Current Topics in
Toxicology
Vol. 9, 2013

Short Communication

Interleukin-6 secretion during *p*-nitrotoluene-induced neurite outgrowth of human neuroblastoma NB-1 cells

Masami Ishido*

Center for Environmental Risk Research, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba 305-8506, Japan

ABSTRACT

Neurite outgrowth of neural cells serves as an index of the neurotoxicity of environmental chemicals. We previously demonstrated the *in vivo* neurotoxicity of *p*-nitrotoluene: exposure of rat pups to *p*-nitrotoluene causes juvenile hyperactivity. To further explore the neurotoxicity of *p*-nitrotoluene *in vitro*, in this study we examined the *in vitro* neurotoxicity of *p*-nitrotoluene using cultured human neuroblastoma NB-1 cells. Addition of *p*-nitrotoluene (0 ~ 1 µM) to NB-1 cells significantly promoted neurite outgrowth ($P < 0.05$); a dose of 0.5 µM *p*-nitrotoluene was as effective as 1 µM, whereas 0.1 µM *p*-nitrotoluene failed to promote outgrowth. The effects of the chemical were stereoisomer-specific. During *p*-nitrotoluene-induced neurite outgrowth, interleukin (IL)-6 was secreted into the culture medium in a dose-dependent manner and increased 1.7-fold following incubation for 48 h with 1 µM *p*-nitrotoluene. Thus, we show for the first time that *p*-nitrotoluene concomitantly promotes neurite outgrowth and IL-6 secretion in human neuroblastoma NB-1 cells.

KEYWORDS: *p*-nitrotoluene, neurite outgrowth, IL-6, human neuroblastoma

INTRODUCTION

Environmental estrogens are a diverse group of synthetic and naturally occurring compounds that mimic the action of steroidal estrogens. These chemicals are called *endocrine disruptors* or *endocrine-disrupting chemicals*. The reproductive effects of endocrine disruptors have been extensively examined.

An emerging body of evidence suggests that endocrine disruptors affect the central nervous system in rodents. Employing the Supermex system, we previously screened endocrine disruptors that cause hyperactivity in rats. The chemicals we tested included several endocrine disruptors such as *p*-nitrotoluene which causes neurodevelopmental disorders that result in hyperkinesia [1].

p-Nitrotoluene is used to synthesize agricultural and rubber chemicals. The carcinogenesis of the chemical has been examined in a number of studies [2, 3]. Several reports showed that *p*-nitrotoluene has neither estrogenic nor androgenic effects in uterotrophic or Hershberger assays [4] or in recombinant yeast screening assays [5]. A two-generation reproductive toxicity study of the chemical also failed to find any effects on the endocrine and reproductive organs [6]. However, our previous data clearly demonstrated that *p*-nitrotoluene causes hyperactivity [7] comparable to that produced by 6-hydroxydopamine [8]. Here, we report the results of further investigation of the neurotoxicity of *p*-nitrotoluene using cultured human neuroblastoma NB-1 cells.

MATERIALS AND METHODS

Cell culture

Human neuroblastoma NB-1 cells were obtained from RIKEN (Tsukuba, Japan) and grown in 45%

*ishidou@nies.go.jp

RPMI-1640 and 45% Eagle's minimum essential medium containing 10% fetal bovine serum, 50 units/ml of penicillin G, and 50 µg/ml of streptomycin sulfate, as described previously [9, 10]. Cells were cultured once a week at a split ratio of 1:6.

Determination of the length of neurite outgrowth

NB-1 cells were exposed to *p*-nitrotoluene at a concentration as indicated for 48 h. For testing the stereoeffects of the chemical, *o*- or *m*-nitrotoluene was added at 1 µM concentration for 48 h. The treated cells were then fixed with 11% glutaraldehyde, stained with crystal violet and photographed under a microscope (DMIRB, Leica Microsystems, Tokyo, Japan) equipped with a digital camera. Neurite length was measured using NIH ImageJ 1.34x software (public domain software) in randomly chosen phase-contrast microscopic fields.

