The Japanese Society of Toxicology

Fundamental Toxicological Sciences
URL : http://www.fundtoxicolsci.org/index_e.html

Original Article

Biphenyl increases the intracellular Ca^{2+} concentration in HL-60 cells

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(Received April 20, 2018; Accepted May 1, 2018)

ABSTRACT — Biphenyl is a universal intermediate agent used as a protectant in various industrial activities. Biphenyl is currently used in postharvest applications as a fungicide in foreign countries to maintain the safety and quality of agricultural products. However, the risk of using biphenyl is in dispute in Japan. The toxicity of biphenyl has been studied in animals, and reportedly affects the liver and kidney especially. However, the toxic effect of biphenyl on cells is currently not well-understood, and the mechanism is unclear. We examined the toxicity of biphenyl on HL60 cells, a human promyelocytic leukemia cell line, by performing flow cytometry analyses with fluorescent probes. Biphenyl at 100 μM or greater significantly increased lethality and the intensity of side scatter on HL60 cells. Moreover, biphenyl at 30 μM or greater increased intracellular Ca^{2+} in a concentration-dependent manner. This increase resulted from free extracellular Ca^{2+} entering into the cells. These results indicate that an increase in intracellular Ca^{2+} may be one of several causes of the cytotoxicity induced by biphenyl. This study will contribute to the safety evaluation of biphenyl in the future.

Key words: Biphenyl, Intracellular Ca^{2+}, Cytotoxicity

INTRODUCTION

Biphenyl (CAS No. 92-52-4) exists naturally as a component of coal tar, crude oil, and natural gas (Adams and Richardson, 1953). Biphenyl is used as a fungistat, most commonly to preserve packaged citrus fruits during transportation and storage (HSDB, 2015; Li *et al*., 2016). The Ministry of Health, Labour, and Welfare in Japan has stated that biphenyl residues in foods should be less than 0.07 g/kg (Ministry of Health, Labour and Welfare, 2009).

Biphenyl is well-absorbed through the gastrointestinal tract, and is excreted rapidly and almost exclusively in the urine (American Conference of Governmental Industrial Hygienists, 2005; Hazleton *et al*., 1956; Sugihara *et al*., 1997). In the liver of humans and animals, biphenyl is hydroxylated to 4-hydroxybiphenyl (30%), biphenyl glucuronide (18.4%), 4,4′-dihydroxybiphenyl (5.3%), 3,4-dihydroxybiphenyl (3.1%), and diphenylmercapturic acid (1.3%) (American Conference of Governmental Industrial Hygienists, 2005). Toxicity of biphenyl has been reported under *in vivo* experimental conditions. For example, the administration of biphenyl to rats induces growth inhibition and pathological changes, including inflammation in the kidneys and decreases

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in hemoglobin in the blood (Shiraiwa *et al*., 1989; Booth *et al*., 1961). In addition, the tumor-promoting potential of biphenyl has been reported in several studies (Shiraiwa *et al*., 1989; Kurata *et al*., 1986; Shibata *et al*., 1989; Williams, 1978). However, the toxic effect of biphenyl on cells is currently not well-understood.

Increased levels of intracellular Ca^{2+} regulate cell death after apoptotic stimuli (Zhou *et al*., 2015; Scotto *et al*., 1998). In our previous study, we reported that food additives such as t-butylhydroquinone and polysorbate 80 elevated intracellular Ca^{2+} levels and increased the population of dead cells (Kamemura *et al*., 2017; Hirama *et al*., 2004). The main purpose of this study was to examine whether biphenyl increased intracellular Ca^{2+} levels and induced cytotoxicity on HL60 cells.

MATERIALS AND METHODS

Chemicals

Biphenyl and dimethyl sulfoxide (DMSO) were purchased from Nacalai Tesque (Kyoto, Japan). Propidium iodide (PI) was obtained from Molecular Probes (Eugene, OR, USA). Fluo-3-AM was obtained from Dojin Chemical Laboratory (Kumamoto, Japan). Biphenyls were initially dissolved in DMSO and then added to cell suspensions.

