Biochemical and Biophysical Research Communications **279,** 270–274 (2000)
doi:10.1006/bbrc.2000.3940, available online at http://www.idealibrary.com on IDEAL®

Effects of *para*-Nonylphenol on 92 kDa Gelatinase Secretion by Human Peripheral Lymphocytes and U937 Cells *in Vitro*

Shunichiro Kubota,*[,1] Sachiko Ohara,* Taeko Miyauchi,* Tomomi Edo,*[,†]
Eiji Takayama,‡ Kousuke Tomita,† and Yousuke Seyama*

**Department of Physiological Chemistry and Metabolism, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan; ‡Department of Parasitology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan; and †College of Engineering, Kanto Gakuin University, Yokohama 236-8501, Japan*

Received November 2, 2000

Much attention has focused on environmental estrogenic chemicals such as *para*-nonylphenol which disrupt various tissues via the estrogen receptor. We studied effects of *para*-nonylphenol on gelatinase secretion by human lymphocytes *in vitro*. *para*-Nonylphenol (0.05–50 μM) dose dependently suppressed 92 kDa gelatinase secretion. The suppressive effect of 25 and 50 μM *para*-nonylphenol was completely blocked by tamoxifen. We also studied the effects of *para*-nonylphenol (0.05–50 μM) on 92 kDa gelatinase secretion by human leukemia U937 cells. *para*-Nonylphenol suppressed 92 kDa gelatinase secretion in a dose-dependent manner. The suppressive effect of 50 μM *para*-nonylphenol was completely blocked by tamoxifen. Estradiol did not significantly suppress 92 kDa gelatinase secretion. Our results suggest that *para*-nonylphenol suppressed 92 kDa gelatinase secretion via the estrogen receptor, however, *para*-nonylphenol interacts with the estrogen receptor in a manner distinct from estradiol. As this assay system is simple and rapid, it may prove useful to evaluate toxic effects of *para*-nonylphenol on human blood cells.

***Key Words:* *para*-nonylphenol; gelatinase; peripheral lymphocyte; leukemia cell; estrogen receptor; tamoxifen.**

Environmental chemicals disrupt endocrine functions of various organs, including the reproductive systems in humans (1–3). Chemicals mimic steroid hormones through interactions with the estrogen receptor. Numerous environmental chemicals which can bind to the estrogen receptor and induce expression of the estrogen receptor-mediated genes have been identified (4, 5). Alkylphenols which are widely used as surfactants in plastics are degraded to *para*-nonylphenol. *para*-Nonylphenol binds to the estrogen receptor and induces estrogen-dependent gene expression (4). Human peripheral mononuclear cells express the estrogen receptor (6, 7).

The interaction between cells and the extracellular matrix is critical for normal development, wound healing, inflammation, and cancer metastasis. Modulation of cell-extracellular matrix interactions occurs through hydrolysis of the extracellular matrix by matrix metalloproteinases (8, 9). Metalloproteinases play a pivotal role in regulation of angiogenesis, cell proliferation, differentiation, and cell death. Uncontrolled proteolysis due to up-regulation or down-regulation of metalloproteinases contributes to abnormal development and to the generation of pathological conditions such as inflammation and cancer metastasis. Metalloproteinases are enzymes that regulate cell-extracellular matrix interactions, and these enzymes are classified into four types according to their substrate specificity, collagenases, stromelysins, elastases, and gelatinases (72 and 92 kDa gelatinases) (9). 92 kDa gelatinase is utilized by myeloid and lymphoid cells for migration across basement membranes (10, 11). Effects of *para*-nonylphenol on 92 kDa gelatinase secretion by cells including peripheral lymphocytes and U937 cells *in vitro* have not been documented. We undertook this study to elucidate effects of *para*-nonylphenol on secretion of 92 kDa gelatinases by human peripheral lymphocytes and U937 *in vitro*.

MATERIALS AND METHODS

Materials. Cell culture media (RPMI 1640) with or without phenol red were obtained from Sigma (Tokyo, Japan), and Gibco BRL (Tokyo, Japan), respectively. *para*-Nonylphenol, obtained from Kanto

[1] To whom correspondence should be addressed. Fax: 81-3-5689-2704. E-mail: kubota@bio.m.u-tokyo.ac.jp.

