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DE-71-Induced Apoptosis Involving Intracellular Calcium and the Bax-Mitochondria-Caspase Protease Pathway in Human Neuroblastoma Cells *In Vitro*

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Polybrominated diphenyl ethers (PBDEs) are used extensively as flame-retardants and are ubiquitous in the environment and in wildlife and human tissue. Recent studies have shown that PBDEs induce neurotoxic effects *in vivo* and apoptosis *in vitro*. However, the signaling mechanisms responsible for these events are still unclear. In this study, we investigated the action of a commercial mixture of PBDEs (pentabrominated diphenyl ether, DE-71) on a human neuroblastoma cell line, SK-N-SH. A cell viability test showed a dose-dependent increase in lactate dehydrogenase leakage and 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl-tetrazolium bromide reduction. Cell apoptosis was observed through morphological examination, and DNA degradation in the cell cycle and cell apoptosis were demonstrated using flow cytometry and DNA laddering. The formation of reactive oxygen species was not observed, but DE-71 was found to significantly induce caspase-3, -8, and -9 activity, which suggests that apoptosis is not induced by oxidative stress but via a caspase-dependent pathway. We further investigated the intracellular calcium ($[Ca^{2+}]_i$) levels using flow cytometry and observed an increase in the intracellular Ca^{2+} concentration with a time-dependent trend. We also found that the N-methyl d-aspartate (NMDA) receptor antagonist MK801 (3μM) significantly reduced DE-71-induced cell apoptosis. The results of a Western blotting test demonstrated that DE-71 treatment increases the level of Bax translocation to the mitochondria in a dose-dependent fashion and stimulates the release of cytochrome c (Cyt c) from the mitochondria into the cytoplasm. Overall, our results indicate that DE-71 induces the apoptosis of $[Ca^{2+}]_i$ in SK-N-SH cells via Bax insertion, Cyt c release in the mitochondria, and the caspase activation pathway.

Key Words: **pentabrominated diphenyl ether (DE-71); apoptosis; Bax; caspase; calcium; cytochrome c; SK-N-SH.**

Polybrominated diphenyl ethers (PBDEs) are used worldwide as flame-retardants in textiles, foams, electronic appliances, building materials, and plastic products (Darnerud *et al*., 2001). PBDEs are included in these materials as additives, rather than being chemically bound to them, and can therefore be easily released into the environment. In 1999, the annual demand for PBDEs was approximately 65,000 tons, of which decabrominated diphenyl ether accounted for more than 85%, octabrominated diphenyl ether for 5%, and pentabrominated diphenyl ether (penta-BDE) for approximately 10% (reviewed by de Wit, 2002). PBDEs are highly lipophilic, persistent in the environment, and can bioaccumulate in aquatic and terrestrial animals (Hale *et al*., 2003; Kierkegaard *et al*., 1999; Liu *et al*., 2005; Norstrom *et al*., 2002; reviewed by Costa and Giordano, 2007; Law *et al*., 2006) and in human blood and breast milk (Bi *et al*., 2006; Ohta *et al*., 2002; Ryan *et al*., 2002; Schecter *et al*., 2003; Sjodin *et al*., 2003; reviewed by Costa and Giordano, 2007; Wang *et al*., 2007). Among the PBDE congeners that have been detected in the environment and in biota, 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47), 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99), 2,2′,4,4′,6-pentabromodiphenyl ether (BDE-100), and 2,2′,4,4′,5,5′-hexabromodiphenyl ether (BDE-153) are the most common (Law *et al*., 2006) and may have the potential to biomagnify along the food chain (Darnerud *et al*., 2001). The levels of PBDEs detected in human samples have also increased rapidly in recent years (Betts, 2002; Petreas *et al*., 2003; Schecter *et al*., 2005; Sjödin *et al*., 2004b). For example, although a human milk monitoring program in Sweden showed a decreasing trend of other persistent organic pollutants (e.g., polychlorinated biphenyls [PCBs], 1,1,1-trichloro-2,2-bis-(4-chlorophenyl) ethane (DDT)), the levels of PBDEs were found to have doubled every 5 years between 1972 and 1997 (Noren and Meironyte, 2000). Likewise, increased concentrations of PBDEs in human samples have been found in Germany (Schröter-Kermani *et al*., 2000), Norway (Thomsen *et al*., 2002), Japan (Akutsu *et al*., 2003), and North America (Sjödin *et al*., 2004a). Notably, the concentrations of BDE-47, -99, -100, and -153 in humans can reach up to 130, 30, 24, and 16 ng/g of lipid, respectively (Betts, 2002; Päpke *et al*., 2001). The persistent and bioaccumulative properties of PBDEs and the rapid increase in the concentrations of these substances in the environment and in biota have generated considerable concern about their toxicological effects on human and wildlife.