Enzyme-linked immunosorbent assay (ELISA) for IL-6

For measurement of IL-6 protein, NB-1 cells were plated in a 24-well plate and treated with *p*-nitrotoluene in the 0-1 µM concentration range for 24 h, after which the culture medium was harvested. The level of rat IL-6 protein in the culture medium was measured using a Quantikine M ELISA kit (R&D System, Minneapolis, MN, USA), according to the manufacturer's protocol.

Statistical analyses

Statistical analyses were carried out by Student's *t*-test using Excel2007 software (Microsoft Co., Tokyo, Japan).

RESULTS

Figure 1 shows that *p*-nitrotoluene significantly induced neurite outgrowth of NB-1 cells. Addition of 0.1 µM *p*-nitrotoluene did not significantly induce neurite outgrowth; however, concentrations of 0.5 and 1 µM promoted an approximately 1.3-fold increase in neurite outgrowth.

To examine the effects of *p*-nitrotoluene stereoisomers on neurite outgrowth, NB-1 cells were cultured for 48 h in the presence of 1 µM *o*-nitrotoluene, *m*-nitrotoluene, or *p*-nitrotoluene. As shown in Figure 2, *m*-nitrotoluene had no effect under the conditions we used, whereas *o*-nitrotoluene did induce an increase in neurite outgrowth of about 1.2-fold.

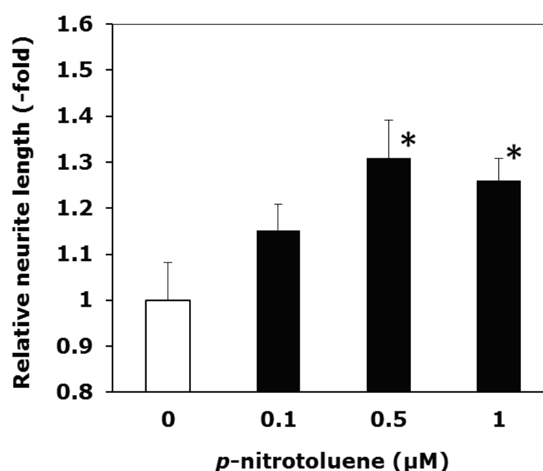

Figure 1. *p*-Nitrotoluene promotes dose-dependent neurite outgrowth of NB-1 cells. NB-1 cells were treated with *p*-nitrotoluene (0 ~ 1 µM) for 48 h, as indicated. Treated cells were then fixed with glutaraldehyde and stained with crystal violet. Neurite extension was quantified with NIH ImageJ 1.34x software. Data are shown as the mean ± standard error (S.E.); (n = 5). $*P < 0.05$.

In addition to exogenous factors such as *p*-nitrotoluene, several endogenous factors including pituitary adenylate cyclase-activating polypeptide (PACAP) have been shown to induce neurite outgrowth in cultured neural cells [11]. In a previous study, we found that PACAP promotes IL-6 secretion by cultured PC12 cells during PACAP-induced neurite outgrowth [12]. We therefore examined whether or not *p*-nitrotoluene promotes IL-6 secretion during *p*-nitrotoluene-induced neurite outgrowth. NB-1 cells were incubated for 48 h in the presence of various concentrations of *p*-nitrotoluene (0 ~ 1 µM), after which the culture medium was harvested and the level of IL-6 protein was measured by ELISA. Figure 3 shows that *p*-nitrotoluene induced IL-6 secretion in a dose-dependent manner. Treatment of NB-1 cells (5×10^4 cells/well in a 24-well plate) with 1 µM *p*-nitrotoluene for 48 h resulted in 1.7 fold higher the level of IL-6 in the control culture.

