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In Vivo Estrogenic Action of Nonylphenol in Immature Female Rats

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Alkylphenol polyethoxylates (APEs) are widely used as components of detergents, paints, herbicides, insecticides and many other formulated products. Recent studies with effluent of sewage-treatment works in England led to the identification of a class of compounds, the alkylphenols, as having estrogenic properties (White et al. 1994, Jobling et al. 1995). Approximately 80 % of APEs are nonylphenol polyethoxylates and 20 % , octaphenol polyethoxylates. It is estimated that 60% of APEs end up in the aquatic environment as nonylphenol (NP) and octaphenol. Nonylphenol was first reported to have estrogenic action based on its induction of proliferation and up-regulation of the progesterone receptor in human estrogen-sensitive breast tumor cells (Soto et al. 1991, White et al. 1994). A majority of these studies involved in vitro systems with few reports on whole animals. In rainbow trout, administration of nonylphenol was shown to up-regulate vitellogenin expression (Ren et al. 1995). In higher vertebrates, except for the reported increase in mitotic index in endometrial epithelium of ovariectomized female rats (Soto et al. 1991), the in vivo effects of nonylphenol are not known.

Estrogens are potent regulatory factors for many developmental and physiological responses. Untimely exposure to natural or synthetic estrogenic compounds may have serious consequences to the reproductive cycle in humans and animals (Korach, 1993; Ginsburg 1994). Estrogen also influences the neuroendocrine and skeletal systems and promote carcinogenic events in target tissues suggesting that the accumulation of alkylphenols can adversely affect human health. We therefore performed the following study to determine if nonylphenol has a uterotrophic effect in immature female rats. In addition, we evaluated the effect of estradiol in comparable immature female rat pups and contrasted with those from nonylphenol exposure.

MATERIALS AND METHODS

Except otherwise stated, all chemicals were from Sigma chemical Company (St. Louis, MO.) ICI 182,780, a specific estrogen antagonist, was a gift from Dr. A Wakeling of Zeneca Pharmaceuticals, Cheshire, England.

Pregnant Sprague-Dawley rats from an inbred colony maintained at the Medical College of Wisconsin were housed in individual cages and maintained

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on a 12-h alternate light-dark cycle. On the expected date of delivery, cages were inspected every 6 hours for birth. The day of birth was regarded as day 0. Pups were allowed to suckle freely until 20-21 days of age. The NIH guidelines for the care and use of laboratory animals were followed to ensure that animals were not subjected to pain and discomfort. Immature female pups at 20-21 days of age were given intraperitoneal (i.p.) injections of estradiol (E) or nonylphenol (NP) at the specified dose 24 hours (unless otherwise stated) before sacrifice. Age matched females used as controls were not injected. Whenever possible, littermates were used for one set of experiment. Otherwise, female pups born on the same date were grouped and then split into subgroups for different treatments in one experiment. At least 3 animals were used for each treatment subgroup. Rats were sacrificed by decapitation. After sacrifice, their uteri were excised, dissected free of connective tissue and fat, weighed and snap-freezed for storage at -75°C. Uteri were thawed and homogenized in 10mM Tris-HCl, pH 7.2. A portion of the homogenate was used for protein and DNA assay. The remainder of the homogenate was centrifuged at 39,000 X g to obtain a pellet which was homogenized in 10 mM Tris-HCl, pH 7.2 containing 0.5M $CaCl_2$ to solubilize the uterotrophic marker enzyme, peroxidase.

Protein concentration was determined by the Bradford method (1976) using the Biorad^R reagent (BioRad Laboratories, Richmond, CA.) with BSA as the standard. DNA was determined by the calorimetric reaction with diphenylamine reagent according to Burton (1956) using highly polymerized calf thymus DNA as the standard. A procedure modified from the method of Croft and Lubran (1965) was adapted to minimize the interference with sialic acid. All results were normalized to 100 gm body weight to account for the difference in body size among different animals. Uterine peroxidase was determined using the procedure of Lyttle and DeSombre (1977) using H_2O_2 as the substrate and guaiacol as the chromogen dissolved in solubilization buffer. One ml of tissue extract was added to 2 ml of substrate in buffer and changes in OD at 470 nm were measured for the initial two minutes. Enzyme activity was expressed as increase in OD/min/gm tissue.

