Regulation of Human CYP2C18 and CYP2C19 in Transgenic Mice:

Influence of Castration, Testosterone and Growth Hormone

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Abbreviations:

BAC, bacterial artificial chromosome; CYP, cytochrome P450; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gDNA, genomic DNA; GH, growth hormone; HPRT, hypoxanthine guanine phosphoribosyltransferase; PCR, polymerase chain reaction; STAT, signal transducer and activator of transcription; wt, wild-type
Abstract

The hormonal regulation of human CYP2C18 and CYP2C19, which are expressed in a male-specific manner in liver and kidney in a mouse transgenic model, was examined. The influence of prepubertal castration in male mice and testosterone treatment of female mice was investigated, as was the effect of continuous administration of growth hormone (GH) to transgenic males. Prepubertal castration of transgenic male mice suppressed the expression of CYP2C18 and CYP2C19 in liver and kidney to female levels, whereas expression was increased for the endogenous female-specific mouse hepatic genes Cyp2c37, Cyp2c38, Cyp2c39 and Cyp2c40. Testosterone treatment of female mice increased CYP2C18 and CYP2C19 expression in kidney, and to a lesser extent in liver, but was without effect in brain or small intestine, where gene expression was not sex-dependent. Continuous GH treatment of transgenic males for 7 days suppressed hepatic expression of CYP2C19 (>90% decrease) and CYP2C18 (~50% decrease), but had minimal effect on the expression of these genes in kidney, brain or small intestine. Under these conditions, continuous GH induced all four female-specific mouse liver Cyp2c genes in males to normal female levels. These studies indicate that the human CYP2C18 and CYP2C19 genes contain regulatory elements that respond to the endogenous mouse hormonal profiles, with androgen being the primary regulator of male-specific expression in kidney, whereas the androgen-dependent pituitary GH secretory pattern is the primary regulator of male-specific expression in liver in a manner that is similar to the regulation of the endogenous sex-specific hepatic genes.
Introduction

Hepatic cytochrome P450 (CYP)-dependent metabolism of steroids and xenobiotics is sexually dimorphic in rodents and some other species (Waxman and O'Connor 2006), whereas in humans sex differences are much less pronounced. Metabolism by sex-specific CYP enzymes could lead to sex-dependent susceptibility to chemical toxicants and carcinogenicity (Aldridge et al., 2003). Some reports have suggested sex differences in activity of CYP2C19 (Xie et al., 1997) and CYP2E1 (Kim and O'Shea 1995), but such differences have been difficult to reproduce by others (Hägg, et al., 2001; Bebia et al., 2004; Kim and O'Shea 1995). CYP3A4, the most important drug metabolizing CYP enzyme in human liver, is more highly expressed in women than in men, as revealed by in vivo measurements of the metabolism of probe drug and by examination of CYP3A4 protein and RNA levels in a large collection of human livers (Diczfalusy et al., 2008; Wolbold et al., 2003). The regulation of the sex-dependent expression of CYP3A4 has been studied in a transgenic CYP3A4/CYP3A7 humanized mouse line, where CYP3A4 mRNA and protein are expressed in livers of adult female mice but not male mice, and where continuous infusion of recombinant growth hormone (GH) in transgenic male mice increased hepatic CYP3A4 mRNA and protein to female levels (Cheung et al., 2006).

The sexually dimorphic, ultradian rhythm of circulating GH levels has been shown to regulate the sex-dependent expression of hepatic monooxygenases in rats and mice (Waxman and O'Connor 2006). In males, the pulsatile GH-secretion pattern activates and stimulates nuclear translocation of signal transducer and activator of transcription (STAT5b) (Waxman et al., 1995), which is required for sex-specific expression of >1000 genes in mouse liver (Clodfelter et al., 2006). The sex-dependent differences in plasma GH profiles first emerge at puberty but are set, and ultimately regulated, by gonadal steroid imprinting during the neonatal period (Jansson et al., 1985). Sex-differences in plasma GH profiles are most dramatic in rodents, but significant male–female differences in the regulation of pituitary GH release also exist in humans (Veldhuis et al., 2001).
In addition to GH, gonadal hormones are essential for the differentiation of many sexually dimorphic CYPs. Whereas estrogens induce the female-specific gene \textit{CYP2C12} in female rat liver (Dannan et al., 1986), androgens are required for expression of the male-specific genes \textit{CYP2A2} (Waxman et al., 1988), \textit{CYP2C11} (Morgan et al., 1985; Waxman et al., 1985), \textit{CYP2C13} (McClellan-Green et al., 1989), \textit{CYP3A2} (Waxman et al., 1985; Ribeiro and Lechner 1992) and \textit{CYP4A2} (Sundseth and Waxman 1992) in male rat liver. Androgens and estrogens act on the hypothalamic-pituitary axis, which controls the sexually dimorphic pattern of GH secretion and thereby influence the expression of the sex-dependent hepatic CYP enzymes in an indirect manner (Mode and Norstedt 1982; Jansson et al., 1985). In contrast, androgens act directly on the kidney to regulate sex-dependent CYP expression (Sundseth and Waxman 1992; Henderson and Wolf 1991; Imaoka et al., 1992).

