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—Original Article—

4-Nitro-3-phenylphenol has both androgenic and anti-androgenic-like effects in rats

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Abstract. To investigate the effect of endocrine disruption of 4-nitro-3-phenylphenol (PNMPP) on immature male Wistar-Imamichi rats, the rat pituitary was exposed to PNMPP (10^{-5}–10^{-9} M) for 24 h with or without gonadotropin-releasing hormone (GnRH) in experiment I. In addition, the Leydig cells (10^{-5}–10^{-9} M) were exposed to PNMPP for 24 h with or without human chronic gonadotropin (hCG) in experiment II. Our results showed that the PNMPP at 10^{-5}–10^{-7} M suppressed follicle-stimulating hormone (FSH) and luteinizing hormone (LH) productions from GnRH-stimulated pituitary cells. At the same time, PNMPP 10^{-5}–10^{-7} M induced an increase in testosterone production from the Leydig cells treated with or without hCG. Based on our results, it can be concluded that that PNMPP might have both androgen agonist action by decreasing FSH and LH production in the pituitary and anti-androgenic action by increasing testosterone production in the Leydig cell.
Key words: 4-Nitro-3-phenylphenol, Gonadotropins, Leydig cell, Pituitary, Testosterone

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There is currently increased interest in the endocrine disruption of diesel exhaust (DE) and diesel exhaust particles (DEPs) [1, 2]. Many studies have demonstrated that DE and DEPs induce problems of organs and hormones related reproduction in both humans [3] and animals [4–10].

It has previously been reported that 4-nitro-3-phenylphenol (PNMPP) isolated from DEPs had estrogenic action and affected reproductive function. Injection of PNMPP into ovariectomized immature female rats induced an increase in uterine weight, oxytocin-induced myometrium contractility [11], and a significant increase in the uterine luminal epithelium [12]. In *in vitro* studies, PNMPP provoked a proliferation of breast cancer cell line MCF-7 [13] and decreased the estradiol concentration but did not affect testosterone and cortisol secretion in human adrenal H295R cells [14]. Furthermore, PNMPP had an anti-androgenic effect by inhibiting 5α-dihydrotestosterone (DHT) binding to the androgen receptor (AR) [1].

We hypothesized that PNMPP might affect hormone synthesis and secretion in reproduction-related organs. Accordingly, this study investigated the effect of PNMPP on gonadotropin synthesis in the pituitary and testosterone synthesis on Leydig cells in cell cultures.

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Materials and Methods

Chemicals

4-Nitro-3-phenylphenol (PNMPP), as shown in Fig. 1, was synthesized by the method described previously [2].

Animals

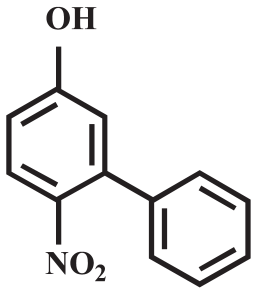
Immature male Wistar-Imamichi rats at 28 days of age were purchased from the Imamichi Institute for Animal Reproduction, Ibaraki, Japan. They were maintained under conditions of controlled lighting (14 h: light 10 h dark, lights on 0500 h), temperature (22 ± 2 C), and humidity (50 ± 5%). Food (CE-2 commercial diet; Clea Japan, Tokyo, Japan) and water were available *ad libitum*. All procedures were carried out in accordance with guidelines established by the Tokyo University of Agriculture and Technology, for use of laboratory animals.

Experimental procedure

The rats were decapitated, and the anterior pituitary gland and Leydig cells were removed immediately.

Experiment I: Effect of PNMPP on hormone secretion from the anterior pituitary

The anterior pituitaries were placed in cold DMEM medium containing 10 g/l M5M, 6 g/l HEPES, 10% $NaHCO_3$, and 10 ml/l



4-Nitro-3-phenylphenol
(PNMPP)

Fig. 1. Chemical structure of 4-nitro-3-phenylphenol (PNMPP), a component of diesel exhaust particles.

MEM nonessential amino acid without enzymes. The pituitaries were minced and incubated with Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen, Burlington, ON, Canada) with 2.8 mg/ml collagenase, 0.8 mg/ml hyaluronidase, 8 mg/ml bovine serum albumin (BSA), and 200 U/ml DNAse in a shaking incubator (150 cycles/min) at 34 C for approximately 20 min. Minced pituitary was washed by centrifugation at 1,500 rpm for 5 min at room temperature and then resuspended with DMEM with 10% Daigo's GF21 solution (inhibin-free serum; Wako Pure Chemical Industries, Osaka, Japan), 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Burlington, ON, Canada). The pituitary suspension was cultured and incubated for 78 h in 96-well culture plates at 37 C under an atmosphere of 95% air and 5% CO_2. Then the culture media were changed, and the cells were exposed to PNMPP (10^{-9}–10^{-5} M) dissolved in media. At 24 h after exposure to PNMPP, the cells were stimulated with and without 10 nm gonadotropin-releasing hormone (GnRH); National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK, Torrance, CA, USA) for 4 h and then collected. The culture media were subsequently stored at –20 C until assayed for FSH and LH.

