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Immunohistological Study for Estrogenic Activities of Nitrophenols in Diesel Exhaust Particles

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We recently isolated 3-methyl-4-nitrophenol (4-nitro-*m*-cresol; PNMC) and 4-nitro-3-phenylphenol (PNMPP) from diesel exhaust particles (DEP) and identified them as vasodilators and xenoestrogens. The estrogenic activity of PNMC and PNMPP was further examined by using immunohistochemical staining of proliferating cell nuclear antigen (PCNA) in uterine luminal epithelium of ovariectomized 25-d-old immature female rats injected with PNMC and PNMPP subcutaneously for 2 d. Significant increases were observed in uterine luminal epithelium in PCNA positive cells of animals receiving 10 and 100 mg/kg PNMC and 0.1 mg/kg PNMPP compared with controls. These results clearly show the estrogenic activity of PNMC and PNMPP by cell proliferation on the uterine luminal epithelium.

Key Words: 3-Methyl-4-nitrophenol; 4-nitro-3-phenylphenol; estrogenic activity; proliferating cell nuclear antigen (PCNA); diesel exhaust particles (DEP); rat.

Introduction

Air pollution is a grave problem throughout the world, and diesel exhaust particles (DEP) are known to be a major contributor to it. Also, DEP are reported to have several hazardous effects on human health, such as lung cancer *(1,2)*, allergic rhinitis *(3,4)*, and bronchial asthma-like diseases *(5,6)*. Furthermore, DEP have exerted toxic effects on both the male and female reproductive systems in vivo *(7–10)*. These in vivo findings show DEP may contain compounds that modulate the activity of estrogen. Taneda et al. *(11,12)* reported that crude DEP and successively extracted hexane, benzene, dichloromethane, and methanol fractions from DEP showed estrogenic activity by the recombinant yeast screen assay. Also, Mori et al. *(13)* reported that 4,6- and 2,8-dimethyldibenzothiophenes isolated from DEP had estrogenic activity in recombinant yeast screen assays as well. However, DEP contains a vast number of organic compounds, and the specific compounds that are responsible for the phenomenon remain unclear. We recently isolated the nitrophenol derivatives 3- and 4-nitrophenol, 2- and 3-methyl-4-nitrophenol, and 4-nitro-3-phenylphenol from DEP, and showed that they have vasodilator activity *(14,15)*. In addition, we demonstrated the estrogenic activities of 3-methyl-4-nitrophenol (PNMC) and 4-nitro-3-phenylphenol (PNMPP) of DEP by using uterotrophic and myometrial contractility assay as functional estrogenic assays *(16)*.

The present study focused on the direct estrogenic activity of PNMC and PNMPP by using the immunohistochemistry technique of proliferating cell nuclear antigen (PCNA) in the uterine luminal epithelium of rats at the histological level.

Results

Immunolocalization in the uterine luminal epithelium of PCNA labeling for PNMC, PNMPP, and estradiol-17β treated rats are shown in Fig. 1.

Using PCNA labeling of uterine tissue sections as a marker of cell proliferation, the doses of PNMC (10 and 100 mg/kg) had a higher uterine luminal epithelium labeling index as compared with controls (277% and 327% of control value; $p < 0.05$); their estradiol-17β (5 μg/kg)–treated rats showed even greater uterine epithelial labeling index (626%; $p < 0.05$; Fig. 2). PNMPP also induced significant increases in positive staining of uterine luminal epithelium at doses of 0.1 mg/kg (164%, $p < 0.05$) but not in the doses of 1 and 10 mg/kg. The estradiol-17β treated rats in the PNMPP groups also showed higher uterine luminal epithelium labeling index (367%, $p < 0.05$; Fig. 3).

Discussion

In this study, we have studied the estrogenic activity of PNMC and PNMPP by immunostaining of PCNA in the rat

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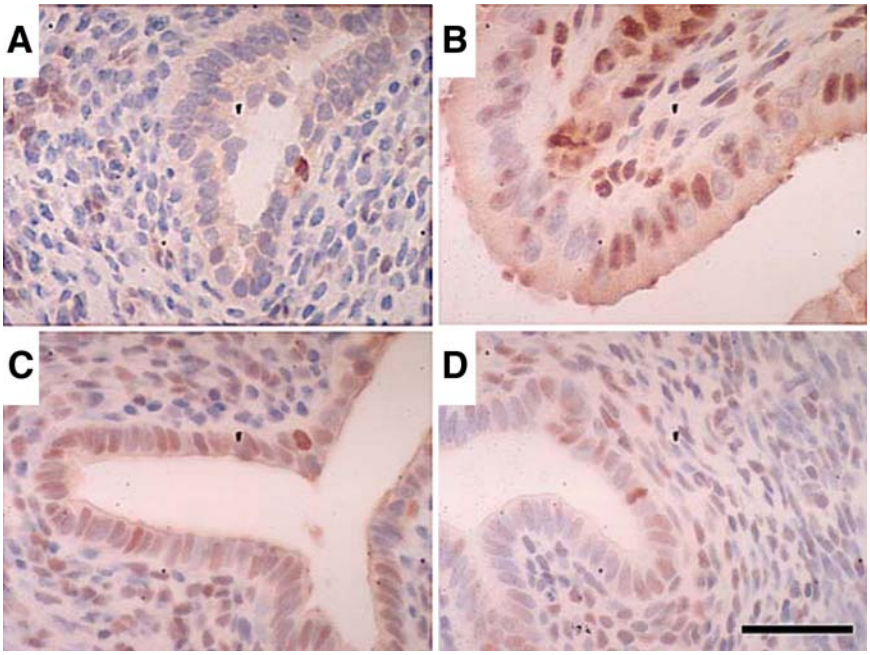

