Apoptosis Inducing and Enhancing Activities of Environmental Estrogenic Compounds

Masatoshi Beppu,* Yoshie Nakadai, and Yu Igarashi

Laboratory of Environmental Health Science, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan

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Effects of estrogenic compounds (bisphenol A, alkyl phenols, phthalate esters, and genistein) on T lymphocyte apoptosis were investigated in vitro. The assays were performed in the absence or presence of low concentrations of apoptosis-inducing agents etoposide or dexamethasone to detect apoptosis-inducing, -enhancing, and -suppressing activities of the test compounds. When T lymphatic Jurkat cells were exposed to 10 μM bisphenol A for 20 hr, apoptosis was not induced, but apoptosis induced by 1 μM etoposide was significantly enhanced. 4-*n*-Nonylphenol (10 μM) showed an apoptosis-inducing activity significantly. 4-*tert*-Octylphenol (10 μM) exhibited an apoptosis-enhancing activity. In contrast, phthalate esters including di-*n*-butyl phthalate and di-2-ethylhexyl phthalate showed neither activity at 10 μM. Genistein (10 μM) significantly exhibited apoptosis-inducing and enhancing activities. On the other hand, 17β-estradiol did not show any of these activities at 10 nM, the concentration exerting the estrogenic activity comparable to or much higher than that of 10 μM of the test compounds. Effects of these estrogenic compounds on apoptosis were also investigated using mouse primary thymocytes. Mouse thymocytes similarly exposed to the estrogenic compounds in the absence or the presence of dexamethasone for 6 hr were characterized. In agreement with Jurkat cells, apoptosis-inducing or/and -enhancing activities were observed for the cells coincubated with bisphenol A, alkyl phenols, and genistein, but not those with di-2-ethylhexyl phthalate or 17β-estradiol. The apoptosis-inducing or/and -enhancing effects of the estrogenic compounds observed here appear to be due to their unidentified properties other than estrogenic activity.

Key words —— apoptosis, endocrine disruptor, estrogenic compound, T lymphocyte, thymocyte

*To whom correspondence should be addressed: Laboratory of Environmental Health Science, School of Pharmacy, Tokyo University of Pharmacy and Life Sciences, 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan. Tel.: +81-42-676-4495; Fax: +81-42-676-4499; E-mail: beppum@ps.toyaku.ac.jp

INTRODUCTION

Endocrine disrupting chemicals (EDCs) or so-called environmental hormones have been suspected to be associated with various disorders of endocrine system in wildlife, particularly disorders in reproduction.[1] Moreover, there is another concern that EDCs may also disrupt other biological phenomena involving intra- or/and extracellular signal transduction.

Apoptosis is the cell death executed by the intrinsic self-killing machinery of the cell. It occurs physiologically in embryogenesis, tissue turnover, and tissue remodeling. It is also caused by pathological or environmental factors such as certain viruses, bacteria, radiations, natural compounds, and chemicals. Various EDCs have been reported to induce apoptosis in various types of cells,[2–14] suggesting that those chemicals exert cell toxicity by triggering the apoptotic signals. However, another possibility that EDCs may enhance or suppress the apoptotic signals induced by physiological or other factors has not been investigated. If some environmental chemicals exert such apoptosis-interfering effect, they could be potentially detrimental to the body.

In the present study, we assessed apoptosis-inducing as well as -enhancing/-suppressing activities of EDCs using human T lymphatic Jurkat cells and mouse thymocytes. The EDCs tested were environmental estrogenic compounds,[1,15] including bisphenol A,[15,16] alkyl phenols (4-*n*-nonylphenol[16,17] and 4-*tert*-octylphenol[15,16]), and phthalate esters (di-*n*-butyl phthalate[18] and di-2-ethylhexyl phthalate[19]). A phytoestrogen genistein[15] and a physiological estrogen 17β-estradiol were also investigated for their apoptosis-inducing and -enhancing/-suppressing activities.

