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Gender-specific induction of cytochrome P450s in nonylphenol-treated FVB/NJ mice

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Abstract

Nonylphenol (NP) is a breakdown product of nonylphenol ethoxylates, which are used in a variety of industrial, agricultural, household cleaning, and beauty products. NP is one of the most commonly found toxicants in the United States and Europe and is considered a toxicant of concern because of its long half-life. NP is an environmental estrogen that also activates the pregnane X-receptor (PXR) and in turn induces P450s. No study to date has examined the gender-specific effects of NP on hepatic P450 expression. We provided NP at 0, 50 or 75 mg/kg/day for 7 days to male and female FVB/NJ mice and compared their P450 expression profiles. Q-PCR was performed on hepatic cDNA using primers to several CYP isoforms regulated by PXR or its relative, the constitutive androstane receptor (CAR). In female mice, NP induced Cyp2b10 and Cyp2b13, and downregulated the female-specific P450s, Cyp3a41 and Cyp3a44. In contrast, male mice treated with NP showed increased expression of Cyp2a4, Cyp2b9, and Cyp2b10. Western blots confirmed induction of Cyp2b subfamily members in both males and females. Consistent with the Q-PCR data, Western blots showed dose-dependent downregulation of Cyp3a only in females and induction of Cyp2a only in males. The overall increase in female-predominant P450s in males (Cyp2a4, 2b9) and the decrease in female-predominant P450s in females (Cyp3a41, 3a44) suggest that NP is in part feminizing the P450 profile in males and masculinizing the P450 profile in females. Testosterone hydroxylation was also altered in a gender-specific manner, as testosterone 16 α -hydroxylase activity was only induced in NP-treated males. In contrast, NP-treated females demonstrated a greater propensity for metabolizing zoxazolamine probably due to greater Cyp2b induction in females. In conclusion, NP causes gender-specific P450 induction and therefore exposure to NP may cause distinct pharmacological and toxicological effects in males compared to females.

Keywords

Nonylphenol; P450; Gender-specific; PXR; CAR; Sexual dimorphism

Introduction

Akylphenol ethoxylates (APEs) are nonionic surfactants used in a variety of products including institutional cleaning agents, textiles, agricultural chemicals, plastics, household cleaning products, paper products, and personal care products. Nonylphenol (NP) is a degradation product of the nonylphenol ethoxylates, which constitute approximately 80% of the alkylphenol ethoxylates (Talmage, 1994; Knepper and Berna, 2003). Research has identified NP as the most critical metabolite of APEs because of its resistance to biodegradation, its ability to bioaccumulate, its toxicity, and its presence/abundance in some food products, cosmetics, and drinking water (Clark et al., 1992; Ahel et al., 1994; Tyler et al., 1998; Fernandes et al.,

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2003;Loyo-Rosales et al., 2004). Over 1 billion pounds of nonylphenol ethoxylates are produced annually, making them one of the most commonly found toxicants in the United States and Europe (Talmage, 1994). The United States Geological Survey showed that NP is found in greater than 50% of our surface waters and when present is often the contaminant found at the highest concentration. Furthermore, the parent compounds to NP, the nonylphenol ethoxylates (NPEs), are also often found at concentrations greater than most or all of the other anthropogenic chemicals (Kolpin et al., 2002). NPEs are also the only priority organic pollutants that are routinely found at greater concentrations than recommended in sewage sludges. Greater than 90% of Europe's sludges have more NPEs than their recommended limit; no other chemical exceeds its limits in more than 8% of the sludges (Abad et al., 2005).

NP is an environmental estrogen that also activates the pregnane X-receptor (PXR) (Masuyama et al., 2000). The PXR regulates the induction of several hepatic cytochrome P450 (CYP) subfamilies, which are important enzymes involved in the phase I metabolism of many chemical contaminants and pharmaceuticals. For example, CYP3A4, the predominant human hepatic P450, metabolizes greater than 60% of pharmaceutical agents (Sonoda and Evans, 2003). Other P450s such as CYP2D6, CYP2B6, CYP2C9, and CYP1A2 are also important in xenobiotic metabolism, as well as steroid catabolism, bile acid metabolism, and bilirubin elimination (Namkung et al., 1988;Baldwin and LeBlanc, 1992;Schuetz et al., 2001;Staudinger et al., 2001b). NP has been shown to induce several P450s in rodents in vivo, including Cyp2b and Cyp3a subfamily members. For example, Cyp2b protein was increased in female FVB/NJ mice fed NP at 50 or 75 mg/kg/day for 7 days (Acevedo et al., 2005). In addition, NP but not E₂, induced CYP3A in male and female rats injected i.p. (Lee et al., 1996;Masuyama et al., 2000). In contrast, some studies have shown downregulation of CYP3A protein or CYP3A activity by NP. FVB/NJ female mice treated for 32 weeks demonstrated reduced Cyp3a activity and protein levels (Acevedo et al., 2005). CD Sprague-Dawley male rats treated orally with 2 or 240 mg/kg/day NP from gestational day 7 through postnatal day 50 also demonstrated reduced CYP3A levels in male rats but not female rats (Laurenzana et al., 2002).

The liver is a sexually dimorphic organ, and the release pattern of growth hormone and its subsequent control of Stat5b regulate several P450s in a gender-specific or gender-predominant manner (Waxman et al., 1991;Park et al., 1999). Other hepatic transcription factors potentially involved in sex-specific expression of P450s include rsl (Krebs et al., 2003), HNF-1 α (Cheung et al., 2003), HNF-4 α (Wiwi et al., 2004), and RXR α (Cai et al., 2003). Sexually dimorphic P450s include CYP2C11, 2A2, 3A2, and 4A2, which are considered male-specific or male-predominant in the rat, while Cyp2d9 and 4a12 are well-characterized male-predominant P450s in the mouse. In contrast, CYP2C12 is female-predominant in the rat, while Cyp2a4, 2b9, 2b10, 2b13, 3a41, and 3a44 are female-predominant P450s in the mouse (Sueyoshi et al., 1999;Anakk et al., 2004;Wiwi et al., 2004).