Cell culture

HL-60 cells, originally derived from a human promyelocytic leukemia (Gallagher *et al*., 1979), were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA), 2 mM L-glutamine (HyClone, Logan, UT, USA), 100 units/mL penicillin, and 100 µg/mL streptomycin in a 37°C incubator with 5% CO_2. HL-60 cells were cultured at a density of 5×10^5 cells/mL and were used to examine the toxic effects of biphenyl.

Fluorescence measurements of intracellular Ca^{2+} levels and cell lethality

Intracellular Ca^{2+} levels and cell lethality were examined using the fluorescent probes Fluo-3-AM and PI, respectively, by a flow cytometer (FACSVerse™, BD Biosciences, Franklin Lakes, NJ, USA). The methods used in this study were similar to those described previously (Kamemura *et al*., 2017; Hirama *et al*., 2004). PI fluorescence was measured at excitation and emission wavelengths of 488 and 600 ± 20 nm, respectively. Fluo-3-AM fluorescence was measured at excitation and emission wavelengths of 488 and 530 ± 20 nm, respectively. Fluo-3-AM fluorescence was measured from cells that were not stained with PI.

Statistical analyses

Statistical analyses were conducted using the Statistical Package for Social Sciences (version 18.0; IBM, Chicago, IL, USA). Values are expressed as means ± standard deviation of four samples. Statistical comparisons were performed using ANOVA followed by Tukey's multiple comparison test. Differences were considered significant at P values of less than 0.05. Asterisks indicate significant differences between the control (0.1% DMSO) and biphenyl-treated groups (*$P < 0.05$, **$P < 0.01$).

RESULTS

Biphenyl increases cell lethality

Exposure to 300 µM biphenyl for 3 hr increased the population of dead HL-60 cells (Fig. 1A). Biphenyl at 100-300 µM for 3 hr significantly increased cell lethality in a concentration-dependent manner (Fig. 1B).

In addition, as shown in Fig. 2A, incubation of cells with 300 µM biphenyl for 3 hr changed the cytogram of PI stained cells (forward scatter versus side scatter). Biphenyl at 300 µM significantly increased the side scatter, but not the forward scatter (Fig. 2A). A significant increase in the intensity of the side scatter was observed with 100-300 µM of biphenyl (Fig. 2B). Therefore, HL60 cells were treated with 10-100 µM biphenyl in subsequent experiments.

Biphenyl induced increases in the intracellular Ca^{2+} level

A relationship between changes in side scatter and disturbance of intracellular Ca^{2+} homeostasis was shown in our previous study (Kinazaki *et al*., 2009). Therefore, to confirm whether biphenyl increased intracellular Ca^{2+} levels, biphenyl-induced changes of Fluo-3 fluorescence were examined. Biphenyl shifted the histogram of Fluo-3 fluorescence toward a higher intensity range (Fig. 3A). Exposure of cells to biphenyl at concentrations of 30 µM or greater significantly increased the intensity of Fluo-3 fluorescence in a concentration-dependent manner (Fig. 3B). This result indicates that biphenyl exposure resulted in increased intracellular Ca^{2+} concentrations in HL-60 cells.

To determine whether the biphenyl-induced increase of Fluo-3 fluorescence was dependent on external free Ca^{2+}, the effect of biphenyl was examined under Ca^{2+}-free conditions. Biphenyl at 100 µM significantly increased the intensity of Fluo-3 fluorescence 3 hr after treatment in normal culture medium. However, treatment with