Differences in diets, drinking and smoking habits, and medication history are all known to affect CYP enzyme expression and/or activity in humans (Thum et al., 2006; Bomsien et al., 2006). These environmental factors can be controlled in animals maintained under defined dietary and environmental conditions, suggesting that animal models, including transgenic mice, may be useful for investigation of the sex-specific expression and hormonal regulation of human CYP enzymes. Thus, recently we found strong sex differences in human \textit{CYP} gene and enzyme expression in a humanized \textit{CYP2C18/CYP2C19} transgenic mouse line, where adult male mice expressed much higher \textit{CYP2C18} and \textit{CYP2C19} mRNA levels in liver and kidney as compared to females (Löfgren et al., 2008).

We considered it of interest to investigate to which extent GH and androgens are responsible for the sexually dimorphic expression of the human genes \textit{CYP2C18} and \textit{CYP2C19}. We examined the influence of castration and continuous GH treatment of male mice, and continuous testosterone treatment of female mice in the transgenic mouse model. Our findings show that exogenous GH administration and prepubertal castration suppress \textit{CYP2C18} and \textit{CYP2C19} expression in liver and kidney of transgenic male mice and stimulate hepatic expression of four
endogenous female specific Cyp2c genes. Testosterone administration to female mice stimulates the expression of \textit{CYP2C18} and \textit{CYP2C19} in both liver and kidney. These results provide insight into the sexually dimorphic regulation of \textit{CYP2C18} and \textit{CYP2C19} expression, suggesting that this animal model can be a valuable tool for studying the regulation of these human \textit{CYP} genes.

\textbf{Materials and Methods}

\textit{Chemicals, Enzymes, and Other Reagents}

Alzet® micro-osmotic pumps, model 1007D, were obtained from Scanbur AB (Sollentuna, Sweden). Recombinant rat GH was obtained from the National Hormone and Peptide Program (Torrance, CA). Oligonucleotide PCR primers were purchased from Invitrogen (Paisley, Scotland). Testosterone 60-day release pellets (Cat. No SA-151) and 60 day placebo control pellets (Cat. No SC-111) were obtained from Innovative Research of America (Sarasota, Florida, USA). All other laboratory chemicals were of analytical grade and obtained from commercial suppliers.

\textit{Animals and Treatments}

\textit{CYP2C18/CYP2C19} hemizygous transgenic mice (Löfgren et al., 2008) and wild-type C57BL/6OlaHsd littermates were housed at the Karolinska Institute in Stockholm and were treated according to research protocols approved by the Swedish Ethical Application Committee. The mice were housed on wood shavings in plastic cages, with water and commercial mouse diet supplied \textit{ad libitum}.

In the first study, transgenic male mice were castrated at \~{} 3 weeks, prior to puberty which occurs between 6-9 weeks of age. Transgenic male littermates underwent sham surgery at the same time. Transgenic female littermates had a 15 mg testosterone pellet (60 day release) or a placebo pellet (60 day release) inserted subcutaneously in the skin of the neck at 3 weeks of age. All animals were necropsied approximately 8 weeks after surgery, at 11 weeks of age. In addition, untreated wild-type littermates of both sexes were euthanized at the same age.
In the second study, male transgenic mice received continuous infusion with recombinant rat GH. An Alzet® 1007D micro-osmotic pump designed to deliver the solution at a rate of 0.5µl/h was implanted subcutaneously at the back of the neck. Adult (8-11 weeks old) transgenic male mice received the osmotic infusion of recombinant rat GH (660 ng/h) dissolved in buffer (91.5%: 30 mM NaHCO₃/0.15 M NaCl, pH 10.3 and 8.5%: 0.5 M NaHCO₃; and rat albumin at a final concentration of 100 µg/ml) or infusions with buffer only. Transgenic female mice and placebo treated males were used as controls. All transgenic male mice were killed either 4 days or 7 days after osmotic pump implantation. The female transgenic controls and placebo treated controls were killed at the same time. For both studies at least four animals were included in each group. Three and nine week old mice used for preliminary measurements of S-mephenytoin 4’-hydroxylation were either reared at the facilities of AstraZeneca (Sweden) or purchased as wild-type C57BL mice (therefore not genotyped) from B&K Universal Limited (East Yorkshire, UK).

