

4-Nitrophenol isolated from diesel exhaust particles disrupts regulation of reproductive hormones in immature male rats

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Abstract In previous studies, we found that 4-nitrophenol (PNP) isolated from diesel exhaust particles exhibited both estrogenic and anti-androgenic activities. This compound is also a degradation product of the insecticide parathion. Here, we investigated the *in vivo* effect of PNP on reproductive function in immature male rats. Twenty-eight-day-old rats were injected subcutaneously with PNP (0.01, 0.1, 1, or 10 mg/kg) daily for 14 days. Plasma concentrations of luteinizing hormone (LH) were significantly lower in all PNP dosage groups than in the control group, and follicle-stimulating hormone (FSH) was significantly decreased in rats treated with 0.1, 1, or 10 mg/kg PNP. However, plasma concentrations of testosterone were significantly increased by 10 mg/kg PNP, and plasma concentrations of immunoreactive (ir)-inhibin were also significantly increased in the 0.1, 1, and 10 mg/kg PNP groups. Plasma concentrations of prolactin were significantly increased by 10 mg/kg

PNP, and plasma concentrations of corticosterone were significantly increased in all treatment groups. These findings clearly show that PNP influences the hypothalamic–pituitary–gonadal axis in immature male rats, with decreased secretion of LH and FSH and increased secretion of testosterone and inhibin. PNP, therefore, appears to disrupt endocrine activity in the immature male reproductive system.

Keywords 4-Nitrophenol · Diesel exhaust particles (DEP) · Hypothalamic–pituitary axis, reproductive function · Male rat

Introduction

Air pollution is a serious problem throughout the world, and diesel exhaust particles (DEP) are a leading contributor to this problem. DEP contain many kinds of compounds that are hazardous to human health, including compounds that have been associated with lung cancer [1, 2], allergic rhinitis [3, 4], and bronchial asthma-like disease [5, 6]. An important aspect of the endocrine-disrupting effect of diesel emission is the potential to adversely affect male reproductive function. Diesel exhaust reportedly suppresses spermatogenesis in adult mice [7] and rats [8–12]. However, because DEP contain carbon nuclei, which can absorb a vast number of chemicals, the specific compound responsible for this phenomenon remains unclear.

4-Nitrophenol (PNP), a nitrophenol derivative that has been isolated from DEP (1 kg of DEP contains an average of 169 mg PNP [13]), is a vasodilator [14, 15]. It has affinity to estrogen and androgen receptors and has shown estrogenic and anti-androgenic activities *in vitro* in a recombinant yeast screening assay [16] and *in vivo* in immature rat

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uterotrophic and Hershberger assays [17]. PNP is also a degradation product of the insecticide parathion [18], which is used as a fumigant, acaricide, and pre-harvest soil and foliage treatment for a wide variety of crops, both outdoors and in greenhouses worldwide. The potential for exposure of humans, livestock, and wild animals to parathion may be higher in both rural and residential environments. Accumulation of PNP from these sources has serious effects on wildlife and human health via the disruption of endocrine and reproductive systems.

No information has yet been published on the effects of PNP on reproductive function in immature male rats. We used immature male rats to examine the *in vivo* effects of PNP on plasma concentrations of testosterone, immunoreactive (ir)-inhibin, corticosterone, basal luteinizing hormone (LH), follicle-stimulating hormone (FSH), and prolactin (PRL).

Results

Body weight and organ weights

We evaluated changes in body weight and the weights of the liver, kidneys, adrenal glands, testes, epididymis, ventral prostate (VP), seminal vesicles plus coagulating glands (SV), levator ani plus bulbocavernosus muscles (LABC), Cowper's gland (COW), and glans penis (GP) of immature male rats treated with PNP for 14 days (Table 1). The rats in all treatment groups grew normally, and during the

14-day treatment with PNP, there were no significant differences in changes of body weight (data not shown) or the organ weights among the groups (Table 1).

Effects of PNP on plasma hormones

We analyzed the plasma concentrations of LH, FSH, PRL, testosterone, ir-inhibin, and corticosterone in immature rats treated with PNP for 14 days (Fig. 1). Plasma concentrations of LH were significantly lower ($P < 0.05$) in all PNP treatment groups than in the control group (Fig. 1a). Plasma concentrations of FSH were significantly lower ($P < 0.05$ or $P < 0.01$) in the 0.1, 1, and 10 mg/kg PNP treatment groups than in the controls (Fig. 1b). Plasma concentrations of PRL were significantly higher ($P < 0.05$) in the 10 mg/kg PNP group than in the controls (Fig. 1c). Plasma concentrations of testosterone were significantly higher ($P < 0.05$) in the 10 mg/kg PNP group than in the controls (Fig. 1d). Plasma concentrations of ir-inhibin were significantly greater ($P < 0.05$ or $P < 0.01$) in the groups treated with 0.1, 1, or 10 mg/kg PNP than in the controls (Fig. 1e). Plasma concentrations of corticosterone were significantly greater ($P < 0.05$ or $P < 0.01$) in all PNP treatment groups than in the controls (Fig. 1f).

