Cytotechnology **36:** 163–169, 2001.

Alkylphenolic compounds and their effect on the injury rate, survival and acetylcholinesterase activity of the rat neuronal cell line PC12

T. P. N. Talorete[1], H. Isoda[2]* & T. Maekawa[1]
[1] *Institute of Agricultural and Forest Engineering;* [2] *Institute of Biological Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba City, Ibaraki, Japan*
(Author for correspondence; E-mail: isoda@bsys.tsukuba.ac.jp)*

Received 22 December 2000; accepted 30 April 2001

Key words: acetylcholinesterase activity, alkylphenols, cytotoxicity, DNA fragmentation, PC12

Abstract

Most studies on hormonally active agents or endocrine disruptors have been limited to polychlorinated biphenyls and dioxins. In this paper, we report results of *in vitro* studies on the effects of alkylphenolic compounds, namely, n-pentylphenol, n-hexylphenol, n-heptylphenol, n-octylphenol, and n-nonylphenol, on the injury rate, survival, and acetylcholinesterase activity of the rat pheochromocytoma cell line PC12. Results using the lactate dehydrogenase cytotoxicity assay to determine cell injury rate reveal that the alkylphenols mentioned did not induce cell necrosis beyond 30%, even at concentrations as high as 300 μM in a 15-min incubation period. Exposing the cells to alkylphenols for 4 hr and testing for DNA fragmentation showed that nonylphenol and octylphenol also did not induce apoptosis, even at concentrations as high as 500 and 100 μM, respectively. However, incubating the cells with the alkylphenols for 24 hr significantly inhibited acetylcholinesterase activity at concentrations as low as 0.8 μM, with n-octylphenol showing the most significant effect Since it is believed that human exposure to nonylphenol from drinking water is around 0.7 μg day^{-1} and that these compounds can accumulate in adipose tissue, this finding may implicate alkylphenols in neurological and behavioral disturbances in both animals and humans.

Introduction

On a worldwide basis, the annual production of alkylphenolic compounds exceeds 450 000 tons, and this volume is expected to show above average growth as plastics continue to replace traditional building materials (Lorenc et al., 1992). However, since these chemicals and their degradation products contaminate the environment, concerns over their biodegradability, toxicity and estrogenic potential have recently been a focus of extensive research.

White et al. (1994) demonstrated that 4-octylphenol (OP), 4-nonylphenol, 4-nonylphenoxycarboxylic acid, and 4-nonylphenoldiethoxylate were each capable of stimulating vitellogenin gene expression in trout hepatocytes, gene transcription in transfected cells, and growth of breast cancer cells. Alkylphenols have also been implicated in the widespread occurrence of intersexuality among wild riverine fish throughout the United Kingdom (Jobling et al., 1998). Gray and co-workers (1999) have also shown that eggs produced from matings of Japanese medaka (*Oryzias latipes)* exposed to as low as 10 μg l^{-1} OP demonstrated various developmental problems, such as circulatory system difficulties, incomplete eye development and failure to inflate swim bladders upon hatch. These examples reveal that alkylphenols, especially OP, have both toxic and estrogenic properties. Surprisingly, human exposure to alkylphenols, esp. nonylphenol from drinking water is around 0.7 μg day^{-1} (Weeks et al., 1996).

In vitro toxicity assays on OP focusing on specific cell types have been reported recently. Nair-Menon and co-workers (1996) demonstrated that concentrations greater than or equal to 10^{-12} M OP significantly decreased the percentages of viable rat or mouse splenocytes after 27 hr of culture, demonstrating the toxicity of OP to these cells. In a related

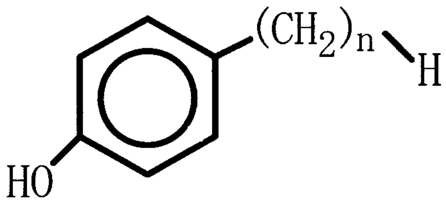

Figure 1. Chemical structure of 4-n-alkylphenols. n = 5 (pentyl), 6 (hexyl), 7 (heptyl), 8 (octyl), 9 (nonyl).