**PCR Genotyping**

Mice were genotyped to confirm the inserted human CYP2C18/CYP2C19 gene segment. Genotyping was performed on genomic DNA (gDNA) extracted from tissues obtained from either a small tail section, or a 2-3 mm diameter ear punch, using a commercially available kit (DNeasy Tissue kit, Qiagen). Amplification of IL-2 (internal control) and CYP2C19 in the extracted gDNA was performed in a 25-µL reaction volume containing 2.5 µL of 10x HiFi PCR MasterMix (ABgene House, Surrey, UK), 400 nM of each primer and 1 µL of gDNA. The mice used for S-mephenytoin hydroxylation measurements were genotyped to confirm the presence of both the CYP2C18/CYP2C19 transgene as well as the BAC3’ and BAC5’ ends of the inserted transgene. Sequences of the gene specific oligonucleotide PCR primers are shown in Table 1. The PCR thermoprofile consisted of an initial denaturation for 2 min at 94°C followed by 33 cycles of 94°C for 10 s, 55°C for 30 s and 72°C for 40 s, followed by a 7 min extension at 72°C. The amplified products were visualized by ethidium bromide/agarose gel electrophoresis.
Analysis of S-Mephenytoin Metabolism

Microsomes from 3 and 9 week old, wild-type and transgenic mice, were prepared from individual male and female mouse livers as described previously (Löfgren et al., 2008), but were ultimately pooled by volume due to the limited amount of microsomal proteins obtained. Metabolism of S-mephenytoin to its major CYP2C19-dependent metabolite 4’-hydroxymephenytoin was measured as described previously (Löfgren et al., 2008) with the exception that a final S-mephenytoin solvent concentration of 1% methanol was used in each microsomal incubation. Michaelis-Menten kinetics was assumed and apparent $K_m$ and $V_{max}$ values were estimated for all groups using Lineweaver-Burke plots. S-mephenytoin concentrations ranged from 10-250 µM and time curves from 0-20 minutes. The intrinsic clearance, $CL_{int}$, was calculated as the $V_{max}$ divided by the $K_m$.

Total RNA Isolation and cDNA Synthesis for Real-Time PCR

Mice were killed by cervical dislocation at age 9-12 weeks. Portions of liver, kidney, brain or small intestine were placed in RNAlater (Qiagen) according to the manufacturer’s recommendations. Total RNA was extracted from 40-100 mg of tissue using a commercially available kit (RNeasy, Qiagen) and DNA was removed by DNase digestion (Qiagen). The concentration of the extracted RNA was determined by absorption at 260 nm and the RNA was then reverse-transcribed into first-strand cDNA using 0.5 µg of total RNA, 4 µL of 5x reaction buffer, 5 µM oligo (dT)$_{18}$, 0.5 mM dNTPs, 10 mM DTT, 1 µL of RNaseOut (Invitrogen, Carlsbad, CA), and 200 U of Superscript II RNase reverse transcriptase (Invitrogen) in a 20-µL reaction volume. Reactions were incubated at 42°C for 60 min followed by inactivation at 70°C for 10 min. Reactions were also run in the absence of reverse transcriptase to address the possibility of gDNA contamination.
Primer Design for Real-Time PCR

The primer pairs used for the amplification of the endogenous murine CYP2C29, CYP2C37, CYP2C38 and CYP2C40 transcripts were targeted to sequences showing relatively low homology amongst both murine CYP2C isoforms and the CYP2C18 and CYP2C19 transgene using a multiple sequence alignment (ClustalW). Multiple potential primers for real-time PCR were evaluated with the following criteria: observation of a single melting curve peak, visualization of a single amplicon of the appropriate length following agarose gel electrophoresis, direct sequencing of amplicons and amplification efficiencies >95%. Primer sequences are shown in Table 1.

Real-Time Quantitative PCR

Real-time PCR reaction mixtures (25 μL) contained 12.5 μL 2xSYBR Green Master Mix (Applied Biosystems, Foster City, CA) or 12.5 μL TaqMan MasterMix (Applied Biosystems); cDNA (0.25 μL in the castration study or 0.125 μL in the GH study) and the appropriate primer pairs (400 nM) or 1.25 μL TaqMan Gene Expression Assay (Applied Biosystems) specific for the mRNA studied. SYBR Green assays were performed using isoform-specific primers (see Table 1). TaqMan Gene Expression Assays with the ID numbers Mm 00656110_gH, Mm00663066_gH, Mm02602271_mH, Mm01205031_mH, Mm01197220_mH were used for detection of CYP2C37, CYP2C50, CYP2C54, CYP2C55 and CYP2C70, respectively. Due to the absence of detectable amplification of CYP2C65 in any liver-derived cDNA sample, this isoform was not studied any further. Murine β-actin was quantified using a VIC-labeled TaqMan Endogenous Control Assay (Applied Biosystems).

PCR was performed using Applied Biosystems 7500 Standard Real-Time PCR system with the following PCR conditions: activation of polymerase at 95°C for 10 min, followed by 40 amplification cycles with denaturation at 95°C for 15 s, and annealing and extension at 60 °C for 1 min. The specificity of the SYBR Green assays was monitored by melting curve analysis of
each amplification product. The optimal combination of genes used to normalize transcript expression level was determined using qBase version 1.3.5. (Hellemans et al., 2007)

In the first study (castration/testosterone treatment) all transcripts were analyzed in triplicate. Murine CYP2C expression levels were normalized using the geometric mean of GAPDH and β-actin whereas GAPDH and HPRT were used for normalization of CYP2C18 and CYP2C19 transcripts. A subsequent survey of established sex-specific genes (Holloway, Laz, and Waxman 2006) was normalized using the geometric mean of GAPDH, HPRT and β-actin. For the GH treatment study, we chose to focus on CYP2C18/CYP2C19, the sexually dimorphic murine CYP2C isoforms and two male and female specific genes identified in the previous survey. Transcripts were analyzed in duplicate, as duplicate samples were considered to be sufficient based on the variation observed in the castration study. Transcript levels were normalized using the geometric mean of GAPDH and HPRT. Relative mRNA expression levels were determined using qBase version 1.3.5. (Hellemans et al., 2007).