Analysis of variance (ANOVA) was used to evaluate the difference of multiple groups. If significant difference was observed among groups, then post-hoc t test was used to compare the means of 2 specific groups. A p value of ≤ 0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Administration of estradiol to immature female rats led to a dose-dependent increase in uterine weight. The increase in uterine mass was accompanied by increases in protein and DNA contents and the marker enzyme, uterine peroxidase (Fig. 1). Similar graded increases in these uterine parameters were also evident when similar immature female rats were given increasing doses of

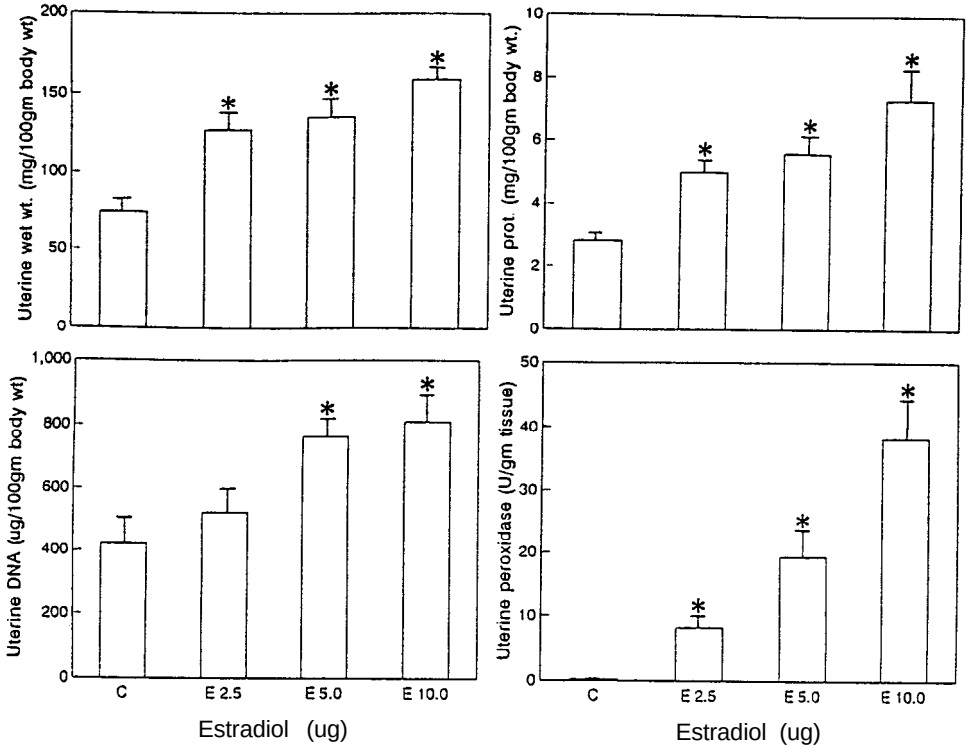

Fig. 1. Dose response of uterine parameters to estradiol administration. Estradiol at 2.5, 5.0, and 10 ug/animal were given by ip injection 24 hours before sacrifice. Values represent means ± S.D. from 3 animals. *denotes values that are significantly different from corresponding control values with $P \leq 0.05$.

nonylphenol (Fig. 2). The dose-response relationship between estradiol and NP however was quite different. Estradiol at a dose of 2.5 ug/animal had a significant effect on the uterus whereas nonylphenol was ineffective when used at a ug range. Significant induction of uterine growth by nonylphenol was found only at a much higher concentration ie ≥ 1.0 mg/animal. These results indicate that NP is estrogenic and is 1/1000 to 1/2000 as potent as estradiol.

We also examined the kinetics of NP induction of uterotrophy compared to estradiol treatment (Figs. 3 and 4). Increase in uterine weight was evident as early as 6 hours after injection. However, the weight increase did not reach a significant level until 12 hours after treatment. Both uterine protein content and peroxidase activity on the other hand were significantly increased even at 6 hours post-injection. DNA content showed a significant increase only at 24 hours. Increases in all the uterine parameters were transient in that they all peaked at 24 hours (except for uterine peroxidase which peaked at 12 hours).