Recently, induction of Cyp2a, Cyp2b, and Cyp3a subfamily members was shown to be regulated by PXR and its relative, the constitutive androstane receptor (CAR), in a sexually dimorphic manner (Sierra-Santoyo et al., 2000;Wei et al., 2002;Anakk et al., 2004;Nelson et al., 2005). For example, PXR-null male mice demonstrate greater Cyp3a activity, while PXR-null female mice demonstrate reduced Cyp3a activity compared to same-sex wild-type mice (Anakk et al., 2004). Dexamethasone downregulates the female-predominant Cyp3a41 in a PXR-dependent manner, while male mice did not demonstrate downregulation of any Cyp3a subfamily members (Anakk et al., 2004). In addition, the CAR activators, phenobarbital and 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) demonstrate male-specific Cyp2a4 induction (Wei et al., 2002). Lastly, St. John's wort mediated induction of CYP3A4 is greater in human females than males (Gurley et al., 2002). Gender differences in P450 induction are important to discern because of potential gender-specific side effects following pharmaceutical or environmental chemical exposure (Meibohm et al., 2002).

The advent of Quantitative real-time PCR (Q-PCR) allows us to monitor the regulation of individual P450 gene isoforms, because small, specific primers can be made that recognize a specific gene in a subfamily (Luo et al., 1998; Zhang et al., 2003; Wiwi et al., 2004). This is generally not true of Northern blotting because the probes are usually large and therefore recognize several P450 isoforms. Similarly, most P450 antibodies recognize several members of a subfamily. We used Q-PCR to examine NP-induced expression of several P450s previously shown to be regulated by CAR and PXR (Cyp2a, 2b, 2c, 3a) in males and females. Results were confirmed by immunoblotting with antibodies recognizing P450 subfamilies. Functional differences between males and females, in respect to NP-mediated P450 induction, were determined with testosterone hydroxylase assays and by measuring zoxazolamine paralysis time.

Materials and methods

Chemicals and reagents

Technical grade NP is a mixture of branched side chains containing 85% *p*-isomers and was obtained from Fluka Chemical Co. (Ronkonkoma, NY). E₂, 1,4-*bis*-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP), dexamethasone (DEX), zoxazolamine (2-amino-5-chlorobenzoxazole) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). All other chemical reagents were purchased from Fisher Scientific (Houston, TX) unless specifically noted.

Animals and NP treatments

All studies were carried out according to NIH guidelines for the humane use of research animals and were approved by the University of Texas at El Paso's Animal Care and Use Committee. Male and female FVB/NJ mice (4–5 weeks) obtained from The Jackson Laboratory (Bar Harbor, ME) were provided water and food ad libitum, and allowed to acclimate for 1 week in individual cages before treatment.

Male mice were fed 0, 25, 50, or 75 mg/kg/day technical grade NP mixed in 100 µl honey for 7 days (*n* = 6). Female mice were fed 0, 50, or 75 mg/kg/day NP, or 100 µg/kg/day 17β-estradiol (E₂) mixed in 100 µl honey for 7 days (*n* = 6). Male mice were treated with 3 different NP doses to determine if a dose response to NP exists; however, 25 mg/kg/day NP typically showed similar results to the controls. Therefore, treatments in females were done similarly except the 25 mg/kg/day NP treatment was replaced with 100 µg/kg/day estradiol (E₂) in the females, to investigate whether NP's estrogenicity is potentially the reason for the P450 alterations. Following treatment, mice were anesthetized by ketamine injection (Sigma-Aldrich) and euthanized by CO₂ asphyxiation. Livers were excised, diced into several pieces, and approximately half of the liver was used for microsome preparation; the other half was placed in TRI-Reagent (Sigma-Aldrich) for RNA extraction. All samples were stored at –80°C.

In addition, FVB/NJ female mice ovariectomized (OVEX) at The Jackson Laboratory (4–5 weeks) were obtained and acclimated for 1 week as described above. Treated mice were fed 0 or 50 mg/kg/day NP, or 100 µg/kg/day E₂ mixed in 100 µl of honey for 7 days (*n* = 6). Following treatment, mice were weighed, euthanized as described above, and the uterus was excised and weighed. The uterosomatic index was determined by dividing the uterine weight by the weight of the mouse.

Sample preparation

Half of the liver was used to prepare microsomes according to previously published protocols (Van der Hoeven and Coon, 1974). Protein concentrations were determined from microsomes resuspended in buffer (0.1 M potassium phosphate, 0.1 mM EDTA, 20% glycerol, pH 7.4)

using the Bio-Rad protein assay according to the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA). Total RNA was extracted from the other half of the liver using a modified phenol/chloroform extraction technique with TRI-Reagent followed by DNase (Promega Corporation, Madison WI) treatment to remove residual genomic DNA. Reverse transcription was performed with 4 µg of RNA to make cDNA using 400 U Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV-RT) (Promega Corporation, Madison, WI), 60 U of RNase inhibitor, a 10 mM dNTP mixture, and 0.1 mg random hexamers.