Experiment II: Effect of PNMPP on hormone secretion from the Leydig cells

The testes were trimmed free of fat and decapsulated. Then the testicular artery was removed from the decapsulated testes to eliminate red blood cells. After that, the testes were dissociated by incubation in M199 medium (Gibco®) containing 0.71 g/l sodium bicarbonate, 2.21 g/l HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 0.1% BSA, and 25 mg/l soybean trypsin inhibitor (STI), pH 7.4, with 0.25 mg/ml of collagenase (Worthington Biochemical, Freehold, NJ, USA) and then horizontal shaking (90 cycles/min) at 34 C for approximately 10–20 min. After dissociation, the seminiferous tubules were removed by filtration through 100 µm nylon mesh. The filtrated was centrifuged at 1,500 rpm for 5 min at room temperature to wash the dissociated cells. To separate the Leydig cells, the cells were mixed with a Percoll suspension and centrifuged at 3,000 rpm for 30 min at room temperature. The Leydig cells were resuspended in M199 medium with 1% fetal bovine serum (FBS) and without STI and collagenase enzymes. The Leydig cells in the medium (10^5 cells/well) were cultured and incubated for 48 h in 96-well culture plates at 37 C under an atmosphere of 95% air and 5% CO_2 . Then the cells were exposed to PNMPP (10^{-9}–10^{-5} M) for 24 h with or without 0.1 IU/ml human chronic gonadotropin (hCG) dissolved in media. After 4 h of hCG stimulation, the medium was collected for testosterone assay.

Hormonal assays

FSH and LH concentrations were measured using an NIDDK radioimmunoassay (RIA) kits (Torrance, CA, USA) for rat FSH and LH. The iodinated preparations were rat FSH-I-5 and LH-I-5. The antisera used were anti-rat FSH-S-11 and anti-rat LH-S-11. Results were expressed as rat FSH RP-2 and rat LH RP-3. The intra- and interassay coefficients of variations were 4.8 and 11.4% for FSH and 5.4 and 6.9% for LH, respectively.

Testosterone concentration was measured using a double-antibody RIA system with ^{125}I-labeled radioligands as described previously [15]. Antisera against testosterone (GDN 250), provided by Dr GD Niswender (Colorado State University, Fort Collins, CO, USA), were used. The intra- and interassay coefficients of variations were 5.9 and 5.8%.

Statistical analysis

The data were expressed as means ± SE. One-way analysis of variance (ANOVA) was used to compare means among groups. Post hoc multiple comparison analyses were performed with the Least Significant Difference (LSD) test when the F ratio for the ANOVA was significant at $P < 0.05$.

Results

Effect of PNMPP on hormone production from the pituitary

As shown in Fig. 2, PNMPP treatment could not increase the concentrations of FSH and LH secreted from the pituitary cells without GnRH stimulation. Conversely, 10^{-5}–10^{-7} M of PNMPP could increase the FSH and LH concentration when the cells were stimulated with GnRH. On the other hand, 10^{-8}–10^{-9} M of PNMPP could not increase FSH and LH concentrations, although the cells were stimulated with GnRH.

Effect of PNMPP on hormone production from Leydig cell culture

Testosterone concentrations were significantly increased, showing an inverted U shape, in cultures of Leydig cells stimulated with and without hCG when the cells were treated with PNMPP (Fig. 3).

Discussion

In the present study, PNMPP (10^{-5}–10^{-7} M) reduced GnRH-stimulated FSH and LH secretions from anterior pituitary cells, but did not have any effect on the pituitary cells without GnRH stimulation. This result indicated that PNMPP played a role in decreasing FSH and LH secretion via GnRH stimulation. As we known, AR is found in the pituitary and affected by androgen administration and castration [16–20]. Androgen also has a role in control of GnRH released from the hypothalamus, as shown by the effect of testosterone treatment on reducing GnRH mRNA [17] and GnRH release [18]. In *in vitro*