Discussion

Administration of PNP to immature male rats caused significant decreases in plasma concentrations of LH and

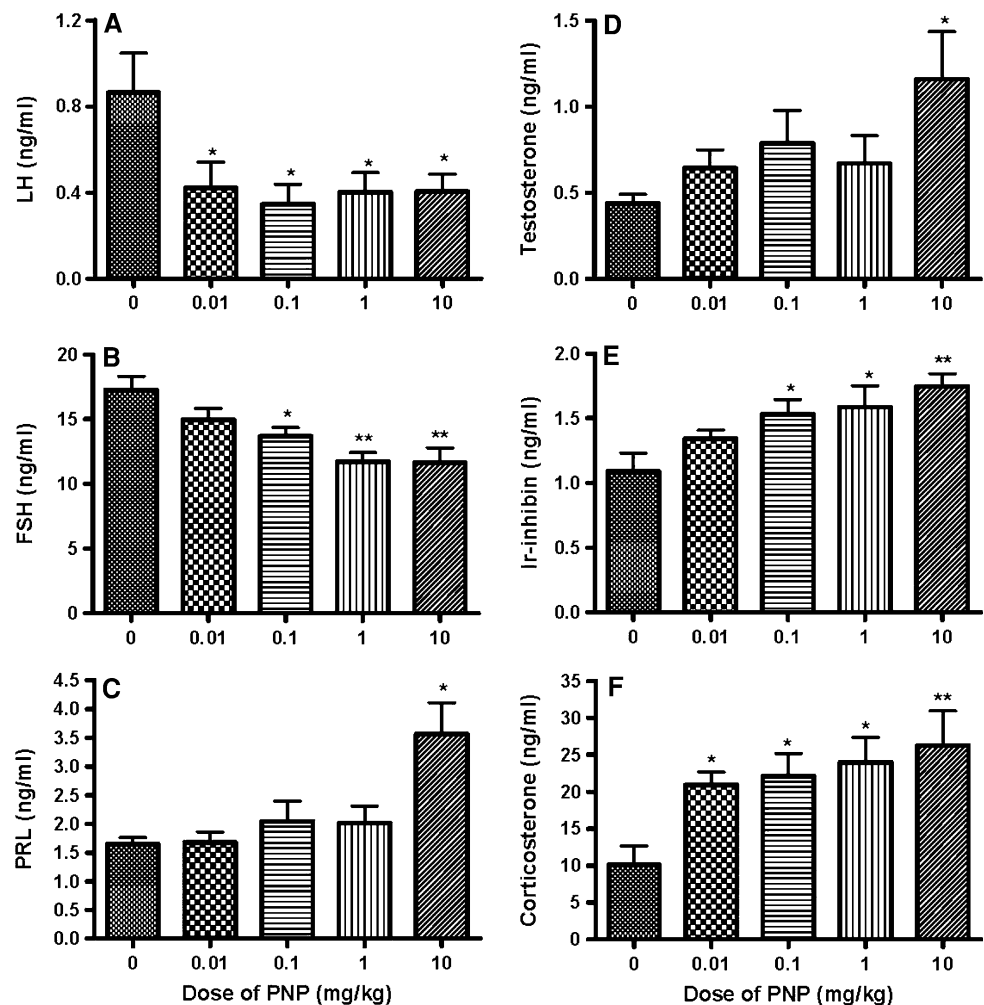
Table 1 Body weights and organ weights of immature rats at the end of treatment with 4-nitrophenol (PNP) for 14 days

