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Interaction of Nonylphenol and Hepatic CYP1A in Rats

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ABSTRACT. Both estradiol and nonylphenol (NP) inhibited hepatic microsomal 7-ethoxyresorufin O-deethylase (EROD) activity of β-naphthoflavone-treated rats. Enzyme kinetic analyses (Lineweaver–Burk plots) using different estradiol and NP concentrations with graded increases in the concentrations of the substrate, ethoxyresorufin, showed that the inhibition was of a competitive nature at all concentrations of estradiol or NP used. Thus, the mechanism by which NP inhibits EROD activity is similar to that of estradiol. NP, however, was much less potent than estradiol. Young rats treated *in vivo* with 80 mg/kg body weight of NP demonstrated a slight but significant decrease in their hepatic microsomal EROD activity and CYP1A protein as measured by western blot analysis. In addition, treatment with NP led to a decrease in the steady-state levels of hepatic CYP1A mRNA in rats, suggesting that NP acted at the pre-translational level. The competitive nature of inhibition by NP on hepatic microsomal EROD activity indirectly suggests that this compound is a possible substrate of the CYP1A enzyme. Furthermore, NP had a moderate modulating effect on the expression of CYP1A in rat liver. BIOCHEM PHARMACOL 52;6:885–889, 1996.

KEY WORDS. nonylphenol; cytochrome P450; rat liver

Alkylphenol ethoxylates have been used extensively in industrial processing and in household and institutional cleaning products for many years. The majority of alkylphenol ethoxylate surfactants are NP§ ethoxylates which yield the stable product, NP. NP appears in the aquatic environment, particularly in sediments that can reach up to 3000 ppb in rivers in the United States [1]. NP has been shown to possess estrogenic properties [2–4] and, therefore, is an "endocrine disrupter" that has significant influence on sexual and reproductive development [5]. To understand the potential for the impact of NP on wildlife and on human health, it is essential to elucidate the effect of NP on the enzymes involved in its metabolism.

The cytochrome P450 monooxygenase system consists of a family of enzymes, found principally in liver, lung, intestine, and kidney, that are important for both drug and endogenous substrate metabolism, including reproductive steroids. Each cytochrome subfamily is functionally distinguishable by the specificity of its substrate and inducer. From an environmental perspective, the most significant aspect of cytochrome P450s is their role in the hepatic metabolism of xenobiotics. Cytochrome P450s activate, inactivate, and facilitate the excretion of most xenobiotics, thus modulating the duration and intensity of their toxicity. The levels of individual forms of cytochrome P450 are normally very low, but are readily induced by specific agents, many of which are also substrates themselves. Other factors affecting hepatic function may also influence the expression of P450 genes. Although CYP3A is normally responsible for steroid metabolism [6], there is indirect evidence suggesting that CYP1A is similarly involved [7, 8]. Conversely, estradiol has been shown to inhibit CYP1A activity *in vitro* [9]. Since NP has estrogen-like properties, it may also affect CYP1A function and/or expression, hence altering the capacity of the exposed animal to metabolize NP and other xenobiotics. The metabolism of NP and its potential to modulate cytochrome function *in vivo* are unknown.

In the present study, we characterized the interaction between NP and the CYP1A1 from rat liver microsomes by *in vitro* inhibition assays of EROD activity. We also investigated the effects of NP on the expression of CYP1A *in vivo* in young rats.

MATERIALS AND METHODS

Chemicals

Unless otherwise stated, all chemicals were from the Sigma Chemical Co. (St. Louis, MO). Monoclonal anti-rat

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§ *Abbreviations:* NP, nonylphenol; CYP, cytochrome P450; and EROD, 7-ethoxyresorufin O-deethylase.

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CYP1A antibodies were obtained from Oxford Biomedical Research Inc. (Oxford, MI). Nitrocellulose membrane and horseradish peroxidase conjugated goat anti-mouse IgG were from Bio-Rad Laboratories (Richmond, CA).

Methods

Microsomes from the livers of rats treated with β-naphthoflavone (40 mg/kg, i.p., daily for 3 days) were prepared by differential centrifugation as described previously [10]. Protein concentration was determined by the method of Bradford [11] using the Bio-Rad® reagent (Bio-Rad Laboratories) with BSA as the standard. EROD activity in microsomes was determined spectrofluorometrically following the method of Burke and Mayer [12] using a Turner spectrofluorometer as previously described [13]. During *in vitro* EROD inhibition studies, NP was added at the beginning of the assay and mixed with buffer and NADPH. Enzymatic reaction was initiated by the introduction of microsomes.

