Endocrine Disruptive Effect of 3-Methyl-4-nitrophenol Isolated from Diesel Exhaust Particles in Hershberger Assay Using Castrated Immature Rats

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To examine the endocrine disruptive effects of 3-methyl-4-nitrophenol (4-nitro-m-cresol; PNMC) in diesel exhaust particles (DEP), the rat Hershberger assay was carried out using castrated immature rats. Castrated 28-d-old immature male rats were implanted with a 5-mm-long silastic tube containing crystalline testosterone and injected with PNMC subcutaneously at doses 1, 10, or 100 mg/kg for 5 consecutive d. The weights of the livers significantly decreased in the 10 and 100 mg/kg PNMC treatment groups as compared with the control group. The weights of the seminal vesicles significantly increased in the 10 mg/kg PNMC treatment group as compared with the control group. The weights of the Cowper’s glands were significantly increased in 1 mg/kg PNMC treatment group compared with the control group. The concentrations of plasma testosterone significantly increased in the 10 and 100 mg/kg PNMC treatment groups, indicating that PNMC induced accumulation of bioactive testosterone released from the implanted tube in circulation. Plasma follicle-stimulating hormone (FSH) and luteinizing hormone (LH) levels significantly decreased under all the doses in the PNMC treatment groups, indicating that PNMC acts on the hypothalamus-pituitary axis.

Key words: 3-methyl-4-nitrophenol; diesel exhaust particles; Hershberger assay; hepatotoxicity

Diesel exhaust particles (DEPs) generated by motor vehicles are a primary cause of air pollution. These particles contain thousands of compounds that have hazardous effects on human health, including lung cancer,1,2 allergic rhinitis,3,4 and bronchial asthma-like disease.5,6 Furthermore, DEPs disrupt endocrine systems, with potential adverse impacts on male and female reproductive functioning.7–12 In vitro findings support these observations; the compounds contained in DEPs modulate the activity of estrogens and anti-androgens.13–16 However, because DEPs contain carbon nuclei can absorb a vast number of chemicals, the specific compound responsible for this phenomenon remains unclear.

We isolated four nitrophenol derivatives, 4-nitrophenol, 2-methyl-4-nitrophenol, 3-methyl-4-nitrophenol (PNMC), and 4-nitro-3-phenylphenol from DEPs.17 PNMC is also a degradation product of the pesticide fenitrothion,18 which is widely used as an organophosphorus insecticide, mainly in agriculture to control chewing and sucking insects in rice, cereals, fruits, vegetables, stored grains, and cotton in many countries.19 The accumulation of PNMC from these sources has serious effects on wildlife and human health via endocrine and reproductive disruption. PNMC exhibited estrogenic activity in vitro and in vivo.20,21 PNMC also possess anti-androgenic activity in vitro and in vivo,22 a unique characteristic of many endocrine-disrupting chemicals,23 but in a preliminary study, some doses of PNMC did not show anti-androgenic activity in vivo. Here, we used the same method: rat Hershberger assays further to prevent the endocrine disruptive effects of PNMC.

Materials and Methods

Chemicals. 3-Methyl-4-nitrophenol (4-nitro-m-cresol; PNMC) was from Tokyo Kasei Kogyo Co., Ltd. (Tokyo), and 2-methyl-N-[4-nitro-3-(trifluoromethyl)phenyl]propanamide was from Sigma (flutamide; St. Louis, MO).

Animals. Immature male Wistar-Imamichi rats, 20-d old, were purchased from the Imamichi Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan). They were kept in a controlled environment under a light regimen of 12 h light/12 h dark (lights on 0700 to 1900 h), temperature (23 ± 2 °C), humidity (50% ± 10%), and ventilation (fresh-air changes hourly). Food (CE-2 commercial diet, Japan Clea Tokyo) and water were available ad libitum. This study was conducted in accordance with the Guiding Principles for the Use of Animals in Toxicology of, and was approved by the Animal Care and
Table 1. Body Weights and Organ Weights of Immature Castrate-T (5-mm silastic tube containing crystalline testosterone implant) Rats at the End of Treatment with PNMC for 5 d

<table>
<thead>
<tr>
<th></th>
<th>Control (T)</th>
<th>PNMC (mg/kg daily)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 + T</td>
<td>10 + T</td>
</tr>
<tr>
<td>Number of animals</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>92.26 ± 1.93</td>
<td>89.35 ± 1.76</td>
</tr>
<tr>
<td>Weight gain (g)</td>
<td>24.19 ± 1.17</td>
<td>23.60 ± 0.71</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>4.86 ± 0.14</td>
<td>4.51 ± 0.12</td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>0.96 ± 0.02</td>
<td>1.02 ± 0.02</td>
</tr>
<tr>
<td>Adrenals (mg)</td>
<td>23.00 ± 1.99</td>
<td>23.63 ± 1.34</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM for eight animals.

