 Expression.** The Cyp2g1-null mice have a mixed genetic background (backcrossed once: 50% B6 and 50% 129/Sv), and the expression and activity of CYP2A5 are known to be different in these two strains, as well as between genders (van Iersel et al., 1994). Sequence analysis of the Cyp2a5 gene from the Cyp2g1-null mice indicated that their Cyp2a5 alleles were derived from the 129/Sv strain (data not shown). Thus, the 129/Sv strain was used as a wild-type control for determining the effects of targeted Cyp2g1 mutation on the expression and activity of CYP2A5. Expression of CYP2A5 mRNA in the liver is also known to be influenced by circadian rhythm (Lavery et al., 1999), although it is not known whether similar effects occur in extrahepatic tissues. Therefore, tissues
were obtained at 9:00 AM or 9:00 PM, when the hepatic 
CYP2A5 mRNA was expected to be at about the lowest 
and the highest level, respectively (Lavery et al., 1999). As shown 
in Table 1, the levels of CYP2A5 mRNA were lower in the 
Cyp2g1-null mice than in the 129/Sv mice, in the liver, kid-
ney, and OM of either males or females, at either 9:00 AM 
or 9:00 PM. However, the magnitude of the strain difference 
was much greater in liver and kidney than in the OM, where 
statistically significant differences were only seen at 9:00 
PM. Interestingly, the circadian effects on CYP2A5 mRNA 
level were dramatic in both liver and kidney, but were not 
significant in the OM. Furthermore, these effects were not 
altered by the disruption of the Cyp2g1 gene, except in the 
kidneys of female mice.

Decreases (25–75%) in CYP2A protein levels were also 
observed when microsomal preparations from liver and kid-
ney of 129/Sv and Cyp2g1-null mice (male and female, at 9:00 
AM or 9:00 PM) were analyzed with an antibody to CYP2A5 
(data not shown). However, because the polyclonal antibody 
used probably reacts with mouse CYP2A4, CYP2A5, and 
CYP2A12, the level of CYP2A5 protein could not be deter-
mined using this approach. Similarly, the level of CYP2A5 
protein could not be determined in the OM by immunoblot 
analysis, because the antibody also reacted with CYP2G1.

Subsequently, the relative levels of CYP2A5 enzyme in the 
129/Sv and Cyp2g1-null strains were estimated by a deter-
mination of microsomal activities in coumarin 7-hydroxy-
lation, which is specific for CYP2A5 (Negishi et al., 1989). As 
shown in Table 2, the extent of decrease in coumarin 7-
hydroxylase activities in liver and kidney of the Cyp2g1-null 
mice was more or less similar to the extent of decrease of the 
CYP2A5 mRNA levels shown in Table 1. However, the dif-
fferences in OM activity between the Cyp2g1-null and the 
129/Sv strains were smaller than the corresponding differ-
ces in the CYP2A5 mRNA levels, and were not seen in 
males at 9:00 AM. Thus, there is a definitive and large 
decrease of CYP2A5 expression and activity in the liver and 
kidney of the Cyp2g1-null mice, but a much smaller decrease 
in the OM. In addition, the activities in liver and kidney were 
only slightly higher at 9:00 PM than at 9:00 AM., in contrast 
to the very large differences seen in mRNA levels; in the OM, 
the activity was apparently lower at 9:00 PM than at 9:00 
AM.

In light of the unexpected, large decreases in CYP2A5 level 
in the livers of the Cyp2g1-null mice, the hepatic expression 
of several other P450s was also examined. Immunoblot ana-
lysis with liver microsomes from adult male and female mice 
sacrificed at about 9:00 AM; microsomes prepared from 
combined tissues of three animals per group) indicated that 
the levels of CYP2B, CYP2C, CYP2E1, CYP3A, as well as CPR 
were similar in microsomes from the Cyp2g1-null mice and 
those from the B6 and 129/Sv strains (data not shown). In 
other experiments not presented, the levels of CYP2F2 
mRNA were determined by RNA-PCR in tissues obtained at 
9:00 AM. Although Cyp2/2 was not detected in the Cyp2g1-
containing BAC clone (data not shown), it is located in the 
Cyp2 gene cluster on chromosome 7 and therefore may also 
be impacted by changes in the Cyp2g1 gene. However, the 
results showed that the level of CYP2F2 mRNA was not 
different among the three strains, although the levels were 
about 3 times as high in males than in females in all strains.