MATERIALS AND METHODS

Materials —— Bisphenol A was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Genistein, 4-*n*-nonylphenol, 4-*tert*-octylphenol, etoposide, dexamethasone, dimethyl sulfoxide

(DMSO), paraformaldehyde, polyoxyethylene (10) octylphenol ether (Triton X-100), diisopropyl fluorophosphate (DFP), phenylmethylsulfonylfluoride (PMSF), and leupeptin hemisulfate monohydrate (leupeptin) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Di-2-ethylhexyl phthalate, and di-*n*-butyl phthalate were the products of Kanto Chemical Co. (Tokyo, Japan). β-Estradiol-watersoluble (17β-estradiol), aprotinin, RPMI 1640 medium, and RPMI 1640 medium (phenol red free) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Fetal calf serum (FCS) and Hanks' balanced salt solution (HBSS) phenol red free were obtained from Biological Industries (Kibbutz Beit Haemek, Israel) and Nissui Pharmaceutical Co. (Tokyo, Japan), respectively. MEBCYTO® apoptosis kit and MEBSTAIN apoptosis kit direct were purchased from Medical & Biological Laboratories (Nagoya, Japan). HEPES and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) were from Dojindo Laboratories (Kumamoto, Japan). Ac-Asp-Asn-Leu-Asp-MCA (Ac-DNLD-MCA) and 7-amino-4-methyl-coumarin (AMC) were obtained from Peptide Institute (Osaka, Japan). All plastic ware including culture dishes, culture plates, and culture flasks used for cell culture were the products of Iwaki Glassware Co. (Tokyo, Japan). According to the manufacturer's information, bisphenol A was not contained in the plastic ware.

Preparation of Reagent Solutions for Cell Culture —— The test compounds except 17β-estradiol were dissolved in DMSO at 50 mM, and stored at –80°C. Before use, aliquots of the stored solutions were diluted with RPMI-1640 medium (phenol red free) supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin and 5% heat-inactivated FCS (RPMI1640-5% FCS) to give 0.1 mM solutions in RPMI1640-5% FCS containing 0.2% DMSO. The 0.1 mM solutions were appropriately diluted with RPMI1640-5% FCS containing 0.2% DMSO, and added to the cell culture at 0.1 volume of the culture.

Etoposide, an apoptosis-inducing agent for Jurkat cells,[20] was dissolved in DMSO at 25 mM, and stored at –80°C. Before use, an aliquot of the stored solution was diluted with RPMI1640-5% FCS to give a 50 μM solution in RPMI1640-5% FCS containing 0.2% DMSO, and added to the cell culture at 0.02 volume of the culture, giving the final concentration of 1 μM.

Dexamethasone, an apoptosis-inducing agent for thymocytes,[21] was also dissolved in DMSO at 625 μM, processed and used similarly, to give the final concentration of 25 nM.

All the cultures were performed at the final DMSO concentration of 0.024%. 17β-Estradiol was dissolved in HBSS (phenol red free) at 0.1 mM, and stored at –80°C. Before use, an aliquot of the stored solution was diluted with RPMI1640-5% FCS containing 0.2% DMSO, and added to the cell culture at 0.1 volume of the culture.

Cell Culture —— All the cell cultures were performed in a 5% CO_2 incubator at 37°C.

Jurkat Cells: Jurkat cells (Riken Cell Bank, Tsukuba, Japan) were maintained in RPMI1640 medium supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin and 5% heat-inactivated FCS (RPMI1640-5% FCS). Experiments were carried out using 24-well plastic plates (1.0 ml cell suspension/well) and culturing the cells (2.0×10^6 cells/ml) in RPMI1640-5% FCS for 20 hr in the presence or absence of the test compounds and the apoptosis-inducing agent etoposide as described above.

Mouse Thymocytes: Thymocytes were prepared from the thymus of ddY male mice 3–6 weeks of age by gently teasing in RPMI-1640 medium (phenol red free) supplemented with 20 mM HEPES, 50 units/ml penicillin, 50 μg/ml streptomycin and 10% heat-inactivated FCS (RPMI1640-HEPES-10% FCS). After washing and resuspending in the same medium, the cells were cultured in 24-well plastic plates (1.0 ml cell suspension/well, 2.0×10^6 cells/ml) for 6 hr in the presence or absence of the test compounds and an apoptosis-inducing agent dexamethasone (25 nM). Animal treatments were performed according to the institutional guideline for animal research at the Tokyo University of Pharmacy and Life Sciences that conforms to official guidelines for the care and use of laboratory animals.

Measurement of Apoptosis —— Extent of apoptosis was measured by the following three different criteria.