T. testosterone

**p < 0.01 as compared with the value for the control group (Dunnett’s multiple comparison test)

Use Committee of the Japanese National Institute for Environmental Studies.

**Hershberger assay.** The Hershberger assay was performed in accordance with the current draft guidelines for the rodent Hershberger assay. Immature male rats aged 21 d were castrated 1 week before the experiment. At 28 d of age each rat was weighed and implanted with a 5-mm-long silastic tube (1.57 mm ID, 3.18 mm OD; Dow Corning, Midland, MI) containing crystalline testosterone. The testosterone-containing tubes were incubated in saline at 37 °C for 24 h before implantation to avoid the possibility of surge-like release after implantation. An implant of this size restores serum testosterone to a physiological level of approximately 1.8 ng/ml. PNMC (1, 10, or 100 mg/kg) was then administered for 5 d by subcutaneous injection. The rats in the negative control group were injected with vehicle alone (phosphate-buffered saline, PBS containing 0.05% Tween 80). Twenty-four h after the last injection, the rats were weighed and killed. Blood samples were collected in plastic tubes containing heparin and were centrifuged at 1,700 × g for 15 min at 4 °C. Plasma was separated and stored at −20 °C until assay for follicle-stimulating hormone (FSH), luteinizing hormone (LH), and testosterone. Five androgen dependent accessory reproductive glands (the ventral prostate, seminal vesicles, levator ani plus bulbocavernosus muscles, Cowper’s gland, and the glans penis) were excised, carefully trimmed of excess adhering connective tissue and fat, and weighed immediately. The liver, kidneys, and adrenal glands were also weighed.

**Radioimmunoassay.** The plasma concentrations of FSH and LH were measured with National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) rat radioimmunoassay (RIA) kits (Torrance, CA, USA) for rat FSH and LH. The antisera used were anti-rat FSH-S-11 and anti-rat LH-S-11. The intra- and inter-assay coefficients of variation were 4.3 and 10.3% for FSH and 5.4 and 6.9% for LH totally.

The plasma concentrations of testosterone were determined by a double-antibody RIA system with I labeled radioligands, as described previously. Antiserum against testosterone (GDN 250) was kindly provided by Dr. G. D. Niswender, Colorado State University (Fort Collins, CO). The intra- and inter-assay coefficients of variation were 6.3 and 7.2% respectively.

**Statistical analysis.** All data are presented as mean ± standard error of the mean (SEM). They 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). Differences were considered statistically significant when the p level was less than 0.05.

**Results**

**Hershberger assay**

The castrated-immature, testosterone-treated male rats in all the treatment groups grew normally, and during the 5-d treatment with PNMC, there were no significant differences in changes in body weight (Fig. 1) or the weights of the kidneys or adrenals (Table 1). However, the weights of the livers decreased significantly (p < 0.01) in the groups treated with 10 or 100 mg/kg PNMC as compared with the control group (Table 1).

The weights of the androgen-dependent accessory reproductive glands (ventral prostate, seminal vesicles, levator ani plus bulbocavernosus muscles, Cowper’s gland, and glans penis) are shown in Table 2. The weight of seminal vesicles significantly increased (p < 0.05) at a PNMC dose of 10 mg/kg compared with the control group. The weight of Cowper’s gland also significantly increased (p < 0.01) at a PNMC dose of 1 mg/kg as compared with the control group. The weights of ventral prostate, levator ani plus bulbocavernosus muscles, and glans penis showed increases although the changes were not significant (Table 2). The weights of ventral prostate, seminal vesicles, levator ani plus bulbocavernosus muscles, and glans penis significantly decreased (p < 0.01) in the castrated rats as compared with the castrated, testosterone-treated rats (the control group).

**Plasma concentrations of FSH, LH, and testosterone**

The plasma concentrations of FSH, LH, and testosterone are shown in Fig. 2. Plasma FSH and LH levels were significantly lower (p < 0.01) at all doses in the PNMC treated groups as compared with the control group (Fig. 2A, B). The plasma concentrations of testosterone were significantly high (p < 0.05 and p < 0.01) in the 10 and 100 mg/kg PNMC-treated groups as compared with the control group (Fig. 2C).
Table 2. Weights of Accessory Sex Organs in Immature Castrate-T (5-mm silastic tube containing crystalline testosterone implant) and Castrate Rats after Treatment with PNMC for 5 d

<table>
<thead>
<tr>
<th>Description</th>
<th>Castrate (no T)</th>
<th>Control (T)</th>
<th>PNMC (mg/kg daily)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of animals</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Ventral prostate (mg)</td>
<td>8 ± 1**</td>
<td>30.00 ± 1.85</td>
</tr>
<tr>
<td></td>
<td>Seminal vesicle (mg)</td>
<td>10 ± 2**</td>
<td>48.25 ± 3.11</td>
</tr>
<tr>
<td></td>
<td>LABC (mg)</td>
<td>39 ± 2**</td>
<td>70.75 ± 4.50</td>
</tr>
<tr>
<td></td>
<td>Glans penis (mg)</td>
<td>37 ± 2**</td>
<td>82.25 ± 3.09</td>
</tr>
<tr>
<td></td>
<td>Cowper’s glands (mg)</td>
<td>No</td>
<td>5.92 ± 0.13</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SEM for six or eight animals.