Table 1: Relative CYP2A5 mRNA Level (Molar Ratio of CYP2A5 to β-Actin mRNA)

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relative CYP2A5 mRNA Level (Molar Ratio of CYP2A5 to β-Actin mRNA)</th>
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<tr>
<td></td>
<td>9:00 AM</td>
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<tr>
<td></td>
<td>129/Sv</td>
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<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>400 ± 130</td>
</tr>
<tr>
<td>Liver</td>
<td>0.87 ± 0.20</td>
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<tr>
<td>Kidney</td>
<td>1.26 ± 0.48</td>
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<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>OM</td>
<td>540 ± 54</td>
</tr>
<tr>
<td>Liver</td>
<td>8.5 ± 2.8</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.40 ± 0.16</td>
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<sup>a</sup> Cyp2g1-null over 129/Sv.

<sup>b</sup> Not significantly different when compared with 129/Sv mice; P > 0.05 (Student’s t test).

<sup>c</sup> Not significantly different when compared with corresponding groups at 9:00 AM (P > 0.05).
identified (Hua et al., 1997), were about 60% lower in the Cyp2g1-null mice than in the 129/Sv mice. The extent of decrease is even greater when compared with rates in the B6 mice. Thus, CYP2G1 clearly plays a major role in microsomal metabolism of these sex steroids in the OM. CYP2G1 also seems to play the dominant role in OM microsomal activation of coumarin: the rates of formation of o-HPA were almost 80% lower in the Cyp2g1-null mice than in the 129/Sv strain. Again, the extent of activity decrease is greater compared with the B6 strain. On the other hand, CYP2G1 may not play a major role in OM microsomal metabolism of DCBN; a statistically significant difference was not found between Cyp2g1-null and 129/Sv mice in the rates of formation of DCBN-protein adduct.

Impact of Cyp2g1 Disruption on AP Metabolism and Toxicity in Vivo. The reduction in CYP2A5 expression in the liver and kidney of the Cyp2g1-null mice suggested that this mouse model might have a decreased systemic clearance of those drugs that are CYP2A5 substrates. In the vivo clearance and toxicity of a known CYP2A5 substrate, AP, was therefore examined. As shown in Table 4, there was no decrease in AP clearance after a single i.p. injection at 400 or 600 mg/kg in the Cyp2g1-null mice. There was also no significant difference in the extent of hepatotoxicity among the three strains, as indicated by the survival rate and by levels of plasma ALT and hepatic total nonprotein thiol at 24 h after treatment (Table 5). Thus, hepatic CYP2A5 does not seem to play a significant role in the clearance or hepatotoxicity of AP in vivo, which was confirmed by in vitro studies (data not shown), indicating that rates of liver microsomal metabolism of AP (at a substrate concentration of 50 μM) to glutathione adduct were not significantly different between Cyp2g1-null mice and B6 or 129/Sv mice.

AP treatment also caused toxicity in the nasal cavity, as has been reported previously (Genter et al., 1998). The most striking lesions were in the LNG (Fig. 3) and consisted of mild to moderate, multifocal to locally extensive necrosis, characterized by loss of glandular epithelial cell granules, as
well as nuclear pyknosis or karyorrhexis of glandular or ductal epithelium. Interestingly, the Cyp2g1-null mice were resistant to AP toxicity in the LNG at a dose of 400 mg/kg (Table 5; Fig. 3). These mice also seemed to be less sensitive to AP toxicity than were the wild-type strains at 600 mg/kg, although the numbers of surviving mice in each group were not sufficient to draw definitive conclusions (Table 5). The site-specific, protective effects of Cyp2g1 gene disruption on the extent of AP cytotoxicity in the LNG were also observed when AP was administered in saline, which yielded lower plasma AP levels than when AP was injected in PEG (data not shown).