Phosphatidylserine (PS) Exposure: Externalization of PS on cell surface was assessed by binding of fluorescein isothiocyanate-labeled annexin V (FITC-annexin V) to the cells using a commercial assay kit (MEBCYTO® apoptosis kit).[22] Briefly, Jurkat cells (2×10^5 cells) or mouse thymocytes (2×10^5 cells) withdrawn from the culture were suspended in a kit buffer containing appropriate concentrations of FITC-annexin V and propidium iodide (PI) and incubated for 15 min at room temperature in the dark according to the manufacturer's in-

struction. The cell suspension was then diluted with another buffer and immediately analyzed by a flow cytometer (FACSCalibur, Becton Dickinson) using a software CELLQUEST, gating for FSC and SSC region of intact Jurkat cells. PI-positive cells were regarded as necrotic cells.

TUNEL Method: Apoptosis-induced DNA fragmentation was assessed by terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) method using a commercial assay kit (MEBSTAIN apoptosis kit direct).[22] Briefly, Jurkat cells (2×10^5 cells) withdrawn from the culture were fixed in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate buffered saline (DPBS(–)) containing 4% paraformaldehyde (4°C, 30 min), washed in DPBS(–) containing 0.2% bovine serum albumin (BSA) (0.2% BSA-DPBS(–)), and permealized in 70% ethanol at –20°C for 30 min. After washing in 0.2% BSA-DPBS(–) again, the cells were treated with TdT and FITC-dUTP for 1hr at room temperature in the dark to label the fragmenting nuclear DNA at the 3′-hydroxyl ends with FITC. The cell suspension was diluted with 500 μl of 0.2% BSA-DPBS(–), and subjected to flow cytometric analysis, gating for FSC and SSC region of intact Jurkat cells.

Caspase Activity: Jurkat cells (2×10^5 cells) withdrawn from the culture were washed twice in chilled DPBS(–), and lysed in a lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 w/v % Triton-X 100, 1 mM PMSF, 5 mM EDTA, and 1 μg/ml leupeptin. Cell lysates were obtained as supernatants after centrifugation of the mixtures. Protein concentrations of the lysates were determined by the dye-binding method,[23] and adjusted to 200 μg/ml. Caspase activity of the cell lysates was assessed by incubation of the lysates (50 μl) with 100 μM Ac-DNLD-MCA, a caspase 3 substrate, in 2-fold concentration of caspase assay buffer [20 mM HEPES (pH7.4), 20 w/v % sucrose, 0.2 w/v % CHAPS, 0.2 mM PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 4 mM dithiothreitol] (50 μl) in 96-well black microplates at 37°C for 2 hr in the dark. After 2 hr of the reaction, fluorescence of the reaction mixture at excitation of 380 nm and at emission of 460 nm was measured using a microspectrometer Safire (Tecan, Maennedorf, Switzerland).

Statistical Analysis —— The data obtained were expressed as the mean ± S.D. of triplicate determinations. Statistical differences between means were determined using an unpaired Student's *t* test.

RESULTS

Effects of Estrogenic Compounds on Apoptosis of Jurkat Cells

Apoptosis-inducing activity of estrogenic compounds was assessed by culturing Jurkat cells for 20 hr in the presence of 10 μM of the compounds. Apoptosis-enhancing or -suppressing activities were assessed by culturing the cells in the presence of 1 μM of an apoptosis-inducing agent etoposide and 10 μM of the compounds.

When Jurkat cells were cultured for 20 hr in the presence of 0 or 10 μM bisphenol A (Fig. 1A, open circles), apoptosis was not induced as measured by PS exposure and DNA fragmentation. Caspase 3 also was not activated. The results suggest that bisphenol A does not have apoptosis-inducing activity. However, in the presence of 1 μM etoposide, where some proportions of the cells become apoptotic (Fig. 1A, closed circles at 0 μM), the proportions of the apoptotic cells were significantly increased by 10 μM bisphenol A (A, closed circles), as measured by PS exposure and DNA fragmentation, suggesting that bisphenol A enhanced the etoposide-induced apoptosis.

4-*n*-Nonylphenol showed an apoptosis-inducing activity at 10 μM (Fig. 1B, open circles), and augmented the apoptosis induced by 1 μM etoposide (closed circles). The latter effect appears to be due to its apoptosis-inducing activity.

4-*tert*-Octylphenol did not show an apoptosis-inducing activity at 10 μM (Fig. 1C, open circles). However, similarly to bisphenol A, it exhibited an apoptosis-enhancing activity toward the etoposide-induced apoptosis (closed circles), although caspase 3 activity appeared not to change.

Di-*n*-butyl phthalate and di-2-ethylhexyl phthalate showed neither apoptosis-inducing nor -enhancing/suppressing activities significantly (Fig. 1D and 1E, respectively).

