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Comparative Effects on Secretion of LH, FSH, Prolactin, and Testosterone by Chronic and Direct Hypothalamic Administration of Nonylphenol to Adult Male Rats

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Nonylphenol (NP) is a widespread environmental pollutant that has been shown to exert both toxic and estrogenic effects on mammalian cells. As the effects of NP on the reproductive system of adult male vertebrates are virtually unknown, we investigated not only the changes of reproductive hormone secretion in serum after chronic exposure to NP but also, in order to identify the site of its action, the reproductive hormone secretion in serum 48 hours after microinfusion of NP within hypothalamic preoptic area (POA). In the chronic exposure, the luteinizing hormone (LH), follicle stimulating hormone (FSH), and testosterone in serum were decreased but prolactin (PRL) concentrations were increased. The LH, FSH, and testosterone in serum were decreased through the direct infusion of NP into POA, while there was no difference in mean serum prolactin between NP and control groups. These observations suggest that NP as endocrine disruptor has modulatory effects on hypothalamo-pituitary-gonadal axis and that the site of action of NP could be hypothalamic POA.

Key Words: Nonylphenol, Alkylphenol, Hypothalamus, Preoptic area, Endocrine disruptor, LH, FSH, Prolactin, Testosterone

INTRODUCTION

The gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH) are secreted by the gonadotrophs in the anterior pituitary in a pulsatile manner and act on the ovaries and testes to promote gametogenesis and reproductive function and to stimulate the production of the sex steroids. In turn, LH and FSH secretion are regulated by the sex steroids acting either directly on the gonadotroph or indirectly by alteration of GnRH pulses from the hypothalamus (Ghaub et al, 1990). GnRH pulses play an important role in the maintenance of a gonadotropin secretion, and the amplitude and frequency of these pulses appear to be changed throughout the reproductive cycle and in response to changes in sex steriod levels (Crowley et al, 1985; Shupnik et al, 1991; Shupnik et al, 1996). Additional gonadal peptides including inhibin, activin, and follistatin also play potentially important regulatory roles, and their effects are limited to FSH (Carroll et al, 1989; Dalkin et al, 1993).

Alkylphenols have been utilized as diluents in pesticides and as components in household laundry detergents, shampoos, and hard surface cleaners (Weinberger et al, 1982) as well as cosmetics and personal care products (Rautuccio et al, 1984). Also, the substantial quantities of alkylphenols have been used in manufacturing phosphate antioxidants, modified phenolic resins, additives to machine oils, and metallurgic oils (Lowenheink & Moran, 1975). Moreover, the majority of the manufactured alkylphenols has been used as intermediates to synthesize alkylphenol polyethoxylates (APEOs), nonionic surfactants applied for washing and cleaning agents, emulsifiers, wetting agents, forming and form reducing agents, and auxiliaries for various branches of industry

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(Cserhati et al, 1995).

Currently, APEOs is the largest source of these environmental alkylphenols, and it is widely used as the second largest group of nonionic detergents in commercial production. Most APEOs enter the aquatic environment after use, often receiving biological treatment before being discharged into rivers (Marcomini et al, 1987). They show a high level of primary degradation in sewage-treatment works leading to relatively stable metabolites (Akel et al, 1987). In the case of aerobic treatment (Van Ginkel, 1996), these APEOs have been shown to decompose to alkylphenol short-chain ethoxylate (Stephanou & Giger, 1982) and corresponding carboxylic acids (Reinhard et al, 1982). These alkylphenols, especially nonylphenol (NP) and octylphenol (OP), accumulate in the environment as manufactured chemicals (Soto et al, 1991). Some of these metabolites such as NP and OP are hydrophobic, whereas other short-chain APEOs and carboxylic acid derivatives (APECs) are more hydrophilic (John & Jobling, 1993).

Human exposure to alkylphenols may occur not only from these environmental contaminants but also through contact with manufactured and metabolic products. NP also has been reported to leach from laboratory polystyrene (Soto et al, 1992), posing potential problems to the laboratory research and analysis.

