Xenobiotic Metabolism in Mice Lacking the UDP-Glucuronosyltransferase 2 Family

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Abbreviations: BAC, bacterial artificial chromosome; bp, base pairs; BPA, bisphenol A; BPAG, bisphenol A glucuronide; dH2O, deionized water; ELISA, enzyme-linked immunosorbant assay; ES, embryonic stem; HPLC, high-performance liquid chromatography; kb, kilobase pairs; napG, naproxen glucuronide; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PGK, phosphoglycerate kinase; RT-PCR, reverse transcription-PCR; qPCR, quantitative PCR; SPF, specific-pathogen free; UDPGA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase; WT, wild type.
ABSTRACT

UDP-Glucuronosyltransferases (UGTs) conjugate a glucuronyl group from glucuronic acid to a wide range of lipophilic substrates to form a hydrophilic glucuronide conjugate. The glucuronide generally has decreased bioactivity and increased water solubility to facilitate excretion. Glucuronidation represents an important detoxification pathway for both endogenous waste products and xenobiotics, including drugs and harmful industrial chemicals. Two clinically significant families of UGT enzymes are present in mammals: UGT1s and UGT2s. Although the two families are distinct in gene structure, studies using recombinant enzymes have shown considerable overlap in their ability to glucuronidate many substrates, often obscuring the relative importance of the two families in the clearance of particular substrates in vivo.

To address this limitation, we have generated a mouse line, termed ΔUgt2, in which the entire Ugt2 gene family, extending over 609 kb, is excised. This mouse line provides a means to determine the contributions of the two UGT families in vivo. We demonstrate the utility of these animals by defining for the first time the in vivo contributions of the UGT1 and UGT2 families to glucuronidation of the environmental estrogenic agent bisphenol A (BPA). The highest activity toward this chemical is reported for human and rodent UGT2 enzymes. Surprisingly, our studies using the ΔUgt2 mice demonstrate that, while both UGT1 and UGT2 isoforms can conjugate BPA, clearance is largely dependent on UGT1s.
Introduction

UDP-Glucuronosyltransferases (UGTs) are membrane-bound phase II enzymes that conjugate a wide array of lipophilic substrates with a glucuronyl group from UDP-glucuronic acid (UDPGA). The highly polar glucuronide conjugate generally decreases the bioactivity of the substrate and increases its water solubility, facilitating excretion through bile or urine. Glucuronidation is important in the metabolism of endogenous wastes and of a wide range of drugs, including many chemotherapeutics, nonsteroidal anti-inflammatory drugs, and opioids, and in protecting against harmful environmental agents such as bisphenol A and benzo-[a]-pyrene (Fang et al., 2002; Hanioka et al., 2008; Kutsuno et al., 2013). Based on evolutionary divergence and homology, mammalian UGTs have been organized into two major families: The UGT1 family and the UGT2 family. The mammalian UGT1 family currently contains only the UGT1A subfamily, while the mammalian UGT2 family contains the UGT2A and UGT2B subfamilies (Mackenzie et al., 2005; Rowland et al., 2013).

The mouse UGT1 family is encoded by the Ugt1a gene. It consists of 9 functional isoforms, each encoded by a unique 5’ exon spliced to a shared set of downstream exons (Mackenzie et al., 2005). The first exon specifies a UGT1A isoform and its substrate specificity, while the downstream exons encode common domains such as a UDPGA binding site and a transmembrane region (Owens et al., 2005). The three Ugt2a and seven Ugt2b genes in mouse are located in a single cluster spanning approximately 600 kb on chromosome 5 (Mackenzie et al., 2005). Ugt2a1 and Ugt2a2, similar to Ugt1a, have unique first exons and a shared set of downstream exons (Jedlitschky et al., 1999). All other Ugt2 genes have a typical structure, with unique exons encoding both the N-terminal substrate-binding domain and a C-terminal UDPGA-binding domain and transmembrane sequence (Hum et al., 1999). Similar to the UGT1 family, the C-terminal domains of the UGT2 family are conserved, while the highly variable N-terminal domains determine substrate specificity (Radominska-Pandya et al., 1999). Many UGT isoforms exhibit activity towards a broad range of substrates, and the specificities of different isoforms often overlap (de Wildt et al., 1999).

Various approaches have been used to determine which UGTs are involved in glucuronidation of particular compounds (Rowland et al., 2013). Assignment using computational approaches is limited by the lack of established structure-activity relations for most UGTs (Kaivosaari, 2010). The majority of
current knowledge comes from assessment of metabolism of substrates by microsomes reconstituted with recombinant enzymes. Such studies have shown that UGT2s are involved in conjugation of a variety of endogenous metabolites, such as retinoic acid and steroid hormones, as well as xenobiotics, such as opioid analgesics (Hum *et al.*, 1999; Radominska-Pandya *et al.*, 2001; Chouinard *et al.*, 2007). While information from these studies can be used to predict the relative importance of various enzymes *in vivo*, extrapolation of *in vitro* results to *in vivo* systems remains an imperfect practice (Lin and Wong, 2002; Miners *et al.*, 2006). Such modeling has to take into account not only the activities of the enzymes towards the substrate under study but also the fact that there are quantitative and qualitative differences in the expression of these enzymes in the tissues exposed to the chemical. Ultimately, *in vivo* metabolism is required to confirm the contribution of particular UGT enzymes to the metabolism of a compound. However, due to the functional overlap of many isoforms, it is often difficult to measure the contributions of one isoform or even one UGT family *in vivo*.