study using cultured rat spermatogenic and Sertoli cells, OP demonstrated direct toxicity by significantly decreasing the percentage of viable cells after 24 hr treatment also at concentrations as low as 10^{-12} M (Raychoudhury et al., 1999). At the molecular level, a recent study has shown that alkylphenols, even at very low doses act as uncouplers of oxidative phosphorylation, suggesting that their preferential target in living organisms are mitochondria (Bragadin et al., 1999).

To determine the possible effects of alkylphenols on neuronal cells, we tested 5 representative compounds on PC12, a rat pheochromocytoma cell line that responds to nerve growth factor (Greene and Tischler, 1976). Expressing neurochemical and ultrastructural properties associated with their normal counterparts, PC12 cells provide a useful model for assessing the effects of these compounds on the mammalian nervous system *in vitro.*

Hence, this study aimed to answer the following questions on PC12 cells: (1) Do alkylphenols cause necrosis? (2) Do alkylphenols cause apoptosis? (3) Do alkylphenols affect the function of PC12 cells by inhibiting acetylcholinesterase activity?

Materials and methods

Chemicals

A series of alkylphenols (4-n-pentylphenol, 4-n-hexylphenol, 4-n-heptylphenol, 4-n-octylphenol, and 4-n-nonylphenol) was purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Figure 1 shows the basic structure of these compounds. Stock solutions (10^{-1} M) were prepared by dissolving appropriate amounts in 1 ml ethanol and diluting in Ca^{2+}, Mg^{2+} free phosphate-buffered saline (PBS(-)) (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) to obtain the required working concentrations.

Cell line and maintenance

The rat pheochromocytoma PC12 cell line was obtained from the National Institute of Bioscience and Human-Technology (Tsukuba, Ibaraki, Japan) and routinely maintained in Dulbecco's Modified Eagle's Medium (DMEM; Sigma) supplemented with 5% fetal bovine serum (Sigma), 10% horse serum (Sigma), and 1% streptomycin (5000 I.U. ml^{-1})-penicillin (5000 μg ml^{-1}) solution (ICN Biomedicals, Inc.) in tissue culture flasks. Cells were incubated at 37 °C in a 95% air-5% CO_2 incubator. Cell passage was carried out at 80% confluence at 1:3 ratio using trypsin.

Lactate dehydrogenase assay

The LDH-Cytotoxic Test kit from Wako (Osaka, Japan) was used for this assay. Sterile 96-well microplates were inoculated with 5000 cells per well in 50 μl of medium and then incubated overnight in a 5% CO_2 incubator at 37 °C. The medium was then carefully removed, and the cells washed twice with PBS(-). After washing, 50 μl of PBS(-) was dispensed into each well, followed by treatment of the cells with 50 μl of the different alkylphenols in varying concentrations. PBS(-) and 0.2% Tween 20 dissolved in PBS were used as negative and positive controls, respectively. After a treatment time of 15 min at room temperature, 50 μl of coloring solution [Nitrotetrazolium blue (3.7 mg/vial), diaphorase, and NADH dissolved in buffer solution (50 g l^{-1} DL-Lithium lactate)] were added into each well and allowed to stand for 30 min at room temperature. The reaction was terminated by the addition of 100 μl of reaction terminator (0.5 N HCl) into each well. The absorbance of the reaction mixture was then measured at 570 nm using a microplate reader. Cell injury rate was calculated using the following equation:

$$\text{Cell injury rate} = (S - N)/(P - N) \times 100$$

S = Absorbance of sample

N = Absorbance of negative control

P = Absorbance of positive control

DNA fragmentation test

Petri plates were inoculated with 1×10^6 cells per plate in 10 ml of medium, followed by overnight incubation in a 5% CO_2 incubator at 37 °C. 4-n-octyphenol and 4-n-nonylphenol were then added into the plates to obtain final concentrations of 50, 100 and 500 μM. The cells were then incubated for 4 hr, after which, the DNA was extracted using the DNA Extractor WB Kit (Sodium Iodide Method) from Wako (Osaka, Japan). The DNA obtained was diluted in 100 μl of TAE buffer. Two μl of the latter was added to 2 μl of loading buffer (Wako) and mixed well. Two μl of this mixture was loaded into wells of a 1.5% electrophoresis-grade agarose gel containing 0.5 μg ml^{-1} ethidium bromide (Wako). Electrophoresis was done for 45 min at 100 V with a molecular weight marker (Marker 1 (λ/Hind III digest) Wako). The complete protocol for resolving large DNA fragments on agarose gels is described elsewhere (Ausubel et al., 1999).

Acetylcholinesterase assay

PC12 cells were inoculated onto 96-well microplates at 1×10^4 cells per well in 100 μl of medium. The cells were incubated with 40 ng ml^{-1} nerve growth factor 2.5S (NGF) (Funakoshi, Tokyo, Japan) and allowed to attach for at least 6 hr before the different alkylphenols were added to obtain the required final concentrations. After incubating for 24 hr, the medium was carefully removed and discarded and the cells carefully washed twice with 200 μl of PBS(-). After washing, 20 μl of 5.6 mM acetylthiocholine iodide and 180 μl of buffer solution, pH 7.5 (0.12 M NaCl, 0.2% TritonX-100, 1 mM EDTA, 50 mM Hepes) were added into each well. After incubating for 2 hr in room temperature, 20 μl of the cell lysates were transferred to another multiwell plate and incubated for 1 hr with 160 μl buffer solution, pH 5.0 (1 mM EDTA, 0.2% TritonX-100, 50 mM acetate buffer) and 20 μl of 0.4 mM 7-diethylamino-3(4′-maleimidyl-phenyl)-4-methylcoumarin in acetonitril. The absorbance in each well was then measured using a fluorescence plate reader (Fluoroskan Ascent, FL) at 460 nm.

Results and discussion

Lactate dehydrogenase assay

A decrease in cell viability is often associated with a damaged cell membrane, leading to the release of enzymes into the medium with a consequent increase in enzyme activity. One enzyme, lactic acid dehydrogenase (LDH), is released in sufficient amounts in short-term cytotoxicity assays to provide a sensitive and quantitative measure of natural cytotoxicity (Korzeniewski and Callewaert, 1983; Decker and Lohmann-Matthes, 1988). The activity of this cytoplasmic enzyme in the cellular supernatant indicates the proportion of killed cells (Decker and Lohmann-Matthes, 1988).

Results in Figure 2 show that the alkylphenols used in this study did not induce cell necrosis beyond 30%, even at concentrations as high as 300 μM in a 15-min incubation period. However, cell injury begins to increase at a concentration of 300 μM for 4-n-hexylphenol and 4-n-pentylphenol. On the other hand, 4-n-heptylphenol, 4-n-octylphenol, and 4-n-nonylphenol do not induce cell lysis beyond 50% until concentrations reach 5 mM (data not shown). The positive controls (plus 0.2% Tween 20) and negative controls (plus PBS(-)) in this experiment indicated 100 and 0% cell injury, respectively, based on differences in absorbance data and direct microscopic examination. 4-n-octylphenol and 4-n-nonylphenol are believed to be estrogenic at 1 μM (Jones et al., 1998); however, other studies show that they are estrogenic at 100 nM and 1 μM, respectively (Soto et al., 1995).