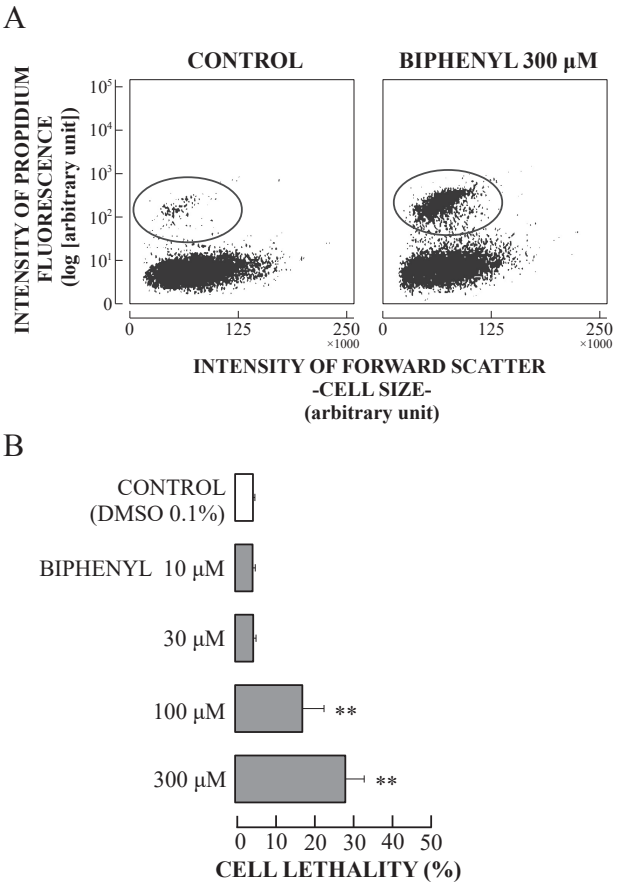

Fig. 1. Changes in HL60 cell survival induced by biphenyl. (A) Changes in the cytograms of propidium iodide (PI) fluorescence after biphenyl exposure. Each cytogram consists of 10,000 cells. (B) Concentration-dependent changes in the population of cells stained with PI following exposure to biphenyl for 3 hr.

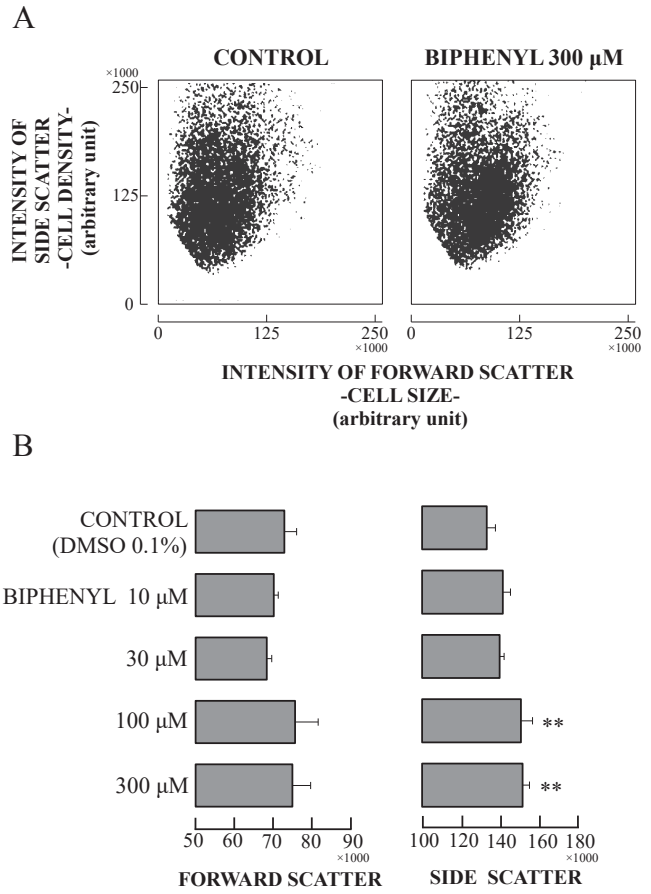

Fig. 2. Effects of biphenyl on the cytograms of HL60 cells. (A) Changes in the cytogram (forward scatter vs. side scatter) induced by biphenyl. Each cytogram consists of 10,000 cells. (B) Biphenyl-induced changes in forward and side scatter (an indicator of Ca^{2+} concentration) are shown in the graphs.

