JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. 2005; **25**: 339–353
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jat.1078

The contribution of hepatic steroid metabolism to serum estradiol and estriol concentrations in nonylphenol treated MMTVneu mice and its potential effects on breast cancer incidence and latency

Ricardo Acevedo,[1] Pamela G. Parnell,[2] Hugo Villanueva,[1] Laura M. Chapman,[1] Tomas Gimenez,[3] Sandra L. Gray[3] and William S. Baldwin[1]*

[1] University of Texas at El Paso, Biological Sciences, El Paso, TX 79968, USA
[2] Clemson University, Veterinary Diagnostic Center, Columbia, SC 29229, USA
[3] Clemson University, Animal and Veterinary Sciences, Clemson, SC 29634, USA

Received 17 August 2004; Revised 6 February 2005; Accepted 1 March 2005

ABSTRACT: The two major pathways for the metabolism of estradiol-17β (E_2) are the 2- and 16-hydroxylase pathways. Research has suggested that the increased production of the estrogenically active 16-hydroxy products such as estriol (E_3) may be involved in increased susceptibility to breast cancer. 4-Nonylphenol (4-NP) is an environmental estrogen that also can activate the pregnane-X receptor (PXR) and induce P-450 enzymes responsible for the production of E_3. It is hypothesized that 4-NP may act in part as an environmental estrogen by increasing E_3 production. Based on its affinity for the estrogen receptor (ER) alone, 4-NP may be more potent than predicted at increasing mammary cancer incidence in the MMTVneu mouse. Female mice were treated *per os* for 7 days at 0, 25, 50 or 75 mg kg^{-1} day^{-1} 4-NP to investigate the effects of 4-NP on hepatic estrogen metabolism after an acute treatment. 4-Nonylphenol increased the hepatic formation of E_3 in a dose-dependent manner. However, serum E_3 concentrations were only increased at 25 mg kg^{-1} day^{-1} presumably due to direct inhibition of E_3 formation by 4-NP. MMTVneu mice were then treated for 32 weeks at 0, 30 or 45 mg kg^{-1} day^{-1} 4-NP to determine its effects on mammary cancer formation and estrogen metabolism. 4-Nonylphenol increased mammary cancer formation in the MMTVneu mice at 45 mg kg^{-1} day^{-1} but not at 30 mg kg^{-1} day^{-1}. Mice treated with an equipotent dose of E_2, 10 μg kg^{-1} day^{-1}, based on the relative binding affinities of nonylphenol and estradiol for ERα, did not develop mammary cancer. This suggests that nonylphenol is more potent than predicted based on its affinity for the estrogen receptor. However, no changes in serum E_3 concentrations or hepatic E_3 production were measured after the chronic treatment. Changes in E_3 formation were correlated with increased CYP2B levels after the 7 day 4-NP treatment, and repression of CYP2B and CYP3A after 32 weeks of 4-NP treatment. Microarray analysis and Q-PCR of liver mRNA from the mice treated for 32 weeks demonstrated a decrease in RXRα, the heterodimeric partner of the PXR, which may in part explain the repressed transcription of the P450s measured. In conclusion, 4-NP treatment for 32 weeks increased mammary cancer formation at a dose of 45 mg kg^{-1} day^{-1}. However, chronic treatment with 4-NP did not increase hepatic E_3 formation or serum E_3 concentrations. The transient induction by 4-NP of hepatic E_3 formation and serum concentrations is most likely not involved in the increased incidence of mammary cancer in MMTVneu mice since E_3 serum concentrations were only increased at 25 mg kg^{-1} day^{-1}, a dose that was not sufficient to induce mammary tumor formation. Nevertheless, the induced hepatic E_3 production in the acute exposures to 4-NP was indicative of an increase in mammary cancer incidence after the chronic exposure. Copyright © 2005 John Wiley & Sons, Ltd.

KEY WORDS: nonylphenol; estradiol; estriol; breast cancer; endocrine disrupter; P-450; CYP3A; CYP2B

Introduction

Breast cancer is the most commonly diagnosed cancer in women and the incidence has risen considerably in developed countries since the industrial revolution (National Center for Health Statistics, 1996). It is estimated that established risk factors such as aging, early menarche, late menopause, no or late parity in life, and genetics account for only 25%–50% of breast cancer cases (Madigan *et al*., 1995; Rockhill *et al*., 1998). Evidence in monozygotic twins suggests that only 27% (CI 4%–41%) of breast cancer has a genetic component and that most breast cancer (73%, CI 59%–96%) is caused by environmental exposures (Lichtenstein *et al*., 2000). These factors may include late onset of pregnancy, high fat diet, exposure to pharmaceuticals and additives, and exposure to environmental pollutants such as the environmental estrogens (Davidson, 1998; Rockhill *et al*., 1998; Bhatt, 2000; Harvey and Darbre, 2004).

* Correspondence to: Dr W. S. Baldwin, University of Texas at El Paso, Biological Sciences, 500 W. University Avenue, El Paso, TX 79968, USA.
E-mail: wbaldwin@utep.edu
Contract/grant sponsor: NIH; Contract/grant number: 2 S06 GM008012-33; ES11694-01; 2G12RR08124.

Nonylphenol is a common persistent, toxic degradation product of alkylphenol ethoxylates (APEs), nonionic surfactants used in a variety of products including institutional cleaning agents, textiles, agricultural chemicals, plastics, paper products, household cleaning agents, and personal care products. Over 1 billion pounds of nonylphenol ethoxylates are produced annually, and over 450 million pounds of APEs were sold in the United States in 1990 (Talmage, 1994). As a consequence of their use in a variety of products, they are quite common in rivers and other aquatic environments that receive sewage discharges (Bennie, 1999). 4-Nonylphenol has been detected in drinking water (Clark *et al*., 1992), and has been reported to leach from tubing for milk processing, and from plastics used in food packaging (Gilbert *et al*., 1992). 4-Nonyphenol increases MCF-7 breast cancer cell growth (Soto *et al*., 1991), and has an estimated threshold dose of 40 mg kg^{-1} day^{-1} *in vivo* for increased development and proliferation of mammary glands in 4–5 week old ovariectomized Noble rats treated orally for 3 or 11 days (Odum *et al*., 1999a, b).

4-Nonylphenol is an environmental estrogen (White *et al*., 1994) with a relative binding affinity (RBA) for ERα, the mammotropic estrogen receptor, of approximately 4500–5500 times less than estradiol (E_2) and 80–100 times less than the phytoestrogen genistein (Kuiper *et al*., 1998; Laws *et al*., 2000; Gutendorf and Westendorf, 2001; Legler *et al*., 2002). 4-Nonylphenol is also approximately 100 times less sensitive than genistein in the mouse uterotropic assay (Milligan *et al*., 1998). However, 4-NP enhances estrogen-dependent transcription of MCF-7 cells equally as well as genistein. While genistein induces transcription of α1-antichymotrypsin (α1-ACT) at lower levels than 4-NP, 4-NP induces transcription of pS2 and transforming growth factor β3 (TGFβ3) at similar levels and induces monoamine oxidase (MAO-A) at lower concentrations than genistein (Jorgensen *et al*., 2000). These observations are not consistent with the affinity data and suggest that 4-NP has other effects on estrogen-related processes in breast cancer cells not explainable by ER binding affinity alone.

Hydroxylation of E_2 occurs primarily at the 2 and 16 positions (Cheng *et al*., 2001; Spink *et al*., 2002). CYP3A family members are responsible for approximately 75% of 16-hydroxylation in humans (Huang *et al*., 1998), while CYP1A family members are responsible for much of the 2-hydroxylation (Dannan *et al*., 1986). 4-Nonylphenol has been shown to induce CYP3A1 levels in rats (Lee *et al*., 1996b; Masuyama *et al*., 2000) by binding to the pregnane X-receptor (Masuyama *et al*., 2000). 16-Hydroxyestradiol (estriol, E_3) and other 16-hydoxylated products of estrogen are estrogen receptor agonists, which is the probable cause of their carcinogenicity (Taioli *et al*., 1999), while the 2-hydroxylated products of E_2 have little biological activity and have been shown to be anti-estrogenic (Schneider *et al*., 1984). Furthermore, the 16α-hydroxylated estrogens such as E_3 induce MCF-7 and T47D cell proliferation, but 2-hydroxylated estrogens do not (Gupta *et al*., 1998).

Evidence suggests that an increase in 16-hydroxylation relative to 2-hydroxylation is a breast cancer risk (Bradlow *et al*., 1995; Telang *et al*., 1997), and pesticides that decrease the ratio of 2-hydroxylation to 16-hydroxylation (2/16E) increase the breast cancer risk (Bradlow *et al*., 1995). Similarly, indole-3-carbinol decreased the breast cancer risk, possibly by inducing 2-hydroxylation of E_2 (Baldwin and LeBlanc, 1992; Michnovicz *et al*., 1997), while reducing the concentrations of 16-hydroxyestrogens (Michnovicz *et al*., 1997b). 4-Nonylphenol at 80 mg kg^{-1} day^{-1} has been shown to down-regulate CYP1A2 mRNA and protein levels (Lee *et al*., 1996a) in addition to its ability to induce CYP3A.

Recent studies indicate that the ratio between 2 and 16-hydroxylation may not be as important as the amount of E_3 produced or E_2 available. When comparing postmenopausal women with breast cancer to healthy controls, it was found that the concentrations of estrone (E_1), E_2 and E_3 were better predictors than the 2/16E ratio (Ursin *et al*., 1997). Furthermore, the urinary concentrations of E_1, E_2 or E_3 reflect the differences in breast cancer risk between Singapore Chinese and United States women, but the 2/16E ratio does not (Ursin *et al*., 2001). Taken together, this work suggests that an increase in available estrogens can result in an increased breast cancer risk and that the increased production of E_3 is a concern.

Lastly, of all the estrogen metabolites tested, E_3 is the most potent (Lippert *et al*., 2003), and it is only about 10 times less potent at binding and activating ERα than E_2. Furthermore, it shows 400 times greater affinity for ERα and 6000 times greater activity in a luciferase reporter gene assay than 4-NP (Gutendorf and Westendorf, 2001), and therefore any increase in E_3 concentrations by 4-NP could have greater effects on mammary gland proliferation and the development of cancer than 4-NP alone.

The purpose of the present study was to (1) determine the effects of 4-NP exposure on hepatic estrogen metabolism and serum E_3 concentrations, (2) establish whether 4-NP could increase mammary cancer incidence in MMTVneu mice susceptible to mammary cancer formation (Guy *et al*., 1992), and (3) determine if there is an association between increased hepatic E_3 production, serum E_3 concentrations and breast cancer incidence in 4-NP treated mice.