0006-291X/00 $35.00

Chemicals Co., Ltd. (Tokyo, Japan) was diluted with dimethyl sulfoxide or RPMI 1640 media to a final concentration. Gelatin, 17β-estradiol and trypan blue solution were obtained from Sigma (St. Louis, MO). Fetal calf serum was obtained from JRH Biosciences (Lenexa, KS). ICI 182780 was purchased from Tocris (Ballwin, MO).

Cells. U937 cells were kindly provided by Dr. K. Kano (The University of Tokyo), and grown in RPMI 1640 media containing 10% fetal bovine serum. Peripheral lymphocytes were prepared using lympho separation medium (ICN, Tokyo, Japan) according to the manufacturer's protocol from heparinized whole blood of a healthy male volunteer. 10^6 cells in 1 ml RPMI 1640 media in 24 well plates were incubated for 24 h. Dimethyl sulfoxide (0.2%) was added to culture media, as a control. The serum free conditioned medium (1 ml) was harvested for zymography.

Zymography. Forty-five microliters out of 1 ml serum free conditioned medium was used for gelatin substrate zymography as described (12). Quantification of 92 kDa gelatinase activity was done by scanning gels densitometrically. The number of pixels was counted using Macintosh Adobe Photoshop 5.0 J and an Histogram analysis program (12). Statistical significance was assessed by Student's *t*-test.

Treatment of cells with nonylphenol. At 24 h incubation after adding *para*-nonylphenol, or *para*-nonylphenol with tamoxifen, or *para*-nonylphenol with ICI 182780, or tamoxifen, or ICI 182780 in serum free RPMI 1640 media, cells were harvested, and centrifuged at 1500 rpm for 10 min. Cell pellets were used for cell counting. Viability of the cells was judged using the trypan blue exclusion method. Cells were mixed with 0.4% trypan blue 1:1 and viable cells were counted using a hemocytometer. Neither *para*-nonylphenol nor phenol red significantly affected cell viability and growth under the conditions in the present study (data not shown). Tamoxifen and ICI 182780 exceeding 4 and 10 μM, respectively, suppressed 92 kDa gelatinase secretion by U937 cells.

RESULTS

Effects of para-Nonylphenol on Gelatinase Secretion by Lymphocytes

Effects of *para*-nonylphenol on 92 kDa gelatinase secretion by human peripheral lymphocytes were investigated, using gelatin zymography. Incubation time with *para*-nonylphenol was 24 h. Peripheral lymphocytes predominantly secrete 92 kDa gelatinase. *para*-Nonylphenol (0.05, 0.5, 5, 25, and 50 μM) dose dependently suppressed the secretion of 92 kDa gelatinases after 24 h (Fig. 1). Tamoxifen (0.5 μM) completely blocked the suppressive effects of 25, and 50 μM *para*-nonylphenol (Fig. 1). A high concentration tamoxifen (5 μM) itself suppressed 92 kDa gelatinase secretion. Quantitative data obtained at 24 h are shown in Fig. 1 (lower panel). *para*-Nonylphenol concentrations at 25 and 50 μM suppressed 92 kDa gelatinase secretion 90.5 $\pm$ 4.1% ($P < 0.05$) and 97.0 $\pm$ 3.8% ($P < 0.05$), respectively, compared to findings in the control. These results suggest that suppressive effects of *para*-nonylphenol on 92 kDa gelatinase secretion were mediated via the estrogen receptor. We did time-dependent experiments, and essentially similar results were obtained with 48 h incubation time (data not shown).