100 μM biphenyl in Ca^{2+}-free medium did not change the intensity of Fluo-3 fluorescence (Fig. 4), indicating that the increase resulted from extracellular Ca^{2+}.

DISCUSSION

Biphenyl is recognized in Japan as a fungicide that is used for postharvest applications. It is important to know the risks of biphenyl because many foods, including citrus fruits, are exposed to biphenyl postharvest and are imported from foreign countries to Japan through the Trans-Pacific Strategic Economic Partnership Agreement (TPP), a trade agreement involving many countries. We examined the toxic effects of biphenyl in HL60 cells, a human promyelocytic leukemia line. Using PI staining, biphenyl increased cell lethality and the intensity of side scatter. Nakagawa *et al*. has also reported cytotoxic effects of 750 μM biphenyl on isolated rat hepatocytes (Nakagawa *et al*., 1993). We showed that biphenyl at 100 μM induced death in HL60 cells. Therefore, we suggest that blood cells have a higher sensitivity to biphenyl.

We determined whether intracellular Ca^{2+} was increased after treating HL60 cells with biphenyl because the change of side scatter intensity involves a change of cell membrane permeability (Murphy and Roederer, 1986). In addition, increases of side scatter intensity are indicative of elevated Ca^{2+} transport from the extra- to intracellular environment (Telford and Miller, 1996).

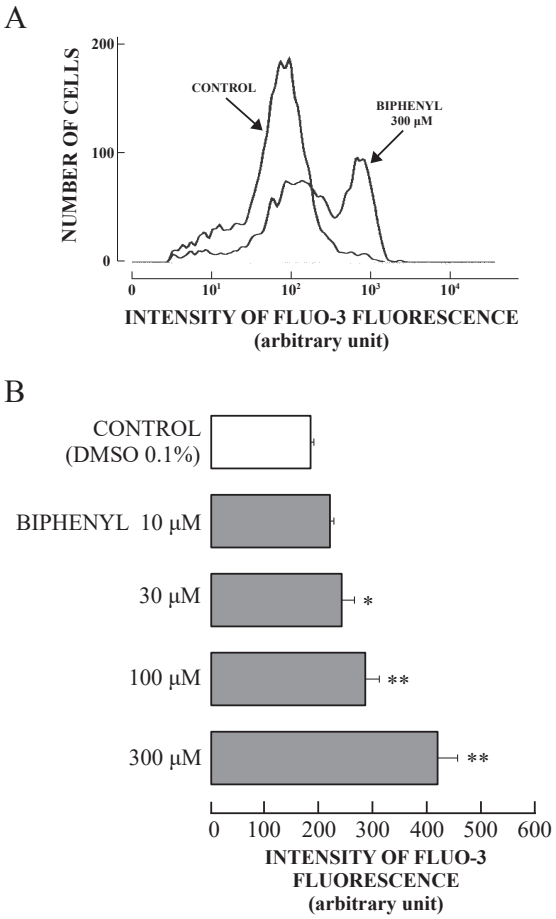

Fig. 3. Biphenyl increases intracellular Ca^{2+} concentrations in HL60 cells. (A) Biphenyl-induced increases in Fluo-3 fluorescence for 1 h are shown in the histograms. (B) Biphenyl-induced changes in Fluo-3 fluorescence (an indicator of Ca^{2+} concentration) are shown in the graphs.