Statistical Analysis

All genotype and treatment associated differences in mRNA expressions between treatment groups were compared with a Student’s t test using Sigma Stat version 2.03 (SPSS Inc., Chicago, IL, USA).

Results

4’-Hydroxymephenytoin Formation

Initially, a developmental study was carried out in which the ontogenecity of CYP2C19-dependent catalytic activity was examined in wild-type and transgenic CYP2C18/CYP2C19 mice. As shown in Table 2, a clear decrease in S-mephenytoin intrinsic clearance, $CL_{int}$, occurred between 3 and 9 weeks of age in female mice and wild-type males but not in transgenic CYP2C18/CYP2C19 male mice. This decrease could be linked to an age-dependent increase in
$K_m$ for both sexes and genotypes and a concomitant decease in $V_{max}$ in females only (both genotypes).

This preliminary study thus indicates that the sex-dependent alterations in CYP2C18 and CYP2C19 gene expression may occur at puberty and we therefore chose to castrate male mice and begin testosterone treatment of female mice before the onset of puberty. The hormonal regulation of CYP2C18 and CYP2C19 was then further studied in mature animals.

**Effect of Male Castration and Female Testosterone Treatment on CYP2C Expression**

Real-time PCR using gene-specific primers was used to monitor the expression of CYP2C18, CYP2C19, and of nine endogenous mouse Cyp2c genes, in adult male mice castrated at 3 weeks of age. As shown in Fig 1, castration dramatically decreased the expression of CYP2C18 and CYP2C19 in adult male mouse liver as compared to sham-operated controls, ($p \leq 0.001$). Both genes were expressed at much lower levels in both placebo and testosterone-treated females, but testosterone-treated females had a slightly higher hepatic expression compared to placebo females ($p \leq 0.05$ for both genes). The same trend was observed in kidney (Fig 1), but here the difference in expression between sham males and castrated males was smaller for CYP2C19 as compared to CYP2C18. The effects of testosterone treatment in female mice were much more pronounced in the kidneys than in the liver, and for CYP2C19 testosterone raised the expression to that of untreated males. No sex or treatment related differences were seen for CYP2C18 or CYP2C19 in either brain or small intestine (data not shown).

Of the nine endogenous mouse Cyp2c genes investigated, four showed a clear female-specific pattern of expression, i.e. Cyp2c37, Cyp2c38, Cyp2c39 and Cyp2c40 both in wild-type and transgenic mice. The transgenic expression of the CYP2C18 and CYP2C19 genes did not influence hepatic mRNA levels of the endogenous mouse Cyp2c genes (Fig. 2). A much smaller sex difference in expression characterized Cyp2c29 and Cyp2c70 when comparing wild-type males and wild-type females ($p \leq 0.01$ for Cyp2c29 and $p \leq 0.05$ for Cyp2c70). When comparing
sham males with placebo females no such sex differences were observed (Fig. 2). This sex dependent expression for all six genes was the opposite of CYP2C18 and CYP2C19, whose expression was higher in males than in females.

Next, we investigated the effect of castration on the expression of five mouse genes showing male- specific expression in liver (Cyp2d9, Cyp7b1, Gsta, Mup1/2/6/8 and Mup3) (Supplementary Table 1). Seven female specific liver genes identified in other studies were also examined (Cyp2a4, Cyp2b9, Cyp3a16, Cyp17a1, Cutl2, Trim 24 and Tox) (Supplementary Table 1) (Wiwi et al., 2007). For all five male-specific genes, castration decreased gene expression in male liver down to female expression levels. A significantly increased expression in castrated males (compared to sham-operated males) was observed for the female-specific genes Cyp2b9 and Tox (p<0.01 for both). A trend of increased expression was also seen for the other female-specific genes, except for Cyp3a16. It should be noted that all of these female genes, with the exception of Cyp3a16, are up-regulated in male liver in the absence of STAT5b (Holloway et al., 2006; Laz et al., 2007).

Effect of GH on mRNA Expression in Liver, Kidney, Brain and Small Intestine

The CYP2C18 and CYP2C19 genes in both liver and kidney showed sexually dimorphic expression as shown in Fig 3 and continuous GH administration to males caused a gradual, time-dependent drop in the expression levels of both genes in both tissues. The GH effect was more dramatic in liver and was much more pronounced for CYP2C19. As expected, no pronounced effect of GH was seen in brain or small intestine (data not shown). As a comparison we investigated the effect of GH treatment on the hepatic expression of the endogenous sex-specific murine CYPs that were affected by castration (Cyp2c37, Cyp2c38, Cyp2c39 and Cyp2c40) (Fig 4). Cyp2c39 showed the most pronounced response to 4 days continuous GH administration to males, with mRNA expression levels being slightly higher than in untreated females. CYP2C37,
CYP2C38 and CYP2C40 transcripts almost reached female expression levels after 7 days of GH treatment.