Other lesions in the nasal cavity included degeneration and necrosis of respiratory epithelium; sloughing of epithelial cells of nasolacrimal duct into lumen; degeneration or detachment of olfactory epithelium; necrosis of submucosal serous glands, including Bowman’s glands; and lympholysis of lymphocytes in the mucosa-associated lymphoid tissue. However, there was no clear difference in the extent of AP toxicity in these structures among the three strains (data not shown). No lesion was found in vehicle-treated B6, 129/Sv, or Cyp2g1-null mice. The apparent lack of effects of Cyp2g1 gene disruption on the extent of AP cytotoxicity in the OM is consistent with results from in vitro studies with OM postmitochondrial S9 fractions, which indicated only a small (about 20–30%) decrease at a substrate concentration of 50 or 100 μM (data not shown), and no decrease at 0.5 mM (Table 6), in the rates of AP metabolism to glutathione adducts in Cyp2g1-null mice, relative to B6 or 129/Sv mice.

The site-specific, protective effects of Cyp2g1 gene disruption on the extent of AP cytotoxicity in the LNG prompted further analysis of P450 expression in this organ. RNA-PCR analysis of dissected LNG detected CYP2A5, but not CYP1A2 or CYP2G1 (Fig. 4A). The identity of the LNG CYP2A5 PCR product was confirmed by sequencing. The level of CYP2A protein in the LNG S9 fraction was much lower in the Cyp2g1-null mice than in the wild-type control mice (Fig. 4B); densitometric analysis indicated that the levels in the Cyp2g1-null mice were less than 10% of the levels detected in the B6 or 129/Sv mice. The reduction in LNG CYP2A protein level was confirmed by decreases in coumarin 7-hydroxylase activity; however, the rates of in vitro metabolic activation of AP by LNG S9 fractions were very low, compared with those of the OM S9 fractions from the same animals, and were not decreased in the Cyp2g1-null mice (Table 6).

**Discussion**

The Cyp2g1−/− mice generated in this study are viable and fertile, and they develop normally and do not show signs of severe olfactory deficits. There seem to be no marked compensatory changes in the expression of the other P450 enzymes in the OM of Cyp2g1-null mice. Thus, CYP2G1 seems to be nonessential in mice for development, reproduction, and olfactory chemoreception. On the other hand, despite the potential for complications by strain- and sex-dependent differences in microsomal metabolic activities, we were able to show that mouse CYP2G1 is the major P450 involved in microsomal metabolism of testosterone and progesterone, and in the metabolic activation of coumarin in the OM. Thus, this animal model will be valuable for assessing the contribution of CYP2G1 to nasal metabolism of other steroid hormones, odorants, drugs, and toxicants. Furthermore, initial studies of the impact of Cyp2g1 knockout on AP toxicity led to an intriguing discovery that the LNG, a previously unidentified target organ for AP toxicity, became

### TABLE 4

Acetaminophen clearance in Cyp2g1-null and wild-type mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose</th>
<th>Body Weight</th>
<th>AUC0–12 g mg·h/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>B6</td>
<td>400</td>
<td>27.4 ± 2.4</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td>129/Sv</td>
<td>400</td>
<td>27.0 ± 2.5</td>
<td>3.6 ± 0.7</td>
</tr>
<tr>
<td>Cyp2g1-null</td>
<td>400</td>
<td>31.4 ± 6.4</td>
<td>2.0 ± 0.3</td>
</tr>
</tbody>
</table>

*There is no significant difference between Cyp2g1-null and 129/Sv mice; *P > 0.05.

*There is no significant difference between Cyp2g1-null mice and either of the corresponding wild-type groups; *P > 0.05.