These challenges have been encountered in defining the contributions of different UGTs to metabolism and clearance of environmental pollutants such as BPA, a monomer commonly used in polycarbonate materials with widespread human exposure. BPA’s reported toxicity in mice at low doses (Richter *et al.*, 2007), possibly through interference in both genomic and non-genomic estrogen responses (Luconi *et al.*, 2002), has made it the subject of intense research (Krishnan *et al.*, 1993). The ability of BPA to impact physiological processes is likely limited by the rapid clearance of the inactive BPA glucuronide (BPAG) (Matthews *et al.*, 2001; Vökel *et al.*, 2002), and studies with recombinant enzymes indicate that human UGT2B15 and rodent UGT2B1 likely play the primary role in clearance (Yokota *et al.*, 1999; Hanioka *et al.*, 2008). This has raised concerns regarding exposure of neonates to BPA (Ginsberg and Rice, 2009), as expression of UGT2 genes during the neonatal period is much lower than in children and adults (Court *et al.*, 1988; de Wildt *et al.*, 1999; Divakaran *et al.*, 2014). Despite the large efforts towards understanding the health impact of BPA exposure, remarkably little is known regarding its metabolic pathways in human or in the model organisms used in assessing its adverse effects.

To address these limitations, we have generated a mouse line homozygous for a targeted deletion of the entire *Ugt2* family. We show that loss of these enzymes does not adversely affect the development of these animals and that microsomes from mice lacking all *Ugt2* genes can be used to establish the role of
the UGT2 enzymes in glucuronidation of xenobiotics. Most importantly, this mouse line can be used to investigate the contribution of UGT2 enzymes to the metabolism of drugs and xenobiotics in vivo.

**Materials and Methods**

**Reagents and Materials.** Alamethicin and (S)-naproxen were purchased from Cayman Chemical (Ann Arbor, MI). BPA, BPAG, UDPGA, and β-glucuronidase were purchased from Sigma-Aldrich (St. Louis, MO). Naproxen glucuronide ((S)-naproxen acyl β-D glucuronide) was purchased from Toronto Research Chemicals (Toronto, ON). All solvents and other reagents were of analytical grade or better. Zorbax Eclipse XDB-C18 and Tosoh TSKGel 80TM columns were purchased from Agilent Technologies (Santa Clara, CA) and Tosoh Bioscience (Tokyo, Japan) respectively. Primer sequences can be found in Supplemental Methods.

**Generation of Mouse Lines.** A replacement type targeting vector was assembled from 129 BACs bMQ357m23 and bMQ37h06. DNA corresponding to the Ugt2 gene cluster was replaced with a neomycin resistance gene driven by the phosphoglycerate kinase promoter. Homologous recombination of the targeting vector with the endogenous Ugt2 locus creates a 609 kb deletion extending from 580 bp downstream of the Ugt2b34 gene to 7.2 kb upstream of the Ugt2a1/2 gene. 129S6-derived ES cells were cultured using standard methods, and DNA was introduced by electroporation. Cells were selected in G418 (Geneticin) and were evaluated by PCR and Southern blot analysis. Correctly targeted ES cells were introduced into C56BL/6 blastocysts and the blastocysts introduced into B6D2F1/J foster mothers to complete their development. All mice were maintained in specific pathogen-free housing in ventilated caging. 129S6 mice purchased from Taconic (Hudson, NY) were bred to chimeras to maintain the deleted locus on this genetic background. All mice used in these studies are therefore 129S6, coisogenic for the deleted Ugt2 locus. For all studies of reproductive function, wild type and null mice were generated by the intercross of heterozygous animals. Serum testosterone levels were measured by ELISA (Endocrine Technologies, Newark, CA). For some experiments mutant mice were age- and sex-matched at weaning and housed together until used in an experiment. All studies were conducted in accordance with the National institutes of Health Guide for the Care and Use of Laboratory Animals as well as the Institutional Animal Care and Use Committee guidelines for University of North Carolina-Chapel Hill.
Gene Expression Assays. Total RNA was isolated from tissue homogenates using a Quick-RNA Miniprep Kit (Zymo Research, Irvine, CA), and reverse transcription PCR was performed using a High Capacity cDNA Reverse Transcription Kit (Life Technologies, Grand Island, NY). cDNA was amplified with FastStart Universal Probe Master with Rox normalization (Roche Applied Science, Indiana, IN), and Ugt1 and Ugt2 expression were measured using SYBR Green qPCR primers and TaqMan qPCR probe mixes (Life Technologies, Grand Island, NY), respectively. Amplifications were performed in duplicate on a QuantStudio 6 Flex Real-Time PCR System (Life Technologies, Grand Island, NY). Data were analyzed using the comparative C_T method as described by Applied Biosystems. Relative expression was determined by normalizing samples to 18S rRNA expression.

Microsome Preparation. Liver microsomes were prepared from 3-5 month old males. Median and left lobes of each liver were harvested after systemic perfusion with PBS and homogenized manually in 0.05 M phosphate buffer (pH 7.4) with 0.154 M potassium chloride and 0.25 M sucrose. Homogenate was centrifuged twice at 10,000 g for 10 min, and the post-mitochondrial fraction was removed and centrifuged at 100,000 g for 45 min. Microsomal pellets were washed with homogenization buffer and resuspended in 0.1 M phosphate buffer (pH 7.4) with 20% glycerol (v/v) and 1 mM EDTA. All preparation steps were performed at 4°C. Microsomal protein concentration was determined using Bio-Rad Protein Assay Dye (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard. Microsomes were stored at -80°C until used and underwent no more than one freeze/thaw cycle before experimentation.

HPLC. HPLC was performed on an Agilent 1200 Series instrument. The system comprised a binary solvent pump with temperature-controlled autosampler, on-line degasser, variable wavelength UV-Vis detector, temperature-controlled column oven, and automated fraction collector. Chromatographic protocols are detailed in Table 1. Identities of all glucuronide peaks were verified by comparison to an authentic standard and by loss of the peak after β-glucuronidase treatment.