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As PBDEs have chemical and physical properties that resemble those of PCBs, they have frequently been associated with neurotoxicity in animals that was initially thought to be due to PCBs (Seegal, 1996; Tilson and Kodavanti, 1997). Neurotoxicity has been demonstrated in both rats and mice subjected to neonatal exposure to PBDEs congeners (e.g., BDE-99, -153, and -209). These neurotoxic effects are characterized by the impairment of spontaneous motor activity, cholinergic transmitters, the disruption of habituation, and the alteration of learning and memory function (Eriksson *et al*., 2001; Viberg *et al*., 2003a,b, 2006). Pre- or postnatal exposure to BDE-99 can cause permanent neurobehavioral disturbances in offspring at doses below those able to cause maternal toxicity in rats and mice (Branchi *et al*., 2002, 2005). *In vitro* studies have also shown that DE-71 (a commercial penta mixture) can induce arachidonic acid release in primary cultured cerebellar cells (Kodavanti and Derr-Yellin, 2002), increase protein kinase C translocation, and inhibit microsomal and mitochondrial Ca^{2+} uptake in cultured rat cerebellar granule cells (CGCs) (Kodavanti and Ward, 2005). Exposure to BDE-99 can also lead to apoptosis in human astrocytoma cells (Madia *et al*., 2004). A recent study showed that DE-71 induces DNA fragmentation and apoptosis in CGC (Reistad *et al*., 2006). However, the signaling mechanisms responsible for these events are not well known. In this study, therefore, a human neuroblastoma cell line (SK-N-SH) was selected as an *in vitro* model to investigate the toxic mechanisms of penta-BDE (DE-71). SK-N-SH cells are derived from the central nervous system and exhibit a neuronal phenotype that expresses multiple neurochemical markers. SK-N-SH cells are often chosen by laboratories as an *in vitro* model for studying potential neurotoxic mechanisms because their cellular characteristics are representative of those in an immature nervous system (Ba *et al*., 2003). We studied (1) cell viability by 3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay and lactate dehydrogenase (LDH) leakage to the culture medium; (2) cell apoptosis by morphological observation, flow cytometry measurement, and DNA laddering; (3) the activation of caspase-3, -8, and -9 activity; (4) the formation of reactive oxygen species (ROS) and the change in intracellular calcium concentration; and (5) the involvement of Bax and cytochrome c (Cyt c). DE-71 was chosen because it contains most of the congeners that have been found in the environment and in biota, such as BDE-47, -99, -100, -153, and -154 (found at ratios of approximately 30.8, 48.1, 8.8, 6.6, and 4.4%, respectively), as its major constituents (Rayne and Ikonomou, 2003), and thus the results should generate some insight into the action of PBDEs in general.

MATERIALS AND METHODS

Reagents. The DE-71 (purity > 99.9%) was obtained from Wellington Laboratories, Inc. (Ontario, Canada), and dimethyl sulfoxide (DMSO) (purity > 99.9%) was obtained from Amresco (Solon, OH). Phenol red-free minimum essential medium (MEM) and fetal bovine serum (FBS) were obtained from Sigma (St Louis, MO) and Hyclone (Logan, UT), respectively. Penicillin-streptomycin solution was obtained from Hyclone, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA), MTT, 3-[(3-cholamidopropyl)dimethylammonio] propanesulfonic acid (CHAPs), β-Nicotinamide adenine dinucleotide, reduced disodium salt (β-NADH), ethidium bromide (EB), propidium iodide (PI), proteinase K, pluronic F-127, and Tween-20 were purchased from Sigma. Fluo-3/AM was procured from Dojindo Laboratories (Kumamoto, Japan). Caspase detection kits were obtained from Keygen Biotech Co., Ltd (Nanjing, Jiangsu, China), and a cell mitochondrial isolation kit was obtained from the Beyotime Institute (Haimen, Jiangsu, China). Anti-human Bax (clone 2D2) antibody was obtained from eBioscience (San Diego, CA), β-actin antibody (clone 4) and Cox II antibody (clone N20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and monoclonal anti-Cyt c antibody (clone 7H8.2C12) was obtained from R&D Systems (Minneapolis, MN). Horseradish peroxidase–conjugated anti-mouse and anti-rabbit were purchased from Amersham Biosciences (Piscataway, NJ), and horseradish peroxidase–conjugated anti-goat was obtained from Pierce (Rockford, IL). Supersignal west pico chemiluminescent substrate was obtained from Pierce. All the chemicals used were of analytical grade.