Discussion

The Cyp2g1−/− mice generated in this study are viable and fertile, and they develop normally and do not show signs of severe olfactory deficits. There seem to be no marked compensatory changes in the expression of the other P450 enzymes in the OM of Cyp2g1-null mice. Thus, CYP2G1 seems to be nonessential in mice for development, reproduction, and olfactory chemoreception. On the other hand, despite the potential for complications by strain- and sex-dependent differences in microsomal metabolic activities, we were able to show that mouse CYP2G1 is the major P450 involved in microsomal metabolism of testosterone and progesterone, and in the metabolic activation of coumarin in the OM. Thus, this animal model will be valuable for assessing the contribution of CYP2G1 to nasal metabolism of other steroid hormones, odorants, drugs, and toxicants. Furthermore, initial studies of the impact of Cyp2g1 knockout on AP toxicity led to an intriguing discovery that the LNG, a previously unidentified target organ for AP toxicity, became

### TABLE 5

Acetaminophen toxicity in Cyp2g1-null and wild-type mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose</th>
<th>24-h Survival Ratea</th>
<th>Plasma ALTb b</th>
<th>Hepatic NPTc b</th>
<th>LNG Toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B6</td>
<td>200</td>
<td>8/8</td>
<td>4000 ± 3800</td>
<td>5.6 ± 1.6</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>7/8</td>
<td>5500 ± 2000</td>
<td>3.2 ± 2.5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>6/8</td>
<td>3700 ± 2600</td>
<td>2.7 ± 1.8</td>
<td>2</td>
</tr>
<tr>
<td>129/Sv</td>
<td>200</td>
<td>8/8</td>
<td>6300 ± 3500</td>
<td>4.7 ± 1.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>7/8</td>
<td>5400 ± 3200</td>
<td>2.8 ± 1.8</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>6/8</td>
<td>3200, 1000</td>
<td>3.3, 3.0</td>
<td>1</td>
</tr>
<tr>
<td>Cyp2g1-null</td>
<td>200</td>
<td>8/8</td>
<td>5200 ± 2400</td>
<td>5.9 ± 1.2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>8/8</td>
<td>3400 ± 2900</td>
<td>4.4 ± 1.5</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>3/8</td>
<td>2300 ± 500</td>
<td>3.1 ± 0.7</td>
<td>2</td>
</tr>
</tbody>
</table>

*ND, not determined.

a Number of mice survived over total number of mice tested.

b Means ± S.D. There is no significant difference between Cyp2g1-null mice and either of the corresponding wild-type groups.
resistant to AP in the Cyp2g1-null mice at AP doses that caused toxicity to the OM and liver, despite the fact that CYP2G1 is not normally expressed in the LNG. We propose that OM CYP2G1 (through a paracrine pathway) or LNG CYP2A5 may indirectly influence resistance of the LNG to AP because CYP2A5 is the major coumarin hydroxylase in the liver (Negishi et al., 1989), and because the Cyp2a5 gene has a strain-related genetic polymorphism that significantly affects coumarin hydroxylase activity (Lindberg et al., 1992). It is likely that the impact of an altered hepatic CYP2A5 expression on OM toxicity will be less worrisome for compounds delivered through the nose. The lack of effect of CYP2A5 down-regulation on systemic clearance and hepatic toxicity of AP is consistent with previous findings indicating that CYP2E1 and CYP1A2 are major contributors to AP metabolism and toxicity in the liver (Genter et al., 1998; Tonge et al., 1998).

Several P450s known to be expressed in the OM are active in the metabolic activation of AP (Gu et al., 1998), which may explain why OM microsomes from Cyp2g1-null mice had only slightly lower turnover numbers for AP than those in wild-type mice (at low substrate concentrations), and why Cyp2g1-null mice were not protected against AP toxicity to the OM.