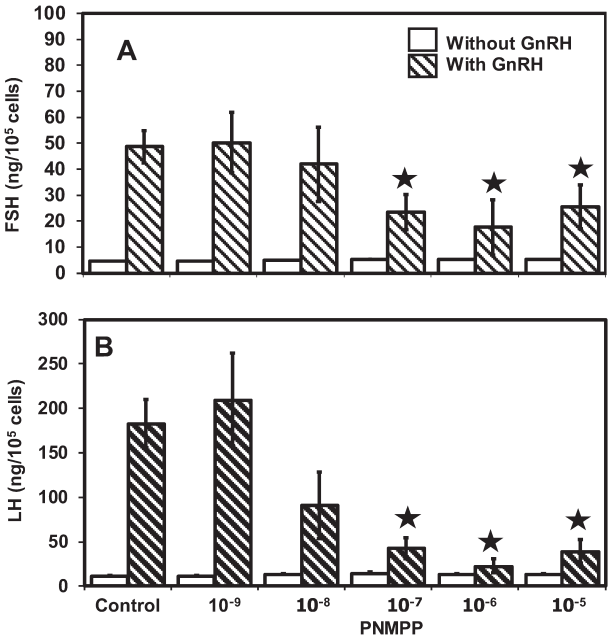


Fig. 2. FSH and LH productions by pituitary cells incubated with PNMPP (10^{-5}–10^{-9} M) in the presence or absence of GnRH. Each graph represents the means ± SE. The F ratio for the ANOVA compared with control was significant at ★ $P < 0.05$.

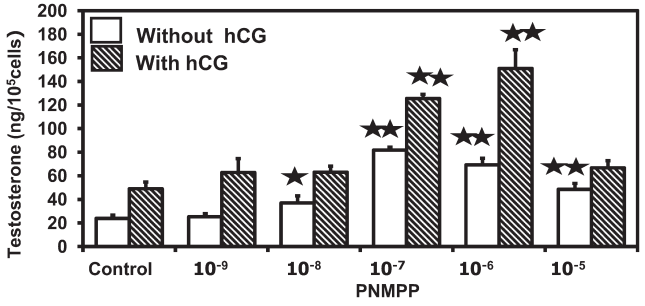


Fig. 3. Testosterone production by Leydig cells incubated with PNMPP (10^{-5}–10^{-9} M) in the presence or absence of hCG. Graph represents the means ± SE. The F ratio for the ANOVA compared with control was significant at ★ $P < 0.05$ and ★★ $P < 0.001$.

studies, androgen was found to suppress pituitary responsiveness to a hypothalamic extract and changed FSH and LH release from the anterior pituitary [20, 21]. Androgen acts by binding at receptor sites and then has a negative feedback action that decreases FSH and LH secretions by slowing the GnRH pulse generator and suppressing FSH and LH syntheses in the pituitary [22].

A single treatment of 3-methyl-4-nitrophenol (4-nitro-m-cresol; PNMC), which was extracted from DEPs, suppressed the plasma LH concentration in Japanese quails [6]. PNMC increased the plasma testosterone concentration and decreased the plasma FSH and LH concentrations, indicating that it acts on the hypothalamus-pituitary axis in adult male rats [23] and immature male rats [24].

Our previous study found that the chemical structure of PNMPP comprises a benzene ring, which is similar to steroid hormones including estrogen and androgen [1]. Hence, in the study of a pituitary cell culture containing GnRH, we might assume that PNMPP acts as androgen and reduce the effect of GnRH action on the secretions of FSH and LH, which would be the same as the effect of PNMC found in previous papers [1, 23, 24].

In the present study, PNMPP induced high secretion of testosterone in the Leydig cells cultured with and without hCG when compared with the control. From previous *in vitro* studies, DEPs have been reported to slightly increase the gene expression of the steroidogenic acute regulatory (StAR) protein in mouse Leydig cells [25]. Exposure to nanoparticle-rich diesel exhaust (NR-DE) enhanced cholesterol synthesis and increased the expression of gene that regulate steroid synthesis along with the testosterone concentration in testicular culture [26]. Consistent with the study *in vitro*, exposures to NR-DE for 1 or 2 months significantly increased StAR and cytochrome P450 side-chain cleavage (P450scc) mRNA and their protein expressions and increased the testosterone concentration in male rats and mice [26, 27]. Either NR-DE or DEPs have a direct effect on testosterone production by increasing mRNA expression and genes associated with testosterone cholesterol synthesis in Leydig cells [23, 27, 28]. Accordingly, we assume that PNMPP may have a direct effect on increasing the testosterone concentration in Leydig cells.

Furthermore, addition of procymidone, an anti-androgenic substance, to a Leydig cell culture stimulated with hCG increased testosterone production by elevating several steroidogenic enzymes including StAR, P450scc and cytochrome P450c17α (P450c17) [29]. Flutamide, an androgen receptor antagonist, also enhanced StAR mRNA expression from Leydig cells of adult rats treated with hCG [30]. Furthermore, it was shown previous that anti-androgen caused hypergonadotropic activation of testicular steroidogenesis [2]. PNMPP has been reported to inhibit DHT action by binding to the androgen receptor in a recombinant yeast screen assay [2]. It can be assumed that PNMPP had an anti-androgenic effect on testosterone production in the Leydig cells.

In summary, the present study clearly demonstrated that PNMPP had androgen agonist action by suppressing the effect of GnRH and then decreasing the FSH and LH concentrations in the pituitary cell culture. In addition, PNMPP had a direct effect on the increase in testosterone concentration in Leydig cell culture without hCG stimulation; moreover, it had androgen antagonist action by increasing the testosterone concentration in Leydig cell culture.

Acknowledgments

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