Bull. Environ. Contam. Toxicol. (2000) 64:804-810

DOI: 10.1007/s001280000074

Bulletin of Environmental Contamination and Toxicology

Changes in Hepatic Cytochrome P450 Enzymes by Biodegradation Products of 4-tert-Octylphenol Polyethoxylate in Rats

N. Hanioka, T. Tanaka-Kagawa, Y.-S. Chung, T. Nishimura, H. Jinno, M. Ando

Division of Environmental Chemistry, National Institute of Health Sciences, l-l 8-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

Received: 22 December 1999/Accepted: 10 March 2000

Alkylphenol polyethoxylates are used extensively as nonionic surfactants in industrial processing and in household and institutional cleaning products (Hannes-Morgan and DeOude 1994). Because of the widespread use of these compounds it generally is accepted that they are major components of organic material in wastewater. Alkylphenol polyethoxylates apparently are degraded microbially to alkylphenol di- and monoethoxylates, and alkylphenol monocarboxylates which can be further degraded to the respective alkylphenols in microbial environments (Ahel et al. 1994). The presence of alkylphenol polyethoxylates, alkylphenol di- and monoethoxylates, alkylphenol monocarboxylates and alkylphenols in aquatic environments is known; however, controversy exists about the environmental concentrations of these compounds (Takada and Eganhouse 1998). The toxicity of alkylphenol polyethoxylates in aquatic organisms has been shown to be less than that of the biodegradation products such as alkylphenols, alkylphenol di- and monoethoxylates, and alkylphenol monocarboxylates. By contrast, the toxicity of alkylphenols to mammalian species is low (Nimrod and Benson 1996). However, there is potential for exposure to alkylphenol ethoxylates and their biodegradation products through the water supply, sewage sludge used for fertilizer, and aquatic flora and fauna as food.

It has been reported that alkylphenols bind to the estrogen receptor and exert estrogenic actions on human breast tumor cells and rainbow trout hepatocytes (White et al. 1994; Nimrod and Benson 1996). Among the alkylphenols, 4-tert-octylphenol (OP) has been shown to be the most potent, and therefore, is an "endocrine disruptor" that has significant influence on sexual and reproductive development (White et al. 1994). Other than in bacteria, little is known about the metabolism and biotransformation of biodegradation products of 4-tert-octylphenol polyethoxylate (OPEO), namely 4-tert-octylphenol di- and monoethoxylate (OP2EO/OP1EO), 4-tert-octylphenol monocarboxylate (OP1EC) and OP. To understand the impact of OPEO biodegradation products on human health, it is essential to elucidate the effects of OPEO on the enzyme involved in its metabolism.

Recently, we have found that OPEO biodegradation products interact with rat hepatic cytochrome P450 (P450 or CYP) enzymes in vitro, and suggested that the P450 enzymes are closely associated with the metabolism and toxicity of OPEO biodegradation products (Hanioka et al. 1999). The metabolism of xenobiotics frequently produces toxic metabolites (Guengerich 1992). The P450 enzymes in liver, kidney, lung and intestine are important in the metabolism of foreign chemicals as well as endogenous substances (Gonzalez 1990). Although OPEO is widely used in industry and in daily life, little is known about the effects of OPEO biodegradation products on P450 enzymes in mammals in vivo. Furthermore, the examination of the relationship between OPEO biodegradation products and cytochrome P450 enzymes in experimental animals is an important aspect of toxicological research. In the present study, to clarify the roles of hepatic P450 isoforms in the metabolism and metabolic activation of OPEO biodegradation products, we have

Correspondence to: N. Hanioka

examined the effects of OP, OP2EO and OPlEC on P450-dependent monooxygenase activities and P450 isoform protein levels in liver microsomes from rats.