DNA fragmentation test

DNA fragmentation is an important part of the cell death mechanism and that it occurs very early in the apoptotic process, first appearing several hours before cell viability starts to decrease (Schwartzman and Cidlowski, 1993). This degradation occurs in a very specific pattern, producing fragments of DNA that are multiples of 180–200 base pairs. This is the length of DNA wrapped around the histone octamer in a nucleosome, which indicates that the chromatin is being cleaved at the linker DNA between nucleosomes, producing oligonucleosomal fragments (Schwartzman and Cidlowski, 1993). Since this fragmentation correlates exactly with cell death, this test is a reliable diagnostic for the occurrence of apoptosis.

Results of the DNA fragmentation test shown in Figure 3 reveal that there is no fragmentation of DNA after 4 hr treatment with 50, 100 and 500 μM of 4-n-nonylphenol (NP) and 4-n-octylphenol (OP). In other words, these two compounds do not cause apoptosis of PC12 cells beyond concentrations where they are believed to be estrogenic to estrogen-responsive cell lines such as MCF-7.

Acetylcholinesterase assay

In the neuromuscular junction, the neurotransmitter acetylcholine binds to the acetylcholine receptor

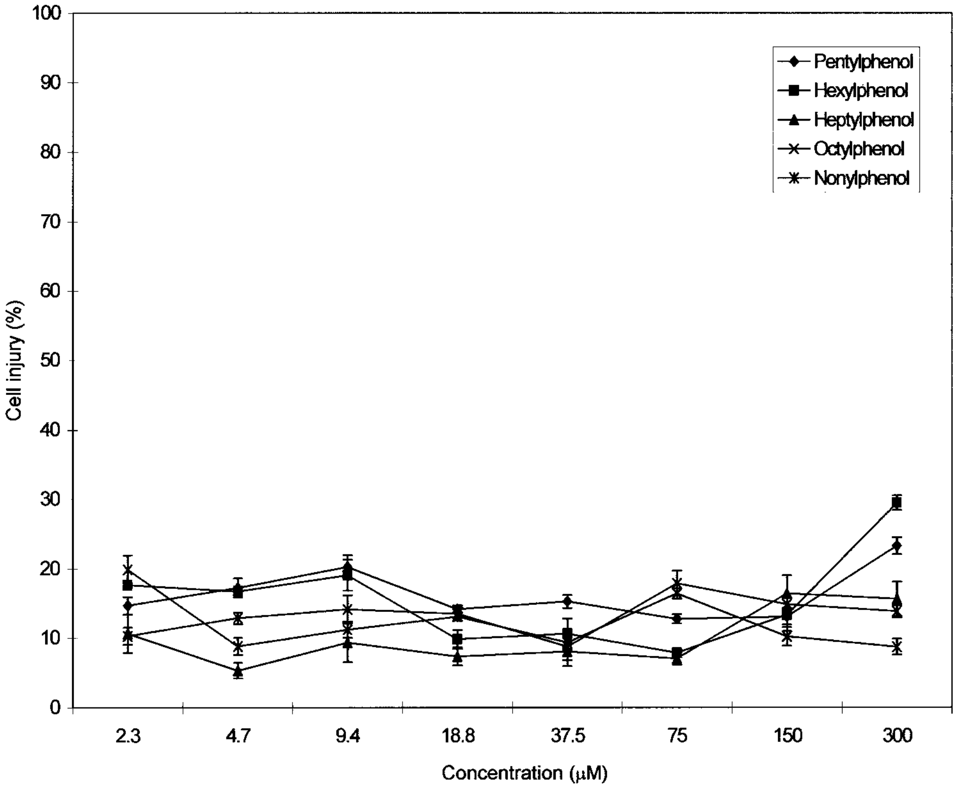

Figure 2. Lactate dehydrogenase activity (expressed as cell injury rate) of PC12 cells after 15 min incubation with varying concentrations of different alkylphenols. Results based on three independent replicates are expressed as percent cell injury ± SEM (see Materials and Methods for calculation).

inducing a conformational change that opens this transmitter-gated ion channel. This short-lived opening is followed by a closed state, whereby the acetylcholine dissociates from the receptor and is hydrolyzed by a specific enzyme called acetylcholinesterase (AChE). Since AChE plays an important role in cholinergic synaptic transmission, its activity in neuronal cells is of particular interest.