Materials and Methods

Mice

All studies were carried out according to NIH guidelines for humane use of research animals according to institutional guidelines. FVB/NJ mice (4–6 weeks of age)

were received from The Jackson Laboratory (Bar Harbor, ME) and allowed to acclimate for 1 week prior to treatment. Mice were fed Harlan Teklad rodent diet and provided water *ad libitum*. Technical grade 4-NP was obtained from Fluka Chemical Co. (Ronkonkoma, NY). This grade of 4-NP is a mixture of branched side chains containing 85% p-isomers.

In the acute study, mice were provided 4-NP orally at 0, 25, 50 or 75 mg kg^{-1} day^{-1} for 7 days in 100 µl honey as a vehicle. Mice were anesthetized with ketamine, blood was collected by heart puncture, and then mice were euthanized with carbon dioxide. The individual livers were sliced into several pieces for cytosol or microsome preparation and stored at −80 °C. Approximately half of each liver was differentially centrifuged to separate cytosol and microsomes, and protein concentrations were determined using a commercially available colorimetric assay (Bio-Rad, Hercules, CA).

In the chronic study, transgenic mice (FVB/N-TgN(MMTVneu)202Mul; also termed MMTVneu mice) that overexpress the neu (Erbb2) proto-oncogene under the control of the MMTV promoter on the FVB/NJ background were obtained from The Jackson Laboratory between the age of 4–6 weeks and acclimated for 1 week prior to 4-NP treatment. The MMTVneu mouse model is an ideal model for examining increased mammary carcinogenesis, because it has increased cancer susceptibility and does not require pregnancy for tumor formation.

Mice were treated with 0, 30 or 45 mg kg^{-1} day^{-1} of 4-NP, or 10 µg kg^{-1} day^{-1} of E_2 (Sigma Chemical Co, St Louis, MO) in 150 µl honey for 32 weeks. The dose of E_2 chosen (10 µg kg^{-1} day^{-1}) was based on the relative binding affinity of 4-NP and E_2 for the ERα, the mammotropic estrogen receptor. E_2 has a relative binding affinity approximately 4500 times greater than 4-NP (Kuiper *et al*., 1998; Laws *et al*., 2000; Gutendorf and Westendorf, 2001; Legler *et al*., 2002) and therefore 45 mg kg^{-1} day^{-1} of 4-NP and 10 µg kg^{-1} day^{-1} of E_2 have equipotent estrogenicity based on their relative binding affinities. Based on the acute study that showed no overt toxicity, and other studies showing the potential estrogenic effects of 4-NP on rats at a threshold dose of 40 mg kg^{-1} day^{-1} (Odum *et al*., 1999a, b), MMTVneu mice on the FVB/NJ background were treated for 32 weeks with 0, 30 or 45 mg kg^{-1} day^{-1} 4-NP. The 30 mg kg^{-1} day^{-1} dose of 4-NP represented a dose below the previously observed estrogenic threshold, while the 45 mg kg^{-1} day^{-1} dose of 4-NP represented a dose slightly greater than the previously observed threshold dose for the estrogenic effects of 4-NP.

Mice were weighed, anesthetized with ketamine, and blood was collected for serum preparation by heart puncture after the 32 weeks of treatment. Mice were then euthanized with carbon dioxide, and livers were excised, sliced into several pieces for cytosol or microsome preparation, and stored at −80 °C. Lungs and mammary tissue were excised and placed in 10% formalin (Fisher Scientific, Fair Lawn, NJ) for histological examination.

Serum Steroid Hormone Concentrations

Blood was drawn monthly from MMTVneu mice and at the end of the 7 day and 32 week treatments described above. Concentrations of E_2 in mouse serum were determined by radioimmunoassay (RIA) using the DSL 3rd generation estradiol RIA (Diagnostic System Laboratory; Webster, TX) with a theoretical level of sensitivity of 0.6 pg ml^{-1}. Concentrations of E_3 and progesterone were determined in serum collected at the end of each study using DSL (Diagnostic Systems Laboratory) Double Antibody Ultra-senstive Unconjugated E_3 RIA with a sensitivity of 0.03 ng ml^{-1} and DSL Progeswteron RIA with a 0.12 ng ml^{-1} sensitivity. RIA methods were validated for use with murine serum by standard curve parallelism studies, spike tests using rat serum and demonstration of linearity under sample dilution. Sample concentrations are means of duplicate determinations.

Vaginal swabs were performed at the time of blood collection and prior to euthanasia, and stained as described previously (Champlin *et al*., 1973) using the Richard-Allan Signature Series™ stains (Kalamazoo, MI) and staining protocol. The maturation index was used to determine mice in estrus by identifying increased cornified epithelium in the vagina and determining the percentage of superficial, intermediate and parabasal cells (Champlin *et al*., 1973; Laws *et al*., 2000). Mice in estrus were not used in hormone determinations or enzyme assays, although most research indicates that steroid metabolism is not affected by estrus cycle (Kashuba *et al*., 1998).

Histological Examination of Lung and Mammary Tissue

Representative sections of formalin-fixed lung and mammary masses were processed by routine methods, embedded in paraffin, sectioned at 4 µm, stained with hematoxylin and eosin, and coverslipped for histological examination by light microscopy. Mammary masses were evaluated for the presence of morphological alterations in tissue structure indicative of a disturbance of growth, and interpreted according to the classification system described previously (Cardiff *et al*., 2000).

Enzyme Assays

Steroid hydroxylation assays were performed as described previously (Quail and Jellinck, 1987; Baldwin and

LeBlanc, 1992) with some modifications. Briefly, 100 μM [^{14}C]estradiol or [^{14}C]testosterone (150 000 dpm) (Perkin-Elmer, Boston, MA.), was incubated at 37 °C for 8 min in 0.1 M potassium phosphate (KPi) buffer, 2 μM ascorbic acid (E_2 metabolism assay only), 200 μg microsomal protein and started with NADPH. The reactions were terminated with 2 ml of ethyl acetate and an additional 2 ml was used to extract the hydroxylated metabolites. The metabolites of E_2 were separated by TLC using 50 ml cyclohexane, 45 ml ethyl acetate and 5 ml ethanol (Sigma Chemical Co., St Louis, MO) (Quail and Jellinck, 1987). The testosterone metabolites were separated by two separate solvent systems. The first solvent system was composed of 80 ml of methylene chloride and 20 ml of acetone, and the second solvent system contained 80 ml chloroform, 20 ml ethyl acetate and 14 ml ethanol (Baldwin and LeBlanc, 1992). All solvents were obtained from Fisher Scientific unless otherwise specified. Metabolite identification of the hydroxylated steroids was determined based on prior research (Quail and Jellinck, 1987; Baldwin and LeBlanc, 1992), and 2-hydroxyestradiol (2-OHE_2) and E_3 standards (Steraloids, Newport, RI) were used to confirm identity by co-migration of the standards with the [^{14}C]estradiol metabolites. E_3 has a R_f value of 0.12 and 2-OHE_2 has a R_f value of 0.24 in this solvent system. The E_3 spot was about 72%–83% E_3 in untreated and 4-NP treated microsomes, respectively, based on co-migration with E_3 in a 2-D TLC plate separated in one-dimension as described above and the second dimension using the testosterone hydroxylase dual solvent system. All steroid hydroxylase assays were performed twice.

Assays to determine the efficacy of 4-NP in inhibiting steroid hydroxylation were performed as described above except 20 μM [^{14}C]testosterone or [^{14}C]estradiol was used to determine the steroid metabolizing enzymes inhibited by 4-NP. Steroid metabolism assays were performed in the presence of 4-NP at concentrations ranging from 2× to 80× (40 μM to 4 mM 4-NP) the E_2 concentration provided in the assay.

Western Blots

Thirty micrograms of microsomal protein was separated by polyacrylamide gel electrophoresis (SDS-PAGE) in a 10% gel (Laemmli, 1970), and then transferred to nitrocellulose for immunoblotting with commercially available rabbit anti-rat CYP3A1 antibodies (Chemicon International, Temicola, CA), or mouse anti-rat CYP2B10 antibody (a gift from Dr Randy Rose, North Carolina State University, Raleigh, NC). Since the anti-rat CYP3A1 antibody may recognize multiple isoforms in the mice, a specific CYP3A family member is not referred to in this manuscript. Alkaline phosphatase coupled secondary antibodies were used to visualize the western blots 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).

Probe Synthesis and Array Hybridization of Liver RNA from 4-NP and Untreated Mice

RNA was extracted using TRI-Reagent (Sigma Chemical Co.) according to the manufacturer's directions. RNA was quantified at 260 nm on a spectrophotometer and RNA integrity was determined by gel electrophoresis. Fifty micrograms of total RNA from each mouse liver sample was enriched for poly A+ RNA using the Atlas™ Pure Total RNA Labeling system (Clontech, Palo Alto, CA), and reverse transcribed in the presence of [^{33}P]dATP, dCTP, dGTP, dTTP and AMV reverse transcriptase to produce cDNA probes. The probes were purified using the ProbeQuant™ G-50 Micro Columns (Amersham Pharmacia Biotech Inc., Piscataway, NJ), and then denatured in the presence of C_ot-1 DNA. The Clontech Atlas™ Mouse 1.2 Arrays (7853-1) (Palo Alto, CA) were prehybridized in 5 ml of ExpressHyb (Clontech) and 0.5 mg of heat denatured sheared salmon sperm for 30 min at 68 °C prior to probe incubation. The probes were added and incubated overnight at 68 °C before washing four times for 30 min each time with 2× SSC, 1% SDS, then once with 0.1× SSC, 0.5% SDS for 30 min, and once with 2× SSC for 5 min at 68 °C. Blots were visualized by placing them on a K-screen for 3–4 days (Bio-Rad).

Arrays were visualized from the K-screen with a phosphoimager (Molecular Imager FX, Bio-Rad), and analysed and quantified using the ResGen Pathways™ Universal Microarray Analysis Software™ (Research Genetics, Carlsbad, CA). Local background was subtracted automatically from the individual spot intensities, and spot intensities were normalized to the mean intensity of all spots on the array. This is called datapoint normalization on the ResGen Pathways Microarray Analysis Software™. Normalized gene intensities on the arrays probed with 4-NP-treated mouse liver and untreated mouse liver cDNA were compared, and fold differences were calculated. Arrays were also visually examined, especially for spots showing statistical significance, to ensure this significance was not attributed to array blemishes or non-specific marks. Differential expression was statistically determined by the Chen test (Chen *et al*., 1997); however, genes were only considered differentially expressed if the changes in expression were greater than 1.50×, similar to previous studies (Eckmann *et al*., 2000; Ranganna *et al*., 2003). The Chen test was selected because type I error was reduced (Chen *et al*., 1997), and it was more stringent than the *t*-test when the statistical

tests were directly compared between treatment groups. Information on the Clontech Atlas™ Mouse 1.2 Array and the genes it contains, as well as bioinformatics on the individual genes is available at the Atlas Bioinformatics website Atlas Info™ at http://atlasinfo.clontech.com/atlasinfo/array-list-action.do. Clontech's bioinformatics tools were used to determine the ontogeny of the differentially expressed genes (Ranganna *et al*., 2003). Data for each gene on the array can be found on GEO (Accession # **GSE1083**).