MATERIALS AND METHODS

OP (>97% pure), OP2EO (Igepal® CA-210, a mixture of 20% OP2EO and 80% OPIEO), 7-ethoxycoumarin, 4-acetamidophenol and ω-hydroxylauric acid were obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). OPlEC (>99% pure) was synthesized by the method of Fujita and Reinhard (1997). Cytochrome c, 7-ethoxyresorufin, 7-pentoxyresorufin, resorufin, lauric acid and 6ß-hydroxytestosterone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Bufuralol, 1'-hydroxybufuralol, 7α–hydroxytestosterone and 2α-hydroxytestosterone were obtained from Salford Ultrafine Chemicals and Research (Manchester, England). NADH and NADPH were obtained from Oriental Yeast Co. (Tokyo, Japan). Goat anti-rat CYP2C11 and CYP3A2 antibodies were purchased from Daiichi Pure Chemical Co. (Tokyo, Japan). The anti-rat antibodies against CYP2C11 and CYP3A2 also cross-reacted with CYP2C6 (CYP2C11/6) and CYP3A1 (CYP3A2/1), respectively. Peroxidase-labeled rabbit anti-goat IgG was purchased from Zymed Laboratories Inc. (South San Francisco, CA, USA). Nitrocellulose membranes (0.45 pm) and 4-chloro-1-naphthol were obtained from Bio-Rad Laboratories (Hercules., CA, USA). All other reagents and solvents were of analytical grade from Wako Pure Chemical Industries (Osaka, Japan).

Male Sprague-Dawley rats weighing 230-250 g from Charles River Japan (Yokohama, Japan) were used. These rats were acclimated for 5 days allowing free access to water and diet before treatment. OP, OP2EO and OP1EC were dissolved with propylene glycol and injected i.p. into rats at a dose of 80 µmol/kg (OP, 16 mg/kg; OP2EO, 21 mg/kg; and OP1EC, 21 mg/kg) on days 1 and 3. Control animals were given an equal volume of vehicle in the same way. Each dose group comprised three animals. The body weights of the animals were determined every day until day 4 and animals were fasted on day 4 and killed on day 5. In the above treatment condition, we have preliminarily observed that a dose of 20 mg/kg OP produce significant changes in P450-related parameters without producing over toxic effects. Therefore, a dose of 80 µmol/kg was selected in the present study. Liver microsomes were prepared by the method reported previously (Hanioka et al. 1998a). The protein content was determined according to Lowry et al. (195 1) using bovine serum albumin as standard.

P450 and cytochrome b_5 (b_5) levels were measured according to Omura and Sato (1964). NADPH-cytochrome c reductase (fp2) and NADH-cytochrome b_5 reductase (fp1) activities were measured as described (Phillips and Langdon 1962; Takesue and Omura 1970). 7-Ethoxyresorufin O-deethylase (EROD) and 7-pentoxyresorufin O-depentylase (PROD) activities were determined from the amount of resorufin produced (Burke et al. 1985). Acetanilide 4-hydroxylase (AA4H), 7-ethoxycoumarin O-deethylase (ECOD), bufuralol 1'-hydroxylase (BF1 'H), 4-nitrophenol 2-hydroxylase (4NP2H) and lauric acid ω-hydroxylase (LAOH) activities were assayed by measuring the formation of 4-acetamidophenol, 7-hydroxycoumarin, 1 '-hydroxybufuralol, 4-nitrocatechol and ω–hydroxylauric acid, respectively (Aoyama and Sato 1988; Kronbach 1991; Liu et al. 1991; Tassaneeyakul et al. 1993; Yamazaki et al. 1999). Testosterone 7α-hydroxylase (TS7AH), testosterone 2α-hydroxylase (TS2AH) and testosterone 6ß-hydroxylase (TS6BH) activities were determined by measuring the formation of 7α–hydroxytestosterone, 2α-hydroxytestosterone and 6ß-hydroxytestosterone, respectively (Hanioka et al. 1998a).

SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970) using 7.5% acrylamide gel. Microsomal proteins were electrophoretically transferred to nitrocellulose membrane according to Towbin et al. (1979). The membrane was treated with anti-rat CYP2C11/6 or CYP3A2/1 antibody, and peroxidase-labeled second antibody. Color development was performed by using 4-chloro- 1 -naphthol and hydrogen

peroxide. Immunoblots were scanned on an image analyzer (Fluor-S, Bio-Rad Laboratories), and relative densities were measured using the software MultiAnalyst v. 1.0.2. (Bio-Rad Laboratories).

