Effects of 3-Methyl-4-nitrophenol on the Suppression of Adrenocortical Function in Immature Male Rats

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In previous studies, we found that 3-methyl-4-nitrophenol (4-nitro-*m*-cresol; PNMC) isolated from diesel exhaust particles, and also a degradation product of the insecticide fenitrothion, exhibited testicular toxicity in the male of both immature rat and adult Japanese quail. It is well established that a functional relationship exists between the gonads and adrenals. The present study investigates the effect of PNMC on the adrenocortical functions of immature male rats. We subcutaneously injected 28-d-old rats with PNMC (1, 10 or 100 mg/kg) daily for 5 d. The adrenal glands weights significantly decreased in rats treated with 10 or 100 mg/kg PNMC. Plasma concentrations of adrenocorticotrophic hormone (ACTH) were significantly increased in animals treated with 100 mg/kg PNMC. In contrast, plasma concentrations of corticosterone were significantly decreased in all PNMC-treated groups, and plasma concentrations of progesterone were significantly decreased in rats treated with 10 or 100 mg/kg PNMC. To investigate the direct effects of PNMC on the secretion of ACTH from the anterior pituitary gland, and on the secretion of corticosterone from the adrenal, we exposed cultured primary anterior pituitary and adrenal cells to PNMC (10^{-8} , 10^{-7} , 10^{-6} , or 10^{-5} M) for 24 h. PNMC did not change basal levels of ACTH released from cultured anterior pituitary cells. However, PNMC significantly inhibited ACTH-stimulated production of corticosterone and progesterone from cultured adrenal cells. These results clearly show that PNMC has a direct effect on the adrenal gland to reduce corticosterone secretion, and the associated increase in plasma ACTH is probably due decreased negative feedback regulation by corticosterone.

Key words 3-methyl-4-nitrophenol; adrenocortical function; rat; diesel exhaust particle; insecticide fenitrothion

Air pollution is a serious problem throughout the world, and diesel exhaust particles (DEP) are the major contributor to it. DEP contain many kinds of compounds that have several hazardous effects on human health, including 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 for adversely affecting male reproductive functions. Diesel exhaust suppresses spermatogenesis in adult mice⁷⁾ and rats,^{8,9)} and pregnant C57BL mice injected with DEP extracts show significant increases in abortion rate and uterine weight.¹⁰⁾ DEP extracts have been found to possess estrogenic, anti-estrogenic, and anti-androgenic activities.^{11–14)} However, because DEP contain carbon nuclei, which absorb a vast number of chemicals, the specific compound responsible for this phenomenon remains unclear.

3-Methyl-4-nitrophenol (PNMC), a nitrophenol derivative isolated from DEP has been shown to be a vasodilator^{15,16)}; it also has estrogenic^{17–19)} and anti-androgenic activities.^{17,20)} In addition, PNMC is a degradation product of the pesticide fenitrothion,^{21,22)} which is widely used as an organophosphorus insecticide and mainly used in agriculture for controlling chewing and sucking insects on rice, cereals, fruits, vegetables, stored grains, and cotton in many countries. Data provided by the Pollutant Release and Transfer Registers (PRTR), reveal that the amount of fenitrothion discharged into the environment in Japan in 2002 was approximately 1.3×10^6 kg, roughly half of which was degraded into PNMC.²²⁾ The accumulation of PNMC from these sources has serious effects on wildlife and human health via endocrine and reproductive disruptions.

It is well known that the adrenal gland is an important endocrine organ, producing and secreting glucocorticoids in response to stress, infection, and trauma, thereby supporting homeostasis of the organism. And, it is well established that a functional relationship exists between the gonads and adrenals.²³⁾ Our previous *in vivo* studies showed that a single administration of PNMC in adult male Japanese quail induced marked testicular atrophy²⁴⁾ and suppression of testicular function in immature rat.²⁵⁾ Furthermore, previous studies have not addressed the effects of PNMC on adrenal function. To this end, we used immature male rats to examine the *in vivo* and *in vitro* effects of PNMC on the pituitary–adrenal axis.

MATERIALS AND METHODS

Chemicals 3-Methyl-4-nitrophenol (4-nitro-*m*-cresol; PNMC) (Fig. 1) was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Collagenase (type IV), hyaluronidase, deoxyribonuclease I (DNase I), fetal bovine serum (FBS), and bovine serum albumin (BSA) were purchased from Sigma (St. Louis, MO, U.S.A.), and Dulbecco's modified Eagle medium (DMEM) and penicillin–streptomycin from Gibco (Grand Island, NY, U.S.A.). Rat adrenocorticotrophic hormone (ACTH; AFPRFR 7890) (NIDDK, NIH, Torrance, CA, U.S.A.) was purchased from the sources indicated.