As a positive control for the effect of continuous GH treatment, we assayed mRNA levels of two male-specific genes (Cyp2d9 and Mup1/2/6/8) and two female-specific genes (Cyp2a4 and Cyp2b9) (Supplementary Table 2). The most pronounced effect was on the male specific genes, where a decrease in mRNA levels was observed after 4 days of continuous GH treatment, and an even greater decrease was seen after 7 days. The effect of continuous GH administration on the female-specific genes, Cyp2a4 and Cyp2b9, was not as pronounced, and the GH-treated male mice never attained full female RNA levels.

Discussion

The sexually dimorphic expression of several hepatic drug-metabolizing CYP enzymes has been demonstrated and studied extensively in mice and rats. The present study shows that in a transgenic mouse model, the human CYP2C19 and CYP2C18 genes are regulated in a sexually dimorphic manner and are under similar regulation by GH and castration as sexually dimorphic endogenous Cyp2c genes. This observation suggests regulatory elements of these genes might also be subject to GH and hormone dependent regulation in humans.

Growth hormone, acting via its sex-specific pituitary profiles, regulates the expression of many sex-specific and sex-predominant genes, including CYP genes. GH is secreted in pulses, but with different levels and frequencies in males and females in several species including humans (van den Berg et al., 1996; Jaffe et al., 1998). Treatment with exogenous GH has been shown to alter CYP2C11 and CYP2C12 expression in rats (Waxman et al., 1991; Kawai et al., 2001); while mouse Cyp2c29 was shown to be sex-independent and GH archetype independent (Jarukamjorn et al., 2006). In humans, CYP2C19 activity has been shown to be stimulated in healthy elderly men by subcutaneous injections of GH once daily (Jürgens et al., 2002). The present study demonstrates that GH is crucial in the sexually dimorphic expression of CYP2C18 and CYP2C19.
in the *CYP2C18/CYP2C19* humanized mouse model. Thus, the human *CYP2C18* and *CYP2C19* genes, when inserted in this transgenic mouse model, contains all the DNA sequences required to respond to the endogenous mouse hormonal environment, including the regulatory elements required for responsiveness to exogenously administered GH.

Signal transducer and activator of transcription (STAT) 5b has been identified as an essential determinant of GH-mediated sexual expression of CYP enzymes (Clodfelter et al., 2006; Holloway et al., 2006). However, the precise intracellular regulatory mechanisms activated by STAT5b, leading to the regulation of hepatic CYP expression, are only partially understood and await more extensive investigation. Although a STAT5b consensus sequence was identified in the promoter region of *CYP2C19*, no protein binding could be detected in EMSA studies despite the fact that a positive control oligonucleotide for STAT5b showed binding which was supershifted with STAT5b antibodies (data not shown). The GH and androgen responsive elements in the *CYP2C18* and *CYP2C19* genes will be the focus of future studies.

Castration caused *CYP2C18* and *CYP2C19* mRNA levels in males to be reduced to female levels, a response that was also seen for several endogenous male-specific mouse liver genes (Supplementary Table 1). This is believed to reflect the role of testicular androgens in maintaining the characteristic sex-dependent patterns of pituitary GH secretion in adult male rats (Jansson, et al., 1985). Continuous GH treatment of intact male *CYP2C18/CYP2C19* transgenic mice suppressed liver *CYP2C19*, as well as the endogenous male-specific mouse genes *Cyp2d9* and *Mup1/2/6/8* to near-female levels. Only partial (~50%) suppression of liver *CYP2C18* expression was achieved, which could be explained if *CYP2C18* mRNA has a long intrinsic half-life, or alternatively, could indicate that other hormonal factors, in addition to continuous GH, may be required to achieve complete feminization. *CYP2C18* and *CYP2C19* were largely unresponsive to GH treatment in the kidney, consistent with earlier studies showing that male-specific CYP gene expression in kidney is primarily regulated by androgen by a mechanism that

Testosterone treatment in female transgenic mice did not have as drastic an effect on the mRNA levels as did castration. In naïve males, testosterone is not secreted at constant levels, but in an episodic manner (Mock et al., 1978), which was not the case in our study. The lack of pulsatility may be the reason for the limited response in testosterone-treated females. Another possible explanation is the continued presence of estrogen in the testosterone-treated females, which may antagonize the effect of androgen treatment. A further possibility is the time period under which the study was conducted. The testosterone capsules used were reported to have a 60 day release period and the necropsies were conducted after 55-56 days. It may be possible that the testosterone levels were lower at the end of the study, or that the transgenic females metabolize testosterone faster causing testosterone levels to fall below the required male physiological levels.