Young female and male rat pups at 20 days of age were used for *in vivo* experiments. Unless otherwise stated, only results from female pups are presented. Experiments using male pups yielded essentially similar results. NP was dissolved in DMSO and injected intraperitoneally at various doses, as specified, at 24 or 48 hr before the rats were killed. Control animals received an equal amount of the vehicle (DMSO) only. Estradiol in DMSO was given to some sex- and age-matched rat pups for additional comparison. Microsomal fractions were prepared from livers, and their EROD activities and CYP1A1 and 1A2 protein concentrations were determined. Western blots for the determination of CYP1A1 and 1A2 protein were carried out as described previously [10], using an antibody that reacts with both CYP1A1 and 1A2 from rats.

For the quantitation of CYP1A mRNA, a modification of the procedure of Simpson [14] was used to isolate high molecular weight RNA from fresh liver samples, as previously outlined [15]. Quantitative changes of CYP1A mRNA were measured by northern blots using total RNA prepared from rat liver obtained from the various treated groups. Hybridization was performed as previously described [15] using cDNA probes containing the gene sequence for CYP1A1 and 1A2 (clones pmP1450-3′ and pmP3450-3′ from ATCC, Rockville, MD). The density and total area of the corresponding CYP1A and β-actin bands in the autoradiogram obtained were quantitated by a JANDEL video analysis system (Jandel Scientific, Corte Madera, CA). Relative concentrations of CYP1A mRNAs were expressed as ratios of CYP1A mRNA/β-actin mRNA from the same sample to correct for loading differences.

Statistics

Results are reported as means ± SD. ANOVA was used to evaluate the difference between multiple groups. If significance was observed between groups, then a post-hoc *t*-test was used to compare the means of two specific groups, with $P \leq 0.05$ considered as significant.

TABLE 1. Effect of E and NP on rat hepatic microsomal EROD activity *in vitro*

Addition	EROD activity (pmol/min/mg)
None	81.3 ± 3.9
+ E (1.25 μM)	62.4 ± 3.6*
+ E (2.50 μM)	50.4 ± 3.0*
+ E (5.00 μM)	35.4 ± 2.4*
+ NP (1.25 μM)	72.9 ± 3.3
+ NP (2.50 μM)	63.3 ± 2.1*
+ NP (5.00 μM)	50.4 ± 2.7*

Values are means ± SD from at least 3 separate determinations. E = estradiol. The concentration of the substrate ethoxyresorufin was 0.25 μM.
* Significantly different from the value with substrate alone ($P \leq 0.05$).

RESULTS

Co-incubating estradiol at various concentrations with the substrate ethoxyresorufin decreased the microsomal EROD activities (Table 1) in a concentration-dependent manner. Co-incubation of NP with ethoxyresorufin also showed a concentration-dependent decrease in EROD activity (Table 1). Compared with estradiol, NP was a less potent inhibitor in that the reduction in EROD activity by NP at 5 μM was similar to that obtained with estradiol at 2.5 μM

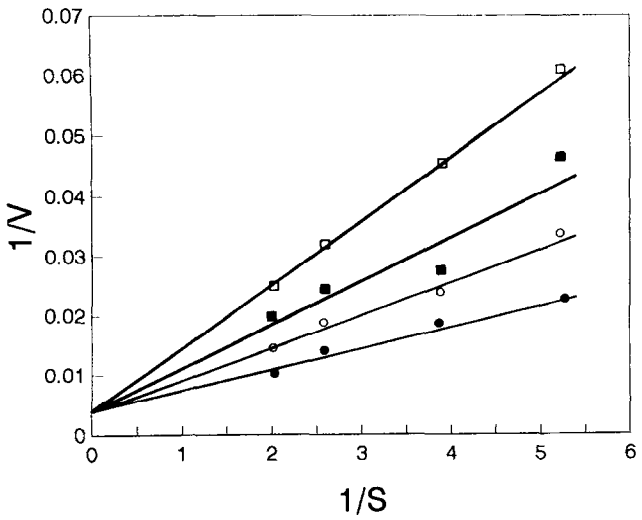

FIG. 1. Representative Lineweaver–Burk plots showing inhibition kinetics of CYP1A catalyzed EROD activity by estradiol. Liver microsomes from β-naphthoflavone-treated rats (40 μg protein) were incubated with 7-ethoxyresorufin in the absence (●) or presence of various concentrations of estradiol [(○) 1.25 μM, (■) 2.5 μM, and (□) 5.0 μM] at increasing concentrations of ethoxyresorufin (0.185 to 0.5 μM). Resorufin formation was determined spectrofluorometrically. Three separate determinations were performed with different microsomal fractions. All yielded similar plots.