AChE is regulated by the nerve growth factor (NGF) in the PC12 cell line and that the rate of release of AChE from PC12 cells is greatly stimulated following NGF treatment (Lucas et al., 1980). When PC12 cells are exposed to NGF, a protein that affects the growth and development of sensory and sympathetic neurons (Levi-Montalcini and Angeletti, 1968), they cease cell division and acquire a number of properties of sympathetic neurons, including extensive neurite outgrowth (Greene and Tischler, 1976), electrical excitability, and increased responsiveness to acetylcholine (Dichter et al., 1977; Greene and Rein, 1977). NGF is also associated with maintenance of survival of PC12 cells (Greene, 1978).

Results in Figure 4 show that even at concentrations as low as 0.8 μM, alkylphenolic compounds can suppress the NGF-induced AChE activity in PC12 cells following a 24 hr incubation period. Results in this experiment are expressed as relative acetylcholinesterase activity, which is obtained by dividing the mean AChE activity of cells incubated with alkylphenolic compounds and NGF by the mean AChE activity of cells incubated with NGF alone (positive control). By expressing in relative terms, we eliminate the differences in cell plating efficiency and cell washings during replicates. The AChE activity of cells incubated without NGF (negative control) indicated 63% less AChE activity compared to the positive control (data not shown). This difference is comparable to that obtained by Lucas and co-workers (1980). Since OP was of particular interest, we incubated the cells with NGF and much lower concentrations (100 to 800 nM) of 4-n-octylphenol and found out that AChE activity