Quantitative real-time polymerase chain reaction (Q-PCR)

Quantitative real-time PCR (Q-PCR) was performed using primers for specific isoforms of Cyp2a, Cyp2b, Cyp2c, and Cyp3a subfamily members. To generate a standard curve and determine the PCR efficiency of each reaction, a composite sample of cDNA from treated and untreated mice was made and 2×, 1:1, 1:10, 1:100 and 1:1000 dilutions were prepared. 18S cDNA was diluted 1:10 prior to Q-PCR. Q-PCR was performed by incubating 80 ng of cDNA with 0.33 mM gene-specific primers, 0.33 mM dNTPs, and 1 U of Taq polymerase (Qiagen, Valencia, CA). During PCR, samples were denatured at 95 °C for 30 s, lowered to the appropriate annealing temperature for 30 s, and extended at 72 °C for 30 s. Table 1 shows the forward and reverse primers designed to each gene and the annealing temperature. Amplifications were performed in triplicate using a 96-well MyiQ Real-Time PCR Detection System (Bio-Rad) with 0.25× SybrGreen (Sigma-Aldrich) as the fluorescent double strand-intercalating agent to quantify gene expression. A minimum of forty cycles was run on all real-time samples to ensure a log based growth curve. Relative quantities of each transcript were determined as described previously (Muller et al., 2002; Roling et al., 2004) using the equation ($NE = \{E_{ref}\}^{Ct-ref} / \{E_{target}\}^{Ct-target}$). Where NE is the normalized gene expression, E is the efficiency of amplification for a particular gene, C_t is the threshold cycle, ref is the housekeeping gene (18 s rRNA), and target is the gene of interest. Amplified products from each primer set were also cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

Western blotting

P450 protein levels were quantified by Western blotting. 50 µg of microsomal protein was separated on 10% SDS-PAGE gels according to Laemmli (1970) and then transferred to nitrocellulose (Bio-Rad) for blotting with the appropriate primary antibody. Primary antibodies were obtained from a variety of sources. The rat anti-mouse Cyp2b10 antibody is a generous gift from Dr. Randy Rose at North Carolina State University, Raleigh, NC. Commercially available rabbit anti-rat CYP3A1 and CYP2C8/9/19 were obtained from Chemicon International (Temecula, CA). Mouse anti-human CYP2A6 was obtained from Gentest Corporation (San Jose, CA). Rabbit anti-mouse β-actin (Sigma Aldrich, St. Louis, MO) was used as a housekeeper to ensure equal loading of samples. All primary antibodies were diluted 1:1000. Goat anti-rabbit IgG (Bio-Rad) alkaline-phosphatase coupled secondary antibodies were used for recognizing CYP3A1 and CYP2C8/9/19 primary antibodies. Goat anti-rat (Bio-Rad), rabbit anti-mouse (Gentest Corporation), and goat anti-mouse (Bio-Rad) IgG were used to recognize the Cyp2b10, CYP2A6 and β-Actin primary antibodies, respectively. Secondary antibodies were diluted 1:500. Western blots were visualized colorimetrically with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) as substrates (Bio-Rad). Bands were scanned on a GS-710 densitometer (Bio-Rad) and quantified with LabWorks image analysis software (UVP laboratory products, Upland, CA). Pre-stained protein standards (Bio-Rad) were used as molecular weight markers.

Testosterone hydroxylase assays

P450 activity was measured using the testosterone hydroxylase assay as previously described using thin-layer chromatography to separate the testosterone metabolites (Baldwin and LeBlanc, 1992), except 200 µg of microsomal protein was used. Quantification of the testosterone metabolites was performed spectrophotometrically with a LS5801 liquid scintillation counter (Beckman, Fullerton, CA).

Zoxazolamine paralysis challenge

Male and female FVB/NJ mice were obtained from The Jackson Laboratory (4–5 weeks) and acclimated as described above. Both male and female mice were treated orally with honey or NP at 50 or 75 mg/kg/day mixed in honey for 1 week. Mice were injected with 440 mg/kg zoxazolamine 24 h after the last treatment with NP or honey. Time of injection was noted as was initial paralysis. Paralysis time was measured by placing paralyzed mice on their backs and measuring the time until they were able to consistently right themselves. In addition, two separate groups of mice were used to act as positive controls for P450 induction. One group was injected once i.p. with 3 mg/kg 1,4-bis-[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) in corn oil 24 h prior to the zoxazolamine challenge, while the other group received 75 mg/kg/day dexamethasone (DEX) in corn oil on 2 consecutive days prior to the zoxazolamine treatment.

Statistical analysis

Statistical tests were performed with StatView software (SAS Institute Inc., Cary, NC). ANOVA was used to compare multiple treatments followed by Fisher's PLSD as the post hoc statistical test in all experiments except for the zoxazolamine study. Kruskal–Wallis nonparametric test for independent variables followed by the least significant difference (LSD) post hoc test using ranks was used to analyze the zoxazolamine data. A *P* value ≤0.05 was regarded as significantly different from controls and is shown with an asterisk in the figures.

Results

Comparative estrogenicity of nonylphenol and estradiol in FVB/NJ mice

We used the uterotrophic assay to compare the *in vivo* estrogenicity of E₂ and NP at the doses provided. OVEX mice were treated with E₂ at 100 µg/kg/day, or NP at 0 or 50 mg/kg/day, and the uterosomatic index was determined. Uterosomatic index was calculated by dividing uterine weight by total body weight. OVEX mice treated with E₂ showed a significant increase in the uterosomatic index when compared to untreated OVEX mice (Table 2). E₂ treatment of OVEX mice resulted in uterosomatic index values comparable to those of untreated intact mice. However, NP did not show estrogenicity at 50 mg/kg/day NP in the uterotrophic assay (Table 2). This indicates that this dose of NP is not estrogenic or is weakly estrogenic in the uterus and suggests that NP's estrogenicity is not the cause for the measured changes in P450 expression. We also measured the uteri from intact female mice treated with E₂ and NP that were used for the purpose of investigating P450 levels. These mice did not show statistical differences in the uterosomatic index when compared to controls, although E₂ treated mice showed a 60% increase in uterine weight. Mice in estrus were eliminated from the analysis.