	Control	PNP (mg/kg daily)			
	0	0.001	0.1	1	10
Number of animals	10	10	10	10	10
Body weight (g)	196.0 ± 3.0	201.7 ± 1.5	196.0 ± 2.2	189.5 ± 2.8	193.0 ± 3.1
Weight gain (g)	117.8 ± 2.7	124.6 ± 1.7	120.2 ± 1.8	113.4 ± 2.3	115.2 ± 2.3
Liver (g)	9.5 ± 0.2	9.8 ± 0.2	9.8 ± 0.2	9.3 ± 0.3	9.4 ± 0.3
Kidneys (g)	1.88 ± 0.04	1.85 ± 0.02	1.74 ± 0.04	1.82 ± 0.06	1.85 ± 0.04
Testes (g)	1.56 ± 0.05	1.58 ± 0.06	1.54 ± 0.03	1.55 ± 0.04	1.52 ± 0.08
Epididymis (mg)	246.9 ± 9.5	271.6 ± 14.9	232.0 ± 19.3	267.5 ± 14.7	257.2 ± 14.7
VP (mg)	100.4 ± 8.3	93.1 ± 8.7	84.1 ± 6.9	87.6 ± 4.8	90.7 ± 7.6
SV (mg)	150.2 ± 18.2	166.0 ± 14.9	128.2 ± 9.6	125.4 ± 14.0	127.6 ± 10.8
LABC (mg)	250.7 ± 8.4	263.4 ± 12.2	237.2 ± 10.7	237.8 ± 10.5	248.8 ± 16.2
COW (mg)	21.3 ± 1.4	18.8 ± 2.1	21.4 ± 1.8	19.2 ± 1.2	18.5 ± 1.1
GP (mg)	73.5 ± 3.4	76.1 ± 7.0	77.6 ± 4.6	73.8 ± 4.2	69.5 ± 5.3
COW (mg)	21.3 ± 1.4	18.8 ± 2.1	21.4 ± 1.8	19.2 ± 1.2	18.5 ± 1.1

Values are expressed as mean ± SEM for 10 animals

Weight gain, after treatment of body weight – before treatment of body weight; VP ventral prostate; SV seminal vesicles plus coagulating glands; LABC levator ani plus bulbocavernosus muscles; COW Cowper's gland; GP glans penis

Fig. 1 Plasma concentrations of LH (a), FSH (b), PRL (c), testosterone (d), immunoreactive (ir)-inhibin (e), and corticosterone (f) in immature rats treated with 4-nitrophenol (PNP) at doses of 0, 0.01, 0.1, 1, or 10 mg/kg/day for 14 days. Each bar represents the mean \pm SEM of 7 or 10 rats per group. * $P < 0.05$, ** $P < 0.01$ compared with control rats (Dunnett's multiple comparison test)



FSH, together with increases in the plasma concentrations of testosterone, ir-inhibin, corticosterone, and PRL (Fig. 1). These results clearly demonstrate that PNP has endocrine-disruptive effects on the hypothalamic–pituitary–testicular axis, reducing LH and FSH secretion from the pituitary gland and increasing testosterone and inhibin secretion from the testis. Testosterone is synthesized mainly in the Leydig cells of the rat testis, and LH is the major stimulant of testosterone secretion by these cells [19, 20]. Inhibin is secreted mainly by the Sertoli cells of the testes in the male rat, and FSH is the major stimulant of inhibin secretion by these cells [21]. These findings suggest that the decreased circulating levels of LH and FSH may be due to negative feedback by the high testosterone and inhibin levels, although the suppressive effects of PNP on gonadotropin-releasing hormone (GnRH) is not excluded. The present findings also suggest that PNP has a suppressive effect on the hypothalamic–pituitary axis.

Administration of PNP significantly increased plasma concentrations of corticosterone in a dose-dependent manner (Fig. 1e). This elevation of plasma levels of corticosterone may suppress the sensitivity of gonadotroph cells to

GnRH, thereby inhibiting gonadotrophin secretion [22]. The low plasma levels of gonadotropins observed may therefore be due to increased secretion of glucocorticoids from the adrenal glands, as evidenced by the high corticosterone levels in PNP-treated rats. Previous study showed that together with the increased plasma PRL concentration, a high level of plasma corticosterone in response to restraint stress was observed [23]. Our results also showed that administration of PNP significantly increased plasma concentrations of PRL and corticosterone. In addition, after PNP treatment at 10 mg/kg both the plasma testosterone concentration and plasma PRL concentration were significantly greater than in the controls (Fig. 1c, d). Previous reports have shown that testosterone increases PRL mRNA levels and stimulates PRL synthesis and release by lactotrophs in the anterior pituitary of the male rat [24, 25]. Previous studies have reported that PNP isolated from DEP have estrogenic activity in vivo and in vitro [16, 26, 27]; estradiol treatment increases plasma corticosterone concentrations in male rats [28]; and the estrogenic chemical bisphenol-A stimulates PRL release in vitro and in vivo [29]. In addition, estrogens could stimulate PRL secretion

by acting directly on the lactotrophs [30]. All of these results suggest that the estrogenic effects of PNP are involved in the impairment of hormone secretion in PNP-treated immature male rats.