Quantitative Real-time PCR (Q-PCR)

Primers were designed to several orphan nuclear receptors and nuclear receptor cofactors involved in the expression of cytochrome P450s. 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 30–45 s. The oligonucleotide primer sequences for each gene are below with their corresponding annealing temperatures in parentheses: GAPDH forward 5′-CATGACCACAGTCCATGC-3′, GAPDH reverse 5′-GTCATACCAGGAAATGAGC-3′ (55 °C); CAR forward 5′-CCAAGGATCTGCCGCTCTT-3′, CAR reverse 5′-TGGTTGTTCAGAATCAGCGC-3′ (51 °C); ERα forward 5′-CTGCCAAGGAGACTCGCTAC-3′, ERα reverse 5′-CCAACAAGGCACTGACCATC-3′ (62 °C); RXRα forward 5′-ATGGGGTATACAGTTGTGAGGG-3′, RXRα reverse 5′-TTTGAGAGCCCCTTAGAGTCAG-3′ (50 °C); PXR forward 5′-GCTGATGGACGCTCAGATG-3′, PXR reverse 5′-GGTCCTCGATGGGCAAG-3′ (62.5 °C).

Q-PCR was performed using a Cepheid Smart Cycler® System with Smart Cycle Software 1.2b (Applied Biosystems, Foster City, CA). 0.25X SybrGreen was used as a fluorescent double strand-intercalating agent to quantify gene expression. The Q-PCR results are the average of two separate PCR runs, divided by the average of the housekeeping gene, GAPDH. Relative quantities of each transcript were determined as described previously (Muller *et al*., 2002; Roling *et al*., 2004).

Statistics

All data are expressed as mean ± SEM. A value of $P < 0.05$ was regarded as statistically significant as determined by ANOVA followed by Scheffe's multiple comparison test as the post-hoc test when comparing multiple treatments. Student's *t*-tests were performed when comparing two treatments. Fisher's exact test compares two independent binomial proportions and was used to determine the probability that 4-NP increased tumor incidence. StatView software® (SAS Institute, Inc. Cary, NC) was used to perform all statistical tests.

Results

Steroid Metabolism following Acute 4-NP Exposure

Mice were treated for 7 days with 4-NP at 0, 25, 50 or 75 mg kg^{-1} day^{-1} to (1) determine the acute effects of 4-NP on E_2 metabolism and steroid hormone concentrations, and (2) ensure that 4-NP is not toxic to the FVB/NJ mouse strain. These concentrations of 4-NP did not cause noticeable toxicity nor were significant histological hepatic lesions observed in the sections of liver examined. 4-Nonylphenol did increase the hepatosomatic index of mice (17%–20%) at 25 and 75 mg kg^{-1} day^{-1} 4-NP (Table 1).

The estradiol metabolites 2-hydroxyestradiol (2-OHE_2) and E_3 were quantified after incubation of hepatic microsomes with [^{14}C]estradiol. 50 and 75 mg kg^{-1} day^{-1} 4-Nonylphenol increased E_3 production 3.2- and 4.3-fold, respectively (Fig. 1a). Therefore, the 2/16E ratio decreased 3.3- and 4.3-fold at 50 and 75 mg kg^{-1} day^{-1}, respectively, solely because of the increase in E_3 production. The formation of E_3 and the decrease in 2/16E ratio is consistent with our hypothesis that 4-NP can increase the concentration of available estrogens.

Testosterone hydroxylase assays were performed to determine the P450s induced by 4-NP since the P450s responsible for the regiospecific and stereospecifc hydroxylation of testosterone have been relatively well characterized (Waxman, 1988). Quantification of the testosterone metabolites revealed that the formation of 16β-hydroxytestosterone, an indicator of CYP2B induction (Ohmori *et al*., 1993), was increased, but

Table 1. Hepatosomatic indices of mice treated with 4-NP. Mice were treated with 4-NP in their honey for 7 days (A) or 32 weeks (B), and then euthanized. Mouse livers were excised, weighed and compared with the weight of the mouse to determine the hepatosomatic index (HIS). Data are presented as mean ± standard deviation ($n = 6$ for the 7 day study and 13–14 for the 32 week study). *Statistical difference by ANOVA followed by Scheffe's multiple comparison post-hoc test ($P < 0.05$)

A

Treatment	7-Day 4-NP HIS
0 mg kg^{-1} day^{-1} 4-NP	0.041 ± 0.004
25 mg kg^{-1} day^{-1} 4-NP	0.048 ± 0.003*
50 mg kg^{-1} day^{-1} 4-NP	0.046 ± 0.003
75 mg kg^{-1} day^{-1} 4-NP	0.049 ± 0.004*

B

0 mg kg^{-1} day^{-1} 4-NP	0.054 ± 0.005
30 mg kg^{-1} day^{-1} 4-NP	0.056 ± 0.005
45 mg kg^{-1} day^{-1} 4-NP	0.057 ± 0.008
10 mg kg^{-1} day^{-1} E_2	0.056 ± 0.005

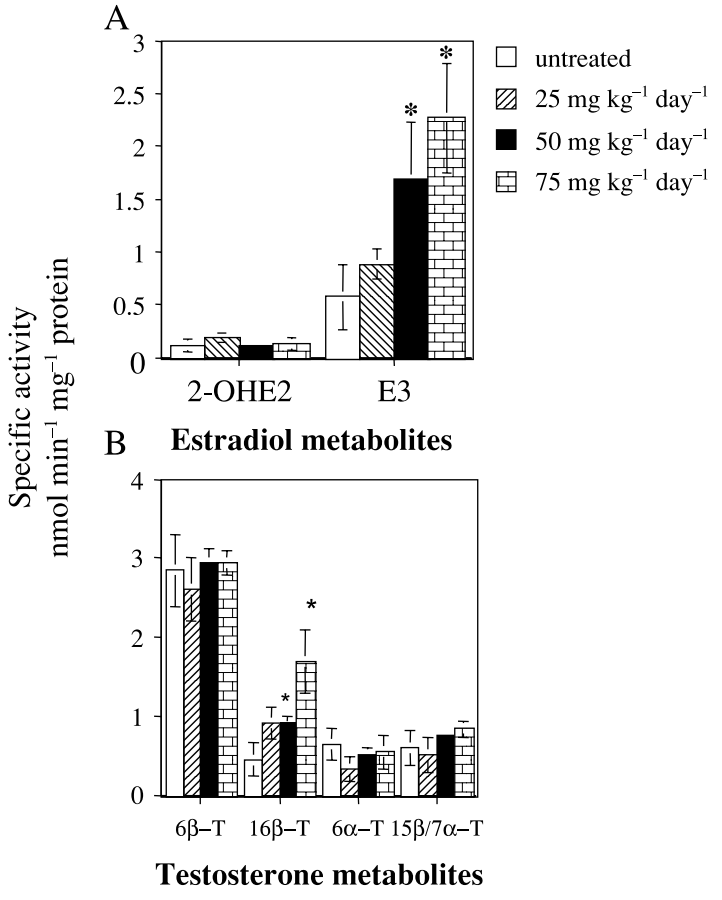

Figure 1. Steroid hydroxylation in mice treated with 4-NP for 7 days. Hydroxylation of estradiol (A) or testosterone (B) was measured in hepatic microsomal fractions from mice treated with 4-NP. Data are expressed as mean ± SEM (n = 5–6). *Statistical difference using ANOVA followed by Scheffe's multiple comparison test as the post-hoc test ($P < 0.05$)

the formation of 6β-hydroxytestosterone, an indicator of CYP3A induction, was not altered (Namkung *et al*., 1988) (Fig. 1b).

Serum Steroid Concentrations after 4-NP Treatment for 7 days

Serum E_3 concentrations were measured to determine if they were associated with hepatic E_3 formation (Fig. 1). 25 mg kg^{-1} day^{-1} 4-NP increased serum E_3 concentrations after the 7 day treatment (Fig. 2a), which is consistent with the increased E_3 formation measured during the estradiol hydroxylase assays. However, serum E_3 concentrations were not increased at 50 or 75 mg kg^{-1} day^{-1} 4-NP in the 7 day study, which is not consistent with our hypothesis.

Estradiol and progesterone concentrations were also measured because E_2 is the predominant estrogen and increased E_2 concentrations have been correlated with increased incidence of breast cancer (Ursin *et al*., 1997). Progesterone has been shown to activate the MMTV promoter (Bradham and Bolander, 1989) and therefore increased concentrations of progesterone may increase the expression of neu and in turn induce cancer formation by an unintended mechanism. 4-Nonylphenol treatment for 7 days did not alter either serum estradiol or progesterone concentrations (Fig. 2b,c).

Inhibition of Steroid Metabolism by 4-Nonylphenol

Since serum E_3 concentrations were not increased at 50 or 75 mg kg^{-1} day^{-1} 4-NP, it was hypothesized that 4-NP may also inhibit hepatic E_2 metabolism. E_2 metabolism inhibition assays were performed with increasing concentrations of 4-NP. The formation of E_3 was preferentially inhibited by 4-NP when compared with inhibition of 2-OHE_2 (Fig. 3a) and the other unidentified E_2 metabolites (data not shown).