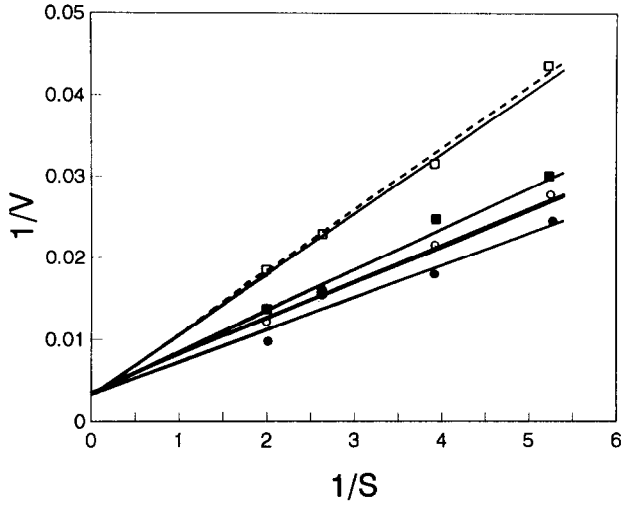

FIG. 2. Representative Lineweaver–Burk plots showing inhibition kinetics of CYP1A catalyzed EROD activity by NP. Liver microsomes from β-naphthoflavone-treated rats (40 μg protein) were incubated with 7-ethoxyresorufin in the absence (●) or presence of various concentrations of NP [(○) 1.25 μM, (■) 2.5 μM, and (□) 5.0 μM] at increasing concentrations of ethoxyresorufin (0.185 to 0.5 μM). Resorufin formation was determined spectrofluorometrically. The dotted line indicating EROD activity with 2.5 μM estradiol added to the assay mixture was included in the figure for comparison. Three separate determinations were performed with different microsomal fractions. All yielded similar plots.

(Table 1). Lineweaver–Burk plots yields a V_{max} of 279.8 ± 10.5 pmol/min/mg and a K_m of 1.0 ± 0.08 μM for the EROD activity of the microsomes used. The plots also showed that both NP and estradiol act competitively with the ethoxyresorufin substrate (Figs. 1 and 2).

To evaluate the modulating effect of NP on microsomal CYP1A1 in rat liver, rats were treated for 24 hr with various concentrations of NP. NP led to a slight decrease (15–20%) in EROD activity only at high concentrations (>40 mg/kg body weight) (Table 2). Estradiol when given to age-matched animals at a dose equivalent to NP at 80 mg/kg body weight, in terms of its effectiveness in eliciting uterotrophic responses in immature female rat pups [16], did not lead to any change in EROD activity.

TABLE 2. Microsomal EROD activity from liver of rats treated with E or graded increases of NP

Treatment	EROD activity (pmol/min/mg)
Control	80.1 ± 6.2
E (100 μg/kg)	83.1 ± 5.1
NP (10 mg/kg)	78.6 ± 8.5
(20 mg/kg)	80.7 ± 8.0
(40 mg/kg)	78.3 ± 7.3
(80 mg/kg)	69.3 ± 6.9*

Values are means ± SD from 3 separate determinations. E = estradiol. NP or E was given at the specified dose 24 hrs before the rats were killed.
* Significantly different from control value with $P \leq 0.05$.

FIG. 3. Western blots of microsomal fractions from rat livers of control (lanes 1 and 2) and NP (80 mg/kg body wt) treated animals. Estradiol treatment (100 μg/kg body wt, lane 3) was included for comparison. NP was administered 24 (lanes 4 and 5) or 48 hr (lanes 6 and 7) before the animals were killed. An equal amount of microsomal protein (30 μg each) was applied to each lane. Blots were developed with monoclonal anti-rat CYP1A serum that recognized both 1A1 and 1A2 isozymes. Lane "m" indicates marker protein with the arrow indicating the position of 50 kDa molecular size.