All data were analyzed using the software StatView v.5.0 (SAS Institute Inc., Cary, NC, USA) and expressed as the mean ± SD of three animals. The statistical significance of differences was calculated using ANOVA with Dunnett's post-hoc test. All differences were considered significant at P values of less than 0.05.

RESULTS AND DISCUSSION

Control, OP-, OP2EO- and OP1EC-treated rats remained healthy in appearance and normal in behavior during the study. Table 1 summarizes the increased body and organ weights. The body weight increases in rats dosed with OP, OP2EO and OP1EC were significantly different from the control. The relative weight of the thymus was decreased by only OP treatment. However, the relative weights of the liver and spleen were not significantly different from control in any OPEO biodegradation product-treated group. Highly toxic chlorinated compounds such as polychlorinated dibenzo-p-dioxins and polychlorinated biphenyls cause a loss of body weight, liver hypertrophy, and spleen and thymus atrophy (Mason et al. 1986). Thus, the effects of OPEO biodegradation products on organ weights differed among the compounds, and from those of chlorinated compounds. The effects of OP, OP2EO and OP1EC on hepatic microsomal protein, P450 and electron transport components is shown in Table 2. In OP- and OP1EC-treated groups, the P4.50 level was significantly decreased by 25 and 19%, respectively. On the other hand, the levels of microsomal protein, b5, fp2 and fp1 were not affected by any OPEO biodegradation product.

Table 1. Body and organ weights of control, OP-, OP2EO- and OP1EC-treated rats

	Control	OP	OP2EO	OP1EC
Δ Body weight[a)]	9.31±1.32	1.14±0.98†	3.25±3.00†	3.55±0.85*
Organ weight				
Liver[b)]	3.09±0.08	3.26±0.19	3.07±0.26	3.02±0.03
Spleen[b)]	0.27±0.05	0.22±0.02	0.25±0.06	0.27±0.05
Thymus[b)]	0.20±0.01	0.10±0.01†	0.20±0.02	0.22±0.01

Experimental conditions are described in Materials and Methods. Each value represents the mean ± SD of three animals.
a)%.
b)g/100 g body weight.
*Significantly different from control (P<0.05).
†Significantly different from control (P<0.01).

Table 3 shows the effects of OP, OP2EO and OP1EC on various P450-dependent monooxygenase activities in liver microsomes from rats. In testosterone hydroxylation, TS7AH, TS2AH and TS6BH activities in rat liver microsomes are supported by CYP2A1, CYP2C11 and CYP3A2, respectively (Gonzalez 1990; Ryan and Levin 1990). OP, OP2EO and OP1EC significantly decreased the TS2AH activity, each by more than 50%. Furthermore, OP significantly decreased TS6BH activity. By contrast, no change in TS7AH activity induced by OPEO biodegradation products was observed. To investigate whether or not the observed notable decreases of TS2AH and TS6BH activities were accompanied by a change of specific P450 isoforms, immunoblot analysis was performed. P450 isoforms were separated by SDS-polyacrylamide gel electrophoresis and probed with anti-rat CYP2C11/6 and CYP3A2/1 antibodies. The results are shown in Figure 1 and Table 4. The protein levels of CYP211/6 and CYP3A2/1 were significantly decreased by 49 and 43% by OP respectively, although no change

Table 2. Protein, P450, b5, fp2 and fp1 levels in liver microsomes from control, OP-, OP2EO- and OP1EC-treated rats

	Control	OP	OP2EO	OP1EC
Protein[a)]	17.9±0.4	18.0±1.3	18.3±1.2	18.9±1.0
P450[b)]	0.95±0.07	0.71±0.07*	0.79±0.12	0.77±0.02*
b5[b)]	0.20±0.05	0.17±0.03	0.18±0.02	0.18±0.01
fp2[c)]	0.30±0.04	0.23±0.04	0.29±0.03	0.25±0.02
fp1[c)]	8.46±0.66	7.15±0.83	8.28±0.43	8.79±0.44

Experimental conditions are described in Materials and Methods. Each value represents the mean ± SD of three animals.
[a)]mg/liver.
[b)]nmol/mg protein.
[c)]μmol/min/mg protein.
*Significantly different from control ($P<0.05$).