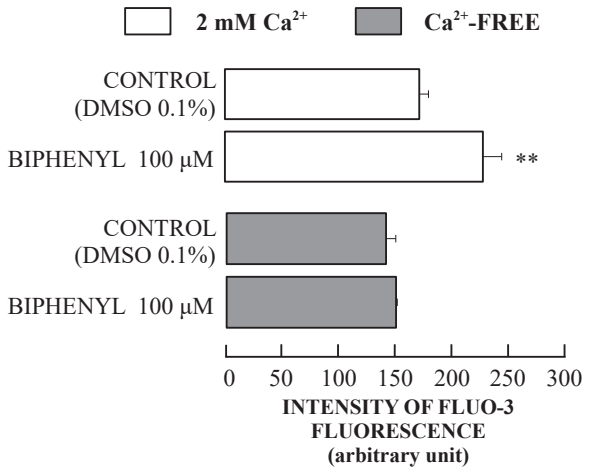

Fig. 4. Effect of biphenyl on the mean intensity of Fluo-3 fluorescence in the presence (2 mM Ca^{2+}) and absence of external Ca^{2+}.

Biphenyl at 30 μM or greater significantly increased intracellular Ca^{2+} (Fig. 3). However, 30 μM biphenyl for 3 hr did not increase cell lethality even though an increase of intracellular Ca^{2+} levels can induce cell death (Zhou *et al*., 2015; Scotto *et al*., 1998). Therefore, the biphenyl exposure concentration must be limited to avoid the risk of cytotoxicity. Moreover, the origin of the increased intracellular free Ca^{+} appeared to be through transport from the extracellular medium because removal of extracellular free Ca^{+2} (Ca^{2+}-free condition) inhibited the increase of intracellular Ca^{+2} by biphenyl (Fig. 4).

Exposure of rats to dietary biphenyl inhibits their growth and induces pathological changes in the kidneys at autopsy, including scarring, inflammation, and tubular atrophy (Booth *et al*., 1961). In the current study, we showed that biphenyl was toxic to HL60 blood cells. The toxicity of biphenyl to humans and animals may be caused by an increase of intracellular Ca^{2+} levels.

ACKNOWLEDGMENTS

This study was supported by a Grant-in-Aid for Scientific Research (C26340039) from the Japan Society for the Promotion of Science.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Adams, N.G. and Richardson, D.M. (1953): Isolation and identification of biphenyls from West Edmond crude oil. Anal. Chem., **25**, 1073-1074.

American Conference of Governmental Industrial Hygienists (2005): Documentation of Threshold Limit Values for Chemical Substances and Physical Agents and Biological Exposure Indices; American Conference of Governmental Industrial Hygienists: Cincinnati, OH, USA.

Booth, A.N., Ambrose, A.M., DeEds, F. and Cox, A.J. Jr. (1961): The reversible nephrotoxic effects of biphenyl. Toxicol. Appl. Pharmacol., **3**, 560-567.

Gallagher, R., Collins, S., Trujillo, J., McCredie, K., Ahearn, M., Tsai, S., Metzgar, R., Aulakh, G., Ting, R., Ruscetti, F. and Gallo, R. (1979): Characterization of the continuous, differentiating myeloid cell line (HL-60) from a patient with acute promyelocytic leukemia. Blood, **54**, 713-733.

Halpaap-Wood, K., Horning, E.C. and Horning, MG. (1981): The

effect of phenobarbital and beta-naphthoflavone induction on the metabolism of biphenyl in the rat and mouse. Drug Metab. Dispos., **9**, 97-102.

Hazleton, L.W., Kundzins, W., Howard, J.W. and Johnston, C.D. (1956) : Studies on biphenyl in the dog. Abstract, 20th Int. Physiol. Congress, **20**, 412-413.

Hirama, S., Tatsuishi, T., Iwase, K., Nakao, H., Umebayashi, C., Nishizaki, Y., Kobayashi, M., Ishida, S., Okano, Y. and Oyama, Y. (2004): Flow-cytometric analysis on adverse effects of polysorbate 80 in rat thymocytes. Toxicology, **199**, 137-143.