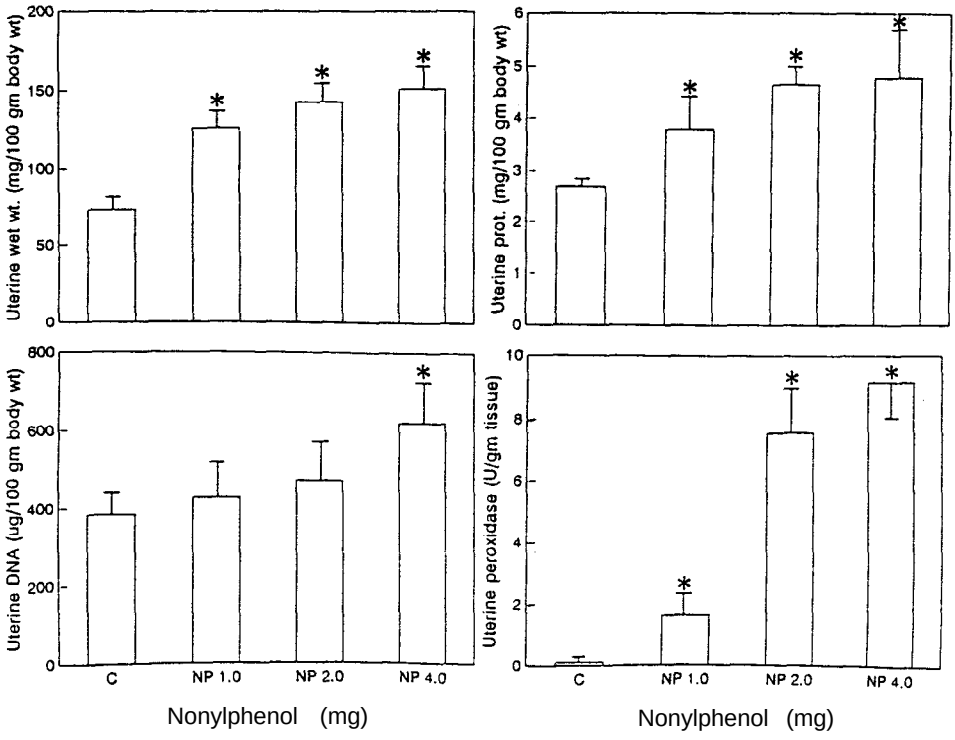

Fig. 2. Dose response of uterine parameters to nonylphenol (NP) administration. Nonylphenol at 1.0, 2.0 and 4.0 mg/animal were given by ip injection 24 hours before sacrifice. Values represent Means ± S.D. from 3 separate animals. *denotes values that are significantly different from corresponding control values with $p \leq 0.05$.

The temporal changes of uterine parameters exposure to NP were similar to those after exposure to estradiol (Fig. 4). Administration of the vehicle dimethylsulfoxide (DX) did not lead to any significant change in any of the uterine parameters when measured at 24 hour after administration, a time point when maximal effects were seen with both estradiol and NP treatment. The tapering off of uterine parameters, particularly that of DNA content which returned to control levels at 48 hour, is particularly intriguing.

Estrogen has a temporal effect on the uterus with an initial proliferation phase, where DNA synthesis peaked at 24 hours after estrogen administration. This is followed by a stage when apoptosis starts to set in reaching a peak around 48 hours after estrogen treatment (Kirkland et al. 1995). Although the exact timing is difficult to know, changes in DNA content observed in our experiments agreed with the bi-phasic response (peak DNA synthesis and peak apoptosis) of the uterus to estrogen challenge. The same temporal changes in