Genistein exhibited an apoptosis-inducing activity at 10 μM (Fig. 1F, open circles), and markedly enhanced the apoptosis induced by 1 μM etoposide (closed circles). The latter enhancement by genistein may be, at least partly, due to its apoptosis-inducing activity.

17β-Estradiol showed neither apoptosis-inducing nor -enhancing/suppressing activities at 10 nM (Fig. 1G), the concentration roughly comparable to or much higher than the estrogenic activity of the test compounds.[15]

When necrosis of Jurkat cells treated as above

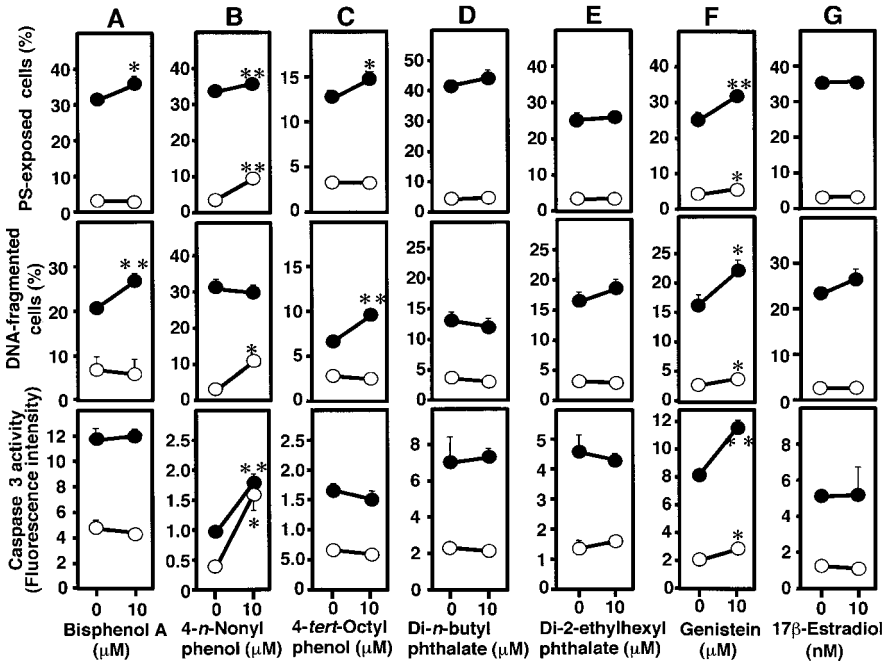

Fig. 1. Effects of Various Estrogenic Compounds on Nonapoptotic and Etoposide-Induced Apoptotic Jurkat Cells

Jurkat cells were cultured with 0 or 10 μM of estrogenic compounds for 20 hr in the presence (closed circles) or absence (open circles) of etoposide (1 μM), and the extent of apoptosis was assessed by PS exposure (annexin V-binding method), DNA fragmentation (TUNEL method), and caspase 3 activity measured using a fluorescence-generating substrate Ac-DNLD-MCA as described in MATERIALS AND METHODS. A) Bisphenol A, B) 4-*n*-nonylphenol, C) 4-*tert*-octylphenol, D) di-*n*-butyl phthalate, E) di-2-ethylhexyl phthalate, F) genistein, and G) 17β-estradiol. Each point is the mean ± S.D. of triplicate determinations. Points showing no error bars are the points with errors less than the size of the symbols. * and **, significantly greater than control (0 μM), $p < 0.05$ and $p < 0.01$, respectively.

was assessed by PI staining, some proportions of the cells treated with 10 μM of 4-*n*-nonylphenol or genistein were found to be necrotic (Fig. 2B and 2F). This necrosis may be the secondary necrosis following the apoptotic sprocess.

Effects of Estrogenic Compounds on Apoptosis of Thymocytes

To see whether the apoptosis-inducing or the apoptosis-enhancing activities of the estrogenic compounds observed above using Jurkat T cell line are also observable in primary cells, effects on mouse thymocytes were studied. Mouse thymocytes were cultured with the estrogenic compounds for 6 hr in the presence or the absence of an apoptosis-inducing agent dexamethasone, and the extent of apoptosis was assessed by PS exposure. As shown in Fig. 3, in agreement with the observations in Jurkat cell culture, weak but significant apoptosis-inducing or/and -enhancing activities were observed for the cells co-incubated with bisphenol A (A), 4-*n*-nonylphenol (B), 4-*tert*-octylphenol (C) and genistein (E), but not those co-incubated with di-2-ethylhexyl phthalate (D) and 17β-estradiol (F).