Fig. 1. Immunohistochemical staining of PCNA of the uterine luminal epithelia in immature OVX rats treated with (**A**) control, (**B**) estradiol-17β, (**C**) PNMC 100 mg/kg, or (**D**) PNMPP 0.1 mg/kg. Counterstained with hematoxylin. Bar = 50 μm.

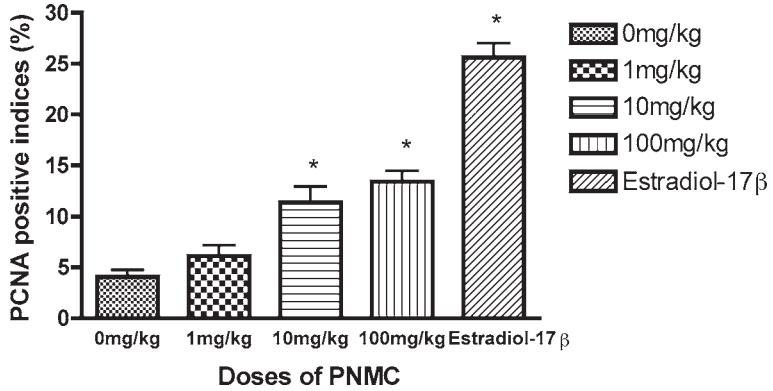

Fig. 2. PCNA positive indices (%) of uterine luminal epithelium in immature OVX rats treated with PNMC. Each bar represents the mean ± SEM of 8 rats per group. *$p < 0.05$ compared with control rats (Tukey–Kramer test).

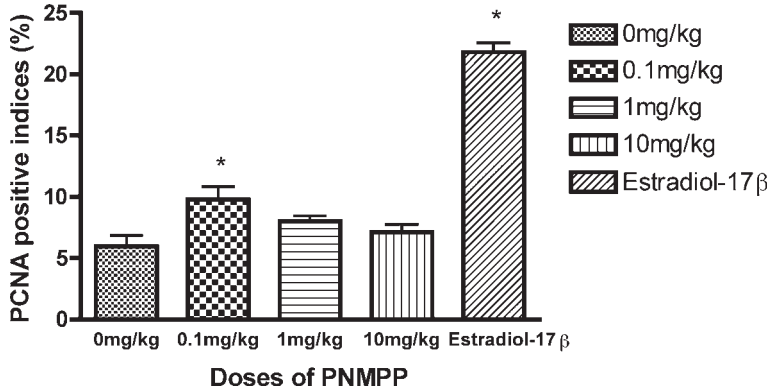

Fig. 3. PCNA positive indices (%) of uterine luminal epithelia in immature OVX rats treated with PNMPP. Each bar represents the mean ± SEM of eight rats per group. *$p < 0.05$ compared with control rats (Tukey–Kramer test).

uterine luminal epithelium. PCNA has been widely used as a method for the detection of proliferating cells in tumors *(17)*, developing tissues *(18)*, and uterus *(19)*. Given adequate fixation and tissue processing, the results of PCNA immunohistochemistry directly reflect the proliferating status of the cells and by the fact that PCNA-labeled nuclei

are observed during the G1 to the S phase of the cell cycle *(20)*. The present study evaluated the localization of PCNA in the cell nuclei in cellular proliferating activity of the uterine luminal epithelium.