As PACAP signaling is reportedly mediated by kinase cascades, we examined the effect of kinase

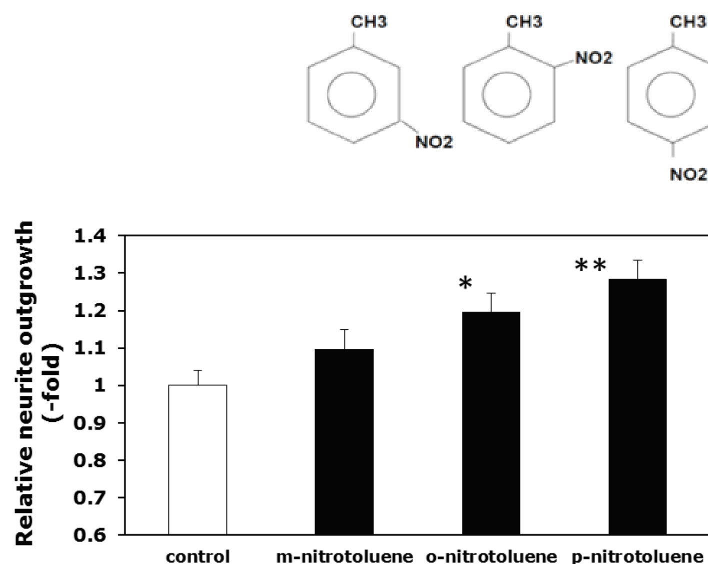

Figure 2. Effects of isomers of nitrotoluene on neurite outgrowth of NB-1 cells. NB-1 cells were treated with *o*-, *m*-, or *p*-nitrotoluene (1 μM) for 48 h, as indicated. The treated cells were then fixed with glutaraldehyde and stained with crystal violet, after which neurite extension was quantified with NIH ImageJ 1.34x software. Data are shown as the mean ± S.E. (n = 6). **P* < 0.01; ***P* < 0.001. *o* : ortho-; *m* : meta-; *p* : para-.

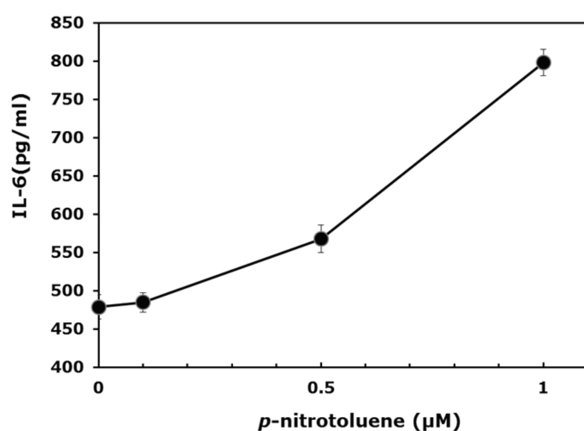

Figure 3. *p*-Nitrotoluene-induced secretion of IL-6 in NB-1 cells. NB-1 cells (5×10^4) were treated with *p*-nitrotoluene (0 ~ 1 μM) for 48 h, after which the medium (50 μl) was harvested and the IL-6 level was determined. Data are shown as the mean ± S.E. (n = 5).

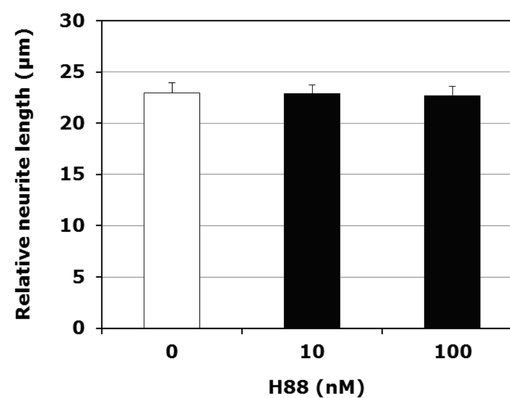

Figure 4. No effect of the kinase inhibitor H88 on neurite outgrowth of NB-1 cells. NB-1 cells were treated with *p*-nitrotoluene (1 μM) for 48 h in the presence of H88 (0 ~ 100 nM), as indicated. Quantification of neurite extension was performed with NIH ImageJ 1.34x software, as described for a legend to Figure 1. Data are shown as the mean ± S.E. (n = 5).

inhibitors on neuritogenesis. Figure 4 shows the effect of H88, a protein kinase A inhibitor. NB-1 cells were treated with or without 1 μM *p*-nitrotoluene for 48 h in the presence of H88 (0 ~ 100 nM), after which neurite length was measured. Up to 100 nM, H88 had no effect on *p*-nitrotoluene-induced neurite extension, indicating that it was possible to distinguish both signaling pathways regulating neurite outgrowth.