Table 3. P450-dependent monooxygenase activities in liver microsomes from control, OP-, OP2EO- and OP1EC-treated rats

	Control	OP	OP2EO	OP1EC
EROD[a)]	67.8±11.3	79.4±19.9	82.9±15.3	92.7±21.1
AA4H[b)]	0.95±0.21	0.74±0.10	0.99±0.28	1.08±0.17
TS7AH[b)]	0.20±0.03	0.24±0.02	0.22±0.05	0.21±0.01
ECOD[b)]	0.48±0.03	0.35±0.09	0.46±0.05	0.44±0.06
PROD[a)]	6.51±0.70	5.83±1.21	8.35±0.96	8.74±1.63
TS2AH[b)]	1.40±0.55	0.17±0.09†	0.68±0.23*	0.51±0.06*
BF1'H[b)]	1.73±0.17	1.47±0.19	1.64±0.24	1.58±0.09
4NP2H[b)]	0.78±0.05	0.72±0.22	0.83±0.08	0.84±0.10
TS6BH[b)]	1.91±0.32	0.96±0.24*	1.50±0.40	1.45±0.34
LAOH[b)]	2.37±0.34	2.31±0.26	2.52±0.51	2.32±0.22

Experimental conditions are described in Materials and Methods. Each value represents the mean ± SD of three animals.
[a)]pmol/min/mg protein.
[b)]nmol/min/mg protein.
*Significantly different from control ($P<0.05$).
†Significantly different from control ($P<0.01$).

in CYP2C11/6 and CYP3A2/1 protein levels caused by OP2EO or OP1EC was observed. However, the question arises as to why OP2EO and OP1EC significantly decreased TS2AH activity, while the CYP2C11/6 protein level was not affected by either OPEO biodegradation product. PROD activity in rat liver microsomes is regarded as being mainly catalyzed by CYP2B1/2 (Burke et al. 1985). Furthermore, CYP2C6 has been reported to be a phenobarbital inducible P450 isoform (Gonzalez 1990; Ryan and Levin 1990). OP2EO and OP1EC increased PROD activity, although the induction was not significant. As described in Materials and Methods, the anti-rat CYP2C11/6 used in the present study cross-reacted with both CYP2C11 and CYP2C6 isoforms. Therefore, we consider that the contradiction in the changes in CYP2C11 associated enzyme activity and protein level caused by OP2EO and OP1EC is due to multiple cross-reacting of the antibody. Of the other P450-dependent monooxygenases, the activities of EROD (CYP1A1-dependent) (Burke et al. 1985), AA4H (CYP1AZ-dependent) (Liu et al. 1991), ECOD (CYP1A1-, CYP2B1/2-, CYP2A2-, CYP2C11- and CYP2E1-dependent) (Gonzalez 1990; Ryan and Levin 1990), BF1'H (CYP2D1-dependent) (Mimura et al. 1994), 4NP2H (CYP2E1-dependent) (Tsutsumi et al. 1993) and LAOH (CYP4A1/2/3-

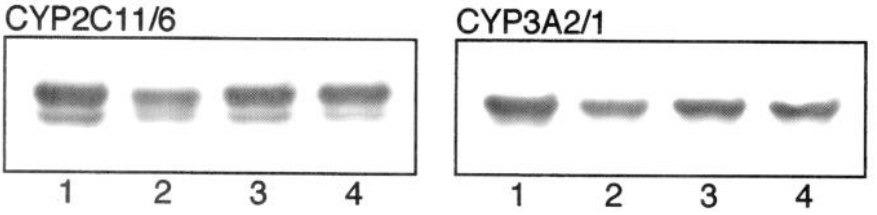

Figure 1. Immunoblotting of liver microsomes from control, OP-, OP2EO- and OP1EC-treated rats with anti-rat CYP2C11/6 and CYP3A2/1 antibodies. Experimental conditions are described in Materials and Methods. Lanes are: 1, control; 2, OP; 3, OP2EO; and 4, OP1EC. Microsomal protein levels were 4 and 6 μg for anti-rat CYP2C11/6 and CYP3A2/1 antibodies, respectively.