Several groups of investigators have voiced their concerns about the presence of alkylphenols in the environment for the following two main reasons. First, the toxicity of these chemicals to animal cells. Lethal threshold for NP for shrimp or salmon was approximately 10^{-6} M, and the concentration of the hydrophobic alkylphenols occured in adipose tissue was greater in fish (McLeese et al, 1989). Thus, the concentration of alkylphenols in vivo that may be increased through bioaccumulation may reach to a hazardous level to animal cells. Second, the contribution of the alkylphenols to the environmental estrogenic pool. Alkylphenols, including NP, have been shown to bind to the estrogen receptor and to exert estrogenic actions on avian and mammalian cells (Jobling & Sumpter, 1993; White et al, 1994), and estradiol receptor-binding assays have shown that NP and OP can compete with 17β-estradiol for the estradiol receptor (Sonnenshein & Soto, 1998). Even though alkylphenols are markedly less estrogenic than 17β-estradiol, the widespread use of these compounds and their abilities to bioaccumulate in lipid suggest that they may contribute substantially to the environmental estrogenic pool.

In fact, it has been hypothesized that these estrogens may well be involved in inducing human breast tumors (Davis & Bradlow, 1995) and exerting deleterious effects on the male reproductive system (Sharpe et al, 1995). Moreover, it is likely that NP and other environmental estrogens when combined have potentiating effects (Rodale, 1976). Therefore, alkylphenols as well as APEOs may also be potentially harmful to exposed humans and the environment (Harrison et al, 1997).

In many studies to localize the estradiol receptors in brain, the POA contains estradiol receptors (Akema et al, 1983; Herbison, 1995), making this anatomical structure a putative site to regulate reproductive hormone secretion. Although the estrogenic effects of NP in vitro were well-known, those of NP on the reproductive hormone regulation in adult male vertebrates are unknown. Therefore, we investigated the effects of NP on the hypothalamo-pituitary-gonadal function by comparing the changes of serum LH, FSH, PRL, and testosterone concentration by chronic systemic administration and direct microinjection of NP into the hypothalamic POA of the adult male rats.

METHODS

Animals

Adult Sprague-Dawley male rats (body weight 200~230 g) were purchased from the Korea Research Institute of Chemical Technology (Taejeon, Korea). Three animals per hanging wire cage were housed in a room. Standard laboratory chow and tap water were supplied ad libitum.

Chemicals

Corn oil was purchased from Sigma Co. (St. louis, MO, USA). NP was purchased from Aldrich Chemical Co. (Milwaukee, WI, USA; 98.1% pure). Fresh solutions of NP diluted with corn oil were made weekly and sealed until used.

Administration of NP and vehicle for 1 month

Rats were either not treated, injected s.c. with 0.2 ml corn oil (vehicle), or injected s.c. with 100 mg/kg

of NP in vehicle. Injections were administered three times per week (on Monday, Wednesday and Friday) at different sites in the nape region for 1 month. There were 8 rats in each group and rats were anesthetized with chloral hydrate 400 mg/kg for 2 days after the last injection, and blood samples (about 4 ml) were collected by cardiac puncture between 09 : 00 and 12 : 00 hr.

Serum was stored at $-20^{\circ}C$ to analyse LH, FSH, PRL and testosterone concentrations.

Microinjection into hypothalamic preoptic area

Rats (270~320 g) were anesthetized with chloral hydrate (400 mg/kg, i.p.) and placed in a stereotaxic apparatus. The skull was exposed and the small hole was drilled through the skull at the appropriate location following coordinates. The stereotaxic coordinates according to the rat brain atlas (Paxinos & Watson, 1986) for the POA were 1.7 mm posterior to bregma, 0.7 mm from lateral from the midline, and 8.0 mm ventral from the dura. A CMA/12 microdialysis probe (CMA/Microdialysis, Stockholm, Sweden) with 2.0 mm tip length (ID = 400 μm, OD = 500 μm) was used after detaching the microdialysis membrane and glue sticking around outlet to apply only for injection. The infusion probe was connected by PE-60 silastic tubing to 100 μl Hamilton syringe held in the CMA/100 Microinjection Pump (CMA/Microdialysis, Stockholm, Sweden). The connecting tubing and syringe were filled with NP or vehicle. The microinjection probe was slowly inserted into the POA. The infusion was begun with 0.2 μm/min of flow rate for 5 minutes, thereby total 1.0 μl of corn oil or 0.5 μg of NP in 50% corn oil were infused. More than 5 minutes after the end of infusion, the probe was slowly withdrawn from the POA, and skin sutured after the cranioplastic cement (Plastic One, VA, USA) was applied on the cranial hole to prevent CSF and heat loss. Small air bubbles were placed in connecting tubing to determine whether the fluid was in fact passing down infusion probe.