Microsomal Glucuronidation Assays. Glucuronidation reaction mixtures contained 0.1 M Tris buffer (pH 7.5), 4 mM magnesium chloride, substrate, and the indicated amount of microsomes in a final volume of 500 µL. Total organic solvent concentrations were less than 0.7% (v/v). Naproxen glucuronidation reactions contained 25 µg/mL alamethicin; BPA glucuronidation was performed both with and without
alamethicin. After preincubation on ice and preheating at 37°C for 5 min, reactions were initiated by addition of UDPGA to a final concentration of 5 mM and incubated at 37°C. Reactions were terminated by addition to an equal volume of ice-cold 4% acetic acid in methanol (v/v) and vortex mixing. Microsomal protein was removed by centrifugation at 10,000 g for 10 min at 4°C, and 30 µL of supernatant was analyzed by HPLC. Controls included baseline measurements and blanks lacking UDPGA. Glucuronide cleavage was performed by incubation of samples with 1,000 units of β-glucuronidase for 4h at pH 5-6. Retention times for naproxen and its glucuronide, napG, were 8.4 and 4.7 min, respectively. Consistent with previous studies (Bowalgaha et al., 2005), the napG standard was unstable, and napG was quantified based on a standard curve for naproxen, which was linear in the range 1 µM to 100 µM ($r^2>0.999$). The lower limit of detection was 1 µM for naproxen and napG. Retention times for BPA and BPAG were 15.2 and 12.8 min, respectively. Standard curves for BPA and BPAG were linear in the range 0.5 µM to 1 mM ($r^2>0.999$ for both), and the lower limit of quantification was 0.5 µM for BPA and BPAG. Kinetics data were analyzed using Microsoft Excel 2010 and GraphPad Prism v4.0 software.

**In Vivo BPA Glucuronidation.** Age and weight-matched WT and ΔUgt2 mice were anesthetized with urethane after which the bile duct was cannulated. After collection of a sample of bile prior to dosage, mice received BPA intravenously as a bolus in 10% ethanol/water (v/v). Two BPA dosage levels were examined: 2 mg BPA/kg bodyweight and 20 mg BPA/kg bodyweight. Bile fractions were collected in 10 min intervals over the next 50 min. Samples were diluted 1:40 in dH₂O, and 30 µL of each sample were analyzed by HPLC. Retention time for BPAG was 5.7 min. Standard curves for BPAG were linear in the range 0.5 µM to 1 mM ($r^2>0.999$) and the lower limit of quantification was 0.5 µM for BPA and BPAG.

**Results**

**Generation of Mouse Line and Verification of ΔUgt2 Locus.**

The Ugt2 genes are located in a segment of DNA on chromosome 5 between Ythdc1 and Sult1b1 (Fig. 1). While numerous polymorphisms distinguish the Ugt2 locus of the 129 strain from that of C57BL/6, no difference in the number and overall organization of the genes was identified by PCR, sequence, or Southern blot analysis. Therefore, using the organization of the published C57BL/6 locus as a guide, we generated a targeting vector designed to remove the entire gene family in a single recombination event in
129 derived ES cells. The deletion was designed to leave the Ythdc1 and Sult1b1 genes, including regulatory regions, intact (Fig. 1). Sult1b1 encodes a sulfotransferase enzyme which contributes to conjugation and excretion of endo- and xenobiotics, and therefore any compromise in the expression of this gene would complicate interpretation of the phenotypes of the UGT2 deficient animals. ES cells were identified in which the targeting vector had integrated into the Ugt2 locus. These were analyzed to ensure that the recombination event had occurred at the predicted locations in the mouse genome and had resulted in loss of all Ugt2 genes. This analysis included extensive PCR, sequence and Southern blot analysis. ES cells that met these criteria were used to generate a mouse line. For simplicity, we refer to this mutation as the ΔUgt2 allele. The ΔUgt2 allele was maintained on the 129S6 genetic background by breeding transmitting chimeras to purchased 129S6 dams. Mice heterozygous for the deletion were intercrossed to generate mice homozygous for the allele. DNA from these pups was used to further verify the loss of all Ugt2 genes by examination of both DNA and RNA from these animals. A probe was generated by PCR from exon 1 of Ugt2b38 that is expected to bind to multiple sites throughout the deleted segment, including the first exons of Ugt2b5, Ugt2b37, Ugt2b36, and Ugt2b35 as well as the intergenic region between Ugt2a3 and Ugt2b38. Analysis of DNA from pups generated by intercross of heterozygous parents showed that this probe failed to bind to DNA from the homozygous ΔUgt2 offspring (Fig. 2A). As expected, probes corresponding to regions flanking the deletion bound to the DNA from these animals and identified the predicted, novel DNA fragments generated by the recombination event.

RNA was prepared from the nasal epithelium and liver of homozygous ΔUgt2 animals and their wild type littermates. As expected, high levels of Ugt2a1/2 expression are observed in epithelium from wild type animals, while expression is absent in samples collected from the null mice (Fig. 2B). Expression of members of the Ugt2b subfamily and Ugt2a3 were examined in RNA prepared from the liver. Again, expression of these genes is easily detected in the wild type animals, whereas no signal is observed in ΔUgt2 animals (Fig. 2C). Taken together, these data support the DNA analysis indicating that the ΔUgt2 mice lack all Ugt2b and Ugt2a genes.