Since NP inhibits CYP1A1 enzyme activity, measurement of EROD activities alone after NP treatment may not be representative of the CYP1A1 protein concentrations. Western blot assays were done to evaluate the effect of NP on CYP1A proteins. Figure 3 and Table 3 indicate that NP at the concentration used (80 mg/kg body weight) resulted in decreases in EROD activity and CYP1A protein concentration. The effect was more pronounced at 48 hr than at 24 hr after treatment.

We also evaluated the effect of NP on the expression of the *CYP1A* genes in young rats *in vivo* by quantifying the steady-state level of CYP1A mRNAs following treatment of rats with 80 mg/kg body weight of NP. Figure 4 and Table 4 show that NP administration led to decreased levels of CYP1A2 mRNAs at both the 24- and 48-hr time points in both immature female and male rat pups. CYP1A1 mRNA was not quantified because of its extremely low levels.

DISCUSSION

In vivo and *in vitro* studies with rodents and humans implicate a role of polyaromatic hydrocarbon inducible CYP enzymes in estradiol metabolism. Tetrachlorodibenzo-*p*-dioxin (TCDD), a potent inducer of CYP1A1, has been shown to increase 2- and 16α-estradiol hydroxylation in human breast cancer cells [17]. Aoyama *et al.* found 2- and 4-hydroxylation of 17β-estradiol in crude extracts of HepG2 cells expressing the human CYP1A2 cDNA [8].

TABLE 3. Microsomal EROD activity and CYP1A concentrations from liver in DMSO (control) and NP-treated rats

Treatment	EROD activity (pmol/min/mg)	Relative concentrations of:	
		CYP1A1	CYP1A2
Control	115.8 ± 10.3	30.1 ± 5.9	49.6 ± 1.6
NP (24 hr)	92.2 ± 9.3*	27.5 ± 6.9	46.2 ± 5.4
NP (48 hr)	82.6 ± 7.7*	23.5 ± 3.1*	37.8 ± 3.2*

Microsomes were isolated from livers of animals 24 and 48 hr after administration of NP at 80mg/kg body weight. Values are means ± SD from 3 separate animals.
* Significantly different from corresponding control value ($P \leq 0.05$).

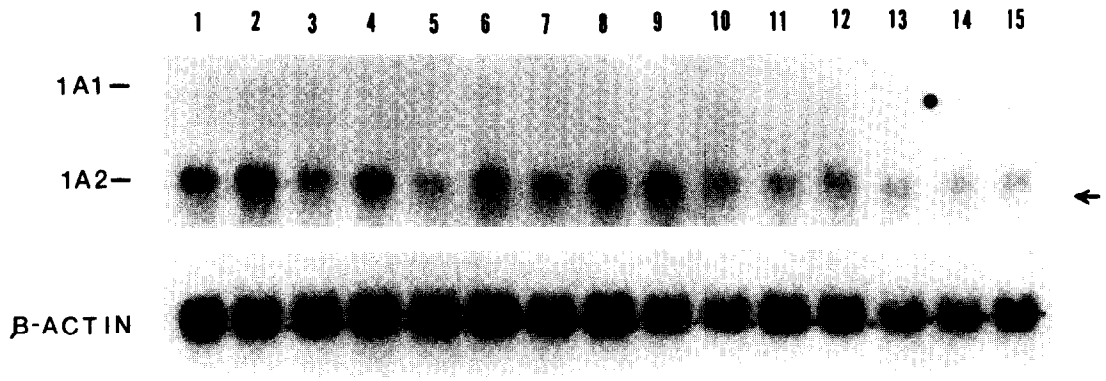

FIG. 4. Northern blots of total RNAs isolated from livers of control (C) and NP (80 mg/kg body wt) treated animals, using CYP1A and β-actin cDNA as probes. Lanes 1–7 were from female pups: Lanes 1 and 2 = C; lanes 3 and 4 = NP after 24 hr; lanes 5–7 = NP after 48 hr. Lanes 8–15 were from male pups: Lanes 8 and 9 = C; lanes 10–12 = NP after 24 hr; lanes 13–15 = NP after 48 hr. The arrow indicates the position of the 18S rRNA band.