### Table 6

<table>
<thead>
<tr>
<th>Strain</th>
<th>Coumarin 7-Hydroxylation</th>
<th>AP-Glutathione Adduct Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>LNG</td>
<td>OM</td>
</tr>
<tr>
<td>B6</td>
<td>8</td>
<td>700</td>
</tr>
<tr>
<td>129/Sv</td>
<td>14</td>
<td>2300</td>
</tr>
<tr>
<td>Cyp2g1-null</td>
<td>5</td>
<td>2100</td>
</tr>
</tbody>
</table>

Fig. 3. Histological analysis of AP toxicity in the LNG. Male, 3-month-old wild-type and Cyp2g1-null mice were treated with AP as described in the legend to Table 5. All sections were at the level of the second palatal ridge (level 5 in Young, 1986). A, cross section of the nasal cavity (of a 129/Sv mouse) at low magnification. The rectangular box marks the area shown in B and C at 100× magnification. ET, ethmoturbinate; MS, maxillary sinus; SP, nasal septum. B, LNG in this AP-treated Cyp2g1-null mouse had normal, palely staining cuboidal duct cells (DC) with open face nuclei and plump secretary cells (SC) with bright eosinophilic cytoplasmic granules. A 400× view is shown in D, C, LNG in this AP-treated 129/Sv mouse had dark, shrunken duct cells (+), congested vessel (CV), and pale, ragged secretary cells (PSC). At 400× magnification (E), additional signs of cytotoxicity can be seen, including apoptotic glandular epithelial cells (arrowheads), karyorrhexis (arrows), cytoplasmic fragmentation with loss of secretory granules (circles), and nuclear condensation in the shrunken ductal epithelial cells (+). Scale bar, 400 μm in A, 30 μm in B and C, and 10 μm in D and E.

Fig. 4. P450 expression in the LNG. A, detection of CYP1A2, CYP2A5, and CYP2G1 mRNA by RNA-PCR. RNA from OM (as a positive control) and LNG of B6 mice was analyzed as described under Materials and Methods. The positions of selected fragments of a 100-bp DNA marker are indicated. B, immunoblot analysis of CYP2A expression in the LNG of Cyp2g1-null (KO) and wild-type C57BL/6 and 129/Sv mice. S9 fractions were prepared from pooled LNG of eight 2-month-old female mice in each group. Equal amounts of protein (10 μg) were analyzed in each lane with an antibody to CYP2A5.

Table 6

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</tr>
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<td>Cyp2g1-null</td>
<td>5</td>
<td>2100</td>
</tr>
</tbody>
</table>
However, the resistance of the LNG to AP toxicity in the Cyp2g1-null mice is intriguing. On one hand, the down-regulation of CYP2A5, which is active in the metabolic activation of AP (Gu et al., 1998), seems to provide a logical explanation; the absence of CYP2G1 and CYP1A2 would make the contribution of CYP2A5 to local AP metabolism much more significant in the LNG than in the OM. However, in vitro analysis indicated that activity of AP metabolic activation was very low in the LNG, and was apparently not reduced in the Cyp2g1-null mice. Thus, the protective effect seen in the LNG of the Cyp2g1-null mice may not be related to a decreased local metabolic activation of AP. Instead, the loss of CYP2G1 in the OM or the down-regulation of CYP2A5 in the LNG (or both) may have resulted in altered metabolism and homeostasis of an endogenous substrate(s), such as a steroid hormone, that is important for the defense of the LNG against AP toxicity. Further studies to confirm this hypothesis are warranted, and the results may have general implications on the endogenous functions of microsomal “drug-metabolizing” P450 enzymes in various extrahepatic tissues.

The mechanism of chemically induced LNG toxicity is not clear. Although in vitro activity of LNG S9 fractions seemed to be very low, the LNG has been found to have significant bioactivation activities in vivo; many compounds have been shown to be bioactivated in the LNG after systemic administration (Castonguay et al., 1983). Few studies have examined P450 expression in the LNG (Adams et al., 1991; Chen et al., 1992). In this study, we have obtained definitive evidence for the expression of CYP2A5, and for the lack of expression of CYP1A2 and CYP2G1, in mouse LNG. Nevertheless, the role of LNG P450s in target tissue toxicity remains to be determined. Additionally, the potential impact of chemically induced LNG degeneration on the production of odorant-binding proteins or on the local immune function will be interesting topics to pursue.