Body weight

To evaluate the potential general toxic effects of NP, body weights were recorded on day 0, 7, 14, 21, and 28 for 1 month treated and untreated groups.

Hormone assays

Serum samples were assayed for LH and FSH concentrations by the method of Niswender et al (1968) using ^{125}I-labeled ligands and rat materials supplied by the Biocode Co. (Belgium). Serum testosterone levels were determined by using the RIA kit with coat-A-count total testosterone obtained from Diagonstic Products (Los Angeles, CA, USA). Serum PRL concentrations were determined by EIA using milenia rat PRL provided by Diagonstic Products (Los Angeles, CA, USA).

All samples were assayed in duplicate using appropriate volumes of sera, and the results were averaged.

Histological finding and verification of probe placement

Animals were anesthetized with chloral hydrate 400 mg/kg i.p. The blood was collected by cardiac puncture and transcardially perfused with 10% formalin in phosphate buffer. The microinjected brains were removed and stored in the same fixative over a day, and paraffin blocks were made. A microtome was used to prepare 5 μm thick coronal sections, and hematoxylin-eosin stain was applied to verify the location of microinjection probe and to evaluate the direct toxicity of corn oil or NP to the surrounding tissues.

Statistical analysis

Results were reported as mean and one standard error of mean (SEM). Body weight changes were analyzed by performing one-way analysis of variance (ANOVA). Unpaired t-test were used for the comparisons between NP and corn oil groups in terms of secretions of reproductive hormones. A *P*-value of less than 0.05 was considered significant.

RESULTS

Body weight

Rats in the untreated group and those injected with corn oil or NP had progressvely gained body weight. The body weight increase of untreated rats was slightly more than that of corn oil or NP-treated rats.

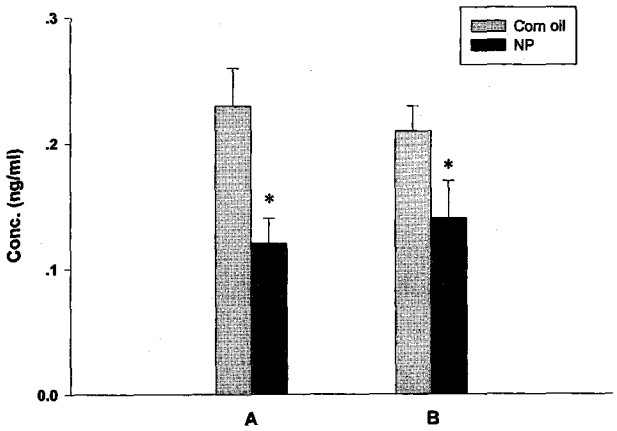

Fig. 1. The concentatin of sera LH 2 days after (A) the chronic administration for 1 month, (B) the microinjection within POA. *: $P<0.05$, **: $P<0.01$

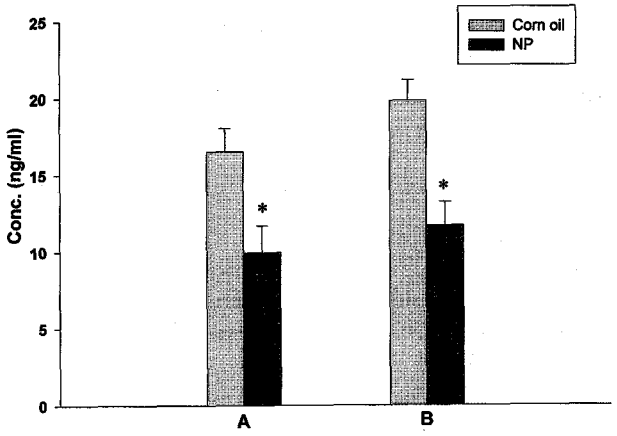

Fig. 2. The concentration of sera FSH 2 days after (A) the chronic administration for 1 month, (B) the microinjection within POA. *: $P<0.05$, **: $P<0.01$