Because estrogen-induced increase in uterine weight reflects both water production as well as cell proliferation, we evaluated histological sections of the prepubescent uterus of the rats using PCNA techniques to determine if true growth was being affected by the injection of PNMC and PNMPP from DEP. PCNA was the most meaningful technique to show increased growth in the uterine luminal epithelium, because dry weight and other methods of determining DNA synthesis include cellular components that may obscure changes in the epithelial compartment. The uterine epithelial labeling index increased among those receiving 10 and 100 mg/kg PNMC and 0.1 mg/kg PNMPP. There was a dose-dependent increase of the labeling index in the PNMC-treated rats, whereas in the PNMPP-treated rats the lowest dose of 0.1 mg/kg showed greater labeling index. This may be explained by the recombinant yeast screen assays and the well-characterized nature of the estrogen receptor-α ligand-binding cavity of these two chemicals showing that PNMPP has stronger estrogenic activity than PNMC *(16,21–23)*. However, in the PNMPP-treated rats, the higher doses did not show any estrogenic activity, and this might be explained as the low-dose effect, which is one of the phenomenon reported on xenoestrogens such as bisphenol A *(24)*, but the mechanisms of this remains unclear.

We have previously reported estrogenic activities of PNMC and PNMPP by using uterotrophic and myometrial contraction assays *(16)*, but they were only functional approaches to detect estrogenicity. In this study, we further revealed estrogenic activity of PNMC and PNMPP by cell proliferation on the uterine luminal epithelium at the histological level. Together we have shown that PNMC and PNMPP, compounds isolated from DEP have estrogenic activity in vivo and in vitro.

Materials and Methods

Chemicals

The chemicals 3-methyl-4- nitrophenol (4-nitro-*m*-cresol, PNMC) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan), and 4-nitro-3-phenylphenol (PNMPP) were synthesized by the method described previously *(15)*.

Animals and Samples

Eighty immature female Wistar–Imamichi rats were purchased from Imamichi Institute for Animal Reproduction (Kasumigaura, Ibaraki, Japan). They were kept under conditions of controlled lightning (lights on from 07:00 to 19:00 h), temperature (22 ± 2°C), humidity (50% ± 5%), and ventilation (25–30 filtered air changes hourly). Food (CE-2 commercial diet; Japan Clea Co., Tokyo, Japan) and water were available *ad libitum*. The rats at 25 d of age were ovariectomized (OVX) and were injected subcutaneously with PNMC (1, 10, and 100 mg/kg) and PNMPP (0.1, 1, and 10 mg/kg) for 2 d, on the day of surgery and the day after that. Rats were injected with vehicle (in PBS containing 0.05% Tween 80) as negative control, and estradiol-17β in sesame oil (5 μg/kg) as positive control. Twenty-four hours after the last injection, rats were weighed and sacrificed by decapitation. The uteri were collected and the left uteri horns were immediately fixed in 4% paraformaldehyde (Sigma Chemical Co, St. Louis, MO, USA) in 0.05 *M* phosphate-buffered saline (PBS) pH 7.4, and embedded in paraffin. The paraffin-embedded uterine tissues were serially sectioned at a thickness of 6 μm and placed on poly-L-lysine-coated (Sigma Chemical Co.) slide glasses (Dako Japan Co, Kyoto, Japan) for the use of immunohistochemistry.

This study was conducted in accordance with the Guiding Principals for the Use of Animals in Toxicology and was approved by the Japanese National Institute for Environmental Studies Animal Care and Use Committee.

Immunohistochemistry of Proliferating Cell Nuclear Antigen (PCNA)

After deparaffinization with xylene, the tissue sections were subjected to antigen retrieval by autoclaving in 0.01 *M* sodium citrate buffer (pH 6.0) at 121°C for 15 min. The sections were then incubated in 7.5% H_2O_2 in methanol at room temperature for 1 h followed by 0.5% casein-Tris saline (CTS; 0.05 *M* Tris-HCl with 0.15 *M* NaCl, pH 7.6) at 4°C for 1 h to quench nonspecific staining. Then they were incubated at 4°C for 16–18 h with monoclonal antibody raised against PCNA (BIOMEDA, Forster City, CA, USA) at a dilution of 1:200 in CTS. After incubation with the antibody, sections were treated with 0.25% (v/v) biotinylated goat anti-mouse secondary antibody (Elite ABC Kit; Vector Labs. Burlingame, CA, USA) in CTS at room temperature for 1 h. These sections were subsequently incubated with 2% (v/v) avidin–biotin complex (Elite ABC Kit) in CTS at room temperature for 30 min. The reaction products were visualized by treating with 0.025% (w/v) 3,3'-diaminobenzidine tetrachloride (DAB, Sigma Chemical Co.) in 0.1 m*M* Tris-buffered saline containing 0.01% H_2O_2 for 1–15 min. Sections were counterstained with hematoxylin for 1 second to better visualize the cell types.

Histological Analysis

The PCNA labeled uterine luminal epithelium were counted under a light microscope (Nikon, Tokyo, Japan). The optional 100 number of positive and negative stained uterine luminal epithelium were counted and counts were taken from optional five areas in each uterine sample.

Statistical Analysis

Analysis of variance was performed to evaluate the results. When ANOVA was significant, the differences between groups were assessed by means of the Tukey–Kramer test.

A provability value (p) of <0.05 was considered to be statistically significant.

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