In addition, testosterone hydroxylase assays were performed in the presence of 4-NP to gain a better understanding of the P-450s inhibited. 4-NP preferentially

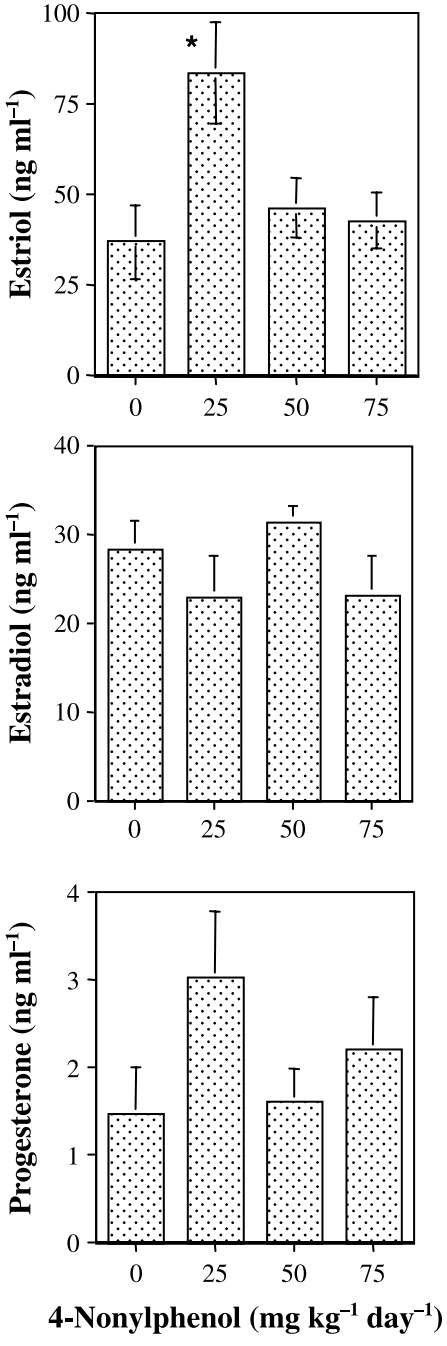

Figure 2. Serum steroid hormone concentrations in mice treated with 4-NP for 7 days. Serum was prepared and estriol (A), estradiol (B) and progesterone (C) concentrations were determined by RIA in mice that were not in estrus. Data are expressed as mean ± SEM (n = 4–6). *Significant difference using ANOVA followed by Scheffe's multiple comparison test as the post-hoc test ($P < 0.05$)

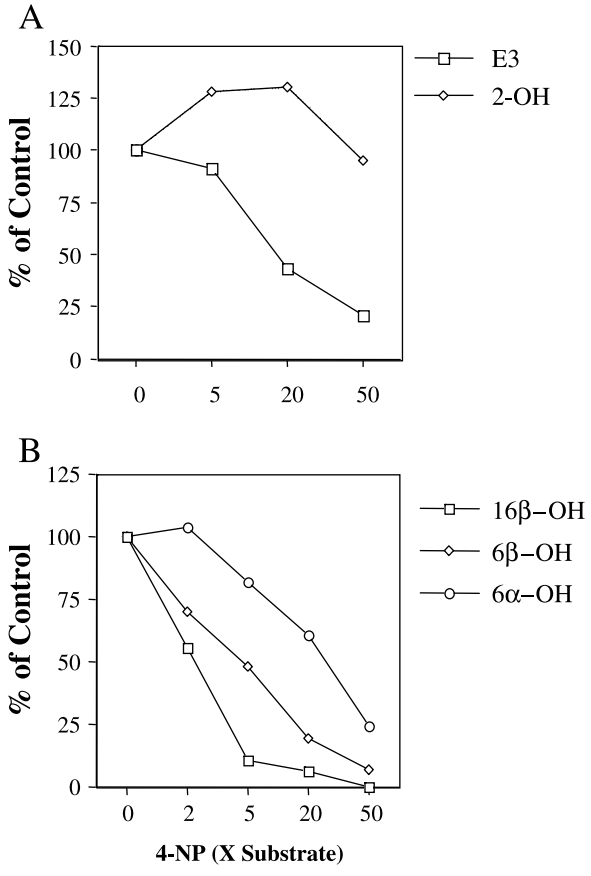

Figure 3. Inhibition of estradiol and testosterone hydroxylation by 4-NP *in vitro*. Estradiol (A) and testosterone (B) hydroxylation by hepatic microsomes from control mice were measured in the presence of 4-NP at 2–50 times the concentration of the [^{14}C] steroids. Data are expressed as relative inhibition of two replicates

inhibited testosterone 6β-, and 16β-hydroxylase activities, compared with 6α-hydroxylase activity (Fig. 3b) and the other NADPH-dependent metabolites (data not shown). This indicates that 4-NP preferentially inhibits CYP2B and CYP3A family members, which is consistent with the previously published literature (Lee *et al*., 1996b, 1998), and with the inhibition of enzymes important in the metabolism of E_2 to E_3 (Huang *et al*., 1998; Horn *et al*., 2002).

Gross Pathology and Tumor Incidence in Chronically 4-NP treated FVB-MMTVneu Mice

MMTVneu mice were treated with 4-NP and E_2, and hepatic E_3 production as well as serum E_2 and E_3 concentrations were compared with tumor incidence. One mouse in the 45 mg kg^{-1} day^{-1} 4-NP group died during the treatment phase of the study. The cause of death was not investigated. Furthermore, 4-NP had no effect on the hepatosomatic index in the 32 week study (Table 1b), nor were physical signs of distress or significant histological lesions observed in the sections of liver examined from mice treated for 32 weeks.

Mammary tumor incidence and latency was monitored during the 32 weeks of 4-nonylphenol and E_2 treatment, and verified by histological examination of lesions identified visually and by palpation upon dissection of the mammary and subcutaneous tissues. Morphological changes in the mammary tissue varied from slight proliferations of epithelium confined to the lumen of ducts, to large focal nodular expansile proliferations of mammary epithelium. In intraductular proliferations, epithelium was 2–4 cell layers thick with very slight nuclear atypia. Rare mitotic figures were noted (mammary intraepithelial neoplasia – MIN). In nodular lesions, epithelial cells formed solid sheets with minimal stroma, but with moderate capillary vascularization. At the interface with capillary stroma, the epithelial cells often were slightly more cuboidal in differentiation. In both types of lesions, the epithelial cells were relatively uniform cells with medium-sized round to occasionally oval nuclei containing stippled chromatin and indistinct nucleoli. Nuclear atypia was mild. Mitotic figures were noted at a frequency of 1–2 per 400× field. Cytoplasm was scant to moderate, faintly eosinophilic and nongranular. Cell margins were relatively indistinct. Intravascular, expansile proliferations of epithelium forming solid sheets were observed in lung tissue multifocally from some mice. Cellular morphology of these epithelial cells was identical to that described for the mammary neoplasms. Based upon morphological characteristics and proven malignant behavior, the nodular masses were classified as solid carcinomas and representative images are shown in Fig. 4.

Five mice in the 45 mg kg^{-1} day^{-1} 4-NP group formed tumors, while no mice in the 30 mg kg^{-1} day^{-1} 4-NP group, one mouse in the control group and no mice treated with E_2 formed mammary tumors (Table 2). One

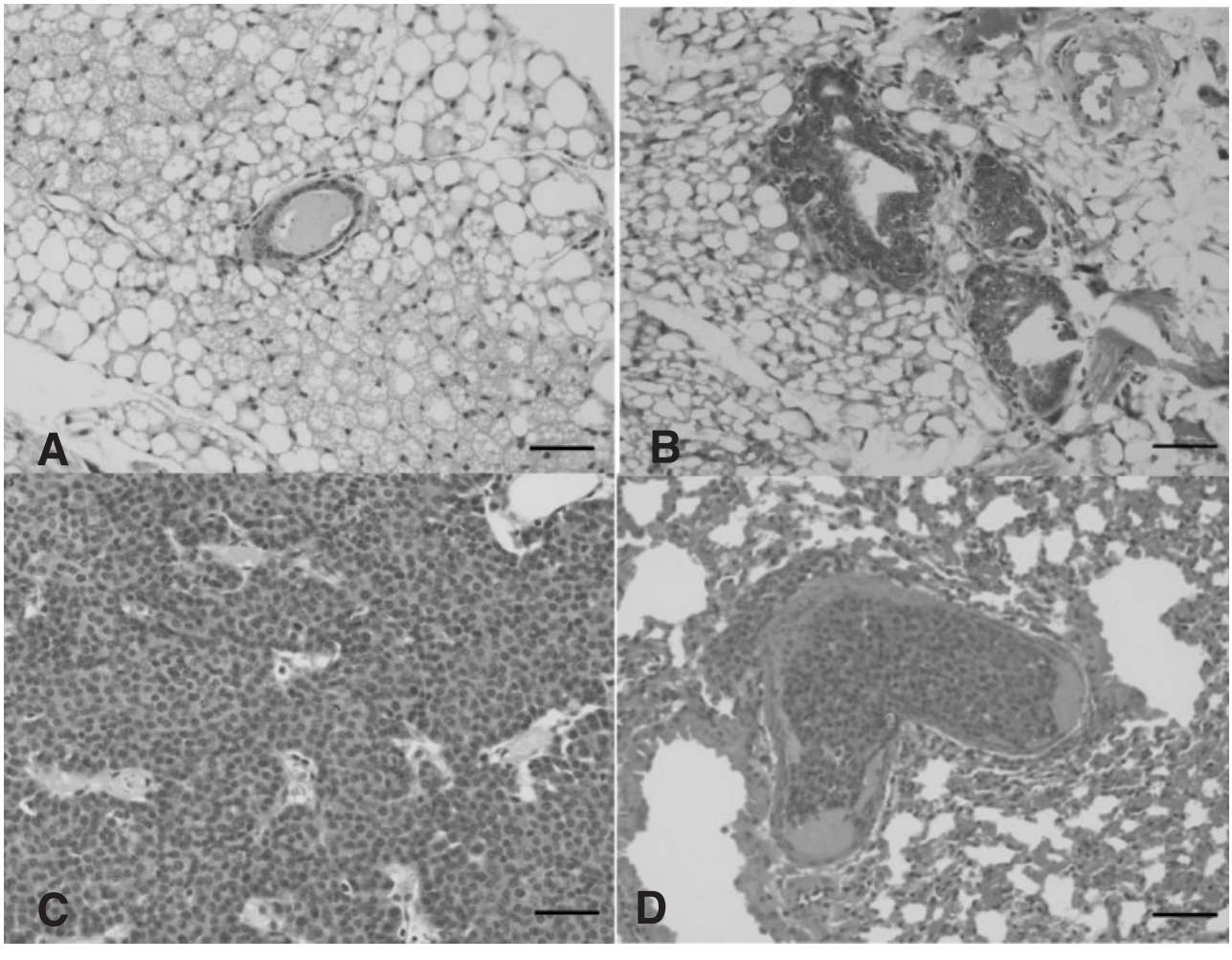

Figure 4. Images of H&E stained mammary glands and mammary tumors. (A) Mammary gland: Normal mammary gland in adipose tissue-simple cuboidal epithelium. (B) Mammary gland: Mammary intraepithelial neoplasia (MIN). The epithelium is dysplastic, with stratification of the epithelium and mild to moderate anisokaryosis. (C) Mammary gland: Solid carcinoma. Sheets of neoplastic epithelial cells, occasionally with slight cuboidal differentiation at the interface with vascular structures. (D) Lung: Metastatic solid carcinoma within a pulmonary vessel. Bar = 400 μm

Table 2. Tumor formation in 4-NP treated MMTVneu mice. MMTVneu transgenic mice were treated daily with 0, 30 and 45 mg kg^{-1} day^{-1} 4-NP, or 10 μg kg^{-1} day^{-1} E_2, and tumor incidence was monitored over the next 32 weeks by palpation and verified histologically (n = 13–14). *Statistical difference from controls by Fisher's exact test ($P < 0.02$)

Treatment	Tumors/Mice[a]	MIN/Mice[b]	Metastasis[c]
0 mg kg^{-1} day^{-1} 4-NP	1/14	1/14	0
30 mg kg^{-1} day^{-1} 4-NP	0/14	1/14	0
45 mg kg^{-1} day^{-1} 4-NP	5/13*	6/13*	2
10 μg kg^{-1} day^{-1} E_2	0/5	0/5	0

[a] Number of mice with tumors/total number of mice.
[b] Number of mice with tumors or severe hyperplasia with possible tumor/total number of mice.
[c] Number of mice that demonstrated metastasis to the lung.