The GH-dependent changes in hepatic CYP expression described here may significantly alter the pharmacokinetics and pharmacodynamics of drugs metabolized by CYP2C19. The interactions between GH and CYP-metabolized drugs may lead to changes in drug efficacy, and as a consequence, to adverse effects. The CYP-humanized mouse model presented here and the GH responsive CYP3A4/CYP3A7 transgenic model described earlier (Cheung et al., 2006) may contribute to the understanding of GH regulation of human CYP genes. Use of these models in medical testing may reduce differences in drug metabolism caused by interspecies variation in CYP-mediated metabolism. However, other physiological parameters and hormone profiles also vary between mice and humans and there are metabolic differences due to the involvement also of the endogenous mouse enzymes. Although rodents have more drastic differences in their GH plasma levels than humans, humans do show significant differences in pituitary GH release patterns between sexes. These species differences, as well as other physiological parameters, can make extrapolation from animal models to humans difficult and thus must be taken into consideration when evaluating results obtained from these types of models.
In conclusion, the human CYP2C18/CYP2C19 transgenic mouse line described here provides a novel tool for studying the regulation and function of human CYP2C18 and CYP2C19 genes. Sexually dimorphic expression of CYP2C18 and CYP2C19 as well as murine Cyp2c37/38/39 and Cyp2c40 genes was observed in the mice, which may lead to significant sex differences in drug metabolism, disposition, pharmacokinetics, and pharmacodynamics. Although the underlying mechanism for regulation of these sexually dimorphic genes could not be fully determined, this animal model may help in studying the regulation of human CYP2C18 and CYP2C19 in a whole-animal system.

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References


Morgan ET, MacGeoch C and Gustafsson JA (1985) Hormonal and developmental regulation of expression of the hepatic microsomal steroid 16α-hydroxylase cytochrome P-450 apoprotein in the rat.


Waxman DJ, Pampori NA, Ram PA, Agrawal AK and Shapiro BH (1991) Interpulse interval in


Footnotes

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Figure legends

**FIGURE 1.** Relative CYP2C18 and CYP2C19 mRNA expression in transgenic mice approximately 11 weeks old. Reverse transcribed total RNA and gene specific primers were used as described in *Materials and Methods*. The geometric mean of two reference transcripts (GAPDH and HPRT) was used to normalize expression levels. Data are presented relative to the lowest individual expression level for each gene transcript and organ (mean ± SE). Each group contains 4 or 5 mice (sham treated males n=4, all other groups n=5). Analyses were performed in triplicate. Experimental groups were compared using Student’s *t* test. The location of the statistical symbol for each respective comparison is denoted by bold face type:

* and *** denote p ≤ 0.05 and p ≤ 0.001, respectively, for **sham-operated males** vs. placebo treated females or **castrated males** vs. testosterone treated females.

#, ## and ### denote p ≤ 0.05, p ≤ 0.01 and p ≤ 0.001, respectively, for sham-operated males vs. **castrated males** or placebo treated females vs. **testosterone treated females**.

¤¤ and ¤¤¤ denote p ≤ 0.01 and p ≤ 0.001, respectively, for sham-operated male vs. **testosterone treated females** or placebo treated females vs. **castrated males**.

**FIGURE 2.** Relative murine hepatic CYP2C mRNA expression in transgenic (sham-operated, placebo and testosterone treated) and wild-type mice approximately 11 weeks old. Reverse transcribed total RNA from liver and gene specific primers were used as described in *Materials and Methods*. The geometric mean of two reference transcripts (GAPDH and β-actin) was used to normalize expression levels. Data are presented relative to the lowest individual expression level for each gene transcript (mean ± SE). Each group contains 3-5 mice (sham-operated males n=3, castrated males n=4, all other groups n=5). Analyses were performed in triplicate. Experimental groups were compared using Student’s *t* test. The location of the statistical symbol for each respective comparison is denoted by bold face type:
*, ** and *** denote $p \leq 0.05; p \leq 0.01$ and $p \leq 0.001$, respectively, for wild-type males vs. wild-type females or sham-operated males vs. placebo treated females or castrated males vs. testosterone treated females.

#, ## and ### denote $p \leq 0.05; p \leq 0.01$ and $p \leq 0.001$, respectively, for sham-operated males vs. castrated males or placebo treated females vs. testosterone treated females.

¤, ¤¤ and ¤¤¤ denote $p \leq 0.05; p \leq 0.01$ and $p \leq 0.001$, respectively, for sham-operated males vs. testosterone treated females or placebo treated females vs. castrated males.

+ and ++ denote $p \leq 0.05$ and $p \leq 0.01$, respectively, for wild-type males vs. sham-operated males or wildtype females vs. placebo treated females.

**FIGURE 3.** Relative CYP2C18 and CYP2C19 mRNA expression in transgenic mice, approximately 11 weeks old. Reverse transcribed total RNA and gene specific primers were used as described in *Materials and Methods*. The geometric mean of two reference transcripts (GAPDH and HPRT) was used to normalize expression levels. Data are presented relative to the lowest individual expression level for each gene transcript and organ (mean ± SE). Each group contains 4-8 mice (placebo treated males 4 and 7 day exposures n=4, GH treated males 4 day exposure n=5, GH treated males 7 day exposure n=6 and transgenic females n=7). Analyses were performed in duplicate. Experimental groups were compared using Student’s *t* test. The location of the statistical symbol for each respective comparison is denoted by bold face type:

* and *** denote $p \leq 0.05$ and $p \leq 0.001$, respectively, for placebo treated males (4 days) vs. transgenic female, placebo treated males (7 days) vs. transgenic female, GH treated males (4 days) vs. transgenic females or GH treated males (7 days) vs. transgenic females.