Sexually dimorphic expression of P450s in FVB/NJ mice

Q-PCR was used to compare basal expression of several P450 isoforms in males and females. Earlier studies have investigated sexually dimorphic expression of some, but not all, P450 isoforms in mice (Burkhart et al., 1985; Wiwi et al., 2004). Furthermore, sexually dimorphic P450 isoform expression has not been examined in FVB/NJ mice. We used FVB/NJ mice in this study because we initially observed NP-mediated hepatic induction of Cyp2b in transgenic

MMTVneu mice susceptible to mammary cancer [FVB/N-TgN(MMTVneu)202Mul] (Acevedo et al., 2005). FVB/NJ mice are the background strain for the MMTVneu mice.

Determination of the sexually dimorphic forms allows us to evaluate whether gender-predominant P450s are subject to NP-mediated induction. The relative expression of each P450 in males and females was determined by comparing its expression to 18 S rRNA as described in the Materials and methods, using primers for one Cyp2a, three Cyp2b, two Cyp2c, and four Cyp3a genes. Eight of the ten P450s investigated appeared more highly expressed in females than males, including Cyp2a4, Cyp2b9, Cyp2b10, Cyp2b13, Cyp2c29, Cyp2c40, Cyp3a41, and Cyp3a44 (Table 3). None of the P450 isoforms we measured are male-predominant. Cyp3a11 and Cyp3a25 are not considered differentially expressed between the genders (Table 3)(Wiwi et al., 2004).

Some P450 isoforms are exclusively expressed in one gender; Cyp3a44 was only detectable in females in the FVB/NJ mice. Cyp2b9 and Cyp3a41 are strongly female-predominant and are considered female-specific (Wiwi et al., 2004). Cyp2a4, Cyp2b10, and Cyp2b13 are also considered female-predominant, being expressed at only slightly higher levels in females, which is consistent with previous data (Burkhart et al., 1985; Wiwi et al., 2004). Interestingly, all of the measured Cyp2b subfamily members are female-predominant in FVB/NJ mice (Table 3). However, while our data demonstrated a reasonably large difference between Cyp2b10 levels in males and females, the data were not statistically different (Table 3). This is not consistent with previous data (Wiwi et al., 2004) and may be due to high variability, differences in primer sensitivity, or mouse strain variance. To our knowledge, the sexually dimorphic character of Cyp2c29 and Cyp2c40 has not been previously examined. Both of these P450s show female-predominance, but Cyp2c29's female predominance is not as robust as the other female-predominant P450s (Table 3).

Q-PCR of P450 isoforms following NP treatment

NP is known to alter the expression of a subset of P450s (Lee et al., 1996; Laurenzana et al., 2002; Acevedo et al., 2005), and gender differences have been observed in respect to the induction of Cyp3a subfamily members by NP (Laurenzana et al., 2002). Therefore, we performed a more comprehensive study and investigated NP-mediated alterations in several individual P450 isoforms within the different subfamilies by Q-PCR in both male and female mice.

NP induced all of the Cyp2a and Cyp2b subfamily members tested. Male mice exhibited induction of Cyp2a4, Cyp2b9 and Cyp2b10; female mice exhibited induction of Cyp2b10 and Cyp2b13 in a dose-dependent manner (Fig. 1). Only Cyp2b10 is induced significantly in both genders; Cyp2a4, Cyp2b9 and Cyp2b13 are induced in a gender-specific manner by NP (Fig. 1). Interestingly, Cyp2a4 and Cyp2b9 are female-predominant P450s, and both showed significant induction only in males. The expression of two Cyp2c subfamily members was also measured following NP treatment. In the Cyp2c subfamily, both males and females exhibited similar trends; however, the response in females appeared more robust (Fig. 1). In males and females, Cyp2c29 expression is induced, but Cyp2c40 expression is only downregulated in females following NP treatment (Fig. 1).

The regulation of the different Cyp3a isoforms by PXR has been well studied (Anakk et al., 2004; Matheny et al., 2004). Cyp3a11 is strongly induced by PXR activators (Staudinger et al., 2001a). Surprisingly, Cyp3a11 transcript levels were not altered in either male or female mice treated with NP, a known PXR agonist, and Cyp3a25 was induced less than a 2-fold in males treated with 75 mg/kg/day NP (Fig. 1). Previous studies have shown PXR-dependent downregulation of the female-specific Cyp3a member, Cyp3a41 (Anakk et al., 2004), and we observed down-regulation of Cyp3a41 by NP only in female mice (Fig. 1). Current research

suggests female-specific Cyp3a44 is not regulated by PXR (Anakk et al., 2004), but its expression was also reduced about 2-fold in female mice treated with NP. This suggests that NP is causing reductions in Cyp3a44 expression through a PXR independent mechanism.

Protein quantification of P450s following NP treatment

Western blotting was performed to quantify the protein levels of the different P450 subfamilies measured by Q-PCR and to confirm the Q-PCR data from NP-treated male and female mice. Due to the fact that antibodies may recognize more than one isoform within a particular subfamily; we refer only to the subfamily. The female-predominant P450 Cyp2a tended to be increased in a dose-dependent fashion by NP only in male mice, but the data were not statistically significant (Fig. 2). The increase in Cyp2a protein corresponds well to the dose-dependent increase in Cyp2a4 measured by Q-PCR only in males. Cyp2c protein concentrations were not significantly different in NP treated mice compared to the controls but did show a downward trend indicative of Cyp2c40 downregulation.

Western blots demonstrated opposing effects of NP on Cyp2b and Cyp3a subfamily members. NP treatment induced Cyp2b protein levels in male and female mice in a dose-dependent fashion. NP increased Cyp2b protein levels by 3.48- and 3.90-fold in 50 and 75 mg/kg/day NP treated females and 1.96- and 2.84-fold in NP treated males (Fig. 2). These data also confirmed the Q-PCR data that demonstrated induction of several Cyp2b subfamily members by NP.