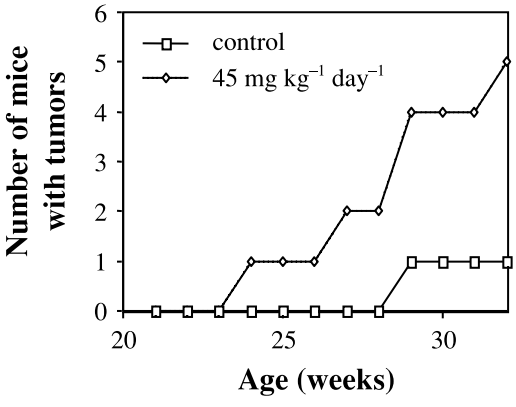

Figure 5. Tumor incidence and latency in 4-nonylphenol treated MMTV mice. MMTVneu transgenic mice were treated daily with 0, 30 or 45 mg kg^{-1} day^{-1} 4-nonylphenol, or 10 μg kg^{-1} day^{-1} estradiol, and tumor incidence was monitored over the next 32 weeks (n = 13–14)

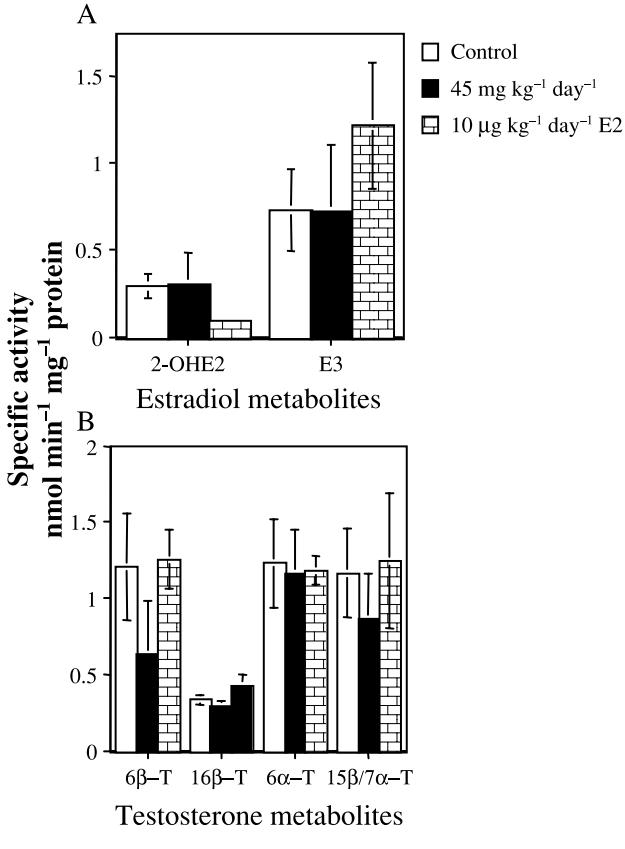

Figure 6. Steroid hydroxylation in mice treated with 4-NP for 32 weeks. Hydroxylation of estradiol (A) or testosterone (B) was measured in hepatic microsomal fractions from mice treated with 4-NP. Data are expressed as mean ± SEM (n = 5–6). *Statistical difference using ANOVA followed by Scheffe's multiple comparison test as the post-hoc test ($P < 0.05$)

additional mouse in the 30 and the 45 mg kg^{-1} day^{-1} 4-NP treatment groups demonstrated MIN. 4-Nonylphenol treated mice also showed a reduced latency period before developing palpable mammary tumors compared with the untreated mice (Fig. 5). Lastly, two mice in the 45 mg kg^{-1} day^{-1} 4-NP group had metastatic lesions in the lungs consistent morphologically with solid carcinoma of mammary origin, while no other treatment group had mice identified to have metastatic pulmonary lesions (Table 2).

Steroid Metabolism Following Chronic 4-NP Exposure

In contrast to the 7 day study, there were no changes in the hydroxylation of E_2 following treatment with 4-NP or E_2 for 32 weeks (Fig. 6a). Similarly, the hepatic microsomal hydroxytestosterone profile was unchanged in mice treated for 32 weeks compared with control mice. However, there was a decrease, albeit not statistically significant, in 6β-hydroxylation of testosterone after the 32 week treatment with 45 mg kg^{-1} day^{-1} 4-NP (Fig. 6b).

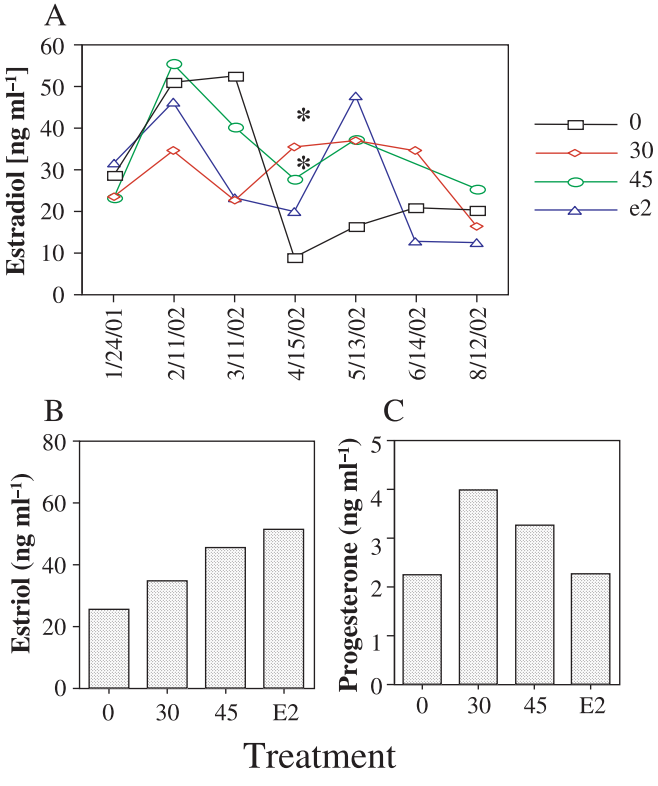

Figure 7. Serum steroid hormone concentrations in mice treated with 4-NP for 32 weeks. Serum was prepared and estradiol (A), estriol (B) and progesterone (C) concentrations were determined by RIA in mice that were not in estrus. Data are expressed as mean ± SEM (n = 10–12). *Statistical difference using ANOVA followed by Scheffe's multiple comparison test as the post-hoc test ($P < 0.05$)

Serum Steroid Concentrations after 4-NP Treatment for 32 Weeks

Blood was drawn monthly from MMTVneu mice during and after the 32 week treatments described above. E_2 concentrations were determined monthly, and E_3 and progesterone concentrations were determined after the termination of the study. 4-Nonylphenol or E_2-treatment did not alter E_2 serum concentrations during the 32 week study, with the exception of the month of April when estradiol levels were induced (Fig. 7a). The biological significance of these data is unknown; however, E_2 concentrations were relatively stable for most of the study and therefore April's data appear to be an anomaly suggesting that 4-NP does not have an effect on serum estradiol concentrations. Serum E_3 concentrations were not increased after 4-NP and E_2 treatments in the 32 week study, however, there was a dose-dependent increase in serum E_3 concentrations (Fig. 7b). Progesterone concentrations also remained unchanged following the 32 week study (Fig. 7c).

Western Blots

Changes in steroid metabolism after the 7 day exposure indicated that CYP2B family members were induced. An immunoblot demonstrated that the 7 day treatment with 50 or 75 mg kg^{-1} day^{-1} 4-NP induced CYP2B10 by 7.7× and 9.2×, respectively (Fig. 8a), but induction of CYP2B10 did not occur following the 32 week treatment at 45 mg kg^{-1} day^{-1} 4-NP (Fig. 8b).

Furthermore, 6β-hydroxylation of testosterone was reduced after 4-NP treatment for 32 weeks, suggesting that CYP3A levels were either reduced by 4-NP treatment or 6β-hydroxylation was inhibited. An immunoblot demonstrated that CYP3A, which was not affected by the 7 day treatment, was down-regulated nearly 50% following the 32 week study (Fig. 8d). The immunoblot confirms that the reduction in 6β-hydroxylation of testosterone is consistent with reduced CYP3A protein and not enzyme inhibition.