Table 1. Body weights (g) of rats untreated and treated with corn oil or NP three times per week (mean±SEM)

Days	Untreated	Corn Oil	100 mg NP
0	221.8±9	219±13.3	217.9±9.9
7	242±8.2	236.4±12.6	235.9±6.7
14	260±7.1	252±11.1	248.3±7.2
21	272.3±6.9	263.4±9.8	260±8.4
28	284.2±5.3	273.9±8.1	269.2±8.8

No statistical difference in mean body weight was present between the subgroups of rats at the same time (Table 1).

Serum LH, FSH, and PRL concentrations

The mean serum concentrations of reproductive hormones between untreated group and the one which were treated with corn oil for 1 month showed no difference (data not shown). The mean serum concentrations of LH and FSH were significantly decreased both by 1 month administration and direct hypothalamic POA infusion of NP (Fig. 1, 2). The serum concentration of PRL was increased markedly about 160% (from 52 ng/ml to 131 ng/ml) by 1 month administration of NP but showed no change by direct POA infusion (Fig. 3). This discrepancy in the secretion of PRL by central and peripheral administration implies that the PRL secretion by chronic exposure of NP is not mediated by POA or NP

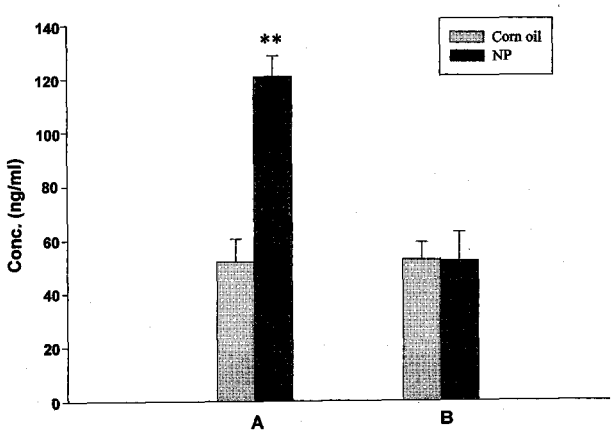

Fig. 3. The concentration of sera prolactin 2 days after (A) the chronic administration for 1 month, (B) the microinjection within POA. *: $P<0.05$, **: $P<0.01$

probably has another mechanism besides estrogenic activity as an endocrine disruptor.

Serum testosterone concentrations

The mean serum concentration of testosterone was markedly decreased by 1 month administration of NP (about 50%) and also by direct POA infusion of NP (about 32%). These data assume that NP's mechanism of action as an endocrine disruptor could be mediated by its action on the hypothalamo-anterior pituitary-gonadal axis. The above data about LH and FSH secretion support this assumption. However, the related effects of NP on the PRL secretion and direct toxicity on testis should be further evaluated.

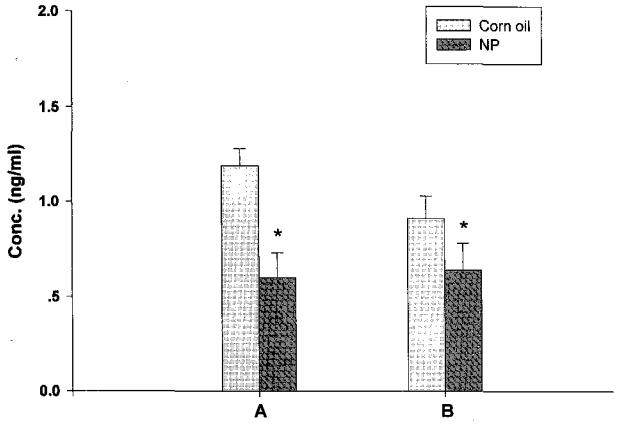

Fig. 4. The concentration of sera testosterone 2 days after (A) the chronic administration for 1 month, (B) the microinjection within POA. *: $P<0.05$, **: $P<0.01$