We next investigated effects of *para*-nonylphenol on gelatinase secretion by human promyelocytic leukemia

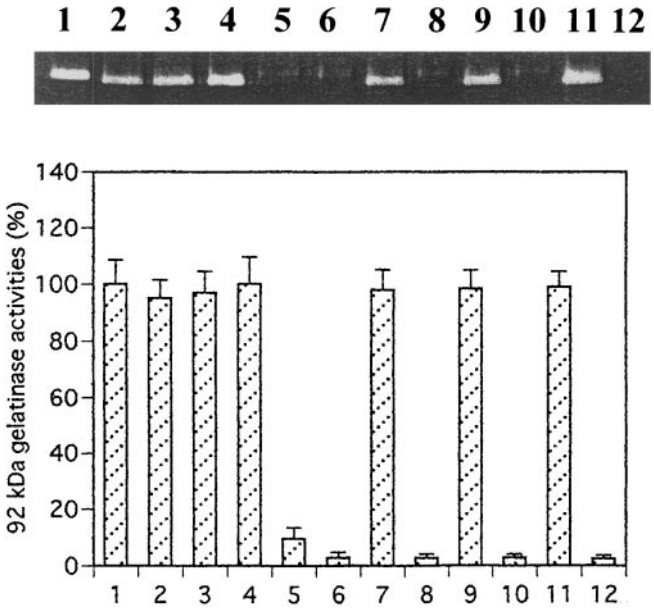

FIG. 1. Effects of *para*-nonylphenol on 92 kDa gelatinase secretion by peripheral lymphocytes. Effects of *para*-nonylphenol (0.05, 0.5, 5, 25, and 50 μM) on 92 kDa gelatinase secretion by human peripheral lymphocytes were analyzed using gelatin zymography. Serum free conditioned media were collected 24 h after the addition of *para*-nonylphenol. A typical zymography is shown in the upper panel. The experiments were done three times, and quantitative data are shown in the lower panel. Quantification was done as described under Materials and Methods, and values, expressed as percentages compared to control (100%), are means $\pm$ SD of three experiments. Lane 1, 0.2% DMSO (control); lane 2, RPMI 1640 media alone; lanes 3–6, *para*-nonylphenol 0.5, 5, 25, and 50 μM, respectively; lanes 7 and 8, tamoxifen 0.5 and 5 μM, respectively; lane 9, *para*-nonylphenol 25 μM + tamoxifen 0.5 μM; lane 10, *para*-nonylphenol 25 μM + tamoxifen 5 μM; lane 11, *para*-nonylphenol 50 μM + 0.5 μM tamoxifen; lane 12, *para*-nonylphenol 50 μM + tamoxifen 5 μM.

U937 cells. U937 cells predominantly secrete 92 kDa gelatinase. As shown in Fig. 2 (upper panel), at 24 h incubation time *para*-nonylphenol (0.05, 0.5, 5, 25, 50, 100, 200, and 300 μM) dose-dependently suppressed the secretion of 92 kDa gelatinase. Results of the quantification are shown in Fig. 2 (lower panel). *para*-Nonylphenol concentrations at 50, 100, 200, and 300 μM suppressed 92 kDa gelatinase secretion 45.0 $\pm$ 5.2% ($P < 0.05$), 66.0 $\pm$ 4.9% ($P < 0.05$), 84.5 $\pm$ 6.2% ($P < 0.05$), and 84.0 $\pm$ 5.0% ($P < 0.05$), respectively, compared to control. Tamoxifen (0.5 μM) completely blocked the suppressive effects of 50 μM *para*-nonylphenol, but not the effects of 100, 200, and 300 μM *para*-nonylphenol. Therefore, we next determined if a higher concentration (4.0 μM) of tamoxifen would block the suppressive effects of *para*-nonylphenol (100, 200, and 300 μM), but it did not do so (Fig. 2). We also determined if ICI 182780 (10 μM), an anti-estrogen would block the suppressive effects of *para*-nonylphenol (100, 200, and 300 μM), but it did not do so (Fig. 2). We did time-dependent experiments, and essen-