Arrays and Q-PCR

Microarrays were performed with mouse liver from the 32 week study because 4-NP had different effects on mice treated for 7 days than mice treated for 32 weeks. The Clontech™ arrays indicated that 13 genes out of the 1176 on the array were altered statistically (Table 3). Transcriptional activators or repressors accounted for 4 of the 13 genes altered (DBP, RXRα, SHFDG1, NDKB),

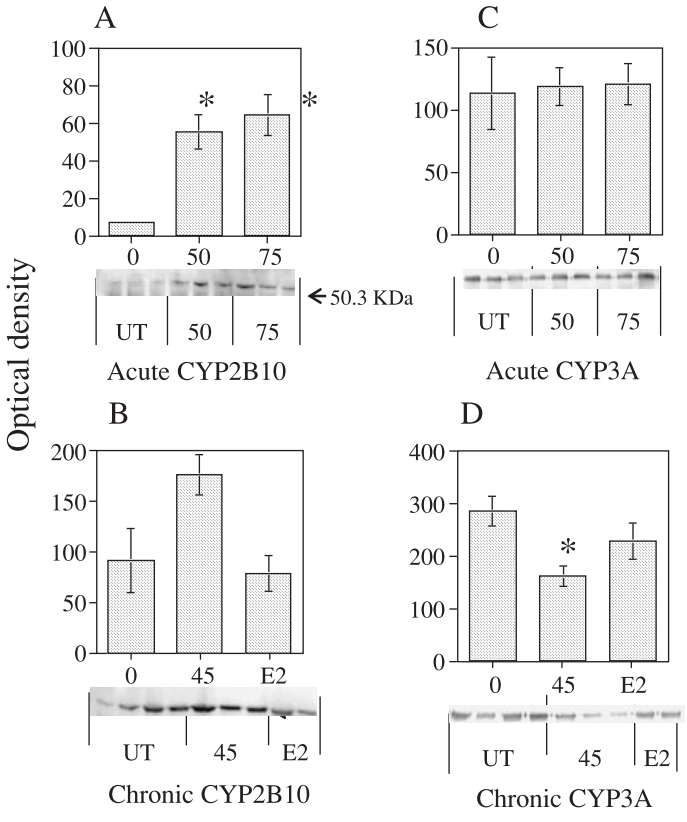

Figure 8. Immunoblotting for CYP3A and CYP2B10 proteins in 4-NP treated mice. Western blots were performed to quantify the relative levels of CYP2B10 (A,B) or CYP3A proteins (C,D) from untreated (UT) and treated mice. Data are expressed as mean ± SEM (n = 2–3). *Statistical difference using ANOVA followed by Scheffe's multiple comparison test as the post-hoc test ($P < 0.05$)

Table 3. Alteration in gene expression in response to exposure to 4-NP for 32 weeks. Radiolabeled cDNA probes were produced from liver mRNA from control and treated MMTVneu mice and hybridized to the Clontech 1.2 I Mouse array. Statistics were performed using the Chen test and significant data ($P < 0.05$, expression changes greater than 1.5) are presented (n = 2)

Gene[a]	Ratio	*P* value	Accession #
D-binding protein (DBP)	−2.15	<0.01	U29762
G1/S specific cyclin D1 (CCND1)	−1.97	<0.01	S78355
Phenylalanine 4-hydroxylase (PAH)	−1.69	<0.01	X51942
Retinoid X-receptor alpha (RXRα)	**−1.90**	**<0.01**	**M84817**
Cathepsin B (CTSB)	−1.75	<0.01	M14222
Serine protease inhibitor 2.4 (SPI 2.4)	−1.96	<0.01	X69832
Ribosomal protein S29 (RSP29)	+2.66	<0.01	L31609
Src proto-oncogene (Src)	+1.78	<0.01	M17031
Split hand foot deleted gene (SHFDG1)	+1.77	<0.05	U41626
Transcription termination factor 1 (TTF1)	+1.86	<0.05	X83974
Nucleotide diphosphate kinase B (NDKB)	+1.82	<0.05	X68193
Defender against cell death (DAD1)	+1.87	<0.05	U83628
Non-myelin light chain 3 (MLC3NM)	+1.71	<0.05	U04443

[a] Gene name is followed by its Hugo nomenclature in parentheses.
[b] GenBank accession number.

and TTF1 is involved in the termination of transcription. An additional 3/13 genes are oncogenes (NDKB, SRC, TTF1), and several other genes are involved in protein turnover or associated with apoptosis (DAD1, NDKB, CTSB, SPI 2.4).

Of special interest was the down-regulation of RXRα by −1.90×. RXRα is the required heterodimerization partner of PXR, CAR, and several other nuclear receptors. Q-PCR was used to confirm RXRα down-regulation, and demonstrated a −2.5× reduction in RXRα transcript

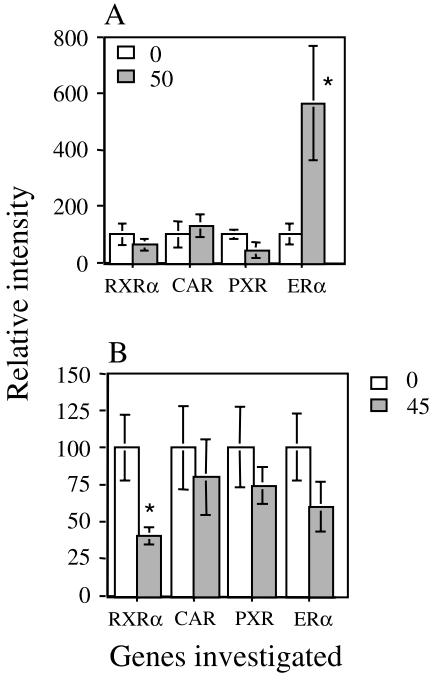

Figure 9. Changes in the expression of nuclear receptors following exposure to 4-NP for 7 days (A) or 32 weeks (B). Control mice are shown by the white bars and 4-NP treated mice by the dark bars. Data are expressed as relative intensity ± SEM (n = 4 for the 7 day study; n = 6–7 for the 32 week study). *Significant difference by Student's t-tests ($P < 0.05$)

levels after the 32 week exposure (Fig. 9b). RXRα was not down-regulated after the 7 day treatment with 4-NP (Fig. 9a). Down-regulation of RXRα after 32 weeks of treatment may in part explain why chronic exposure to 4-NP attenuates CYP2B10 induction, represses CYP3A levels, and did not increase the hepatosomatic index.

Changes in the gene expression of PXR, CAR and ERα were also examined by Q-PCR after the 7 day and 32 week treatment with 4-NP. Previous research indicates that estrogens may control the levels of some nuclear receptors (Katzenellenbogen *et al*., 1987). Furthermore, acute phase reactions have been shown to down-regulate several nuclear receptors, including RXR, PXR and CAR, as well as the P450s controlled by these receptors (Beigneux *et al*., 2002). ERα was induced 5.6-fold following treatment with 4-NP after the 7 day treatment, but not PXR or CAR (Fig. 9a). ERα was not affected by the 32 week exposure (Fig. 9b). Neither the PXR or CAR were altered by 4-NP treatment.

The two methods of measuring gene expression (microarray analysis and Q-PCR) demonstrated high concordance. PXR, RXRα and ERα are on the Clontech array. PXR was decreased 22.3% on the array and 26.0% via Q-PCR following the 32 week treatment with 4-NP, and ERα was decreased 36.2% on the array and 39.6% via Q-PCR. As reported above, RXRα was reduced according to both the array data (47.4%) and Q-PCR (60%).

Discussion

MMTVneu mice treated with 4-NP for 32 weeks demonstrated increased breast cancer incidence and metastasis, and decreased latency (Fig. 5, Table 2). After 32 weeks, only one tumor was grossly visible or histologically detectable in the control group. Tumor formation was observed in five mice at the 45 mg kg^{-1} day^{-1} 4-NP treatment group, and no tumors were present in the 30 mg kg^{-1} day^{-1} 4-NP group (Table 2). MIN were also observed histologically but not grossly, in a single mouse in both the 30 and 45 mg kg^{-1} day^{-1} 4-NP treatment groups (Table 2). These lesions are consistent with the typical phenotypes of solid carcinomas and MIN described for MMTV-neu transgenic mice (Fig. 4) (Cardiff *et al*., 2000). This suggests that there is a threshold dose for tumor formation in 4-NP treated mice of approximately 45 mg kg^{-1} day^{-1}, which is consistent with previous observations that demonstrate a 40 mg kg^{-1} day^{-1} threshold dose for proliferation of mammary tissue in rats (Odum *et al*., 1999a, 1999b).

In contrast, none of the mice treated with 10 µg kg^{-1} day^{-1} estradiol showed tumor formation (Table 2). Only five mice were treated with estradiol and the low number of animals in the treatment group may have contributed to these data. However, it is also possible that 10 µg kg^{-1} day^{-1} estradiol was below the threshold dose necessary to cause tumor formation in MMTVneu mice. The 10 µg kg^{-1} day^{-1} E_2 dose provided to mice was based on its relative binding affinity for ERα compared with those of nonylphenols (about 4500× greater for estradiol). Subsequent studies performed in our lab have demonstrated that 100 µg kg^{-1} day^{-1} E_2 increased uterosomatic index, breast tumor formation, and decreased tumor latency relative to the controls (data not shown).

It is hypothesized that 4-NP would be more potent than expected based on its relative binding affinity for ERα, and that increased hepatic production and increased serum concentrations of E_3 would be the primary basis for the increased potency of 4-NP. Estriol is a potent estrogen unlike most other estradiol metabolites (Taioli *et al*., 1999), and because of its estrogenicity is an indicator of poor prognosis for breast cancer (Telang *et al*., 1997). 4-Nonylphenol treatment of mice for 7 days increased the hepatic production of E_3 from E_2 in a dose-dependent manner (Fig. 1a). However, no increased serum concentrations of E_3 were observed at the doses that induced mammary cancer formation (Fig. 2a). Serum E_3 concentrations were only increased at 25 mg kg^{-1} day^{-1} 4-NP (Fig. 2a), presumably due to the preferential inhibition by 4-NP of E_3 formation in liver microsomes (Fig. 3a). Furthermore, increased serum E_3 concentrations

were not observed in the chronic study (Fig. 7b), and E_2 serum concentrations were relatively stable throughout both the acute and chronic 4-NP studies (Figs 2, 7).

While serum E_3 concentrations were not increased in a manner consistent with our hypothesis, hepatic E_3 production in mice exposed for 7 days (Fig. 1a) was associated with increased mammary cancer incidence and decreased latency (Fig. 5, Table 2). This is consistent with previous experiments with 4-nonylphenol (Baldwin *et al*., 1997) and other compounds that indicate an association between alterations in steroid metabolism and physiological changes such as reduced fecundity, repressed steroid hormone concentrations and reductions in breast cancer incidence (Bradlow *et al*., 1995; Michnovicz *et al*., 1997; Wilson and LeBlanc, 1998). However, our results indicate that 4-NP's ability to induce hepatic E_3 formation is transient in FVB/NJ mice. Furthermore, the association between steroid metabolism and mammary cancer does not appear to reflect a potential mechanism for the mammotropic effects of 4-NP, since serum E_2 and E_3 concentrations were not induced by 4-NP at doses that increased mammary cancer (Figs 2, 7).

4-Nonylphenol is more potent at increasing mammary cancer incidence in MMTVneu mice than predicted based on its affinity for ERα; however, the mechanism of this increased potency is unknown. The increased tumor formation in 4-NP-treated mice compared with E_2-treated mice may be due to increased retention of 4-NP. However, the possibility cannot be excluded that 4-NP may have other effects on the mammary glands that differentiate it from estradiol and enhance its ability to cause tumor formation, such as the ability to bind other nuclear receptors found in the mammary gland (Dotzlaw *et al*., 1999; Masuyama *et al*., 2000), recruit co-activators (Hall *et al*., 2002), or enhance interactions with different estrogen response elements (Hall and Korach, 2002; Hall *et al*., 2002).