T. testosterone; LABC, levator ani plus bulbocavernous muscles
*p < 0.05, **p < 0.01 as compared with the value for the control group (Dunnett’s multiple comparison test)

Fig. 2. Plasma Concentrations of FSH, LH, and Testosterone.
Plasma concentrations of FSH (A), LH (B), and testosterone (C) in immature castrate-T (5-mm silastic tube containing crystalline testosterone implant) rats treated with 3-methyl-4-nitrophenol (PNMC) at doses of 0, 1, 10, or 100 mg/kg/d for 5 d. Each bar represents the mean ± SEM for eight rats per group. **p < 0.01, *p < 0.05 as compared with the control rats (Dunnett’s multiple comparison test).

Discussion

The present study used the Hershberger assay to examine the endocrine disruptive effects of PNMC based on the responses of androgen-dependent tissues and hormones in castrated immature rats. Treatment with PNMC decreased the liver weight and increased the weights of seminal vesicles and Cowper’s glands, and the plasma concentration of testosterone, but the plasma FSH and LH levels decreased. Testosterone has been found to have an androgenic effect by increasing the accessory reproductive organ weight, which has been studied using both castrated immature and adult male rats.28) In the present study, although the organ weights, including those of the ventral prostate, levator ani plus bulbocavernous muscles, glans penis, kidneys, and adrenal glands showed no significant changes, they tended to increase in the PNMC-treatment groups as compared to the control group. In the present study, we supposed that PNMC at high doses might decrease the testosterone-stimulated weights of the accessory reproductive organs, in agreement with our previous study,22) but 1, 10, and 100 mg/kg doses of PNMC caused increases in the weights of the accessory reproductive organs, and did not show anti-androgenic activity in the present study.

In this study, the plasma concentrations of testosterone increased on treatment with 10 and 100 mg/kg of PNMC with 5-mm-long silastic testosterone tube implantation as compared with 5-mm silastic testosterone tube implantation alone. We found the same testosterone level in the plasma of castrated-immature rats implanted with the same size 5-mm-long silastic tube because it contained the same concentration of testosterone, but the results were that the concentration of testosterone increased in PNMC-treated group. It is possible that PNMC disrupts the androgen-metabolizing enzymes in castrated rats.29) Hepatic P450 enzyme modulation might alter the metabolism and elimination of testosterone, and this lead to accumulation in circulating testosterone levels and hormonal activity.30,31) In addition, treatment with 10 and 100 mg/kg of PNMC decreased liver weights, but there was no change from our previous study.22) These results suggest that the increasing plasma testosterone levels of PNMC are due to hepatotoxicity which reduces metabolic deactivation in the liver.

Plasma LH and FSH levels decreased with increased testosterone levels in the 10 and 100 mg/kg PNMC treated groups in the present study. On the other hand, the 1 mg/kg PNMC treated castrated rats showed low plasma levels of LH, FSH, although concentrations of testosterone were unchanged. These results suggest that PNMC acts directly on the hypothalamus–pituitary axis to reduce LH and FSH secretion from the anterior pituitary gland. Confirming the present results, a previous study in Japanese quails and male rats clearly demonstrated that PNMC acts on the hypothalamus-
pituitary axis by reducing circulating LH and subsequently reducing testosterone secretion in vivo and in vitro. In addition, previous studies have reported that PNMC had estrogenic activity in vivo and in vitro and that estradiol treatment decreased plasma LH and subsequently reducing testosterone secretion. All of these results and that estradiol treatment decreased plasma LH and subsequently reducing testosterone secretion. Pituitary axis by reducing circulating LH and subsequently reducing testosterone secretion. Plasma FSH and LH levels in castrated-immature rats treated with implanted testosterone tubes, but plasma testosterone levels were relatively constant. These results indicate that 0.1 mg/kg of PNMC has anti-androgenic effects on the hypothalamus-pituitary axis and the accessory reproductive glands, but the doses of PNMC of the present study did not.

In conclusion, the present study suggests that PNMC has toxic effects on the hypothalamus-pituitary axis and the liver. Its toxic effects on the hypothalamus-pituitary axis and the liver reducing gonadotropin secretion from the pituitary gland, and inhibits androgen-metabolizing enzymes in the liver.

Acknowledgments

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