Necrosis was not occurring under the culture conditions employed as detected by PI (data not shown).

DISCUSSION

In the present study, we tried to measure not only the apoptosis-inducing activity but also the apoptosis-enhancing or -suppressing activities using Jurkat T cells, and found that some estrogenic compounds not showing apoptosis-inducing activity exhibited apoptosis-enhancing activity (bisphenol A and 4-*tert*-octylphenol). When the test compounds showing the apoptosis-inducing activity potentiated the etoposide-induced apoptosis, it was difficult to assess whether the potentiation was simply due to the additive effect of the test compound and etoposide or due to the latent enhancing activity of the test compound (4-*n*-nonylphenol and genistein). Primary mouse thymocytes also showed similar tendency, suggesting that the observed cellular response is not confined to tumor cells but can be seen in normal T cells.

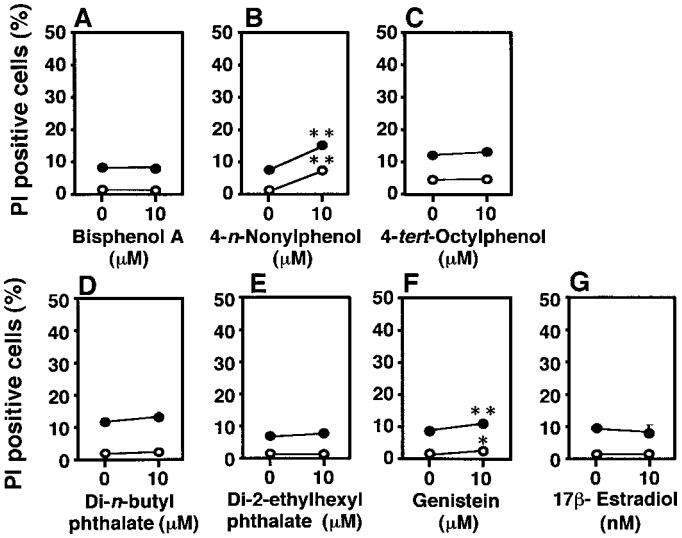

Fig. 2. Effects of Various Estrogenic Compounds on Necrosis of Nonapoptotic and Etoposide-Induced Apoptotic Jurkat Cells

Jurkat cells were cultured with the indicated concentrations of estrogenic compounds for 20 hr in the presence (closed circles) or absence (open circles) of etoposide (1 μM), and the extent of necrosis was assessed by PI staining as described in MATERIALS AND METHODS. A) bisphenol A, B) 4-*n*-nonylphenol, C) 4-*tert*-octylphenol, D) di-*n*-butyl phthalate, E) di-2-ethylhexyl phthalate, F) genistein, and G) 17β-estradiol. Each point is the mean ± S.D. of triplicate determinations. Points showing no error bars are the points with errors less than the size of the symbols. * and **, significantly greater than control (0 μM), $p < 0.05$ and $p < 0.01$, respectively.

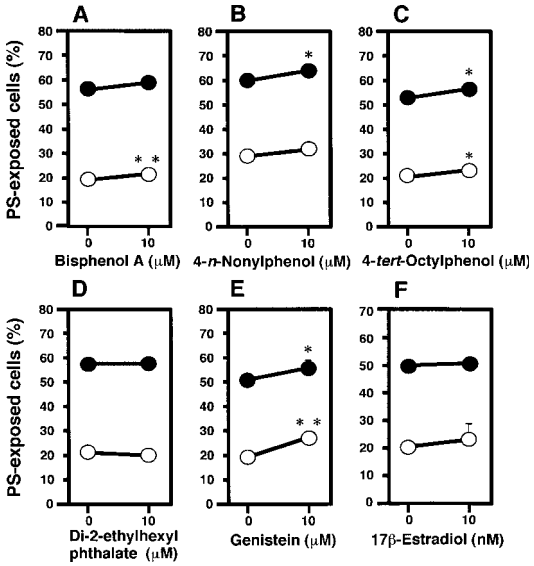

Fig. 3. Effects of Various Estrogenic Compounds on Nonapoptotic and Dexamethasone-Induced Apoptotic Mouse Thymocytes

Mouse thymocytes prepared as described in MATERIALS AND METHODS were cultured with the indicated concentrations of estrogenic compounds for 6 hr in the presence (closed circles) or absence (open circles) of dexamethasone (25 nM), and the extent of apoptosis was assessed by PS exposure as described in MATERIALS AND METHODS. A) Bisphenol A, B) 4-*n*-nonylphenol, C) 4-*tert*-octylphenol, D) di-2-ethylhexyl phthalate, E) genistein, and F) 17β-estradiol. Each point is the mean ± S.D. of triplicate determinations. Points showing no error bars are the points with errors less than the size of the symbols. * and **, significantly greater than control (0 μM), $p < 0.05$ and $p < 0.01$, respectively.