Animals We purchased 21-d-old male Wistar–Imamichi rats from the Imamichi Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan). They were kept in a controlled environment (lights on, 0700–1900 h; temperature,

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Fig. 1. Chemical Structure of 3-Methyl-4-nitrophenol (4-Nitro-*m*-cresol; PNMC)

PNMC, a component of diesel exhaust particles and a degradation product of the insecticide fenitrothion.

23±2 °C; humidity, 50±10%; air exchanged 20 times per hour). Food (CE-2 diet, Japan Clea Co., Tokyo, Japan) and water were available *ad libitum*. This study was conducted in accordance with the Guiding Principles for the Use of Animals in Toxicology and was approved by the Animal Care and Use Committee of the Japanese National Institute for Environmental Studies.

Administration of PNMC We subcutaneously injected 28-d-old rats with 1, 10, or 100 mg/kg PNMC (in phosphate-buffered saline [PBS] containing 0.05% Tween 80) daily for 5 d. Additional rats were injected with vehicle alone as the negative-control group. At 24 h after the last injection, rats (9 per group) were weighed and decapitated. Blood samples were collected into plastic tubes containing heparin and centrifuged at 1700×*g* for 15 min at 4 °C. Plasma was separated and stored at −20 °C until assayed for adrenocorticotrophic hormone (ACTH), progesterone, and corticosterone. The adrenal glands were excised, carefully trimmed of excess adhering tissue and fat, and immediately weighed.

Preparation of Anterior Pituitary Cells and Cell Cultures Anterior pituitary cells were prepared from 28-d-old male rats. All culture media contained 100 U/ml penicillin and 100 μg/ml streptomycin. The methods used for the preparation of pituitary cell cultures were similar to those described previously,²⁶ with minor modifications. Briefly, anterior pituitaries were minced into DMEM with 2.8 mg/ml collagenase, 0.8 mg/ml hyaluronidase, 8 mg/ml BSA, and 200 U/ml DNase I incubated the reactions at 37 °C for 30 min. The cells were then washed three times with DMEM containing 10% FBS and the supernatant cells were filtered through cell strainers (pore size 70-μm nylon mesh; Falcon, BD Biosciences, Bedford, MA, U.S.A.). The viability of the cells was evaluated by means of the trypan blue exclusion test and found to be approximately 90%. Pituitary cells (2×10⁴ cells/well) were cultured in DMEM with 10% FBS in 96-well culture plates at 37 °C under an atmosphere of 95% air–5% CO₂. After 78 h of preculture and a change of medium, cells were exposed for 24 h to 10^{−8}, 10^{−7}, 10^{−6}, or 10^{−5} M PNMC dissolved in DMEM, and then the medium was collected from each well. Culture media were stored at −20 °C until assayed for ACTH.

Isolation and Culture of Primary Adrenal Cells

Adrenal glands were obtained from 28-d-old male rats. Animals were killed by decapitation, and adrenal glands were removed immediately. For isolation of adrenal cells, whole glands were used, and the procedures used followed previously described methods²⁷ with minor modifications. Briefly, the isolation and dissociation of adrenal cells were performed at 37 °C for 30 min in DMEM with collagenase (2 mg/ml) and DNase I (200 U/ml) in a shaking water bath. The cells then were washed three times with DMEM containing 2% FBS, and the cells in the supernatant were filtered through cell strainers (70-μm nylon mesh; Falcon). Cells were used in suspension at a concentration of 10⁵ cells/ml, in DMEM medium supplemented with 2% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The viability of the cells was evaluated by means of the trypan blue exclusion test and found to be approximately 93%. Adrenal cells (2×10⁴ cells/well) were cultured in 96-well culture plates for 24 h at 37 °C under an atmosphere of 95% air–5% CO₂. The isolated adrenal cells were cultured for 24 h and then incubated for an additional 24 h in the presence of 10^{−8}, 10^{−7}, 10^{−6}, or 10^{−5} M PNMC dissolved in DMEM. After final incubation with PNMC, culture media were replaced with fresh media with 1 nM ACTH (to stimulate cells) or vehicle only, the cells were incubated for another 4 h, and then the culture medium was collected from each well. Culture media were stored at −20 °C until assayed for corticosterone and progesterone.

Radioimmunoassay Concentrations of ACTH in samples of plasma and culture medium were measured by double-antibody RIAs using ¹²⁵I-labeled radioligands, as described previously.²⁸ Synthetic rat ACTH 1-39 (Sigma) was used as the reference standard. The intra- and inter-assay coefficients of variation were 11.3% and 11.9%, respectively. Concentrations of progesterone²⁹ and corticosterone³⁰ in samples of plasma and culture medium were determined by use of double-antibody RIA systems with ¹²⁵I-labelled radioligands as described previously. The intra- and inter-assay coefficients of variation were 6.9% and 11.2% for progesterone and 9.5% and 16.4% for corticosterone, respectively.