NP treatment downregulated Cyp3a protein levels in both males and females, but only females showed a dose-dependent effect of NP exposure and the decrease was greater in female mice (Fig. 2). Cyp3a Western blots with microsomes from male livers show a predominant top band and a much weaker bottom band that sometimes does not resolve well from the top band. In contrast, Cyp3a Western blots of female livers show two close, distinct bands that are similar in intensity. Other laboratories have also shown two bands in females (Schuetz et al., 2000; Yamada et al., 2002) and have attributed these bands to Cyp3a11 on top and Cyp3a41 on the bottom (Schuetz et al., 2000). Both of these bands appear downregulated with greater downregulation of the lower band. However, the bands were quantified together because the resolution was not high enough to quantify them separately. The decrease in Cyp3a protein expression in females (Fig. 2) is consistent with the decrease in expression of both female-specific P450s (Cyp3a41, Cyp3a44) as determined by Q-PCR (Fig. 1).

Testosterone hydroxylase assays to determine enzyme activity of NP responsive P450s

Testosterone hydroxylation is often used as a biomarker for multiple P450s, with special attention to the steroid metabolizing P450s. Both males and females treated with NP demonstrated increased 16 β -hydroxylation of testosterone (Fig. 3), indicative of Cyp2b induction (Waxman, 1988; Ohmori et al., 1993). Testosterone hydroxylation in the 6 β -position was not altered in either males or females (Fig. 3), even though Cyp3a protein levels were significantly decreased in females in a dose-dependent manner. It is possible that other inducible P450s are weakly involved in hydroxylating testosterone at this position. Males showed a marginal increase in 6 α -hydroxylation (Fig. 3), coinciding with data indicating that Cyp2a4 was increased only in males (Figs. 1, 2). Lastly, 16 α -hydroxylation of testosterone was significantly increased, but only in males (Fig. 3). Male rats also show induction of testosterone 16 α -hydroxylase activity following phenobarbital treatment (Waxman et al., 1983). In mice, 16 α -hydroxylation has been assigned to Cyp2b and Cyp2c (Yamada et al., 2002) subfamily members, especially Cyp2b9 (Paolini et al., 1999). We hypothesize that the male-specific increase in 16 α -hydroxylase activity is due to the male-specific induction of Cyp2b9 by NP (Fig. 1).

NP alters the pharmacological effects of zoxazolamine

The muscle relaxant zoxazolamine (ZOX) is a classical P450 substrate used to determine the overall in vivo effects of P450 inducers/inhibitors on metabolism, as increased paralysis indicates inhibition of P450s and decreased paralysis indicates induction. We used ZOX to elucidate the pharmacological effects of NP on P450 metabolism and its potential to cause drug–toxicant interactions. In addition, groups of mice were treated with TCPOBOP (prototypical Cyp2b inducer) or DEX (prototypical Cyp3a inducer) as positive controls.

Male mice were more sensitive to the ZOX treatment and four untreated males died following ZOX-induced paralysis (Fig. 4, Table 4). Furthermore, the males did not respond as robustly as the females following treatment with the P450 inducers. TCPOBOP and 50 mg/kg/day NP significantly decreased paralysis time, but the 75 mg/kg/day NP and the DEX treatment did not reduce paralysis time compared to the untreated mice (Fig. 4, Table 4). Repeating the male ZOX protocol produced similar results that demonstrated only the 50 mg/kg/day NP and the TCPOBOP treatment significantly reduced paralysis time (data not shown). We are uncertain why DEX did not reduce ZOX paralysis time in males but speculate that DEX must have been cleared quickly in the male mice. In contrast, female mice responded robustly to all of the P450 inducers. Female mice treated with 50 or 75 mg/kg/day NP, TCPOBOP, or DEX showed reduced paralysis time compared to untreated female mice (Fig. 4). One female mouse did not recover from ZOX-induced paralysis and was euthanized. Because some male and female mice did not recover from paralysis within 6–8 h, a numeric time (minutes) could not be assigned to these mice. This made nonparametric statistics necessary for the analysis of the ZOX paralysis data (Table 4). Overall, both males and females treated with NP showed a pharmacologically relevant alteration in paralysis time, but females were more likely to recover from ZOX treatment and showed a greater response to NP.

Discussion

To our knowledge, this is the first comprehensive report detailing the effects of nonylphenol on the gender-specific expression of P450s in mouse liver. Our findings indicate that nonylphenol affects P450s in a gender-specific manner and in turn alters ZOX metabolism, suggesting that NP and other similar acting environmental chemicals may alter drug metabolism and clearance in a gender-specific manner. QPCR and Western blots indicate that the Cyp2b's are highly induced by NP and probably involved in the decreased ZOX paralysis time observed following NP treatment. Q-PCR also indicates that the changes in P450 expression are most likely regulated at the transcriptional level. A summary of the observed effects of nonylphenol on transcript, protein, and activity in males and females is provided in Table 5.

P450 induction by NP has been observed previously (Lee et al., 1996; Masuyama et al., 2000; Acevedo et al., 2005), and NP has been shown to activate the PXR (Masuyama et al., 2000). Several studies have suggested that greater induction of Cyp2b family members relative to Cyp3a family members indicates a phenobarbital-type induction and suggests CAR activation (Kocarek et al., 1993; Sueyoshi and Negishi, 2001). We also observed a P450 induction profile of Cyp2b>Cyp3a following NP treatment; therefore, activation of CAR should be considered another potential mechanism by which NP may induce P450s.