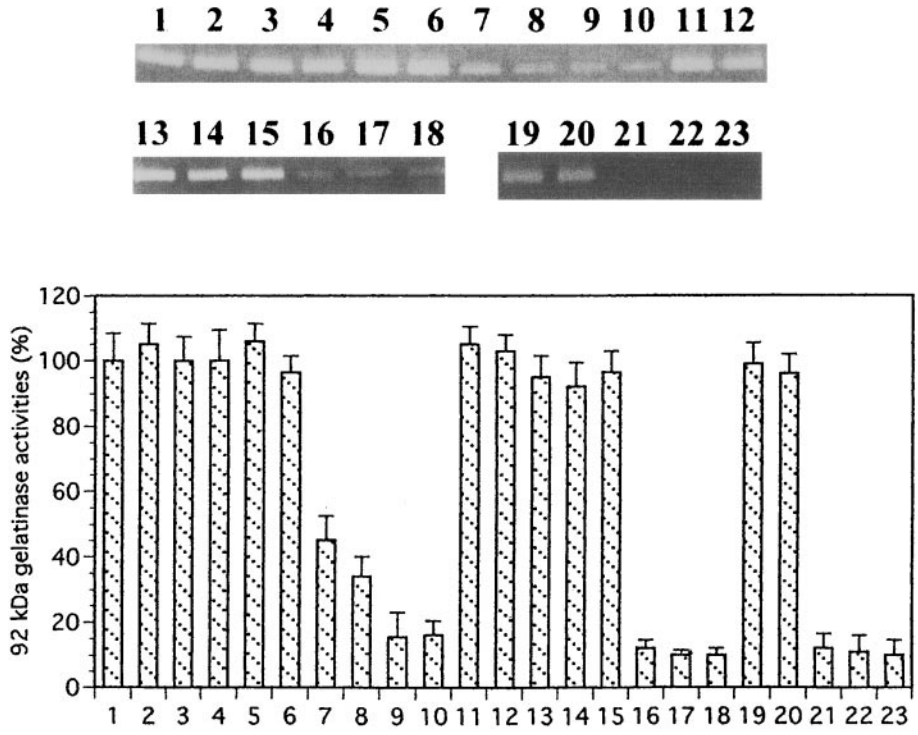

FIG. 2. Effects of *para*-nonylphenol on 92 kDa gelatinase secretion by U937 cells. Effects of *para*-nonylphenol (0.05, 0.5, 5, 25, 50, 100, 200, and 300 μM) on 92 kDa gelatinase secretion by U937 cells were analyzed using gelatin zymography. Serum free conditioned media were collected 24 h after the addition of *para*-nonylphenol. A typical zymography is shown in the upper panel. The experiments were done three times, and quantitative data are shown in lower panel. Quantification was done as described under Materials and Methods, and values, expressed as percentages compared to control (100%), are means ± SD of three experiments (lower panel). Lane 1, 0.2% DMSO (control); lane 2, RPMI 1640 media alone; lanes 3–10, *para*-nonylphenol 0.05, 0.5, 5, 25, 50, 100, 200, and 300 μM, respectively; lane 11, tamoxifen 0.5 μM; lane 12, *para*-nonylphenol 50 μM + tamoxifen 0.5 μM; lanes 13 and 14, tamoxifen 3 and 4 μM, respectively; lanes 15–18, tamoxifen 4 μM + *para*-nonylphenol 50, 100, 200, and 300 μM, respectively; lanes 19 and 20, ICI 182780 1 and 10 μM, respectively; lanes 21–23, ICI 182780 10 μM + *para*-nonylphenol 100, 200, and 300 μM, respectively.

tially similar results were obtained with 48 h incubation time (data not shown).

Effects of 17β-Estradiol on Gelatinase Secretion by Lymphocytes and U937 Cells

We next studied effects of 17β-estradiol on 92 kDa gelatinase secretion for 24 h by lymphocytes and U937 cells. 17β-estradiol (5 nM, 50 nM, 1 μM, 5 μM, and 10 μM) did not significantly suppress 92 kDa gelatinase secretion by lymphocytes (Fig. 3). Results of the quantification are shown in Fig. 3 (lower panel). We also tested effects of physiological and supraphysiological concentrations of 17β-estradiol (5 nM, 50 nM, 1 μM, 2.5 μM, 5 μM, 50 μM, 100 μM, and 200 μM). 17β-estradiol did not significantly suppress 92 kDa gelatinase secretion by U937 cells (Fig. 4). Results of the quantification are shown in Fig. 4 (lower panel). We also studied effects of 17β-estradiol on 92 kDa gelatinase secretion for 48 h by lymphocytes and U937 cells, but it did not significantly suppress 92 kDa gelatinase secretion (data not shown).