**Development and Reproductive Behavior of the ΔUgt2 Mouse.**

Mice lacking the Ugt2 genes are present in litters at expected Mendelian ratios. The growth and development of the mice is normal, and they cannot be distinguished by observation from their wild type
littermates. The human UGT2B enzymes, including UGT2B4, UGT2B7, UGT2B15, and UGT2B17, have been shown to participate in the glucuronidation of endogenous steroids as well as xenobiotics (Hum et al., 1999). Alteration in steroid metabolism could dramatically alter the development of primary and secondary sex organs and thus impair the fecundity of mice. We therefore intercrossed mice homozygous for the ΔUgt2 locus with wild type littermates. The number of pups born to dams was within the normal size range (Supplemental Fig. 1). Furthermore, when ΔUgt2 males were presented with fertile WT females, the interval until the first mating, the number of matings, the percentage of matings resulting in pregnancies, and the size of the resulting litters were not significantly different when compared to WT males (Supplemental Fig. 2). To further examine this point, male mice were sacrificed at 8 weeks, and their body weight and the sizes of their testes and seminiferous tubules were examined. No difference in the development of these organs was observed, consistent with the normal fertility of these animals (Supplemental Fig. 3). Serum testosterone levels measured in individually housed, 5-6 month old male mice did not differ significantly from controls (Supplemental Fig. 4).

**Gene Expression**

The health of the ΔUgt2 animals could reflect compensation for the loss of Ugt2 genes by increased expression of Ugt1s, as substantial overlaps in the activities of these two enzyme families have been documented in both humans and rats (Radominska-Pandya et al., 1999; Lin and Wong, 2002; Bock, 2010). Increased expression of Ugt1 isoforms in response to loss of the Ugt2 genes would impact any conclusions drawn regarding observed differences in the metabolism of chemicals by the ΔUgt2 animals. As mentioned above, the Ugt1 isoforms each have a unique 5’ exon. Primers specific for the first exons of Ugt1a1, Ugt1a7, and Ugt1a9 were generated and each was paired with a common reverse primer corresponding to the shared second exon. qPCR analysis using these primers revealed that expression patterns for these three Ugt1 mRNAs are similar to those reported previously (Buckley and Klaassen, 2007). When compared to wild type mice, ΔUgt2 mice show no significant difference in expression of any of the three mRNAs in the adrenal gland, liver, prostate, epididymis, or testes (Fig. 3). This indicates that the normal development of these mice does not reflect compensation for loss of these genes by increased expression of UGT1 isoforms. These results also support the general usefulness of this mouse line for determining the contribution of the Ugt2 genes to xenobiotic metabolism.
Naproxen Glucuronidation.

To demonstrate the usefulness of the ΔUgt2 mice in evaluation of the role of UGT2 enzymes in xenobiotic metabolism, we examined the ability of liver microsomes from these mice to metabolize naproxen. We chose this assay because a number of lines of evidence indicate that human and rat UGT2 isoforms show selectivity toward this substrate (Mouelhi et al., 1987; Pritchard et al., 1994; Bowalgaha et al., 2005; Sanoh et al., 2012). Microsomes from ΔUgt2 and co-isogenic wild type controls were incubated with naproxen for 2h, and supernatant was analyzed by HPLC for the presence of the glucuronide. Microsomes from wild type mice formed 46.2 ± 1.9 (mean ± S.E.M., n=3) nmol of naproxen glucuronide per mg microsomal protein while microsomes from ΔUgt2 mice showed no detectable amount of glucuronide formation (Fig. 4). These data indicate loss of activity against UGT2-specific substrates in the ΔUgt2 animals and support the utility of this mouse line in assigning glucuronidation of xenobiotics to the Ugt2 gene family.

BPA Glucuronidation

We next asked whether ΔUgt2 mice could be used to measure the contribution of UGT2 enzymes to the metabolism of xenobiotics metabolized by both UGT families. Studies using recombinant human and rat UGTs have shown that BPA is a substrate for both enzyme families, with greater activity assigned to UGT2 enzymes (Yokota et al., 1999; Hanioka et al., 2008, 2011). We therefore first determined whether UGT2 enzymes are exclusively responsible for the metabolism of BPA by mouse microsomes. Microsomes prepared from pairs of wild type and ΔUgt mice were incubated with BPA and the cofactor UDPGA for 2h. The quantities of BPA and BPAG in the reaction products were determined by HPLC. Under these conditions, rapid and complete metabolism of BPA was observed in reactions carried out using both wild-type and mutant microsomes. No difference was observed between ΔUgt2 and WT microsomes in the total amount of BPAG produced (Fig. 5D). Thus, glucuronidation of BPA occurs in the absence of all UGT2 enzymes. Presumably, this conjugation is carried out by UGT1 enzymes. However, as the reactions were allowed to go to completion, these experiments could not differentiate between the contributions of UGT1s and UGT2s to BPA glucuronidation.

By examining the kinetics of BPA glucuronidation in WT and ΔUgt2 microsomes, the activities of the two enzyme families can be examined. The activity of WT microsomes is expected to represent the sum of
the activities of the UGT1 and UGT2 families, whereas the activity of ΔUgt2 microsomes represents that of the UGT1 family alone. Reaction conditions were optimized to allow measurement of differences in activity between WT and ΔUgt2 microsomes by increasing the substrate-to-enzyme ratio and by removal of alamethicin. Alamethicin increases the rate of glucuronidation in microsomes by facilitating the movement of substrate and UDPGA through the microsomal lipid bilayer. Thus, removal of alamethicin from glucuronidation reactions decreased reaction rates and facilitated kinetic analysis of the rapidly metabolized substrate. As shown in Figure 5, under these conditions, the ability of UGT2 enzymes to contribute to BPA metabolism is easily observed. BPA glucuronidation exhibited Michaelis-Menten kinetics in both WT and ΔUgt2 microsomes. Curves were fit to plots of glucuronidation rate (pmol/min/mg microsomal protein) vs BPA concentration (Fig 5E). Kinetic parameters are shown in Table 2. The $V_{\text{max}}$ measured for ΔUgt2 microsomes, which contain only UGT1 enzymes, is approximately 50% lower than that of WT microsomes, which contain UGT1s and UGT2s. This 50% decrease is attributed to the absence of the UGT2 enzymes in ΔUgt2 microsomes. We thus concluded that 50% of the activity in WT microsomes was due to UGT2s and 50% to UGT1s, suggesting the activities of the two families are approximately equal. $K_m$ did not differ significantly between the WT and the ΔUgt2 microsomes. This suggests that, under these conditions, the two murine UGT families have approximately equal activity towards and affinity for BPA. To ensure that the exclusion of alamethicin did not alter the relative activities of microsomes derived from the two mouse lines, WT and ΔUgt2 microsomes were incubated with 100 µM BPA either in the presence or absence of alamethicin. Significant differences between WT and ΔUgt2 microsomes are present under both alamethicin-activated and non-activated conditions, and the ratio of WT activity to ΔUgt2 activity is comparable in both cases (Supplemental Fig. 5). This result is consistent with previous studies (Fisher et al., 2000) suggesting that alamethicin has an isoform-independent activating effect on UGTs.