It is known that peripheral blood T lymphocytes and thymocytes express estrogen receptors,[24,25] and that Jurkat cells also express low levels of the receptors.[26] However, 17β-estradiol did not affect apoptosis of Jurkat cells at 10 nM. Its estrogenic activity at this concentration is comparable to or much higher than the estrogenic activities of 10 μM of the test compounds, because the ratios of the estrogenic activity of 17β-estradiol to that of bisphenol A, 4-*n*-nonylphenol, 4-*tert*-octylphenol, di-*n*-butyl phthalate, di-2-ethylhexyl phthalate, and genistein are approximately 1 to 1×10^{-4}, 1 to $< 3 \times 10^{-7}$, 1 to 1.5×10^{-3}, 1 to $< 3 \times 10^{-7}$, 1 to $< 1.5 \times 10^{-7}$, and 1 to 3×10^{-3}, respectively, based on the data of the yeast two-hybrid reporter gene expression assay.[15] Accordingly, the observed apoptosis-inducing and -enhancing activities of the test compounds cannot be ascribed to their estrogenic activity.

Genistein is known to be a tyrosine kinase inhibitor[27] and a DNA topoisomerase II inhibitor.[28] It was also shown to induce apoptosis.[29,30] Therefore, its apoptosis-inducing and -enhancing activities observed here are very likely to be due to the tyrosine kinase inhibitor and DNA topoisomerase II inhibitor activities.

Bisphenol A, 4-*n*-nonylphenol, and 4-*tert*-octylphenol exerted apoptosis-inducing or/and -enhancing activities, while di-*n*-butyl phthalate and di-2-ethylhexyl phthalate did not. Considering that the

former three compounds contain phenolic hydroxyl groups in their structures but the latter two do not, the phenolic hydroxyl groups may play an important role in the induction or/and enhancement of apoptosis.

In some previous papers, induction of apoptosis in vitro or in vivo by di-*n*-butyl phthalate,[11] di-2-ethylhexyl phthalate,[12–14] and 17β-estradiol[26,31–33] were reported. These studies were generally carried out using much higher concentrations of the compounds than those used in our present study. Therefore, the possibility that these compounds may exert apoptosis-inducing or/and -enhancing activities at much higher or extreme concentrations cannot be excluded.

It is not known how some of the test compounds enhanced the apoptosis. However, considering that apoptosis is executed and regulated through intracellular signal transduction, several possibilities are conceivable for the mechanisms. i) A possibility that the compounds affected the intracellular apoptotic signal transduction initiated by etoposide or dexamethasone, causing the enhancement of apoptosis. ii) A possibility that the compounds are apoptosis-inducing agents having thresholds higher than the concentrations used, but which exceeded the thresholds when used in combination with etoposide or dexamethasone. iii) A possibility that the compounds inhibited some apoptosis-suppressing factor. iv) In the case of 4-*n*-nonylphenol that caused necrosis at 10 μM, the cell membrane damages may have caused an increase in the influx of etoposide into the cells, and apoptosis may have been augmented.

Another point to be clarified in understanding the mechanism of the apoptosis enhancement is the role of caspases. In the cases of bisphenol A and 4-*tert*-octylphenol, caspase activity of Jurkat cells was not necessarily increased, as judged by caspase 3, while PS exposure and/or DNA fragmentation were increased. This may indicate that the apoptosis-enhancing effect of the two compounds is mediated by caspase-independent pathways.[34] However, further studies are required to clarify the role of caspases in the observed phenomena.

Our present study indicated that some environmental chemicals exert not only apoptosis-inducing activity but also -enhancing activity. Such latent activities of environmental chemicals may cause unnecessary cell death that would be disadvantageous or toxic to the body. Further studies, including in vivo experiments, are necessary to properly understand and assess the effect of the environmental chemicals on cell death in the living bodies.

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