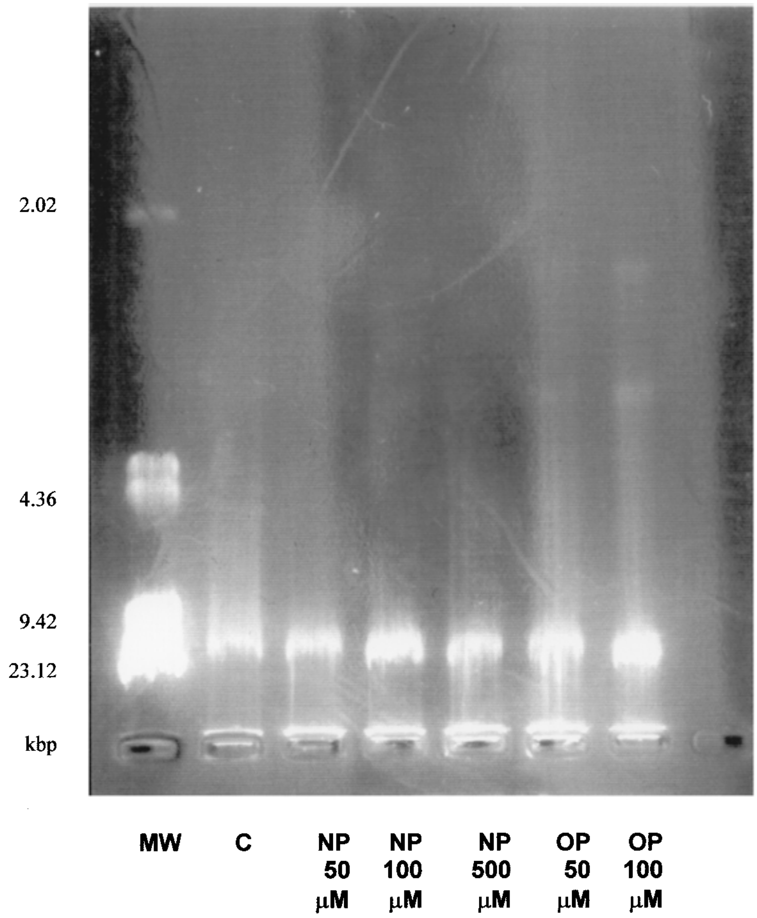

Figure 3. Ethidium-bromide-stained agarose gel showing genomic DNA from the rat pheochromocytoma cell line PC 12. Results show no fragmentation of DNA after 4 hr treatment with the indicated concentrations of 4-n-nonylphenol (NP) and 4-n-octylphenol (OP). PC12 DNA was extracted using the Sodium Iodide Method and electrophoresed on a 1.5% agarose gel. C, control; MW, molecular weight marker. Results represent three independent replicates.

decreased significantly ($p < 0.05$, t-test) beginning at 400 nM (data not shown).

Extremely low concentrations of camptothecin and actinomycin, two inhibitors of RNA synthesis, were also reported to effectively block the action of NGF on acetylcholinesterase activity (Greene and Rukenstein, 1981). Microtubule inhibitors (colchicine, taxol and nocodazole) as well as tunicamycin, an inhibitor of protein glycosylation, also suppress acetylcholinesterase release (Lucas and Kreutzberg, 1985). According to Lucas and Kreutzberg (1985), (1) NGF acts on acetylcholinesterase secretion via increased synthesis of mRNA; (2) Microtubule inhibitors act by preventing intracellular transport; and (3) Tunicamycin acts by decreasing the intracellular activity of acetylcholinesterase, possibly via enhanced proteolytic breakdown. Some of the alkylphenols used in this study have been reported to bind to the nuclear estrogen receptor in *in vitro* binding assays, suggesting that they can act as xenoestrogens (White et al., 1994). Moreover, they sometimes induce protein expression and enzyme activities in other cell lines such as MCF-7 cells. Hence, they could not have acted in the same way as the aforementioned compounds in suppressing AChE activity. In PC12 cells, NGF interacts with two distinct plasma membrane receptor proteins: $p75^{NGFR}$, a cysteine-rich glycoprotein having a relatively low affinity for NGF (Chao et al., 1992) and $p140^{trk}$ (Klein et al., 1991), a receptor tyrosine kinase which binds NGF with a high affinity, resulting in the