DISCUSSION

The current study revealed that *para*-nonylphenol significantly suppressed 92 kDa gelatinase secretion by peripheral lymphocytes and U937 cells. To our knowledge this is the first report that *para*-nonylphenol affects the secretion of 92 kDa gelatinase by human lymphocytes and U937 cells *in vitro.* Since both cells express estrogen receptors (6, 7, 13) which we confirmed by RT-PCR (data not shown), suppression of 92 kDa gelatinase may have been mediated via estrogen receptors. This was supported by findings that tamoxifen blocked the effects of *para*-nonylphenol on 92 kDa gelatinase secretion by these lymphocytes and U937 cells. The suppressive effect of 25 and 50 μM *para*-nonylphenol was completely blocked by the estrogen receptor antagonist tamoxifen (0.5 μM) in case of lymphocytes, and of 50 μM *para*-nonylphenol was completely reduced by tamoxifen (0.5 μM) in case of U937 cells. These results strongly suggest that the suppressive effect of *para*-nonylphenol was mediated via the estrogen receptor. However, the suppressive effect of higher doses (100, 200, and 300 μM) of *para*-nonyl-

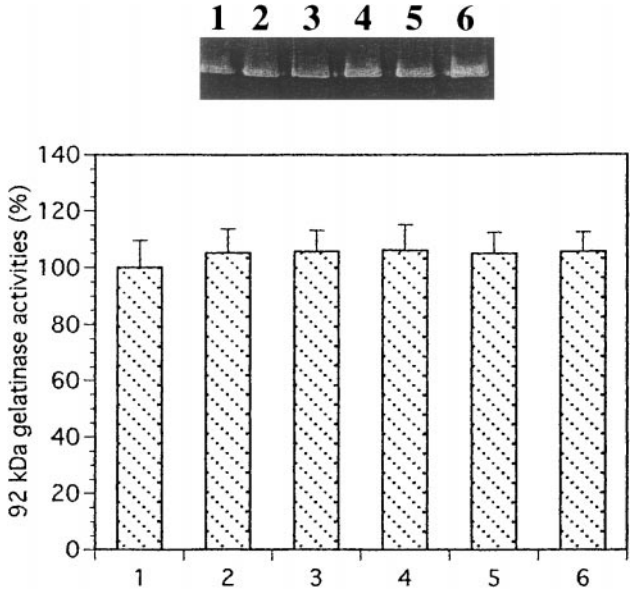

FIG. 3. The effects of 17β-estradiol on 92 kDa gelatinase secretion by human lymphocytes. Effects of 17β-estradiol (5 nM, 50 nM, 1 μM, 5 μM, and 10 μM) on 92 kDa gelatinase secretion by human lymphocytes were studied. Serum free conditioned media were collected 24 h after the addition of 17β-estradiol. A typical zymography is shown in the upper panel. Quantification was done as described under Materials and Methods, and values, expressed as percentages compared to control (100%), are means ± SD of three experiments (lower panel). Lane 1, control; lanes 2–6, 17β-estradiol, 5 nM, 50 nM, 1 μM, 5 μM, and 10 μM, respectively.

phenol in case of U937 cells was not blocked by tamoxifen (4 μM). Therefore, we tested another antiestrogen, ICI 182780 whether it would block the suppressive effects of higher doses (100, 200, and 300 μM) of *para*-nonylphenol, but it did not do so. There are at least two possibilities; one is that the suppressive effect of higher doses (100, 200, and 300 μM) of *para*-nonylphenol was not mediated via estrogen receptor. The other one is that the dose of *para*-nonylphenol used was too high to cause a suppression. Since a higher concentration of tamoxifen (>4 μM) and ICI 182780 (>10 μM) suppressed 92 kDa gelatinase secretion by U937 cells, it was not feasible to test whether a higher concentration of tamoxifen and ICI 182780 would block the suppressive effects of *para*-nonylphenol (100, 200, and 300 μM). Physiological and supraphysiological concentrations of 17β-estradiol did not significantly suppress 92 kDa gelatinase secretion by lymphocytes and U 937 cells. The results suggest that with regard to 92 kDa gelatinase regulation, *para*-nonylphenol interacts with the estrogen receptor and transduces signals in a manner distinct from that of estradiol. The different action from estradiol at the estrogen receptor was reported in case of bisphenol, an estrogenic chemical (14). Downstream signal transduction after the estrogen receptor remains to be elucidated. Crowe and Brown recently reported that transcriptional inhibition of 92 kDa gelatinase activity by a *c-fos*/estrogen receptor fusion protein is mediated by the proximal AP-1 site of the 92 kDa gelatinase promoter (15). We are currently studying whether the AP-1 site of the 92 kDa gelatinase promoter is involved in *para*-nonylphenol-induced suppression of 92 kDa gelatinase.