To further evaluate the relative contributions of the two UGT families to microsomal BPA glucuronidation, we used our data sets to derive theoretical kinetic parameters for the UGT1 and UGT2 enzymes, assuming that all BPA glucuronide detected was formed by these two enzyme families. As ΔUgt2 mice express only UGT1 enzymes, kinetic parameters of the ΔUgt2 microsomes are assigned to the UGT1 family. Glucuronidation of BPA in WT microsomes was assumed to represent the sum of the activities of
both UGT2 and UGT1 enzymes, and a theoretical \(K_m\) and \(V_{\text{max}}\) for the UGT2 family was derived using the two-enzyme Michaelis-Menten equation (Segel, 1993):

\[
v_{\text{WT}} = \frac{(V_{\text{max,UGT1}})[S]}{K_m^{\text{UGT1}} + [S]} + \frac{(V_{\text{max,UGT2}})[S]}{K_m^{\text{UGT2}} + [S]}
\]

In this equation, \(V\) is the rate of reaction, \([S]\) is the concentration of substrate, and \(K_m\) and \(V_{\text{max}}\) are the Michaelis constant for and maximum rate attributed to each UGT family, respectively. As shown below, UGT2 activity can be represented by the difference in glucuronidation rate between WT mice and \(\Delta\text{Ugt2}\) counterparts:

\[
\frac{(V_{\text{max,UGT2}})[S]}{K_m^{\text{UGT2}} + [S]} = v_{\text{WT}} - \frac{(V_{\text{max,UGT1}})[S]}{K_m^{\text{UGT1}} + [S]} = v_{\text{WT}} - v_{\Delta\text{Ugt2}}
\]

Thus, UGT2-mediated BPA glucuronidation was modeled using a Michaelis-Menten curve fitted to a plot of the difference in rate between paired WT and \(\Delta\text{Ugt2}\) microsomes at each substrate concentration (Fig. 6). Kinetic parameters are shown in Table 3. Based on this analysis, the activities and affinities of the UGT1 and UGT2 enzymes towards BPA are predicted to be very similar.

**BPA Metabolism In Vivo**

Evaluation of BPA glucuronidation by \(\Delta\text{Ugt2}\) and wild type microsomes indicates that UGT1 and UGT2 enzymes contribute equally to BPA metabolism. To determine whether this predicts the activities of these gene families in vivo, we examined the metabolism of BPA in wild type and mutant animals. The bile ducts of paired WT and \(\Delta\text{Ugt2}\) male mice matched for weight and age were cannulated. After collection of a pre-dose bile sample for use as a baseline control, each animal received a single bolus of BPA delivered intravenously. Fractions of bile were collected over the next 50 min, and the quantity of BPAG in each bile fraction was determined by HPLC. No significant difference was seen in the volume or rate of bile production by the \(\Delta\text{Ugt2}\) and wild type animals, and BPAG accumulation in the bile was linear over the 50 min collection in both mouse lines. The biliary excretion rate was therefore expressed as the fraction of the initial BPA dose excreted per minute.

As expected based on the study of \(\Delta\text{Ugt2}\) microsomes, BPAG was detected in the bile of the \(\Delta\text{Ugt2}\) mice, clearly indicating that the UGT1 family glucuronidates BPA in vivo. More surprisingly, following a dose of
2 mg BPA/kg bodyweight, no significant difference between the rate of biliary BPA excretion in wild-type and Δ\textit{Ugt2} mice was observed (Fig. 7). This result suggests that the impact of the UGT2 family on BPA clearance is minimal at this exposure level and that the UGT1 family has significant capacity for BPA conjugation \textit{in vivo}. When the BPA dose was increased to 20 mg/kg bodyweight, a significant decrease between average biliary excretion rates for WT and Δ\textit{Ugt2} mice was observed. Approximately 0.31% of the BPA dose was glucuronidated and excreted per minute by WT mice. However, in mutants only 0.22% of the initial dose was glucuronidated and excreted per minute, approximately a 28% reduction in excretion rate (Fig. 7). A significant contribution of the UGT2s to BPA clearance was only observable at this extremely high dose of BPA. Male mice were used in these studies to avoid potential increases in animal-to-animal variation due to the estrous cycle in females. However, examination of hepatic BPA glucuronidation after dosage of 2 mg BPA/kg bodyweight in female mice also failed to reveal a significant difference between WT and Δ\textit{Ugt2} animals (Supplemental Fig. 6). Thus, we conclude that the UGT1 family is primarily responsible for the rapid glucuronidation and clearance of BPA in male and female mice.