Both Cyp2a5 and Cyp2g1 are predominantly expressed in the OM (for review, see Ding and Dahl, 2003). The genes are adjacent to one another on chromosome 7, with Cyp2g1 immediately (less than 20 kb) upstream of Cyp2a5 (Wang et al., 2003). The mechanism of Cyp2a5 suppression in the Cyp2g1-null mice has not been identified. However, neighboring effects of gene targeting have been documented previously for other genes (for review, see Olson et al., 1996). In the present case, the neo gene, which replaces exon 3 of Cyp2g1, was retained in the targeted allele; it was oriented in the same transcriptional direction as the Cyp2g1 gene and the downstream Cyp2a5 gene. It is likely that active transcription of the neo gene in tissues that normally have an inactive Cyp2g1 allele (such as the liver and the LNG) disturbed the chromosomal structure at the Cyp2a5 promoter, thereby leading to suppression of Cyp2a5 transcription. On the other hand, because the Cyp2g1 locus was normally active in the OM, the effects of neo insertion on Cyp2a5 expression would be less pronounced. Further studies on this tissue-selective neighboring effect, such as studies on the nature and sites of chromosomal alternations in the liver and OM, may shed new light on the mechanisms of tissue-selective expression of the CYP2A genes.

The neighboring effect of Cyp2g1 disruption was most likely limited to the Cyp2a5 gene. This is supported by the observation that hepatic expression of Cyp2f2, which is about 250 kb downstream of Cyp2a5 (Wang et al., 2003), was not affected. Although it is not clear at present which of the CYP2B proteins were detected in liver microsomes by the polyclonal anti-CYP2B antibody, the lack of alteration in the overall intensity of the detected CYP2B bands suggests that the expression of the Cyp2b genes, which are about 40 to 950 kb upstream of Cyp2g1 (Wang et al., 2003), was not affected either. The first identified gene downstream of Cyp2a5 seems to be a Cyp2a pseudo-gene (Cyp2a23p), about 65 kb away (Wang et al., 2003).

The dramatic circadian variation in the level of hepatic and renal CYP2A5 mRNA was not altered by the disruption of the Cyp2g1 gene (except in case of female kidney), despite overall decreases in CYP2A5 mRNA levels in these tissues. Previous studies by Lavery et al. (1999) had indicated that, in the liver, this circadian effect was partly controlled by the transcription factor albumin D-site-binding protein. Interestingly, we observed that the circadian variation in CYP2A5 mRNA level was essentially undetectable in the OM, a fact which implies tissue differences in the role of this transcription factor at the Cyp2a5 promoter.

The complex and poorly characterized strain variations in the expression and activity of various microsomal Cyp genes and enzymes remain a major challenge for studies using Cyp-null mouse models. In our Cyp2g1-null model, which is on a mixed B6 and 129/Sv background, the close linkage between Cyp2g1 and Cyp2a5 dictates that the Cyp2a5 gene is derived from the 129/Sv strain, from which the Cj7 ES cells used for gene targeting were derived. In contrast, the Cyp2a5 gene in the wild-type littermates is derived from the B6 strain. Other nearby Cyp genes will also likely cosegregate with the targeted Cyp2g1 allele, and therefore will likely be derived from the 129/Sv strain. Both Cyp2a5 (Lindberg et al., 1992) and Cyp2g1 (Zhuo et al., 2001) have coding region polymorphisms that may have contributed to the strain differences in microsomal activities seen in this study. Therefore, it was necessary to use both parental strains as controls in most of the experiments, which may have resulted in an underestimation of the contributions of CYP2G1 in some cases.

Acknowledgments

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