Matrix metalloproteinases are a family of extracellular matrix degrading zinc-dependent proteinases comprising 24 species (9), and secreted by cells in a latent form. 92 kDa gelatinase is a latent form, and can be activated by other proteinases, or by autocatalytic cleavage. 92 kDa gelatinase degrades preferentially collagen type IV, V, and VII (16, 17). The migration of white blood cells including lymphocytes from the blood stream into tissues or inflammatory tissues is considered to be facilitated by 92 kDa gelatinase secreted by migrating cells (10, 11, 18). 92 kDa gelatinase activities are also correlated with the ability of migrating cells during tumor metastasis and wound healing processes (9). Therefore, either suppressed degradation or a lack of degradation of extracellular matrix components such as collagen type IV, V, and VII due to suppressed 92 kDa gelatinase secretion by *para*-nonylphenol may affect processes of inflammation, tumor metastasis, and wound healing.

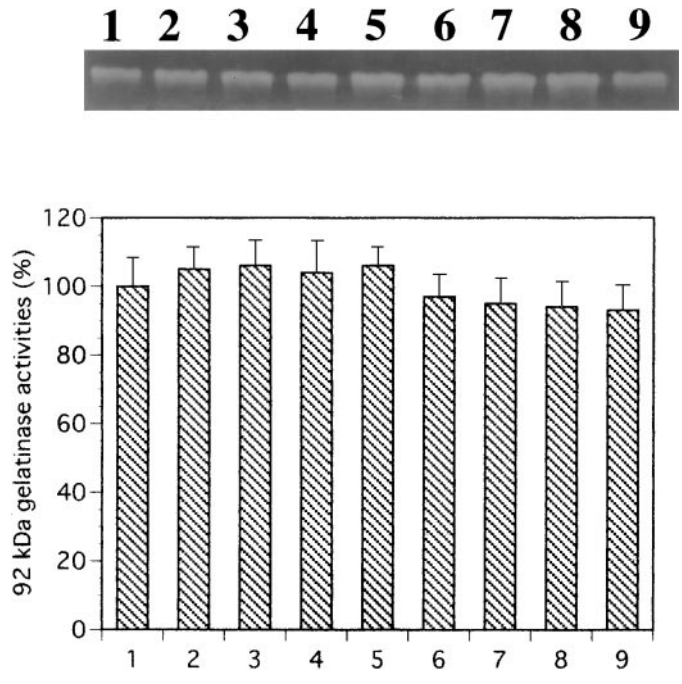

FIG. 4. The effects of 17β-estradiol on 92 kDa gelatinase secretion by U937 cells. Serum free conditioned media were collected 24 h after the addition of 17β-estradiol. A typical zymography is shown in the upper panel. Quantification was done as described under Materials and Methods, and values, expressed as percentages compared to control (100%), are means ± SD of three experiments (lower panel). Lane 1, control; lanes 2–9, 17β-estradiol, 5 nM, 50 nM, 1 μM, 2.5 μM, 5 μM, 50 μM, 100 μM, and 200 μM, respectively.

In summary, we found that *para*-nonylphenol suppressed 92 kDa gelatinase secretion by human peripheral lymphocytes, and U937 cells, and that this suppression was mediated via the estrogen-receptor pathway. As this system is simple and rapid, it may prove useful for evaluating the toxicity of *para*-nonylphenol or other environmental chemicals on human blood cells.

ACKNOWLEDGMENT

This study was supported by Health Science Research Grants for Research on Environmental Health from the Ministry of Health and Welfare of Japan.

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