#, ## and ### denote $p \leq 0.05; p \leq 0.01$ and $p \leq 0.001$, respectively, for placebo treated males (4 days) vs. GH treated males (4 days) or placebo treated males (7 days) vs. GH treated males (7 days).
FIGURE 4. Relative hepatic expression of sex-specific murine CYP2C transcripts in transgenic mice, approximately 11 weeks old. Reverse transcribed total RNA from liver and gene specific primers were used as described in Materials and Methods. The geometric mean of two reference transcripts (GAPDH and HPRT) was used to normalize expression levels. Data are presented relative to the lowest individual expression level for each gene transcript (mean ± SE). Each group contains 4-8 mice (placebo treated males (4 and 7 days) n=4, GH treated males (4 days) n=5, GH treated male (7 days) n=6, transgenic females n=7 and wild-type males n=8). Analyses were performed in duplicate. Experimental groups were compared using Student’s t test. The location of the statistical symbol for each respective comparison is denoted by bold face type: *, ** and *** denote p ≤ 0.05; p ≤ 0.01 and p ≤ 0.001, respectively, for placebo treated males (4 days) vs. transgenic female, placebo treated males (7 days) vs. transgenic female, GH treated males (4 days) vs. transgenic females or GH treated males (7 days) vs. transgenic females. # and ## denote p ≤ 0.05 and p ≤ 0.01, respectively, for placebo treated males (4 days) vs. GH treated males (4 days) or placebo treated males (7 days) vs. GH treated males (7 days). + and ++ denote p ≤ 0.05 and p ≤ 0.01, respectively, for wild-type male vs. placebo male (4 days) or wild-type male vs. placebo male (7 days).
TABLE 1. Primer sequences used for Real-Time PCR and genotyping.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Strand</th>
<th>Primer Sequence (5'→3')</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2C19</td>
<td>Sense</td>
<td>GCC ATT TCC CAC TGG CTG AAA G</td>
<td>(Löfgren et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>ACG AAA CTA GGA GGG AGA TCC</td>
<td></td>
</tr>
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<td>Sense</td>
<td>GCC ATT TCC CAC TGG CTG AAA G</td>
<td>(Löfgren et al., 2008)</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>ACG AAA CTA GGA GGG AGA TCC</td>
<td></td>
</tr>
<tr>
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<td>Sense</td>
<td>GGC AAG AAA CAC TTC ATG AGC ACT</td>
<td>(Löfgren et al., 2008)</td>
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<tr>
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<td></td>
</tr>
<tr>
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<td>Sense</td>
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<tr>
<td></td>
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<td>GAA GAA ATT GGA ACC ATG TCC</td>
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<tr>
<td>BAC (RP11-66J14) 5’end</td>
<td>Sense</td>
<td>TAA CAT TAG CAG GTG AAG CCC AAA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CAA TGT GTT CCA TGA TGG TTG ATG</td>
<td></td>
</tr>
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</tr>
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</tr>
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<td>Sense</td>
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<td>(Löfgren et al., 2008)</td>
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<td>Sense</td>
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<td>(Klose et al., 1999; Löfgren et al., 2008)</td>
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<tr>
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<td>Antisense</td>
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<td></td>
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<tr>
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<td>Sense</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CGCTTCATACCGTGCAATATG</td>
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</tr>
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<tr>
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<td>Antisense</td>
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</tr>
<tr>
<td></td>
<td>Antisense</td>
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</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>Cyp2d9</td>
<td>Sense</td>
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<td>(Wiwi et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>CGC AAG AGT ATC GGG AAT GC</td>
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<tr>
<td>Cyp7b1</td>
<td>Sense</td>
<td>TGA GTT CTT GAG GCT GTG CTC</td>
<td>(Holloway et al., 2006)</td>
</tr>
<tr>
<td></td>
<td>Antisense</td>
<td>TCC TGC ACT TCT CGG ATG ATG</td>
<td></td>
</tr>
<tr>
<td>Gstn</td>
<td>Sense</td>
<td>TGA GGA TGG AGA CCT CAC CC</td>
<td>(Wiwi et al., 2004)</td>
</tr>
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<td>Gene/Primer</td>
<td>Sense/Antisense</td>
<td>Sequence</td>
<td>Year</td>
</tr>
<tr>
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<td>Mup1/2/6/8*</td>
<td>Sense</td>
<td>GAC TTT TTC TGG AGC AAA TCC ATG (Holloway et al., 2006)</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>GAG CAC TCT TCA TCT CTT ACA G</td>
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<td>Mup3</td>
<td>Sense</td>
<td>GAA GAG TGC ACC GAA ATG ACT G (Holloway et al., 2006)</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>TGC CAG CCT TTT CTG TTT GTT</td>
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<td>Cyp2a4</td>
<td>Sense</td>
<td>AGC AGG CTA CCT TCG ACT GG (Wiwi et al., 2004)</td>
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<tr>
<td></td>
<td>Antisense</td>
<td>GCT GCT GAA GCC TAT GCC AT</td>
<td>2004</td>
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<td>Cyp2b9</td>
<td>Sense</td>
<td>CTG AGA CCA CAA GCG CCA C (Wiwi et al., 2004)</td>
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<td></td>
<td>Antisense</td>
<td>CTT GAG CAT GAG CAG GAC TCC</td>
<td>2004</td>
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<td>Cyp3a16</td>
<td>Sense</td>
<td>CAA ACC GGC AGG ATT TTT TC (Holloway et al., 2006)</td>
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<td>GAG AGA TGG ATT TAC TCA TCC CC</td>
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<td>Sense</td>
<td>TGC CCC TGG TGG TGA GTC TA (Holloway et al., 2006)</td>
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<td>CAT GCA TAT GAC CAC TGC TGG</td>
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<td>Cut2</td>
<td>Sense</td>
<td>CCT CAA GAC GAA CAC CGT CAT (Laz et al., 2006)</td>
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<td>GCG CAT CCT GGA CCT GTA GT</td>
<td>2007</td>
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<td>Sense</td>
<td>GAG GCC TCC GTC AAA CAG AAC (Laz et al., 2007)</td>
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<td>Sense</td>
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<td></td>
<td>Antisense</td>
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<td>Mouse HPRT</td>
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<td>TGG GGC TGT ACT GCT AAC C</td>
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<td>Sense</td>
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<td></td>
<td>Antisense</td>
<td>AAG ATG GTG ATG GGC TTC CCG</td>
<td>2001</td>
</tr>
</tbody>
</table>