Histological finding and verification of probe placement

The position of microinjection probes were ascertained after H-E staining, If the probe tips were not located in the POA, the data were discarded. In photomicroscopic fields, the probe tracts and the location of probe tips were identified. POA injected with corn oil had no significant histological changes. POA injected with NP, however, showed some necrotic changes such as swelling (small arrow) and coagulation (arrowhead), but most cells were intact (Fig. 5). These results suggested that the possibility of direct toxicity of NP for mammalian cells or its higher affinity for sex hormone release regulating cells in hypothalamus or anterior pituitary gland.

DISCUSSION

As Sharpe and Skakkebaek state (1993), a wide range of compounds is known to be estrogenic, including many organochlorine compounds such as dichloro-diphenyl-trichloroethane (DDT) and polychlorinated biphenyls. Man is inevitably exposed to some of these compounds. These kinds of compounds now are extended to another wholly different group of chemicals, the APEOs, which degrade to environmentally-persistent estrogenic compounds, and the possibility that APEOs may be related with reduced sperm counts and other testicular disorders of men should be seriously considered (Nimrod & Benson, 1996).

The results of the present experiments show that the exposure of NP can adversely affect the secretion of hormones that control the reproductive system. The NP inhibited secretion of LH, FSH, and testosterone secretion, while serum PRL secretion was increased. This finding supports the previous reports that NP binds to estrogen receptors and exerts estrogenic actions in vitro (Soto et al, 1991; 1992). The effects of NP on LH and FSH secretion by central administration provide strong evidence that NP acts in vivo at the level of the hypothalamus and anterior pituitary gland (APG), but the effect on PRL secretion suggested that the NP's effect on hypothalamo-pituitary-gonadal axis could be mediated by mechanism other than estrogenic activity.

The possibility that NP might exert non-estrogenic toxic actions must also be considered as an additional explanation for the effects of NP on APG reproductive hormone secretion. We do not know whether NP reached APG cells in sufficient concentration to kill some cells without measuring the circulating levels or APG concentrations of NP. However, it is unlikely that NP exerted any marked toxic effects on lactotrophs in vivo on the present study, because PRL secretion by the APG was neither compromised in rats treated with NP and hypothalamic tissues around microinjection probe nor damaged severely in histological findings.

The reductions in mean serum testosterone concentration in rats treated with NP may be due to more than one mechanism. First, the suppressive effects in serum testosterone levels can be explained on the basis of the lowered serum LH concentration. In the male rat, circulating LH is responsible for maintaining a normal elevation of serum testosterone concentrations (Kalra, 1983). Second, NP may also have acted directly on Leydig cells to inhibit testosterone synthesis as exerted by OP and estrogen (Barke et al, 1977) and reduced sizes of testes and male accessory sex organs (Sharpe et al, 1995; Bookfor & Blake, 1997). We did not determine whether these treatments have reduced Leydig cell number as an additional contributing factor to compromise testosterone release. Nevertheless, the results clearly demonstrate that NP can exert dramatic effects on reproductive hormone secretion by a central mechanism. Because of alkylphenols' ability to bioaccumulate in lipids (Shiraishi et al, 1989) and the possibility of environmental estrogens to act alone, alkylphenols have