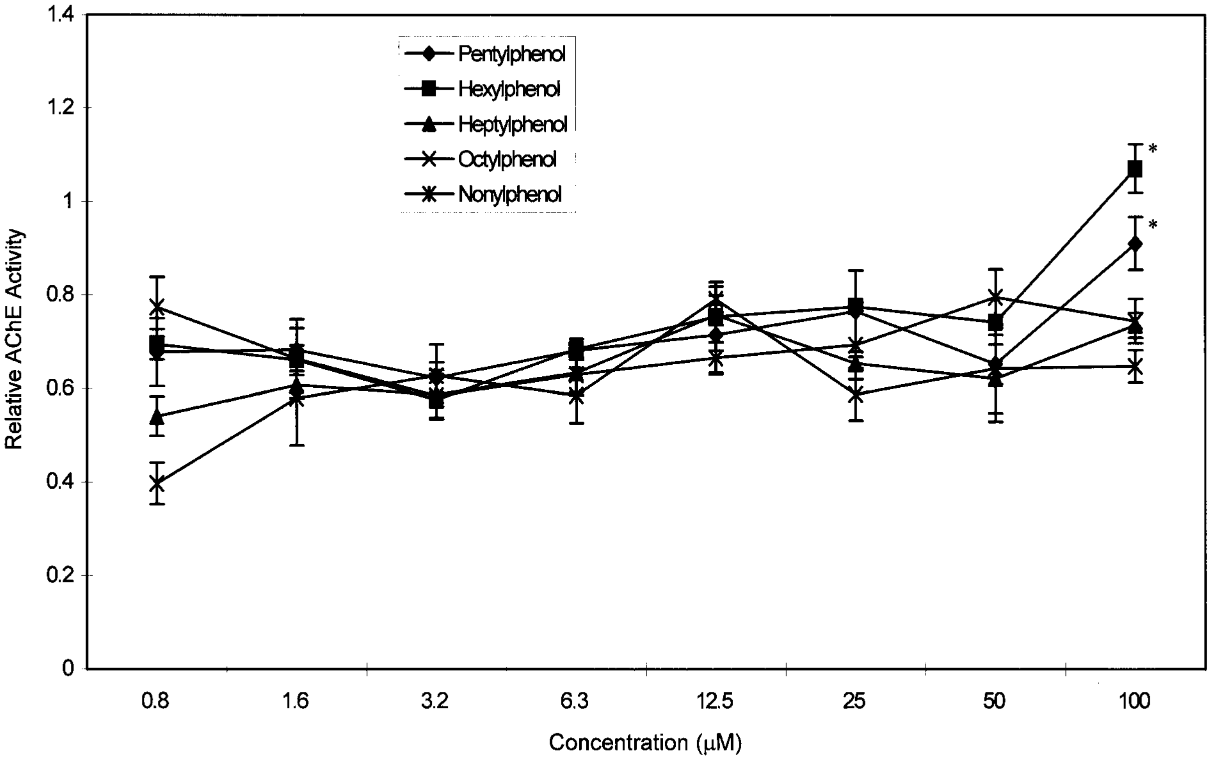

Figure 4. Relative acetylcholinesterase (AChE) activity of PC12 cells after 24 hr incubation with 40 ng ml^{-1} nerve growth factor (NGF), which stimulates AChE release, and varying concentrations of different alkylphenols. Results based on three independent replicates are expressed as relative acetylcholinesterase activity, which is obtained by dividing the mean AChE activity of cells incubated with alkylphenolic compounds and NGF by the mean AChE activity of cells incubated with NGF alone (positive control), ± SEM. Relative AChE activity of positive control is therefore 1. Results show significant decreases compared to control ($p < 0.05$, t-test), except for those with an asterisk (*). Cells incubated without NGF and alkylphenolic compounds (negative control) showed 63% lower AChE activity compared to positive control. This is equivalent to a relative AChE activity of 0.37. (Data not shown in figure.)

rapid tyrosine autophosphorylation of the receptor and activation of signal transduction proteins (Kim et al., 1991; Maher et al., 1988; Soltoff et al., 1992). The authors suggest two possible mechanisms by which alkylphenolic compounds suppress AChE activity: (1) it is possible that regulation of AChE activity requires a high degree of receptor occupancy by NGF and that the alkylphenols act as antagonists by competing with NGF for the NGF binding sites on the plasma membrane; and, (2) it is also possible that the alkylphenolic compounds, like the plant estrogen genistein, directly inhibit protein kinase activity by blocking autophosphorylation in the receptor tyrosine kinase, thus preventing further signal transduction. Further studies are indeed required to accurately determine whether alkylphenolic compounds suppress AChE activity with or without the involvement of the NGF receptor.

Conclusion

This research has shown that the alkylphenols used in this study did not induce necrosis of PC12 cells even at concentrations as high as 300 μM in a 15-min incubation period. Apoptosis was also not induced when the cells were exposed to nonylphenol and octylphenol for 4 hr at concentrations as high as 500 and 100 μM, respectively. However, the acetylcholinesterase activity of the PC12 cells was significantly inhibited at concentrations as low as 0.8 μM., with octylphenol showing the most significant effect. This suppression may be due to competition with the NGF binding site or inhibition of protein kinase activity by alkylphenols. Since some alkylphenols are known to be estrogenic at concentrations as low as 1 μM and also cause developmental problems in animals at higher concentrations, these findings on PC12 cells may further implicate alkylphenols in neurological and behavioral disturbances in both animals and humans.

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