CYP2B but not CYP3A enzymes were induced by the acute treatment with 4-NP (Fig. 8). The increased hepatic E_3 formation observed following the 4-NP treatment was associated with the induction of CYP2B (Figs 1, 6). The role of CYP2B10 in the 16-hydroxylation of E_2 to E_3 is unknown; however, E_2 is a potent activator of CAR and CYP2B10 transcription in mice (Kawamoto *et al*., 2000) and this suggests a role for CYP2B10 in the metabolism of E_2 in mice. Furthermore, indole-3-carbinol increased the production of E_3 from E_2 in rats demonstrating induction of CYP2B1/2, but no induction of CYP3A family members, suggesting that CYP2B is important in the formation of E_3 (Horn *et al*., 2002).

The induction of several P-450s and the concomitant increase in steroid metabolism following the 7 day study is probably elicited by transcriptional regulation by either the PXR or the constitutive androstane receptor (CAR) since 4-NP has been shown to activate these receptors (Masuyama *et al*., 2000; Baldwin *et al*., 2004). However, we are uncertain why the 32 week exposure to 4-NP did not cause similar effects to the 7 day exposure on P-450 levels. Previous research has imparted similar results demonstrating that 4-NP induces CYP3A after acute treatment (Lee *et al*., 1996b; Masuyama *et al*., 2000) and decreases CYP3A levels in rats treated chronically from gestational day 7 through post-natal day 50 (Laurenzana *et al*., 2002).

Furthermore, results demonstrating initial induction of enzymes followed by repression were obtained in a recent study investigating glutathione S-transferases (GSTs) in rainbow trout (*Onchorynchus mykiss*) exposed to 220 $\mu g\ l^{-1}$ 4-NP. Fish exposed for 1 week demonstrated an increase in GST activity; however, fish exposed for 3 weeks demonstrated a decrease in GST activity. This was associated with increased bioaccumulation of 4-NP in the liver (Uguz *et al*., 2003). Studies in rodents have also indicated that 4-NP bioaccumulates and may saturate liver enzymes causing toxic effects (Green *et al*., 2003).

RXRα is the requisite heterodimeric partner required for PXR and CAR activation of transcription (Blumberg *et al*., 1998). Down-regulation of RXRα has been shown to reduce P450 expression (Beigneux *et al*., 2002). RXRα deficient mice have reduced expression of CYP3A1 and CYP2B10 (Cai *et al*., 2002). Furthermore, hamsters treated with lipopolysaccharide (LPS), the cytokines tumor necrosis factor (TNF), or interleukin-1 (IL-1) to mimic an acute phase response have reduced RXRα, and reduced CYP3A and 2B levels (Beigneux *et al*., 2000, 2002). These mice form fewer RXR dimers with their nuclear receptor partners and show a reduced ligand-dependent transcriptional response (Beigneux *et al*., 2000).

It is possible that long-term exposure to 4-NP causes an acute phase response or directly mediates a reduction in RXRα, and thus reduces CYP3A levels. However, the acute phase response also reduces the levels of several other nuclear receptors, including PXR and CAR (Beigneux *et al*., 2002) and while the levels of these receptors were repressed they were not reduced significantly (Fig. 9b), nor was liver damage observed.

It cannot be ruled out that P450 levels are being repressed by a different mechanism. Estrogens have been shown to repress the expression of the major mouse hepatic CYP3A family member, CYP3A11, and CYP3A11 expression increased in aromatase deficient mice that are unable to produce estradiol (Yamada *et al*., 2002). Furthermore, the female predominant CYP3A family member, CYP3A41, is down-regulated by dexamethasone activation of the PXR (Anakk *et al*., 2004) and this may explain the down-regulation of CYP3A after long-term treatment with 4-NP.

In conclusion, 4-NP increased tumor formation in MMTVneu mice in a manner consistent with its estrogenicity. Consistent with our hypothesis 4-NP was more

potent than expected compared with estradiol based on their respective affinities for ERα. There are several instances in the literature where chemically mediated alterations in the rate of steroid hormone biotransformation are presumed to cause physiological changes. Our results suggest that in the case of 4-NP, there is an association between changes in hormone biotransformation, E_3 formation and mammary cancer formation. However, observed increases in hepatic E_3 production, or serum E_2 or E_3 concentrations are not a likely mechanism for the increased mammary cancer incidence in this study.

Acknowledgements—Research for this study was provided by NIH grants 2 S06 GM008012-33, ES11694-01, and 2G12RR08124. The authors would like to thank Dr Randy Rose at North Carolina State University for donating his CYP2B10 antibody, and Richard Robinson for optimizing the steroid metabolism assays. Additionally, we would like to thank Dr William C. Bridges, Jr at Clemson University for help in the statistical design of the cancer study and Dr Julia Bader at UTEP for her help with the Fisher's exact test.

References

Anakk S, Kalsotra A, Kikuta Y, Huang W, Zhang J, Staudinger JL, Moore DD, Strobel HW. 2004. CAR/PXR provide directives for Cyp3a41 gene regulation differently from Cyp3a11. *Pharmacogenomics J*. **4**: 91–101.

Baldwin WS, Acevedo R, Chapman LM, Villanueva H. 2004. Comparison of acute and chronic exposure to nonylphenol reveals that chronic exposure attenuates P-450 induction and RXRa levels. Society of Toxicology annual meeting. *Toxicologist* **73**: 353.

Baldwin WS, Graham SE, Shea D, LeBlanc GA. 1997. Metabolic androgenization of female *Daphnia magna* by the xenoestrogen 4-nonylphenol. *Environ. Toxicol. Chem.* **16**: 1905–1911.

Baldwin WS, LeBlanc GA. 1992. The anti-carcinogenic plant compound indole-3-carbinol differentially modulates P450-mediated steroid hydroxylase activities in mice. *Chem. Biol. Interact.* **83**: 155–169.

Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR. 2000. The acute phase response is associated with retinoid X receptor repression in rodent liver. *J. Biol. Chem.* **275**: 16390–16399.

Beigneux AP, Moser AH, Shigenaga JK, Grunfeld C, Feingold KR. 2002. Reduction in cytochrome P-450 enzyme expression is associated with repression of CAR (constitutive androstane receptor) and PXR (pregnane X receptor) in mouse liver during the acute phase response. *Biochem. Biophys. Res. Commun.* **293**: 145–149.

Bennie D. 1999. Review of the environmental occurrence of alkylphenols and alkylphenol ethoxylates. *Water Qual. Res. J. Can.* **34**: 79–122.

Bhatt RV. 2000. Environmental influence on reproductive health. *Int. J. Gynaecol. Obstet.* **70**: 69–75.

Blumberg B, Sabbagh WJ, Juguilon H, Bolado JJ, van Meter CM, Ong ES, Evans RM. 1998. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev.* **12**: 3195–3205.

Bradham BM, Bolander FFJ. 1989. The role of sex steroids in the expression of MMTV in the normal mouse mammary gland. *Biochem. Biophys. Res. Commun.* **159**: 1020–1025.

Bradlow HL, Davis DL, Lin G, Sepkovic D, Tiwari R. 1995. Effects of pesticides on the ratio of 16 alpha/2-hydroxyestrone: a biologic marker of breast cancer risk. *Environ. Health Perspect.* **103** Suppl 7: 147–150.

Cai Y, Konishi T, Han G, Campwala KH, French SW, Wan YJ. 2002. The role of hepatocyte RXR alpha in xenobiotic-sensing nuclear receptor-mediated pathways. *Eur. J. Pharm. Sci.* **15**: 89–96.

Cardiff RD, Anver MR, Gusterson BA, Hennighausen L, Jensen RA, Merino MJ, Rehm S, Russo J, Tavassoli FA, Wakefield LM, Ward JM, Green JE. 2000. The mammary pathology of genetically engineered mice: the consensus report and recommendations from the Annapolis meeting. *Oncogene* **19**: 968–988.

Champlin AK, Dorr DL, Gates AH. 1973. Determining the stage of the estrous cycle in the mouse by the appearance of the vagina. *Biol. Reprod.* **8**: 491–494.

Chen Y, Dougherty ER, Bittner ML. 1997. Ratio-based decision and the quantitative analysis of cDNA microarray images. *J. Biomed. Opt.* **2**: 2364–2374.

Cheng ZN, Huang SL, Tan ZR, Wang W, Zhou HH. 2001. Determination of estradiol metabolites in human liver microsome by high performance liquid chromatography-electrochemistry detector. *Acta Pharmacol. Sin.* **22**: 369–374.

Clark LB, Rosen RB, Hartman TG, Louis JB, Suffet IH, Lippincott RL, Rosen JD. 1992. Determination of alkylphenol etoxylates and their acetic acid derivatives in drinking water by particle beam liquid chromatography/mass spectrometry. *Int. J. Environ. Anal. Chem.* **47**: 167–180.

Dannan GA, Porubek DJ, Nelson SD, Waxman DJ, Guengerich FP. 1986. 17-beta estradiol 2- and 4-hydroxylation catalyzed by rat hepatic cytochrome P450: Roles of individual forms, inductive effects, developmental patterns and alterations by gonadectomy and hormone replacement. *Endocrinology* **118**: 1952–1960.

Davidson NE. 1998. Environmental estrogens and breast cancer risk. *Curr. Opin. Oncol.* **10**: 475–478.

Dotzlaw H, Leygue E, Watson P, Murphy LC. 1999. The human orphan receptor PXR messenger RNA is expressed in both normal and neoplastic breast tissue. *Clin. Cancer Res.* **5**: 2103–2107.

Eckmann L, Smith JR, Houseley MP, Dwinell MB, Kagnoff MF. 2000. Analysis by high density cDNA arrays of altered gene expression in human intestinal epithelial cells in response to infection with the invasive enteric bacteria salmonella. *J. Biol. Chem.* **275**: 14084–14094.

Gilbert MA, Shepherd MK, Startin JR, Wallwork MA. 1992. Identification by gas chromatography-mass spectrometry of vinylchloride oligomers and other low molecular weight components of poly(vinylchloride) resins for food package applications. *J. Chromatogr.* **237**: 249–261.

Green T, Swain C, Van Miller JP, Joiner RL. 2003. Absorption, bioavailability, and metabolism of para-nonylphenol in the rat. *Regul. Toxicol. Pharmacol.* **38**: 43–51.

Gupta M, McDougal A, Safe S. 1998. Estrogenic and antiestrogenic activities of 16alpha- and 2-hydroxy metabolites of 17beta-estradiol in MCF-7 and T47D human breast cancer cells. *J. Steroid Biochem. Mol. Biol.* **67**: 413–419.

Gutendorf B, Westendorf J. 2001. Comparison of an array of *in vitro* assays for the assessment of the estrogenic potential of natural and synthetic estrogens, phytoestrogens and xenoestrogens. *Toxicology* **166**: 79–89.

Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. 1992. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl Acad. Sci. USA* **89**: 10578–10582.