Table 4. CYP2C11/6 and CYP3A2/1 protein levels in liver microsomes from control, OP-, OP2EO- and OP1EC-treated rats

	Control	OP	OP2EO	OP1EC
CYP2C11/6	1.00±0.13	0.51±0.04†	0.82±0.10	0.80±0.20
CYP3A2/1	1.00±0.16	0.57±0.12*	0.78±0.13	0.67±0.17

Experimental conditions are described in Materials and Methods. The immunoblotting results were normalized to those of control rats. Each value represents the mean ± SD of three animals.
*Significantly different from control ($P<0.05$).
†Significantly different from control ($P<0.01$).

dependent) (Gonzalez 1990) were not significantly affected by any OPEO biodegradation product. It has been reported that CYP2C11 and CYP3A2 are expressed only in male rats (Gonzalez 1990, Ryan and Levin 1990). Therefore, the findings in the present study suggest that OPEO biodegradation products selectively reduce the functions and levels of male-specific P450 isoforms in rat liver, and that the order of potency is OP>OP1EC≥OP2EO.

Alkylphenols have been shown to have endocrine disruption effects as do polychlorinated dioxins, phthalates and pesticides (White et al. 1994, Nimrod and Benson 1996; Golden et al. 1998). Furtheremore, it has been reported that polychlorinated dioxins and phthalates extensively induce CYP1A1/2 and CYP4A1/2/3 in rat liver, respectively (Lake et al. 1984, Mason et al. 1986). The present study showed that OPEO biodegradation products markedly decrease the catalytic activity and protein level of the male-specific P450 isoform, CYP2C11, in rat liver, and the profile of the changes in P450 enzymes induced by OPEO biodegradation products differed from that produced by polychlorinated dioxins and phthalates. We have recently found that endocrine disrupting chemicals such as atrazine, simazine and bisphenol A decrease the enzyme activity of CYP2C11 in rat liver in vivo (Hanioka et al. 1998a, 1998b). Thus, sex-specific P450 isoforms may indirectly help to elucidate the endocrine disruption effects of alkylphenols, and the information on changes in P450 enzymes described herein is meaningful.

In conclusion, catalytic and immunochemical studies in rat liver microsomes showed that OP, OP2EO and OP1EC markedly decreased the catalytic activity and protein level of CYF2C11, and that the order of potency is OP>OP1EC≥OP2EO. The catalytic activity and protein level of CYP3A2 was significantly decreased by only OP. Therefore, the changes in male-specific P450 isoform(s) in liver may be an important factor in elucidating the toxicity mechanisms of alkylphenols.

Acknowledgments. This study was supported by a grant from the Ministry of Health and

Welfare of Japan.

REFERENCES

Ahel M, Giger W, Koch M (1994) Behaviour of alkylphenol polyethoxylate surfactants in the aquatic environment: I. Occurrence and transformation in sewage treatment. Water Res 28: 1131-1142

Aoyama T, Sato R (1988) High pressure liquid chromatography methods for separation of ω- and (ω-1)-hydroxy fatty acids: Their applications to microsomal fatty acid ω–oxidation. Anal Biochem 170: 73-82

Burke MD, Thompson S, Elcombe CR, Halpert J, Haaparanta T, Mayer RT (1985) Ethoxy-, pentoxy- and benzyloxyphenoxazones and homologues: A series of substrates to distinguish between different induced cytochromes P-450. Biochem Pharmacol 34: 3337-3345

Fujita Y, Reinhard M (1997) Identification of metabolites from the biological transformation of the nonionic surfactant residue octylphenoxyacetic acid and its brominated analog. Environ Sci Technol 31: 1518- 1524

Golden RJ, Noller KL, Titus-Emstoff L, Kaufman RH, Mittendorf R, Stillman R, Reese EA (1998) Environmental endocrine modulators and human health: An assessment of the biological evidence. Crit Rev Toxicol 28: 109-227

Gonzalez FJ (1990) Molecular genetics of the P-450 superfamily. Pharmacol Ther 45: l-38

Guengerich FP (1992) Metabolic activation of carcinogens. Pharmacol Ther 54: 17-61

Hanioka N, Jinno H, Tanaka-Kagawa T, Nishimura T, Ando M. (1998a) Changes in rat liver cytochrome P450 enzymes by atrazine and simazine treatment. Xenobiotica 28: 683-698