NP induced several P450s in a gender-specific manner. Both males and females showed induction of Cyp2b as determined by Western blotting (Fig. 2). In addition, both males and females showed induction of Cyp2b10 as determined by Q-PCR (Fig. 1), but males showed greater relative induction (12-fold) and females showed greater absolute induction (220 relative light units). The induction of Cyp2b10 in NP-treated males reduces the female-predominant

expression from a 32-fold difference to a 2.5-fold difference when compared to untreated females.

Only males showed induction of Cyp2b9 and only females showed induction of Cyp2b13 after NP treatment (Fig. 1, Table 5). Both Cyp2b9 and Cyp2b13 are female-predominant, but Cyp2b9 is strongly female-predominant while Cyp2b13 is weakly female-predominant (Table 3). There is conflicting evidence as to whether Cyp2b9 is inducible by phenobarbital (Rivera-Rivera et al., 2003). However, increases in Cyp2b9 activity as measured by increased testosterone 16 α -hydroxylase activity have been observed in male mice following treatment with bile acids (Paolini et al., 1999), which are weak PXR and CAR agonists (Zhang et al., 2004). Furthermore, a male-specific increase in Cyp2b9 transcript levels was observed following treatment with the CAR activator, TCPOBOP, in wild-type, but not CAR-null mice (Nelson et al., 2005). Our data are consistent with these latter results indicating that the strongly female-predominant P450, Cyp2b9, is only induced in males.

Females, but not males, showed a pronounced decrease in Cyp3a protein levels following NP treatment (Fig. 2) consistent with decreases in Cyp3a41 and Cyp3a44 (Fig. 1). This observation was somewhat surprising as many of the Cyp3a isoforms are under control of PXR, which is activated by NP (Masuyama et al., 2000). However, previous studies have shown PXR-dependent downregulation of the female-predominant Cyp3a member, Cyp3a41 (Anakk et al., 2004). The downregulation of these two female-specific P450s by NP accounts for the pronounced decrease in Cyp3a in female mice (Figs. 1,2). Previous studies have suggested that the two Cyp3a bands produced in immunoblots are Cyp3a11 on top and Cyp3a41 on the bottom (Schuetz et al., 2000). However, both bands appear downregulated by NP and Cyp3a11 was not downregulated according to the Q-PCR data. Therefore, we cannot discount the contributions of Cyp3a13, Cyp3a16, Cyp3a25, Cyp3a44, and Cyp3a57 to these bands. Our data showing the downregulation of Cyp3a44 (Fig. 1) may contribute to the downregulation of the top band.

Males and females also showed differences in respect to the induction of Cyp2a and Cyp2c subfamily members following NP treatment. Cyp2a4 was increased by NP in males as measured by Q-PCR, but not in females (Fig. 1). Previous data have demonstrated that the potent CAR agonist TCPOBOP only induces Cyp2a4 in males and in a CAR-dependent fashion (Wei et al., 2002). Statistically significant increases in Cyp2a were not observed by Western blotting or testosterone 6 α -hydroxylase activity in males. However, both Cyp2a protein levels and testosterone 6 α -hydroxylase activity increased in a fashion consistent with the Q-PCR data (Figs. 2,3, Table 5). Steroid 6 α -hydroxylase activity is attributed to CYP2A1 in rats (Swinney et al., 1987), but Cyp2a is only a putative 6 α -hydroxylase in mice to our knowledge (Baldwin and LeBlanc, 1992).

Overall, the Cyp2c isoforms measured showed few gender-specific alterations in transcript expression and no differences in protein levels. Cyp2c29 is induced in both males and females. However, Cyp2c40 was reduced in both genders, but only in a statistically significant fashion in females (Fig. 1). This decrease in Cyp2c40 may be a gender-specific effect or reflect the increased sensitivity of female mice to NP because of their constitutive expression of Cyp2c40 is 33 $\times$ greater than males. Interestingly, the female-predominant rat P450, CYP2C12 is also downregulated by the PXR agonist, dexamethasone, in both males and females (Thangavel et al., 2004). Therefore, there is precedence for a PXR agonist to downregulate a Cyp2c subfamily member. However, we did not measure a statistically significant change in Cyp2c protein levels (Table 5). This may be caused by the opposing actions of NP on the different Cyp2c subfamily members.

Many of the strongly female-predominant genes were only induced in males, such as Cyp2b9 and Cyp2a4. This may be due to the increased assay sensitivity caused by lower basal transcript levels. Induction of Cyp2b9 and Cyp2a4 P450s in females may not occur or may be difficult to measure because these P450s show high constitutive expression. It is interesting to speculate that the lack of Cyp2b9 induction in females following phenobarbital (Nelson et al., 2005) or NP treatment may be because Cyp2b9 is already expressed at or near its maximum. Similarly, the lack of Cyp2a4 induction in females may be because it is expressed at near maximum levels constitutively. However, not all of the female-predominant P450s are only induced in males as Cyp2b13 is female-predominant and only induced in females.

The overall increase in female-predominant P450s in males (Cyp2a4, 2b9, 2b10, 2c29) and the decrease in female-predominant P450s in females (Cyp3a41, 3a44, 2c40) suggest that NP is in part feminizing the P450 profile in males and masculinizing the P450 profile in females. Chemicals such as DEX and TCPOBOP have also caused gender-specific effects on Cyp2a4, Cyp2b9, Cyp2c29, and Cyp3a41 expression consistent with our results (Wei et al., 2000; Zhang et al., 2003; Anakk et al., 2004; Nelson et al., 2005). It is interesting to hypothesize that under normal conditions, the liver retains its sexually dimorphic nature, but when it encounters a toxicant stress such as NP, it loses some of its gender differences in order to overcome the stressor. Other laboratories have also recognized that several chemicals, including many endocrine disruptors such as endosulfan, ketoconazole, vinclozolin, and indole-3-carbinol, alter the P450 and testosterone hydroxylase profiles in a manner that suggests a reduction in gender specificity, especially as it pertains to feminization of the male liver (LeBlanc et al., 1997; Wilson and LeBlanc, 1998; Wilson et al., 1999).