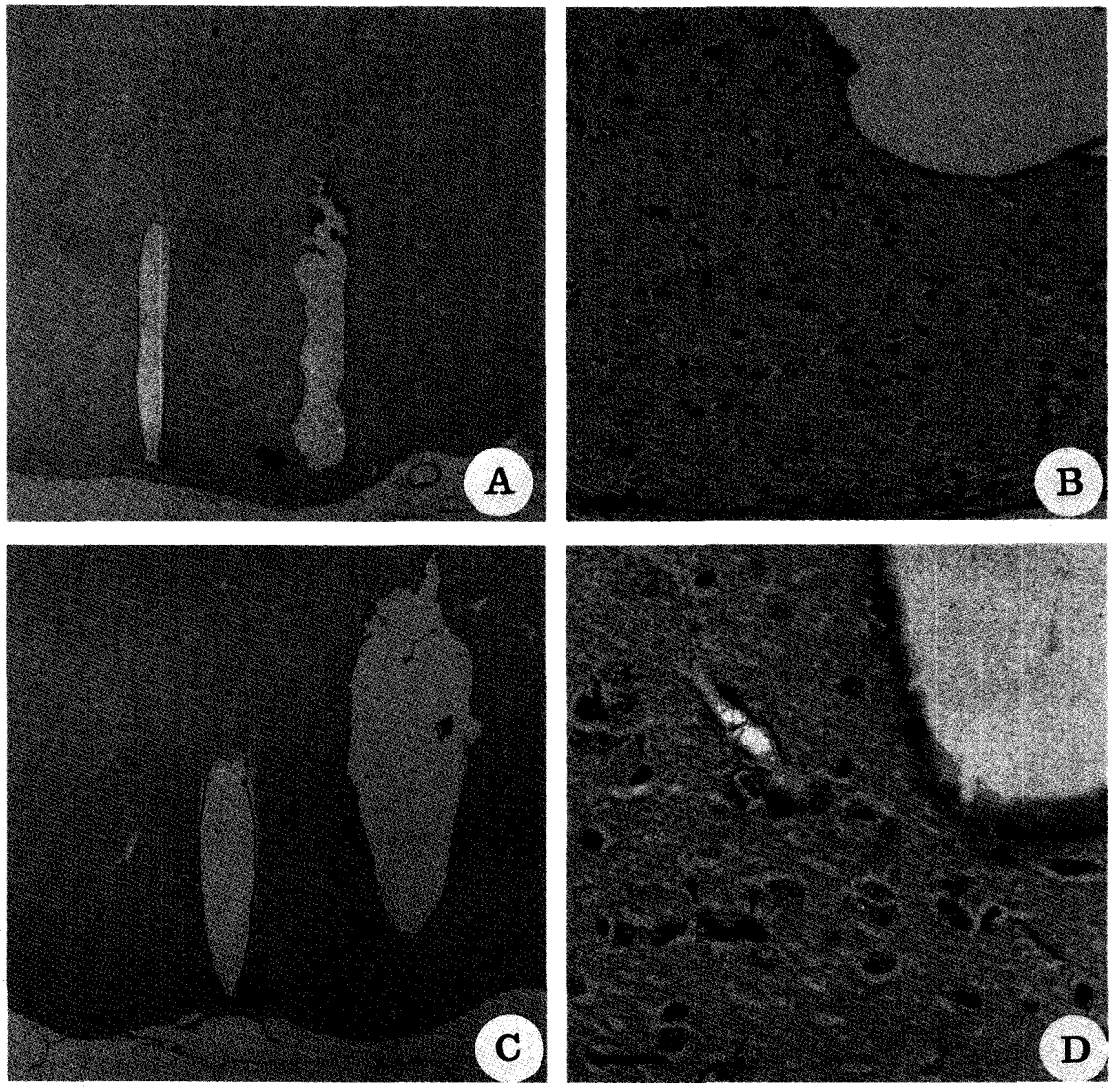

Fig. 5. Light micrographs of 5 μm coronal sections stained with hematoxylin-eosin showing the probe tips in preoptic area (arrow) 2 days after 10 μl of vehicle (A, B) 0.5 μg of nonylphenol injection (C, D). B and D are higher magnification. Calibration bars: 100 μm in B and 50 μm in D.

additive effects (Gaido et al, 1997), or synergize when acting together (Rodale, 1976), it must be considered that NP and other alkylphenols and short-chain polyethoxylate alkylphenols may act as an environmental hazard and affect wildlife and human health. Further studies in the orchidectomized or estrogen antagonist-treated rats are needed to evaluate the exact mechanism of NP in hypothalamus and APG. This experiment also suggests that the direct POA injection with hormonal assay could be available as an *in vivo* screening method for endocrine disruptors especially acting on hypothalamic POA or anterior pituitary gland in mammals.

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