Hydroxylation of 17β-estradiol was also reported using purified rat CYP1A2 enzymes [18, 19]. Co-incubation of estradiol with expressed human CYP1A1 and 1A2 in *Saccharomyces cerevisiae* inhibits enzyme activity in a competitive manner, suggesting estradiol as a possible substrate of CYP1A [9].

The present results confirm the observation that rat microsomal EROD activity is inhibited by estradiol and NP. Compared with estradiol, however, the concentration-response data indicate that NP is a less potent inhibitor of rat hepatic microsomal EROD activity.

Kinetic analyses of the present data (Lineweaver–Burk plots) demonstrated that, as reported previously [9], estradiol exhibits a competitive type of inhibition and further demonstrate that NP inhibition of EROD activity of the rat hepatic microsomal preparation is competitive. Like estradiol, NP may therefore be a substrate of the CYP1A enzyme. Taken together, our results suggest a possible role of CYP1A in the metabolism and biotransformation of NP, at least in the liver. Metabolism of NP in other tissues such as the kidney and lung may also occur since these tissues have been shown to produce CYP1A [20].

The present study also shows that exposure of young rats *in vivo* to a high dose of NP (40 mg/kg body wt) decreased the EROD activity in microsomes subsequently isolated from the liver of these treated rats. These results suggest that NP has some effect in modulating CYP1A in rat liver. Since estradiol at a dose equivalent to NP for its estrogenic action (induction of uterine growth and up-regulation of uterine peroxidase) led to no observable changes in EROD activity or CYP1A protein concentration, it is possible that the CYP1A modulating effect of NP was due to something other than its estrogenic properties. Because of the pharmacological dose of NP used in the treatment (up to 80 mg/kg body wt), and the inhibitory properties of NP on EROD activity in *in vitro* assays, the lowering of EROD activity observed may be due to the residual level of NP in the subsequently isolated microsomal fraction that inhibits, at least partially, the catalytic activity of CYP1A1. To resolve this issue, the effect of NP on CYP1A in rat liver was examined further by western blot analysis. The decrease in EROD activity was accompanied by a decrease in relative concentrations of bands corresponding to the CYP1A1 and 1A2 proteins. The decrease was particularly evident in hepatic microsomes from animals at 48 hr after treatment. Thus, the decrease in EROD activity following treatment with NP is more likely a result of a decrease in CYP1A isozymes and less likely the result of inhibition by residual NP in tissue extracts.

Quantitation of CYP1A mRNA levels in control and NP-treated rats confirmed the CYP1A protein measurement. There was a significant decrease in the steady-state level of CYP1A2 mRNA following NP treatment. CYP1A1 mRNA was barely detectable, but also showed a decrease after NP treatment. Thus, down-regulation of CYP1A occurs at the pre-translational level. The exact site of action of NP, of NP metabolites, or possibly of other metabolites produced by changes in the metabolism of endogenous substrates is unknown. Since the structure of NP contains only a single aromatic ring, it is highly unlikely that it acts through the AH receptor in regulating CYP1A expression. The effects that are seen here are likely to be indirect.

The slight down-regulation of CYP1A by NP treatment, together with the inhibition of the catalytic activity of CYP1A by NP, may have important physiologic and pharmacologic implications in that exposure to high concentra-

TABLE 4. Steady-state levels of hepatic CYP1A2 mRNA in control and NP-treated female rats

	Relative concentrations of CYP1A2 mRNA	
Treatment	**Females**	**Males**
Control	0.46 ± 0.10	0.35 ± 0.02
NP (24 hr)	0.35 ± 0.09	0.23 ± 0.04*
NP (48 hr)	0.21 ± 0.07*	0.20 ± 0.03*

Total RNAs were isolated from the liver of animals 24 and 48 hr after administration of NP (80 mg/kg body wt). All values were normalized against β-actin mRNA and are presented as ratios of CYP1A2 mRNA/β-actin mRNA. Values are means ± SD from 3 separate animals.

*Significantly different from control value ($P \leq 0.05$).

tions of NP may potentially affect the overall function of the CYP1A enzymes. For example, in the liver, NP treatment changes the total capacity of deethylase and hydroxylase activities. In view of the importance of CYP1A in the overall metabolic schema of xenobiotic and endogenous substrate activation and deactivation, the effect of NP on CYP1A and other CYPs warrants additional study.

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