ZOX-induced paralysis time was significantly decreased by NP in female mice, while male mice showed a marginal effect. The greater induction of Cyp2b in female mice treated with NP is probably responsible for much of the difference between male and female ZOX-induced paralysis time. To our knowledge, the P450s responsible for ZOX metabolism have not been published, but increases in Cyp1a, Cyp2b, and Cyp3a activity have been correlated with decreased ZOX-induced paralysis (Kapitulnik et al., 1976; Wei et al., 2000; Staudinger et al., 2001a). Untreated or TCPOBOP-treated CAR-null mice rarely right themselves following ZOX-treatment, indicating that CAR-null mice are extremely sensitive to ZOX. Because CAR is the primary regulator of Cyp2b induction this would indicate Cyp2b is important in ZOX metabolism. Our data also suggest that the induction of Cyp2b by NP nullifies the downregulation of Cyp3a in female mice in regards to ZOX clearance.

Interestingly, the effect of 75 mg/kg/day NP treatment on ZOX-induced paralysis was attenuated compared to 50 mg/kg/day NP treatment in both males and females (Fig. 4). In males, 75 mg/kg/day NP did not significantly decrease ZOX paralysis time. It is not known why NP did not act in a dose-dependent fashion, but it is possible that NP is acting as a P450 inhibitor at higher concentrations. NP has been shown to inhibit P450 activity (Lee et al., 1996; Niwa et al., 2002; Acevedo et al., 2005), including testosterone 16 β -hydroxylase activity (Acevedo et al., 2005), which has been assigned to the Cyp2b subfamily members. NP is extensively metabolized by rat and human Cyp2b family members (Lee et al., 1998), so NP may be competing with ZOX for Cyp2b-mediated metabolism.

Overall, NP caused significantly different effects on P450 gene expression in female mice compared to male mice. Metabolism by the sex-predominant P450s can lead to sex-dependent susceptibility to drugs, chemicals and carcinogens (Waxman and Chang, 1995). Therefore, there is a possibility that NP and other environmental chemicals that induce P450s in a gender-specific manner may cause unexpected drug–drug or drug–toxicant interactions primarily in one sex.

Gender differences in human pharmacokinetics are rare, but when present, may be clinically significant. Only a few drugs, such as metoprolol and other β -blockers, and the serotonin uptake inhibitor, fluvoxamine, are cleared slower in women (Meibohm et al., 2002). However, most drugs are cleared faster in human females than males. Most drugs, such as verapamil, cyclosporine, erythromycin, nifedipine, diazepam, prednisolone, methylprednisolone, ifosfamide, tirilazad, and alfentanil are cleared faster by females than males (reviewed in Meibohm et al., 2002; Wolbold et al., 2003). In a world in which we are exposed to an ever increasing number of pharmaceuticals and environmental chemicals such as nonylphenol, the potential for gender-predominant pharmaceutically induced side effects or drug–toxicant interactions may be increasing. Further studies may need to address whether workplace or environmental exposures may increase our susceptibility to adverse and unexpected drug–drug and drug–toxicant interactions.

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Fig 1.

FVB/NJ female and male mice treated with NP showed sexually dimorphic changes in P450 expression as determined by Q-PCR. Q-PCR was performed as described in the Materials and methods. Data are expressed as mean $\pm$ SD ($n = 4-5$) for each of the different P450s in males and females (a-i). Relative mean differential expression compared to the controls is reported above the columns in each graph. An asterisk indicates a significant difference from the untreated mice by ANOVA followed with Fisher's PLSD as the post hoc test ($P < 0.05$).

Fig 2.

Western blots of hepatic microsomes from NP treated FVB/NJ male and female mice. Western blots were performed and visualized as described in the Materials and methods. Blots were quantified densitometrically and the relative mean differential expression as compared to the controls is reported above the blots. An asterisk indicates a significant difference from the untreated mice by ANOVA followed with Fisher's PLSD as the post hoc test ($P < 0.05$).

Fig 3. Relative testosterone hydroxylase activities in male (A) and female (B) hepatic mouse microsomes following in vivo treatment with nonylphenol. Data are presented as mean $\pm$ SD. An asterisk indicates a significant difference from the untreated mice by ANOVA followed with Fisher's PLSD as the post hoc test ($P < 0.05$).

Fig 4.

Zox-induced paralysis in treated mice. NP significantly decreased paralysis time in male and female mice. The graph shows the paralysis time for each mouse. The individual mice in each treatment group are shown from left-to-right with increasing paralysis time, with d indicating the mouse remained paralyzed for more than 8 h (females) or 6 h (males) and was euthanized. The statistical analysis for Fig. 4 is shown in Table 4.