HSDB (Hazardous Substances Data Bank) (2014): Biphenyl-CAS-RN 92-52-4. 2014 Available: http://toxnet.nlm.nih.gov/cgi-bin/sis/search2/f?./temp/~sj6N5p:1 [accessed 20 October 2015].

Kamemura, N., Oyama, K., Kanemaru, K., Yokoigawa, K. and Oyama, Y. (2017): Diverse cellular actions of tert-butylhydroquinone, a food additive, on rat thymocytes. Toxicol. Res., **6**, 922-929.

Kinazaki, A., Sakanashi Y., Oyama, T.M., Shibagaki, H., Yamashita, K., Hashimoto, E., Nishimura, Y., Ishida, S., Okano, Y. and Oyama, Y. (2009): Micromolar Zn (2+) potentiates the cytotoxic action of submicromolar econazole in rat thymocytes: possible disturbance of intracellular Ca(2+) and Zn(2+) homeostasis. Toxicol. In Vitro, **23**, 610-616.

Kurata, Y., Asamoto, M., Hagiwara, A., Masui, T. and Fukushima, S. (1986): Promoting effects of various agents in rat urinary bladder carcinogenesis initiated by N-butyl-N-(4-hydroxybutyl)nitrosamine. Cancer Lett., **32**, 125-135.

Li, Z., Hogan, K.A., Cai, C. and Rieth, S. (2016): Human health effects of biphenyl: key findings and scientific issues. Environ. Health Perspect., **124**, 703-712.

Ministry of Health, Labour and Welfare. (2009): Risk Assessment; Ministry of Health, Labour and Welfare: Tokyo, Japan.

Murphy, R.F. and Roederer, M. (1986): Flow cytometric analysis of endocytic pathways. In: Applications of Fluorescence in the Biomedical Sciences, Taylor DL (ed). Alan R. Liss. Inc., New York, 1986, pp.545-566.

Nakagawa, Y., Tayama, S., Moore, G. and Moldéus, P. (1993): Cytotoxic effects of biphenyl and hydroxybiphenyls on isolated rat hepatocytes. Biochem. Pharmacol., **45**, 1959-1965.

Scotto, C., Deloulme, J.C., Rousseau, D., Chambaz, E. and Baudier, J. (1998): Calcium and S100B regulation of p53-dependent cell growth arrest and apoptosis. Mol. Cell Biol., **18**, 4272-4281.

Shibata, M.A., Tanaka, H., Yamada, M., Tamano, S. and Fukushima, S. (1989): Proliferative response of renal pelvic epithelium in rats to oral administration of ortho-phenylphenol, sodium ortho-phenylphenate and diphenyl. Cancer Lett., **8**, 19-28.

Shiraiwa, K., Takita, M., Tsutsumi, M., Kinugasa, T., Denda, A., Takahashi, S. and Konish, Y. (1989): Diphenyl induces urolithiasis but does not possess the ability TO promote carcinogenesis by N-ethyl-n-hydroxyethylnitrosamine in kidneys of rats. J. Toxicol. Pathol., **24**, 41-48.

Sugihara, N., Shimomichi, K. and Furuno, K. (1997): Cytotoxicity of food preservatives in cultured rat hepatocytes loaded with linolenic acid. Toxicology., **120**, 29-36.

Telford, W.G. and Miller, R.A. (1996): Detection of plasma membrane Ca^{2+}-ATPase activity in mouse T lymphocytes by flow cytometry using fluo-3-loaded vesicles. Cytometry, **24**, 243-250.

Williams, G.M. (1978): Further improvements in the hepatocyte primary culture DNA repair test for carcinogens: detection of carcinogenic biphenyl derivatives. Cancer Lett., **4**, 69-75.

Zhou, X., Hao, W., Shi, H., Hou, Y. and Xu, Q. (2015): Calcium homeostasis disruption - a bridge connecting cadmium-induced apoptosis, autophagy and tumorigenesis. Oncol. Res. Treat., **38**, 311-315.