Statistical Analysis All data are presented as means±S.E.M. Statistical analysis was performed using one-way analysis of variance followed by Fisher's protected least significant difference test (Fisher's PLSD; StatView 5.0, SAS Institute Inc., Cary, NC, U.S.A.). The criterion for significance was *p*<0.05.

RESULTS

Effects of PNMC on Adrenal Glands Weights The rats in all treatment groups grew normally, and there were no differences in the body weight among the groups. Absolute and relative adrenal glands weights (Table 1) were decreased significantly (*p*<0.05) in the groups treated with 10 and 100 mg/kg PNMC compared with the control group.

Effects of PNMC on Plasma ACTH, Corticosterone and Progesterone Plasma concentrations of ACTH increased significantly (*p*<0.05) in the group treated with 100 mg/kg PNMC daily for 5 d compared with the control group (Fig. 2A). Plasma concentrations of corticosterone decreased significantly (*p*<0.01) in all PNMC-treated groups (Fig. 2B), and plasma concentration of progesterone in the groups treated with 10 or 100 mg/kg PNMC were signifi-

Table 1. Adrenal Gland Weights of Immature Male Rats Treated with 3-Methyl-4-nitrophenol (PNMC) for 5 d

	Control	PNMC (mg/kg/d)		
	0	1	10	100
Number of animals	9	9	9	9
Body weight (g)	97.6±1.7	101.6±1.1	98.0±1.2	99.9±1.6
Weight gain (g)	24.5±0.9	25.2±0.5	23.7±1.3	24.9±1.9
Adrenal glands weight (mg)	32.6±1.7	29.9±1.6	28.6±1.0*	28.0±0.7*
Adrenal glands weight/ body weight (mg/g)	0.335±0.02	0.294±0.01	0.292±0.01*	0.281±0.01*

Values are expressed as means±S.E.M. * $p<0.05$ compared with value for the control group (analysis of variance and Fisher's PLSD).

Fig. 2. Effects of PNMC on Plasma ACTH, Corticosterone and Progesterone

Plasma concentrations of ACTH (A), corticosterone (B) and progesterone (C) in immature male rats treated with PNMC at doses of 1, 10, or 100 mg/kg daily for 5 d. Each bar represents the mean±S.E.M. of 9 rats per group. * $p<0.05$ and ** $p<0.01$ compared with control rats (Fisher's PLSD).

cantly low as compared with the control group ($p<0.05$ and $p<0.01$, respectively; Fig. 2C).

Effect of *in Vitro* Exposure of Anterior Pituitary Cells to PNMC on ACTH Production To determine the direct effects of PNMC on ACTH secretion, we cultured anterior pituitary cells in the presence of PNMC for 24 h and then measured the concentration of ACTH in culture media. High concentrations of PNMC tended to decrease the basal release of ACTH from pituitary cells, but the differences were not statistically significant (Fig. 3).

Effect of *in Vitro* Exposure of Adrenal Cells to PNMC on Corticosterone and Progesterone Production To determine the direct effects of PNMC on adrenal corticosterone and progesterone production, we cultured freshly isolated adrenal cells in the presence of PNMC for 24 h and then measured the concentration of corticosterone and progesterone in the culture media. Compared with ACTH unstimulated control values, basal (unstimulated) corticosterone concentra-

Fig. 3. Effect of PNMC on Adrenocorticotrophic Hormone (ACTH) Secretion from Anterior Pituitary Cells

Anterior pituitary cells were incubated for 24 h with 10^{-8} , 10^{-7} , 10^{-6} , or 10^{-5} M PNMC. Each bar represents the mean±S.E.M. ($n=6$). The experiment was done 3 times, and a representative result is shown.

Fig. 4. Effect of PNMC on Adrenocorticotrophic Hormone (ACTH)-Unstimulated or Stimulated Adrenal Cells on Corticosterone and Progesterone Secretion

Adrenal cells were incubated for 24 h with 10^{-8} , 10^{-7} , 10^{-6} , or 10^{-5} M PNMC either with or without ACTH (1 nM). Each bar represents the mean±S.E.M. ($n=6$). The experiment was done 3 times, and a representative result is shown. ** $p<0.01$ compared with the ACTH unstimulation control, # $p<0.01$ compared with the ACTH stimulation control (Fisher's PLSD).

tions were decreased in cells treated with 10^{-5} M PNMC ($p<0.01$, Fig. 4A), but progesterone production was unchanged (Fig. 4B). Stimulation with ACTH in the absence of PNMC treatment caused a significantly increase in the concentration of corticosterone and progesterone compared with

basal values ($p < 0.01$, Figs. 4A,B). In contrast, PNMC significantly inhibited ACTH-induced corticosterone secretion in adrenocortical cells (Fig. 4A). Similarly to its effect on corticosterone production, PNMC significantly inhibited ACTH-induced progesterone secretion in adrenocortical cells ($p < 0.01$, Fig. 4B).