Table 1

P450 primers used for Q-PCR reactions

Gene	Forward primer	Reverse primer	T_m (°C) ^a	Source
Cyp2a4	AGCAGGCTACCTTCGACTGG	GCTGCTGAAGGCTATGCCAT	63.6	Wiwi et al. (2004)
Cyp2b9	CTGAGACCACAAGCGCCAC	CTTGACCATGAGCAGGACTCC	64.3	Wiwi et al. (2004)
Cyp2b10	CTGAATCCGCTCCTCCACACTC	TGAGCCAACCTTCAAGGAATAT	61.4	This manuscript
Cyp2b13	GAAGTGAAGTACCAGCACCCTCCT	TGAGCATGAGCAGGAAACCACT	63.8	Wiwi et al. (2004)
Cyp2c29	GGCCTCAAAGCCCTACTGTCA	AACGCCAAAACCTTTAATC	53.6	Luo et al. (1998)
Cyp2c40	CATTGAACACTGGCAACATTG	GTCACAGGTTACTTCATGCAC	57.6	Luo et al. (1998)
Cyp3a11	CTTTCCTTACCCTGCATTCC	CTCATCCTGCAGTTTTTCTGGAT	64.2	Wiwi et al. (2004)
Cyp3a25	CACATCATTTGGCGTGAACG	TTTCTGCACAAAGGGATCCTG	64.1	Wiwi et al. (2004)
Cyp3a41	GTGGAGAAAGCCAAAGGGATT	GAAGACCAAGGATCAAAAAAGTCA	63.1	Wiwi et al. (2004)
Cyp3a44	TTGTGGAGGAAGCCAAAAAGTTT	TTGTGGAGGAAGCCAAAAAGTTT	63.9	Wiwi et al. (2004)
18s rRNA	ATGGCCGTTCTTAGTTGGTG	ATGCCAGAGTCTCGTTCGTT	61.7	This manuscript

^a Annealing temperature.

Table 2

Uterosomatic index of mice treated with NP

Treatment	USI ^a OVEX mice	USI intact mice ^{a, b}
Control (0 mg/kg/day)	0.72 ± 0.18	2.11 ± 0.33
NP (50 mg/kg/day)	0.84 ± 0.41	1.79 ± 1.02
NP (75 mg/kg/day)	n.d.	2.58 ± 1.72
E ₂ (100 µg/kg/day)	2.84 ± 0.71*	3.39 ± 0.57

Ovariectomized and intact FVB/NJ female mice were treated with NP or estradiol (E₂) in their honey for 7 days and euthanized.

n.d.: Not done.

Data are presented as mean ± standard deviation ($n = 5-6$). An asterisk indicates a statistical difference from controls by ANOVA followed by Fisher's PLSD as the post hoc test ($P \leq 0.05$).

^aUSI = uterine weight (mg) divided by the mouse weight (g), multiplied by 1000.

^bMice in estrus, as determined by counting pyknotic nucleic, were eliminated from the results.

Table 3

Sexually dimorphic P450s as measured by Q-PCR

Gene	Male expression	Female expression
Cyp2a4	1.00 ± 0.12	26.5 ± 3.70 ^a
Cyp2b9	1.00 ± 0.33	336 ± 155 ^a
Cyp2b10	1.00 ± 0.81	31.6 ± 18.9
Cyp2b13	1.00 ± 0.13	56.9 ± 7.96 ^a
Cyp2c29	1.00 ± 0.09	8.34 ± 1.92 ^a
Cyp2c40	1.00 ± 0.10	33.1 ± 9.30 ^a
Cyp3a11	1.00 ± 0.71	1.74 ± 0.59
Cyp3a25	1.08 ± 0.16	1.00 ± 0.85
Cyp3a41	1.00 ± 0.59	3480 ± 626 ^a
Cyp3a44	n.d.	Infinity ^a

n.d. = not detected.

^a Male and female gene expression were compared by determining the relative expression of each P450 divided by 18S rRNA. Q-PCR for each P450 isoform was performed on male and female samples in triplicate during the same run and is expressed as relative expression ± SEM. An asterisk indicates a statistically significant difference in P450 expression between males and females as determined by Student's *t* test (*n* = 5).

Table 4

Statistical analysis ^a of zoxazolamine paralysis time in male and female mice following treatment with nonylphenol (NP), dexamethasone (DEX), or TCPOBOP (TC)

Treatment	Males		Females	
	Mean rank ^b	number ^c	Mean rank ^b	number ^c
Control	23.38	8	26.00	8
NP (50 mg/kg/day)	12.25*	8	15.50*	8
NP (75 mg/kg/day)	14.56	7	15.71*	8
DEX (75 mg/kg/day)	13.17	4	7.75*	3
TC (3 mg/kg/day)	8.00*	4	4.00*	3

An asterisk indicates statistical significance ($P < 0.05$).

^a Statistical analysis was performed using the Kruskal–Wallis non-parametric test for independent samples followed by the least significant difference (LSD) post hoc test using the ranks (SAS Institute Inc., Cary, NC).

^b Paralysis time was ranked from lowest to highest among the five treatment groups to obtain a Sum rank for each treatment. The sum rank is divided by the number of mice in each treatment to obtain the mean rank.

^c The number of mice per treatment group.

Table 5
Summary of the experimental results obtained using different techniques to determine P450 induction following NP treatment in male and female mice

Subfamily	Isoform	Male		Females			
		Q-PCR ^a	Westerns ^b	T-OH ^c	Q-PCR ^a	Westerns ^b	T-OH ^c
Cyp2a	Cyp2a4	↑	↑ (n.s)	6α ↑ (n.s)	NC	NC	NC 6α
	Cyp2b9	↑	↑	16α ↑	NC	↑	NC 16α
	Cyp2b10	↑		16β ↑	↑		16β ↑
Cyp2b	Cyp2b13	NC			↑		
	Cyp2c29	↑	NC	16α ↑	↑	NC	NC 16α
	Cyp2c40	↓			NC		
Cyp2c	Cyp2c40	↓			NC		
	Cyp3a11	NC	↓	NC 6β	NC	↓	NC 6β
	Cyp3a						
Cyp3a	Cyp3a25	↑			NC		
	Cyp3a41	NC			↓		
	Cyp3a44	ND			↓		

NC not changed.
↑↓ Up- or downregulated respectively.
ND, not detected.
n.s., Not statistically significant.
^aQuantitative PCR results for P450 isoforms.
^bWestern immunoblotting of P450 subfamilies.
^cTestosterone hydroxylation assay, P450 subfamily primary metabolite.