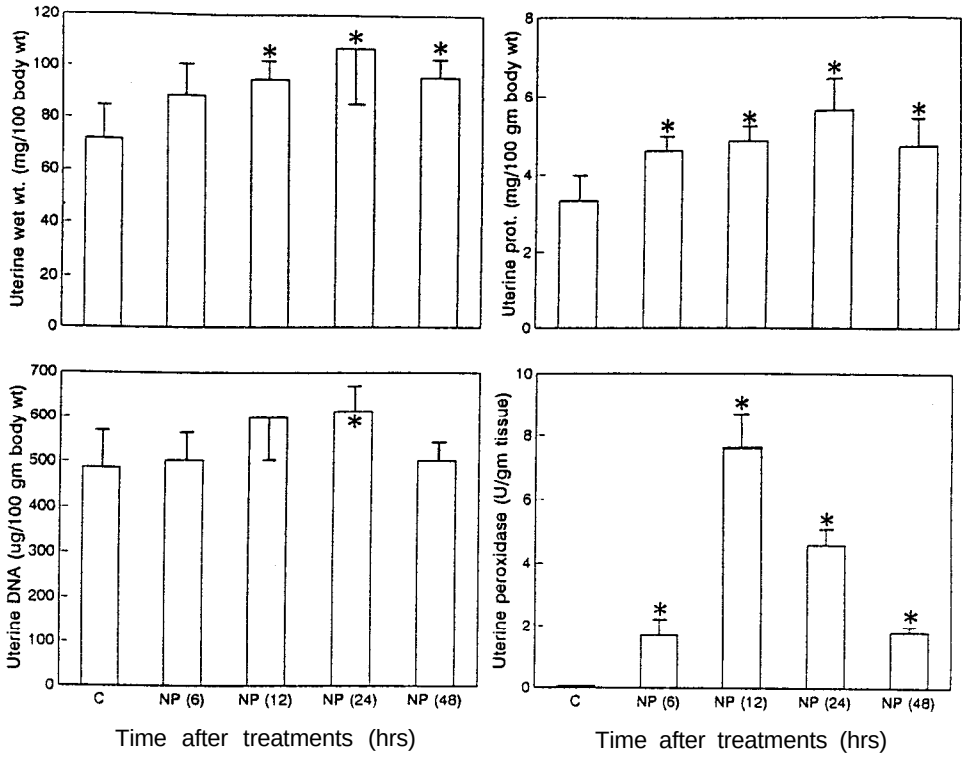

Fig. 3. Time course of uterine response to a single dose of NP (2.0 mg/animal) and sacrifice at the times indicated. Values represent Means ± S. D. from 3 separate animals. *denotes values that are significantly different from the corresponding control values with $p \leq 0.05$.

uterine DNA contents in NP treated animals suggested patterns and stages were similar to estradiol.

We next investigated the effect of estrogen antagonist on the ability of NP to stimulate uterine growth in immature female rats. Table 1 shows that ICI 182,780, a potent estrogen antagonist, when given at 20X the concentration of estradiol completely blocked the increases in uterine weight and protein contents induced by estradiol. The increase in uterine peroxidase, although suppressed drastically, was not completely blocked by co-administration of ICI 182,780. The same dose of ICI 182,780 when given simultaneously with NP also blocked the increases in uterine weight and protein contents. In this case, the increase in peroxidase activity was also completely blocked. Thus, it appears that NP acts through the estrogen receptor in its action on the rat uterus. Our observations are in agreement with those reported for alkylphenol action on in vitro cell cultures (White et al. 1994).
Because of the similarity in action between NP and estradiol, we examined the