DISCUSSION

In light of the large number of known endocrine-disrupting chemicals, there is an increasing demand

to develop rapid screening techniques. Neuronal differentiation is a complex process that involves both morphological and biochemical changes, the most obvious of which are a decrease in cell proliferation and the emergence of extending processes. The growth of axonal and dendritic processes during brain development is a critical determinant of neural connectivity, and disruption of this process could lead to neuronal dysfunction. Neurite outgrowth can be recapitulated *in vitro* using a variety of cell models. These models have become valuable tools for investigating the mechanism underlying the activity of neurotoxicants. Neurite outgrowth can now be used as an endpoint of neurotoxicity due to the development of advanced image acquisition techniques.

In this study, we demonstrated that *p*-nitrotoluene induces IL-6 secretion during chemical-promoted neurite outgrowth in human neuroblastoma NB-1 cells. As the kinetics of *p*-nitrotoluene-induced neurite extension and IL-6 secretion differed, both events seem to be independent of each other: a dose of 0.5 μM *p*-nitrotoluene was as effective as 1 μM, however 0.5 μM *p*-nitrotoluene had much less effects on IL-6 secretion than 1 μM (Figure 1 versus Figure 3).

Brain inflammation is a complex cellular and molecular response to stress, injury, or infection of the central nervous system and functions to defend against insults, clear dead cells and damaged neurons, and return the central nervous system to a normal state [13]. Inflammation in the brain is driven by the activation of resident microglia, astrocytes, and infiltrating peripheral macrophages, which release a plethora of anti- and proinflammatory cytokines, chemokines, neurotransmitters, and reactive oxygen species. IL-6 is a multifunctional proinflammatory cytokine with diverse actions, including regulation of acute phase reactions, immune responses, and cellular differentiation. In the developing brain, IL-6 promotes astrocyte proliferation and neural survival [14], suggesting that it may have a dual role in dictating beneficial versus detrimental responses in neuroinflammation.

Furthermore, several lines of evidence suggest that neuroinflammation plays a role in the degeneration of dopaminergic neurons in Parkinson's disease. A variety of cytokines are expressed at higher levels in the substantia nigra, striatum, and/or cerebrospinal fluid of Parkinson's patients relative to control subjects. It has been reported that levels of IL-6 was elevated in cerebrospinal fluid of Parkinson's disease patients [15, 16]. Particularly, the level of IL-6 was 1.5 fold higher in cerebrospinal fluid of Parkinson's disease patients compared to control subjects [16]. In a previous study, we reported that *p*-nitrotoluene exhibits *in vivo* neurotoxicity and showed that it induces alterations in the expression of various interleukin family genes, such as those encoding IL-10, IL-13, and IL-15 in the striatum and IL-1 beta in the midbrain of rats with chemical-induced hyperactivity, the etiology of which might be a neurodevelopmental deficit of the dopaminergic neurons, as assessed by DNA macroarray analysis [7, 17]. Therefore, it is tempting to measure IL-6 contents in the rat brains with hyperactivity.

Recently, we found that PACAP, which is a 38-amino acid neuropeptide, promotes neurite outgrowth of PC12 cells mediated by the IL6-STAT3 pathway [12]. PACAP acts via two G-protein-coupled receptors, which activate different signal transduction pathways via cAMP elevation and calcium mobilization, leading to the stimulation of several protein kinases, such as protein kinase A and the MAP kinases ERK1/2. These kinases in turn induce or repress transcription of genes associated with cell growth and differentiation [18]. Therefore, H88, a kinase inhibitor blocked PACAP-induced neurite outgrowth of PC12 cells, at least in part (data not shown), whereas the inhibitor at same concentration did not block *p*-nitrotoluene-elicited neurite outgrowth of NB-1 cells (Figure 4), suggesting that it would distinguish both signaling pathways regulating neurite outgrowth. Neurite outgrowth induced by neurotrophins apparently differs from that induced by neurotoxins. Neurite extension induced by neurotoxins might be abnormal, resulting in defective synapse connections *in vivo*.