4-Nitrophenol treatment had no effect on the normal growth of rats, nor did it cause differences in the weights of the testes or the accessory reproductive organs, even though testosterone levels increased. These results suggested that increased plasma testosterone levels did not contribute to the growth of the accessory reproductive organs. These findings also suggested that PNP treatment did not contribute to puberty. In addition, increased plasma testosterone levels may depress the levels of LH and FSH, which are needed to maintain normal testicular function. We reported previously that PNP isolated from DEP showed estrogenic activity *in vivo* and *in vitro* [16, 26, 27]. Previous studies have reported that the estrogenic chemical bisphenol-A inhibits the function of accessory reproductive organs [31], and estrogens such as estradiol and diethylstilbestrol inhibit the development of spermatogonia and the function of Leydig and Sertoli cells in the fetal rat testis [32]. These results suggest that the increase in testosterone levels caused by PNP exposure is balanced by the estrogenic activity of PNP, thus preventing significant changes in the weights of the accessory reproductive organs. In addition, PNP has anti-androgenic activity *in vitro* [16] and *in vivo* [17]. Previous reports have shown that flutamide, a potent androgen antagonist, decreases the weights of the accessory sex organs in rats [33, 34]. These results suggest that both the estrogenic and the anti-androgenic potencies of PNP are involved in the disruption of reproductive organ weights in PNP-treated rats.

In conclusion, our results showed that PNP in DEP impaired reproductive function in immature male rats by disturbing the hypothalamic–pituitary–testicular axis. The present findings clearly demonstrate that PNP has an endocrine-disruptive effect on male reproductive function.

Materials and methods

Chemicals

4-Nitrophenol (*p*-nitrophenol; PNP) was purchased from Tokyo Kasei Kogyo Tokyo, Japan).

Animals

Male Wistar-Imamichi rats (age, 21 days) were purchased from Imamichi Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan). They were kept in a controlled environment under 12 h light–12 h darkness, a temperature of $23 \pm 2^\circ\text{C}$, humidity of $50 \pm 10\%$, and ventilation with

fresh-air changes hourly. Food (CE-2 commercial diet, Japan Clea Co., Tokyo, Japan) and water were available *ad libitum*. This study was conducted with the approval of the Japanese National Institute for Environmental Studies Ethics Committee for Experimental Animals.

Administration of PNP

Twenty-eight-day-old rats were injected subcutaneously with PNP (0.01, 0.1, 1, or 10 mg/kg body weight) daily for 14 days. Rats injected with vehicle alone (PBS containing 0.05% Tween 80) were used as the control group. Twenty-four hours after the last injection, rats were weighed and decapitated. Blood samples were collected in plastic tubes containing heparin and were centrifuged at 1,700g for 15 min at 4°C . Plasma was separated and stored at -20°C until assayed for LH, FSH, PRL, testosterone, corticosterone, and ir-inhibin. Accessory reproductive glands (VP, SV, LABC, COW, GP) were excised, carefully trimmed of excess adhering tissue and fat, and immediately weighed. The adrenals, liver, and kidneys were also weighed.

Radioimmunoassay (RIA)

Plasma concentrations of LH, FSH, and PRL were measured by using NIDDK rat radioimmunoassay (RIA) kits (Torrance, CA, USA) for rat LH, FSH, and PRL. The antisera used were anti-rat LH-S-11, anti-rat FSH-S-11, and anti-rat PRL-S-9. Results were expressed in terms of the NIDDK rat LH-RP-3, FSH-RP-2, and PRL-RP-2, respectively. The sensitivities of the assay were 3.9 pg/tube for LH, 78.1 pg/tube for FSH, and 4.9 pg/tube for PRL, and the respective intra- and interassay coefficients of variation were 5.4% and 6.9% for LH, 4.3% and 10.3% for FSH, and 3.4% and 5.2% for PRL.

Plasma concentrations of ir-inhibin were measured as described previously [35]. The iodinated preparation was 32-kDa bovine inhibin, and the antiserum used was rabbit antiserum against bovine inhibin (TNDH-1). Results were expressed in terms of 32-kDa bovine inhibin. The sensitivity of the assay was 1.95 pg/tube, and the intra- and interassay coefficients of variation were 8.8% and 14.4%, respectively.

Plasma concentrations of testosterone and corticosterone [36] were determined with a double-antibody RIA system with ^{125}I -labeled radioligands, as described previously [37]. The antiserum against testosterone (GDN 250) [38] was kindly provided by Dr. G. D. Niswender of Colorado State University (Fort Collins, CO, USA). The sensitivities of the assay were 0.3 pg/tube for testosterone, 0.6 pg/tube for corticosterone, and the respective intra- and interassay coefficients of variation were 6.3% and 7.2% for testosterone and 9.5% and 16.4% for corticosterone.

Statistical analysis

All data are presented as mean \pm standard error of the mean (SEM) and were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test. Statistical analysis was performed with GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Differences were considered statistically significant when the *P* level was less than 0.05.

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