\textbf{Discussion}

We show here the generation of mice lacking the \textasciitilde{}600 kb DNA segment carrying the \textit{Ugt2} gene family. Unlike mice lacking the UGT1 enzymes (Nguyen \textit{et al.}, 2008), the mice are healthy and indistinguishable from control animals when bred in an SPF animal facility. While the UGT1 family is essential for the conjugation and excretion of endogenous metabolites, our data indicate that the members of the UGT2 family do not play an essential and unique role in this function. Particularly surprising is the fact that the loss of UGT2 enzymes does not significantly alter sexual development or fertility, given the ability of both rodent and human UGT2s to metabolize steroid hormones, particularly androgens (Hum \textit{et al.}, 1999; Chouinard \textit{et al.}, 2008). Studies using mice lacking the androgen receptor or mice treated with antiandrogens such as cyproterone acetate have shown that the development of the secondary sex organs is sensitive to androgen levels (Jean-Faucher \textit{C et al.}, 1984; Yeh \textit{et al.}, 2002). Both of these studies reported impaired development of secondary sex organs observable at a gross anatomical level as well as infertility when androgen metabolism was impaired. This indicates that the normal development
of these organs is a sensitive surrogate marker for normal androgen metabolism in mice. A possible explanation for the lack of a developmental or reproductive phenotype in the mutants is that glucuronidation is not a critical pathway for androgen excretion in mice. There is evidence that sulfonation is the predominant excretory pathway for some substrates in rodents (Sanoh et al., 2012), and a number of steroid-conjugating sulfotransferases, including the estrogen-preferring Sult1e1 and the hydroxysteroid sulfotransferases Sult2a1 and Sult2b1, could contribute to androgen metabolism (Alnouti and Klaassen, 2006). Supporting this possibility, neither the Gunn rat (Nguyen et al., 2008) nor the ΔUgt2 mouse exhibits altered fertility or sexual development, while the Sult1e1 knockout mouse exhibits alterations in both capacities (Qian et al., 2001).

The Ugt2a genes are located in the same cluster as the Ugt2b genes in both humans and rodents. Ugt2a1 and Ugt2a2 are expressed in the nasal epithelium, and studies using recombinant enzymes have shown that they are able to conjugate odorants. This is hypothesized to be important in the termination of odorant signaling and preventing receptor desensitization (Lazard et al., 1991; Sneitz et al., 2009). The ΔUgt2 mouse line should allow this hypothesis to be tested, both in vitro using cultured nasal epithelial cells and in vivo using odor-dependent behavioral paradigms (Friedrich, 2006). While the function of Ugt2a3 remains unclear (Buckley and Klaassen, 2009; Sneitz et al., 2009), its conservation across species suggests a role in excretion of endogenous metabolites (Mackenzie et al., 2005). However, our studies indicate that, at least in mice bred in a vivarium, this gene is not essential for normal development and health.

Previous studies using recombinant UGT2 enzymes indicate that the UGT2 family is important in the metabolism of many NSAIDS in both humans and rats (King et al., 2000). Using microsomes prepared from the ΔUgt2 mice, we show that glucuronidation of naproxen is dependent on the UGT2 enzymes. Even after extended incubation, only the parent compound was detected in ΔUgt2 microsomal reaction products. However, we cannot exclude the possibility that some glucuronidation may be detected at higher substrate concentrations due to low-affinity interaction with UGT1 isoforms. Such interactions have been observed using recombinant human UGT1s (Bowalgaha et al., 2005). Studies using recombinant rat UGT enzymes indicate that UGT2B1 shows high activity towards many NSAIDS, including naproxen.
(Ritter, 2000). While our studies only allow assignment to the UGT2 enzyme family and not individual isoforms, it is likely that naproxen metabolism in the mouse is dependent on Ugt2b1 expression.

Extensive studies have confirmed widespread human exposure to bisphenol A (Vandenberg et al., 2007), and studies carried out in both rats and mice indicate possible adverse effects of BPA on development and health (Kabuto et al., 2004; Richter et al., 2007; Vandenberg et al., 2007; Hanioka et al., 2008). Considerable resources have been directed toward determination of the health risks associated with BPA exposure. Despite the interest in this xenobiotic, the understanding of its metabolism is incomplete. BPA is rapidly glucuronidated in both humans and rodents and excreted through urine and bile, respectively. However, less is known regarding the enzymes primarily responsible for formation of the glucuronide. Assigning this activity to the individual enzymes of the UGT1 and UGT2 families has relied largely on studies using recombinant enzymes. Studies of both human and rat enzymes indicate that the UGT2s, specifically UGT2B15 in human and UGT2B1 in rat, show the highest activity toward BPA. It has therefore been assumed that the UGT2 family is critical for rapid clearance of this xenobiotic. Our results are not consistent with this model and indicate that UGT1 enzymes generate a larger proportion of BPA glucuronide in vivo. One possibility is that Ugt1a9, orthologous to the BPA-conjugating human UGT1A9 (Hanioka et al., 2008), is a pseudogene in rats but functional in mice (Mackenzie et al., 2005). Thus, the UGT1s may make a larger relative contribution to BPA glucuronidation in mice than in rats.

The role of the UGT1s in BPA metabolism became apparent upon incubation of BPA with microsomes isolated from ΔUgt2 mice. When allowed to go to completion, reactions containing wild type and ΔUgt2 microsomes with equal initial concentrations of BPA formed equal concentrations of BPA glucuronide. A contribution of UGT2s to BPA metabolism could be defined by examining the rate of formation of the glucuronide, and a 50% reduction was observed in the Vmax of ΔUgt2 microsomes when compared to wild type controls. The approximately equal contributions of UGT1s and UGT2s to BPA metabolism in these in vitro studies did not accurately predict the impact of UGT2 deficiency on BPA metabolism in vivo. At a dose of 2 mg/kg, orders of magnitude greater than the estimated 1.5 µg/kg exposure for the general human population (EU Report, 2010), there was no significant difference in biliary BPA excretion between wild-type and ΔUgt2 mice. Only when the dosage was increased to 20 mg/kg was a significant difference observed. These data indicate that the UGT1 family has extensive capacity to glucuronidate BPA and that
this enzyme family may in fact be primarily responsible for BPA metabolism in vivo. Barring extreme exposures, the contribution of UGT2s to hepatic conjugation of BPA may be negligible.