1 These primers were used for gDNA/genotyping.

2 These primers were used for gDNA/genotyping of mice used in S-mephenytoin measurements.

* Major Urinary Protein (MUP). Primer will potentially amplify four different MUP mRNAs (if expressed), i.e. MUP1, MUP2, MUP6 and MUP8.
TABLE 2. Apparent enzyme kinetics parameters of S-mephenytoin metabolism in pooled hepatic microsomes from 3 and 9 weeks old wild-type and CYP2C18/CYP2C19 transgenic mice

<table>
<thead>
<tr>
<th>Animal pool</th>
<th>3 weeks old animals</th>
<th>9 weeks old animals</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$V_{\text{max}}$</td>
<td>$K_m$</td>
</tr>
<tr>
<td></td>
<td>(pmol/mg, min)</td>
<td>(µM)</td>
</tr>
<tr>
<td>wt male</td>
<td>12.4</td>
<td>8.5</td>
</tr>
<tr>
<td>tg male</td>
<td>13.2</td>
<td>14.0</td>
</tr>
<tr>
<td>wt female</td>
<td>22.4</td>
<td>11.9</td>
</tr>
<tr>
<td>tg female</td>
<td>16.6</td>
<td>8.7</td>
</tr>
</tbody>
</table>

wt: wild-type C57BL/6OlaHsd mice, tg: hemizygous CYP2C18/CYP2C19 transgenic mice. Each pool contains 5-10 mice (wild-type males and CYP2C18/CYP2C19 transgenic females 3 weeks old n=5, wild-type females 3 weeks old n=6, CYP2C18/CYP2C19 transgenic males 3 weeks old n=10, wild-type males and females 9 weeks old n=6 and CYP2C18/CYP2C19 transgenic males and females 9 weeks old n=8).
Fig 2

Liver Cyp2c29
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- **

Females
- +++

Liver Cyp2c37
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- ***

Females
- 

Liver Cyp2c38
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- **

Females
- ***

Liver Cyp2c39
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- 

Females
- ***

Liver Cyp2c40
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- 

Females
- ##

Liver Cyp2c50
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- 

Females
- 

Liver Cyp2c54
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- 

Females
- 

Liver Cyp2c55
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- *

Females
- **

Liver Cyp2c70
- Wildtype
- Sham/Placebo
- Castrated
- Testosterone

Males
- *

Females
- ++

Relative mRNA levels
Fig 3

Liver CYP2C18: 4 days treatment vs 7 days treatment; Liver CYP2C19: 4 days treatment vs 7 days treatment.

Kidney CYP2C18: 4 days treatment vs 7 days treatment; Kidney CYP2C19: 4 days treatment vs 7 days treatment.

Relative mRNA levels: Placebo males vs GH males vs Females.
Fig 4

Liver Cyp2c37
- 4 days treatment
- 7 days treatment

Liver Cyp2c38
- 4 days treatment
- 7 days treatment

Liver Cyp2c39
- 4 days treatment
- 7 days treatment

Liver Cyp2c40
- 4 days treatment
- 7 days treatment

Relative mRNA levels

Wt Males  Placebo males  GH males  Females

Wt Males  Placebo males  GH males  Females