Hall JM, Korach KS. 2002. Analysis of the molecular mechanisms of human estrogen receptors a and b reveals differential specificity in target promoter regulation by xenoestrogens. *J. Biol. Chem.* **277**: 44455–44461.

Hall JM, McDonnell DP, Korach KS. 2002. Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. *Mol. Endocrinol.* **16**: 469–486.

Harvey PW, Darbre P. 2004. Endocrine disrupters and human health: Could oestrogenic chemicals in body care cosmetics adversely affect breast cancer incidence in women? *J. Appl. Toxicol.* **24**: 167–176.

Horn TL, Reichert MA, Bliss RL, Malejka-Giganti D. 2002. Modulations of P450 mRNA in liver and mammary gland and P450 activities and metabolism of estrogen in liver by treatment of rats with indole-3-carbinol. *Biochem. Pharmacol.* **64**: 393–404.

Huang Z, Guengerich FP, Kaminsky LS. 1998. 16Alpha-hydroxylation of estrone by human cytochrome P4503A4/5. *Carcinogenesis* **19**: 867–872.

Jorgensen M, Vendelbo B, Skakkebaek N, Leffers H. 2000. Assaying estrogenicity by quantitating the expression levels of endogenous estrogen-regulated genes. *Environ. Health. Perspect.* **108**: 403–412.

Kashuba AD, Bertino JSJ, Rocci MLJ, Kulawy RW, Beck DJ, Nafziger AN. 1998. Quantification of 3-month intraindividual variability and the influence of sex and menstrual cycle phase on

CYP3A activity as measured by phenotyping with intravenous midazolam. *Clin. Pharmacol. Ther.* **64**: 269–277.
Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. 1987. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. *Cancer Res.* **47**: 4355–4360.
Kawamoto T, Kakizaki S, Yoshinari K, Negishi M. 2000. Estrogen activation of the nuclear orphan receptor CAR (constitutive active receptor) in induction of the mouse Cyp2b10 gene. *Mol. Endocrinol.* **14**: 1897–1905.
Kuiper GJM, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson J-A. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor b. *Endocrinology* **139**: 4252–4263.
Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**: 680–685.
Laurenzana EM, Weis CC, Bryant CW, Newbold RR, Delclos KB. 2002. Effect of dietary administration of genistein, nonylphenol or ethinyl estradiol on hepatic testosterone metabolism, cytochrome P-450 enzymes, and estrogen receptor alpha expression. *Fd. Chem. Toxicol.* **40**: 53–63.
Laws SC, Carey SA, Ferrell JM, Bodman GJ, Cooper RL. 2000. Estrogenic activity of octylphenol, nonylphenol, bisphenol A and methoxychlor in rats. *Toxicol. Sci.* **54**: 154–167.
Lee PC, Marquardt M, Lech JJ. 1998. Metabolism of nonylphenol by rat and human microsomes. *Toxicol. Lett.* **99**: 117–126.
Lee PC, Patra SC, Stelloh CT, Lee W, Struve M. 1996a. Interaction of nonylphenol and hepatic CYP1A in rats. *Biochem. Pharmacol.* **52**: 885–889.
Lee PC, Patra SC, Struve M. 1996b. Modulation of rat hepatic CYP3A by nonylphenol. *Xenobiotica* **26**: 831–838.
Legler J, Zeinstra LM, Schuitemaker F, Lanser PH, Bogerd J, Brouwer A, Vethaak AD, Voogt PD, Murk AJ, Burg BVD. 2002. Comparison of *in vivo* and *in vitro* reporter gene assays for short-term screening of estrogenic activity. *Environ. Sci. Technol.* **36**: 4410–4415.
Lichtenstein P, Holm NV, Verkasalo PK, Iliadou A, Kaprio J, Koskenvuo M, Pukkala E, Skytthe A, Hemminki K. 2000. Environmental and heritable factors in the causation of cancer: Analyses of cohorts of twins from Sweden, Denmark, and Finland. *N. Engl. J. Med.* **343**: 78–85.
Lippert C, Seeger H, Mueck AO. 2003. The effect of endogenous estradiol metabolites on the proliferation of human breast cancer cells. *Life Sci.* **72**: 877–883.
Madigan MP, Ziegler RG, Benichou J, Byrne C, Hoover RN. 1995. Proportion of breast cancer cases in the United States explained by well-established risk factors. *J. Natl Cancer Inst.* **87**: 1681–1685.
Masuyama H, Hiramatsu Y, Kunitomi M, Kudo T, MacDonald PN. 2000. Endocrine disrupting chemicals, phthalic acid and nonylphenol, activate Pregnane X Receptor-mediated transcription. *Mol. Endocrinol.* **14**: 421–428.
Michnovicz JJ, Adlercreutz H, Bradlow HL. 1997. Changes in levels of urinary estrogen metabolites after oral indole-3-carbinol treatment in humans. *J. Natl Cancer. Inst.* **89**: 718–723.
Milligan SR, Balasubramanian AV, Kalita JC. 1998. Relative potency of xenobiotic estrogens in an acute *in vivo* mammalian assay. *Environ. Health. Perspect.* **106**: 23–26.
Muller PY, Janovjak H, Miserez AR, Dobbie Z. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. *Biotechniques* **32**: 1372–1379.
Namkung MJ, Yang HL, Hulla JE, Juchau MR. 1988. On the substrate specificity of cytochrome P450IIIA1. *Mol. Pharmacol.* **34**: 628–637.
National Center for Health Statistics. 1996. *Health, United States, 1995 Chartbook.* Public Health Service: Hyattsville, Maryland.
Odum J, Pyrah IT, Foster JR, Van Miller JP, Joiner RL, Ashby J. 1999a. Comparative activities of p-nonylphenol and diethylstilbestrol in noble rat mammary gland and uterotrophic assays. *Regul. Toxicol. Pharmacol.* **29**: 184–195.
Odum J, Pyrah IT, Soames AR, Foster JR, Van Miller JP, Joiner RL, Ashby J. 1999b. Effects of p-nonylphenol (NP) and diethylstilboestrol (DES) on the Alderley Park (Alpk) rat: comparison of mammary gland and uterus sensitivity following oral gavage or implanted minipumps. *J. Appl. Toxicol.* **19**: 367–378.
Ohmori S, Taniguchi T, Rikihisa T, Kanakubo Y, Kitada M. 1993. Species differences of testosterone 16-hydroxylases in liver microsomes of guinea pig, rat and dog. *Xenobiotica* **23**: 419–426.
Quail JA, Jellinck PH. 1987. Modulation of catechol estrogen synthesis by rat liver microsomes: Effects of treatment with growth hormone or testosterone. *Endocrinology* **121**: 987–992.
Ranganna K, Yousefipour Z, Yatsu FM, Milton SG, Hayes BE. 2003. Gene expression profile of butyrate-inhibited vascular smooth muscle cell proliferation. *Mol. Cell. Biochem.* **254**: 21–36.
Rockhill B, Weinberg CR, Newman B. 1998. Population attributable fraction estimation for established breast cancer risk factors: considering the issues of high prevalence and unmodifiability. *Am. J. Epidemiol.* **147**: 826–833.
Roling JA, Bain LJ, Baldwin WS. 2004. Differential gene expression in mummichogs (*Fundulus heteroclitus*) following treatment with pyrene: comparison to a creosote contaminated site. *Mar. Environ. Res.* **57**: 377–395.
Schneider J, Huh MM, Bradlow HL, Fishman J. 1984. Antiestrogen action of 2-hydroxyesterone on MCF-7 human breast cancer cells. *J. Biol. Chem.* **259**: 4840–4845.
Soto AM, Justicia H, Wray JW, Sonnenschein C. 1991. p-Nonylphenol: an estrogenic xenobiotic released from 'modified' polystyrene. *Environ. Health Perspect.* **92**: 167–173.
Spink DC, Katz BH, Hussain MM, Spink BC, Wu SJ, Liu N, Pause R, Kaminsky LS. 2002. Induction of CYP1A1 and CYP1B1 in T-47D human breast cancer cells by benzo[a]pyrene is diminished by arsenite. *Drug Metab. Dispos.* **30**: 262–269.
Taioli E, Bradlow HL, Garbers SV, Sepkovic DW, Osborne MP, Trachman J, Ganguly S, Garte SJ. 1999. Role of estradiol metabolism and CYP1A1 polymorphisms in breast cancer risk. *Cancer Detect. Prev.* **23**: 232–237.
Talmage SS. 1994. *Environmental and Human Safety of Major Surfactants: Alcohol Ethoxylates and Alkylphenol Ethoxylates.* Lewis: Boca Raton, FL.
Telang NT, Katdare M, Bradlow HL, Osborne MP. 1997. Estradiol metabolism: an endocrine biomarker for modulation of human mammary carcinogenesis. *Environ. Health. Perspect.* **105** Suppl 3: 559–564.
Uguz C, Iscan M, Erguven A, Isgor B, Togan I. 2003. The bioaccumulation of nonyphenol and its adverse effect on the liver of rainbow trout (*Onchorynchus mykiss*). *Environ. Res.* **92**: 262–270.
Ursin G, London S, Stanczyk FZ, Gentzschein E, Paganini-Hill A, Ross RK, Pike MC. 1997. A pilot study of urinary estrogen metabolites (16alpha-OHE1 and 2-OHE1) in postmenopausal women with and without breast cancer. *Environ. Health Perspect.* **105** Suppl 3: 601–605.
Ursin G, Wilson M, Henderson BE, Kolonel LN, Monroe K, Lee HP, Seow A, Yu MC, Stanczyk FZ, Gentzschein E. 2001. Do urinary estrogen metabolites reflect the differences in breast cancer risk between Singapore Chinese and United States African-American and white women? *Cancer Res.* **61**: 3326–3329.
Waxman DJ. 1988. Interactions of hepatic cytochromes P-450 with steroid hormones: Regioselectivity and stereoselectivity of steroid metabolism and hormonal regulation of rat P-450 enzyme expression. *Biochem. Pharmacol.* **37**: 71–84.
White R, Jobling S, Hoare SA, Sumpter JP, Parker MG. 1994. Environmentally persistent alkylphenolic compounds are estrogenic. *Endocrinology* **135**: 175–182.
Wilson VS, LeBlanc GA. 1998. Endosulfan elevates testosterone biotransformation and clearance in CD-1 mice. *Toxicol. Appl. Pharmacol.* **148**: 158–168.
Yamada H, Gohyama N, Honda S, Hara T, Harada N, Oguri K. 2002. Estrogen-dependent regulation of the expression of hepatic Cyp2b and 3a isoforms: assessment using aromatase-deficient mice. *Toxicol. Appl. Pharmacol.* **180**: 1–10.