This work highlights the limitations of directly extrapolating the results of in vitro studies to in vivo metabolism of xenobiotics, which have been extensively discussed previously (Lin and Wong, 2002; Soars et al., 2002; Miners et al., 2006). In general, in vitro studies are reported to underestimate in vivo enzymatic activities. In the case of the ΔUgt2 mice, the in vitro results underestimated the contribution of the UGT1 family to BPA metabolism. One possible explanation could be differential stability of mouse UGT2s and UGT1s during microsomal preparation or incubation, as has been observed in the glucuronidation of flavonoids (Joseph et al., 2007), thereby exaggerating the contribution of one family over the other in in vitro studies. It is also possible that differential localization of UGT1 and UGT2 enzymes in the liver results in differential access to BPA in an in vivo environment. Additional experiments, including evaluation of BPA metabolism by primary mouse hepatocytes, could help to distinguish between these possibilities.

Several human UGT isoforms are capable of conjugating BPA. UGT2B15 is reported to have the highest activity, with lower activities observed for recombinant UGT1A1, UGT1A3, UGT1A9, UGT2B4, and UGT2B7 (Hanioka et al., 2008). An important issue impacted by the assignment of BPA primarily to the UGT2 family is the ability of neonates to metabolize BPA. Neonates and infants have reduced levels of UGT2 expression until 6-7 months of age. They may therefore represent a subpopulation with increased susceptibility to BPA's adverse effects (Strassburg et al., 2002; Zaya et al., 2006; Divakaran et al., 2014). Concern regarding the ability of the newborn to metabolize BPA is the basis for restricting the use of BPA-containing plastics in infant bottles in some countries. However, if human UGT1 enzymes contribute extensively to BPA metabolism, as we have found to be the case in mice, this concern may not be well-founded. UGT1A9 reaches adult levels by 4 months, and expression of UGT1A1 increases rapidly after birth due to the necessity of bilirubin conjugation, which occurs in the maternal liver via transfer across the placenta prior to birth (Schenker et al., 1964; Onishi et al., 1979; Miyagi et al., 2012). While we cannot rule out the possibility that the predominant role of UGT1s in BPA metabolism is unique to the mouse, the UGT1 family is more closely conserved between species than the UGT2 family, suggesting that members of the UGT1 family may play a major role in BPA glucuronidation in humans.
In summary, we describe here the generation of a mouse line allowing definitive assignment of metabolism of xenobiotics to the UGT1 or UGT2 enzyme family. The normal development and good health of these animals makes them an ideal model for such studies. Reconstitution of the deleted locus with individual mouse or human UGT2 genes will provide a means of refining functional assignments to individual UGT2 enzymes \textit{in vivo}. 
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Conducted experiments: Fay, Nguyen, Snouwaert, Dye, Koller
Contributed new reagents or analytical tools: Bodnar
Performed data analysis: Fay, Nguyen, Snouwaert, Dye, Koller
Wrote or contributed to the writing of the manuscript: Fay, Snouwaert, Grant, Koller
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Footnotes.

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Figure Legends

Figure 1. Schematic showing the structure of the wild-type and ΔUgt2 loci. The locus includes all three Ugt2a and seven Ugt2b genes and is located between Ythdc1 and Sult1b1 on chromosome 5. The 609 kb segment of DNA deleted during the homologous recombination event is indicated, and the structure of the ΔUgt2 locus is shown below. The locus carries marker genes, and vector sequences are shown in red. The blue arrows show primers used in initial screening of ES cell clones to identify those in which the deletion vector inserted by homologous recombination.

Figure 2. Verification of the deletion of Ugt2 genes. A. DNA was prepared from tail biopsies of homozygous WT, homozygous ΔUgt2, and heterozygous pups, digested with BamHI, and analyzed by Southern blot. A probe corresponding to a region just outside the Ugt2 locus was used to verify the genotypes of the mice and the integrity of the DNA. As predicted, it hybridized to 11 kb and 6.45 kb DNA fragments corresponding to the wild type allele and ΔUgt2 locus, respectively (upper panel). The filter was then analyzed with a probe binding to BamHI fragments corresponding to a conserved region in Ugt2a3, Ugt2b1, Ugt2b5, and Ugt2b35 (lower panel). The WT locus yielded four different fragments (15.38 kb, 15.23 kb, 10 kb, and 9.57 kb), as expected. Consistent with the excision of the entire Ugt2 locus during a single recombination event, no hybridization of probe is observed in the lanes corresponding to the mice homozygous for the ΔUgt2 locus. B. RNA from olfactory epithelium was analyzed by qPCR for Ugt2a1/2, normalized to 18S expression. As expected, robust expression is observed in wild type mice, while, as indicated by asterisks, none was detected in the ΔUgt2 animals. The quality and epithelial origin of the RNA was verified by showing that Cft expression was robust and that expression level did not differ between the ΔUgt2 and wild type animals. C. RNA prepared from liver was analyzed by qPCR for expression of Ugt2b1, Ugt2b35, Ugt2b5, and Ugt2a3, normalized to 18S expression. N=3 for all samples and error bars represent S.E.M.

Figure 3. Expression and tissue distribution of Ugt1a mRNAs in WT and ΔUgt2 mice. Ugt1a expression levels were measured by quantitative real-time PCR with a SYBR Green qPCR Master Mix. Primers were designed to amplify across the junction between the unique first exon for each isoform and
the shared exons. cDNA samples were diluted to 10 pg/µL for amplification with the 18S probe and to 2.5 ng/µL for all other probes. The comparative Ct method was used to quantify the relative gene expression. The amount of target was normalized to 18S as an endogenous reference and to WT adrenal gland as a calibrator. Expression of the examined isoforms did not differ significantly in any tissue examined between WT and ΔUgt2 animals. Amplifications were performed in duplicate. Error bars represent S.E.M. (n=3).