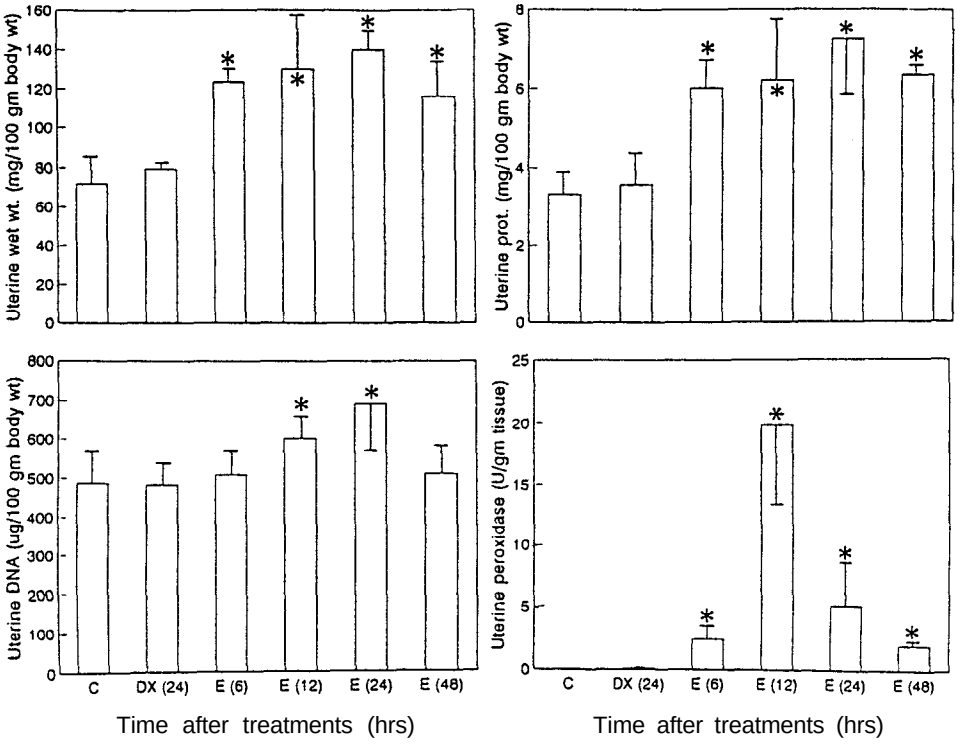

Fig. 4. Time course of uterine response to a single dose of estradiol (5.0 ug/animal) and sacrificed at the times indicated. As an added control, some animals were given an equal volume of the vehicle, dimethylsulfoxide (DX) and sacrificed at 24 hours after injection. Values represent means ± S.D. from 3 animals. *denotes values that are significantly different from corresponding control values.

interaction between these two compounds (Table 2). At optimal concentration of estradiol (10-20 ug/animal), the addition of an optimum concentration of NP (4 mg/animal) had no additional effect on uterine weight. This suggests that both NP and estradiol act through a common pathway in the rat uterus. However, at sub-optimum dose of estradiol (2.5 ug/animal) the addition of NP (4.0 mg/animal) inhibited the estradiol effect in that the combined treatment of NP and estradiol resulted in a lowering of the uterine weight compared to treatment with estradiol alone.

This inhibition by NP in the presence of a sub-optimum dose of estradiol suggests there is direct competition of NP with estradiol for some intermediary pathways. NP being less efficient, recruits and occupies these shared pathways thus preventing the efficient use of these same pathways by estradiol and interfere with its actions. This may have significant physiologic

implication in that exposure to high doses of NP might interfere with the normal function of estrogen.

TABLE 1. Effect of the estrogen antagonist (ICI 182,780) on estradiol or NP induced uterine changes.

TREATMENTS	Wet weight (mg/100gm)	UTERINE Protein (mg/ 100gm)	Peroxidase (U/gm tissue)
Control	74.4 ± 0.3	3.8 ± 0.5	0.03 ± 0.001
Estradiol (2.5 ug)	121.6 ± 5.0*	5.9 ± 0.8*	8.00 ± 0.6*
Nonylphenol (4 mg)	115.5 ± 10.0*	6.4 ± 0.5*	7.70 ± 1.0*
E + ICI (50 ug)	73.6 ± 9.3	3.2 ± 0.2	0.40 ± 0.1*
NP + ICI (50 ug)	71.8 ± 5.5	3.9 ± 0.3	0.02 ± 0.001

Values represent means ± SD from 3 animals.
*denotes values that are significantly different from corresponding control values ($p \leq 0.05$).
E = Estradiol (2.5 ug); NP= Nonylphenol (4 mg). All concentrations refer to are per animal.

TABLE 2. Interaction of nonylphenol and estradiol on uterine growth in immature female rats.

TREATMENTS	Uterine weight (mg/100 gm body weight) Expt. I	Expt. II	Expt. III
Control	70.1 ± 5.0	67.6 ± 10.8	68.4 ± 5.1
Nonylphenol (4 mg)	108.7 ± 12.1*	122.2 ± 12.9*	125.7 ± 15.0*
Estradiol+	(2.5 ug)	(10.0 ug)	(20 ug)
	112.8 ± 14.7*	132,8 ± 13.5*	135.0 ± 10.0*
Estradiol + Nonylphenol (4 mg)	91.7 ± 6.7*#	137.4 ± 16.1*	131.7 ± 14.5*

Each value represents mean ± SD from 3 animals.
*denotes values that are significantly different from corresponding control values ($p \leq 0.05$).
#denotes values significantly different from values of animals treated with estradiol alone. +concentrations of estradiol are specified under each column.

Our results show that NP is estrogenic when administered to rats and it mimics the effects of estradiol in its uterotrophic action. NP is only 1/2000 to 1/1000 as potent as estradiol. The effect of NP appears to be mediated through the estrogen receptor since its action can be blocked by the estrogen antagonist ICI 182,780. It is difficult to ascertain whether NP needs to be metabolized before it expresses its estrogenicity. In vitro experiments indicate this is unlikely since the elaborate systems found in vivo for its metabolism is not present.

Because of NP's presence in the environment including drinking water and its in vivo estrogenic effects, NP may contribute to the reproductive endocrine disruption observed in wildlife. Its effect on humans remains to be studied.

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