Hanioka N, Jinno H, Nishimura T, Ando M (1998b). Suppression of male-specific cytochrome P450 isoforms by bisphenol A in rat liver. Arch Toxicol 72: 387-394

Hanioka N, Jinno H, Chung Y-S, Tanaka-Kagawa T, Nishimura T, Ando M (1999) Inhibition of rat hepatic cytochrome P450 activities by biodegradation products of 4-tert-octylphenol ethoxylate. Xenobiotica 29: 873-883

Hannes-Morgan EC, DeOude NT (1994) Detergent. In: Calow P (ed) Handbook of Ecotoxicology, vol 2. Blackwell Scientific Publications, London, p 130

Kronbach T (1991) Bufuralol, dextromethorphan, and debrisoquine as prototype substrates for human P450IID6. In: Waterman MR, Johnson EF (eds) Methods in Enzymology, vol. 206. Academic Press, New York, p 509

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

Lake BG, Tredger JM, Gray TJ, Stubberfield CR, Hodder KD, Gangolli SD, Williams R (1984) The effect of peroxisome proliferators on the metabolism and spectral interaction of endogenous substrates of cytochrome P-450 in rat hepatic microsomes. Life Sci 35: 2621 -2626

Liu G, Gelboin HV, Myers MJ (1991) Role of cytochrome P450 IA2 in acetanilide 4-hydroxylation as determined with cDNA expression and monoclonal antibodies. Arch Biochem Biophys 284: 400-406

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (195 1) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275

Mason G, Farrell K, Keys B, Piskorska-Pliszczynska J, Safe L, Safe S (1986) Polychlorinated dibenzo-p-dioxins: Quantitative in vitro and in vivo structure-activity relationships. Toxicology 41: 21-31

Mimura M, Yamazaki H, Sugahara C, Hiroi T, Funae Y, Shimada T (1994) Differential roles of cytochromes P450 2D1, 2C11, and 1A1/2 in the hydroxylation of bufuralol by rat liver microsomes. Biochem Pharmacol 47: 1957-1963

Nimrod AC, Benson WH (1996) Environmental estrogenic effects of alkylphenol ethoxylates. Cri Rev Toxicol 26: 335-364

Omura T, Sato R (1964) The carbon monoxide-binding pigment of liver microsomes: I. Evidence for its hemoprotein nature. J Biol Chem 239: 2370-2378

Phillips AH, Langdon RG (1962) Hepatic triphosphopyridine nucleotide-cytochrome c reductase: Isolation, characterization, and kinetic studies. J Biol Chem 237: 2652-2680

Ryan DE, Levin W (1990) Purification and characterization of hepatic microsomal cytochrome P-450. Pharmacol Ther 45: 153-239

Takada H, Eganhouse R (1998) Molecular markers of anthropogenic waste: Their use in determining sources, transport pathways and fate of wastes in the environment. In: Mayers R (ed) The Encyclopedia of Environmental Analysis and Remediation, vol 5. Wiley and Sons, London, p 2883

Takesue S, Omura T (1970) Purification and properties of NADH-cytochrome b_5 reductase solubilized by lysosomes from rat liver microsomes. J Biochem (Tokyo) 67: 267-276

Tassaneeyakul W, Veronese ME, Birkett DJ, Miners JO (1993) High-performance liquid chromatographic assay for 4-nitrophenol hydroxylation, a putative cytochrome P-4502E1 activity, in human liver microsomes. J Chromatogr 616: 73-78

Towbin H, Staehelin T, Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. Proc Natl Acad Sci USA 76: 4350-4354

Tsutsumi M, Lasker JM, Takahashi T, Lieber CS (1993) In vivo induction of hepatic P4502E1 by ethanol: role of increased enzyme synthesis. Arch Biochem Biophys 304: 209-218

Yamazaki H, Tanaka M, Shimada T (1999) Highly sensitive high-performance liquid chromatographic assay for coumarin 7-hydroxylation and 7-ethoxycoumarin O-deethylation by human liver cytochrome P450 enzymes. J Chromatogr B 721: 13- 19

White R, Jobling S, Hoare SA, Sumpter JP, Parker MG (1994) Environmentally persistent alkylphenolic compounds are estrogenic. Endocrinology 135: 175-182