DISCUSSION

To our knowledge, this study is the first to demonstrate that a specific component of DEP directly inhibits adrenocortical function. In rats, corticosterone is the predominant adrenal steroid hormone, and its secretion is directly stimulated by ACTH secretion from corticotrophs in the anterior pituitary gland. In our study, administration of PNMC significantly increased plasma concentrations of ACTH in immature male rats. In contrast, plasma concentrations of corticosterone and progesterone significantly decreased in PNMC-treated animals. Furthermore, whereas PNMC tend to decreased ACTH production in cultured anterior pituitary cells, the pollutant did inhibit corticosterone and progesterone production in cultured primary adrenal cells. This observation is in agreement with the *in vivo* part of our study that showed increased ACTH levels and decreased corticosterone and progesterone levels in PNMC-treated rats. Together our findings demonstrate that PNMC suppresses adrenal function and that the associated increased levels of plasma ACTH might reflect reduced negative feedback regulation by corticosterone.

Although PNMC lacked any effect on the overall growth of rats, it reduced the weights of the adrenal glands and the plasma concentrations of corticosterone and progesterone. This decrease in adrenal weight suggests disruption of the negative feedback system involved in the hypothalamus–pituitary–adrenal axis. Our *in vitro* study clearly showed that PNMC significantly inhibited ACTH-induced corticosterone and progesterone in adrenocortical cells. These results suggest that the inhibitory effect of PNMC on secretion of corticosterone and progesterone maybe mediated *via* the ACTH receptor and by suppressing the steroidogenic enzymatic activity mechanism. We reported previously that PNMC isolated from DEP has estrogenic activity *in vivo* and *in vitro*.^{17–19} Estrogen inhibits steroid production by human fetal adrenal cortical cells,^{31–33} and the phytoestrogen genistein decreases serum cortisol levels in human adrenocortical H295R cells by inhibiting 3β -hydroxysteroid dehydrogenase and cytochrome P450 c21-hydroxylase activity.³⁴ These results suggest that PNMC suppress the adrenal function of rats may through interaction with effects on steroidogenic enzymes.

The present study has great significance in regard to environmental issues. Vast amounts of DEP are emitted into the atmosphere every year. For example, in Japan alone, approximately 5.9×10^7 kg of DEP are released annually,³⁵ an amount we cannot afford to ignore. A kilogram of DEP contains approximately 28 mg PNMC,^{15,16} but the exact concentrations of PNMC in the environment remain unknown. In addition, it is well known that fenitrothion, one of the most common organophosphorus insecticides worldwide, is degraded into PNMC.^{22,36} In Japan the most conspicuous use of fenitrothion is spraying on small areas such as paddy

fields or pinewoods as an insecticide. In one government report, the maximal concentration (5 d average) of fenitrothion was $6.5 \mu\text{g}/\text{m}^3$ (1 h peak; $22 \mu\text{g}/\text{m}^3$) measured at close proximity of a spraying by automatic helicopter on pinewoods.³⁷ The value indicates that a standard adult person (60 kg BW, inhalation volume; 15 m^3) can inhale $48.8 \mu\text{g}$ of PNMC per 24 h, about half of which is from fenitrothion. Baroja *et al.*³⁸ also reported that PNMC was detected with a concentration of $24.1 \text{ ng}/\text{m}^3$ at the sixth day and $54.7 \text{ ng}/\text{m}^3$ at the second week after the spray applications with fenitrothion in forestry atmospheres in Spain. In addition, the amount of PNMC in rainwater in Roskilde, Denmark, peaked at $83 \text{ ng}/\text{l}$ in 2000 and 2001,³⁹ and PNMC is becoming more abundant in the air and soils.⁴⁰ From these data, it is obvious that large amounts of PNMC are present in the environment because of fenitrothion use and diesel exhaust. PNMC is hardly decomposed and is taken in *via* inhalation, orally, and through the skin and this condition could have serious effects on wildlife and humans.

In conclusion, the present results demonstrate that PNMC from DEP affects on adrenal functions. PNMC acts on the adrenal glands directly to reduce corticosterone secretion, and the associated increase in plasma ACTH is probably due decreased negative feedback regulation by corticosterone. Our results suggest that PNMC accumulated in environment may have suppressive effects on adrenal function in wildlife and human beings.

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