Nervous system development involves the coordination of specific cellular events including proliferation, differentiation, migration, neurite outgrowth, synaptogenesis, myelination, and programmed cell death [19]. Although the significance of neurite outgrowth induced by

environmental factors is largely unknown, it should be extended to examine whether additional endocrine disruptors such as phenols and phthalates would also elicit IL-6 secretion for establishing it as a biomarker. The initial hazard identification based on alterations in chemical-induced neurite outgrowth can be used to prioritize compounds for further *in vivo* neurotoxicological testing.

FUNDING

This work was supported by National Institute for Environmental Studies grant 12510 and a Grant-in Aid for Scientific Research (KAKENHI) 24590774.

CONFLICT OF INTEREST STATEMENT

The author has no conflict of interest to declare.

ACKNOWLEDGEMENTS

The author would like to thank Ms. Emiko Nagata and Dr. Shoji Yamamoto for excellent technical assistance and for valuable discussions, respectively.

REFERENCES

1. Masuo, Y. and Ishido, M. 2011, J. Toxicol. Environ. Health B Crit. Rev., 14, 346.
2. National Toxicol. Program, 2002, Tech. Rep. Ser., 498, 1.
3. Dunnick, J. K., Burka, L. T. and Sills, R. 2003, Toxicology, 183, 221.
4. Ministry of Economy, Trade and Industry, Japan. 2002, http://www.meti.go.jp/english/report/data/gEndoctexte.pdf.
5. Nishihara, T., Nishikawa, J., Kanayama, T., Dakeyama, F., Saito, K., Imagawa, M., Takatori, S., Kitagawa, Y., Hori, S. and Utsumi, H. 2000, J. Health Sci., 46, 282.
6. Aso, S., Miyata, K., Ehara, H., Hosyuyama, S., Shiraishi, K., Umano, T. and Minobe, Y. 2005, J. Toxicol. Sci., 30, 117.
7. Ishido, M., Masuo, Y., Oka, S., Niki, E. and Morita, M. 2004, Neurosci. Lett., 366, 1.
8. Masuo, Y., Ishido, M., Morita, M. and Oka, S. 2002, Neurosci. Lett., 335, 124.
9. Pramanik, R., Ishido, M. and Kunimoto, M. 2001, J. Health Sci., 47, 478.
10. Pramanik, R., Ishido, M. and Kunimoto, M. 2002, J. Health Sci., 48, 381.
11. Deutsch, P. J. and Sun, Y. 1992, J. Biol. Chem., 267, 5108.
12. Ishido, M. 2010, J. Mol. Neurosci., 42, 349.
13. Kielian, T. and Drew, P. D. 2005, Cytokines and brain: health and disease. In Inflammatory disorders of the nervous system (A. Minagar and J. S. Alexander, Eds), pp.41-79, Humana Press, New Jersey.
14. Gruol, D. L. and Nelson, T. E. 1997, Mol, Neurobiol., 15, 307.
15. Mogi, M., Harada, M., Kondo, T., Riederer, P., Inagaki, H., Minami, M. and Nagatsu, T. 1994, Neurosci. Lett., 180, 147.
16. Blum-Degen, D., Müller, T., Kuhn, W., Gerlach, M., Przuntek, H. and Reiderer, P. 1995, Neurosci. Lett., 202, 17.
17. Ishido, M. and Masuo, Y. 2004, Regul. Peptides, 123, 15.
18. Vaudry, D., Falluel-Morel, A., Bourgault, S., Basille, M., Burel, D., Wurtz, O., Fournier, A., Chow, B. K., Hashimoto, H., Galas, L. and Vaudry, H. 2009, Pharmacol. Rev., 61, 283.
19. Jacobson, M. 2005, Beginning of the nervous system. In Developmental Neurobiology (R. M. Jacobson, Eds), Kluwer Academic/ Plenum Publishers, pp. 365-413, New York.