Figure 4. Naproxen glucuronidation by liver microsomes from WT and ΔUgt2 mice. Reactions contained 50-150 µg microsomal protein, 25 µg/mL alamethicin, 100 µM naproxen, and 5 mM UDPGA. A. Typical chromatogram of reaction products of WT microsomes. B. Typical chromatogram of reaction products of ΔUgt2 microsomes. C. Typical chromatogram of a control reaction lacking UDPGA. Microsomes from WT mice formed 46.2 ± 1.9 (mean ± S.E.M., n=3) nmol/mg of Naproxen glucuronide while microsomes from ΔUgt2 mice showed no detectable amount of glucuronide formation.

Figure 5. BPA glucuronidation by liver microsomes from WT and ΔUgt2 mice. A. Typical chromatogram of products of a reaction terminated immediately after addition of UDPGA. B. Typical chromatogram of products of a reaction terminated after 10 min of incubation. C. The identity of the BPAG peak was verified by its loss after treatment with β-glucuronidase. D. BPAG formation after 2-hour incubation of BPA and 25 µg/mL alamethicin with WT and ΔUgt2 microsomes. Complete substrate conversion was observed, and BPAG concentrations were not significantly different. E. Michaelis-Menten curves for glucuronidation of BPA by microsomes prepared from WT (squares) and ΔUgt2 (triangles) mice in the absence of alamethicin. Each data point represents microsomal preparations from four mice. Error bars represent standard deviation. r²>0.95 for WT mice and r²>0.85 for ΔUgt2 mice. Reactions contained 20-60 µg microsomal protein, 5-100 µM BPA, and 5 mM UDPGA.

Figure 6. Enzyme kinetics of UGT1- and UGT2-mediated BPA glucuronidation. The glucuronidation activity remaining in mutant mice after deletion of the Ugt2s was assigned to the UGT1 family (circles). The difference in glucuronidation rate measured between microsomes from WT and ΔUgt2 animals was
used to represent activity of the UGT2 family (triangles). The calculated kinetics of glucuronidation of BPA by both enzyme families were consistent with Michaelis-Menten models. Calculations assumed equal concentrations of UGT1 and UGT2 enzymes in WT microsomes. $r^2>$0.85 for UGT1s and $r^2>$0.75 for UGT2s. Error bars represent S.E.M., n=4 microsomal preparations of each genotype.

**Figure 7. In vivo BPA Glucuronidation in WT and ΔUgt2 mice.**

**A.** Typical chromatogram of bile collected from a ΔUgt2 mouse prior to dosage. **B.** Typical chromatogram of a bile fraction collected from a ΔUgt2 mouse from 0-10 min after dosage. **C.** Treatment with β-glucuronidase causes loss of the BPAG peak and changes in the chromatographic profile due to deconjugation of endogenous glucuronides. BPAG peak areas were corrected for the area of a small interfering peak (presumably due to a co-eluting endogenous metabolite; marked by an asterisk) in bile obtained immediately before dosage. The interfering peak remained after β-glucuronidase treatment, indicating that it does not represent BPA exposure prior to experimentation. **D.** Average BPAG excretion after dosage with 2 mg/kg BPA over the collection period for WT and ΔUgt2 male mice, measured as percentage of dose/min. Average excretion rates for WT and ΔUgt2 mice were 0.306 ± 0.030 (mean±S.E.M., n=3) and 0.325 ± 0.039 (mean±S.E.M., n=3) respectively. **E.** Average BPAG excretion after dosage with 20 mg/kg BPA over the collection period for WT and ΔUgt2 mice. Average excretion rates for WT and ΔUgt2 mice were 0.313 ± 0.019 (mean±S.E.M., n=6) and 0.224 ± 0.024 (mean±S.E.M., n=7) respectively. Average percent excretion/min was significantly different at the $p<$0.05 level in an unpaired Student’s $t$ test. BPAG excretion was linear over the time range examined (plots of percent excretion versus time had $r^2>$0.93 for all animals).
Tables

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Table 1. Chromatographic protocols. The aqueous solvent (A) was 0.1% acetic acid in water (v/v) and the organic solvent (B) was 0.1% acetic acid in acetonitrile (v/v). The autosampler chamber was held at 6°C, and columns were held at 25°C for all experiments. The protocol for BPA in vivo is based on that of Yokota et al. (Yokota et al., 1999).
### Table 2. Kinetic parameters for BPA glucuronidation in liver microsomes from WT and ΔUgt2 mice.

Values are expressed as mean ± S.D. (n=4). $V_{max}$ is significantly different at the p<0.0001 level and $K_m$ is not significantly different in unpaired Student’s $t$ tests.

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Table 3. Kinetic parameters for BPA glucuronidation by mouse Ugt1s and Ugt2s. Values are expressed as mean ± S.E.M. (n=4). Neither \( V_{\text{max}} \) nor \( K_{\text{m}} \) is significantly different in an unpaired Student's \( t \) test.
Endogenous Ugt2 locus

609 kb deletion

Δ Ugt2 locus

Figure 1
Figure 2

A

WT/∆Ugt2

∆Ugt2/∆Ugt2

WT/WT

11 kb

6.45 kb

15.38 kb

15.23 kb

10 kb

9.57 kb

B

Fold change, relative to 18S

Wild-type

△Ugt2

Ugt2a1/2

Cftr

18S

C

Ugt2b1

Ugt2b5

Ugt2b35

Ugt2a3

18S

* * * * *

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Figure 3

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Figure 4
Figure 6