Cell culture and exposure. A human neuroblastoma cell line (SK-N-SH) was obtained from the China Center for Type Culture Collection (Wuhan University, China). The cells were maintained in a single layer in flasks in an incubator in 5% CO_2 at 37°C. The culture medium consisted of MEM that was made up of 10% FBS, 100 μg/ml of streptomycin, 100 U/ml of penicillin, 2mM of L-glutamine, 1.0mM of sodium pyruvate, and 0.1mM of MEM nonessential amino acids.

Cell viability assay. Cell viability was assessed by examining MTT reduction and LDH leakage. DE-71 was dissolved in DMSO as a stock solution, and for both types of assay the exposure and control groups were dosed with 0.1% DMSO as a vehicle control. Five replicates were produced in each treatment. In the MTT assay, SK-N-SH (1×10^4) cells were seeded into 96-well plates. The cells were then exposed to 8.9, 22.1, 44.3, 66.4, and 88.5μM of DE-71 for 20 h, after which 25 μl of MTT stock solution (5 μg/l) was added to each well. The wells were then incubated for 4 h. The culture medium was removed and 100 μl of DMSO added to each well. After incubation for another 20 min, the extent to which the MTT had reduced to a formazan product was determined using a microplate reader (M_2, Molecular Device, Union City, CA) at 550 nm. The cell viability was expressed as a percentage of the cell survival rate compared with the control. In the LDH assay, 200 μl of NADH (in 50mM of Tris buffer; pH 7.4, final concentration 0.244mM) and 30 μl of culture medium were exposed for 24 h and then mixed in a UV-transparent microtiter cuvette to which 40 μl of 9.76mM pyruvate had been added. The reduction of NADH was recorded for 3 min with a microplate reader (Molecular Device, M_2) at 340 nm and 25°C. The LDH release was expressed as a percentage of the LDH release of the control.

Apoptosis. The SK-N-SH cells (4×10^4) were seeded onto a sterile glass cover slide, plated onto a 24-well microplate, and cultured at 37°C for 24 h. After 48 h of exposure to 25.6μM of DE-71, the cells were washed twice with PBS (pH 7.4) and stained with PBS containing acridine orange (AO, final concentration 100 μg/ml) and EB (final concentration 100 μg/ml) for 1 min at room temperature. The solution was then removed and the occurrence of apoptosis examined under a fluorescence microscope (Olympus IX 71, Olympus Corp., Tokyo, Japan).

DNA degradation was determined by using a hypotonic solution of PI. Cells entering the sub-G_1 phase were used as the marker for apoptotic cell death. The cells were treated with 6.4, 12.8, and 25.6μM of DE-71 and 0.1% DMSO for 24 and 48 h. After trypsinization (0.05% trypsin/0.53mM of EDTA), the cells were washed twice with Ca^{2+}-free PBS and stored in 70% ethanol overnight at −20°C. The cells were then pelleted by centrifugation and resuspended in 500 μl of 50 μg/ml PI solution that contained 100 μg/ml of RNase, after which they were incubated for 30 min at 37°C. The cell suspensions were assessed immediately for PI fluorescence intensity by flow cytometry (Beckman-Coulter

Epic AltraEpics Altra, Coulter Corp., Miami, FL), the data from which were analyzed using the CellQuest program. Approximately 10,000 cells were analyzed from each treatment.

In the DNA-laddering experiment, the cells were exposed to 25.6μM of DE-71 for 24 and 48 h, respectively, and were collected and washed once with PBS and lysed in a buffer (10mM of Tris; 10mM of EDTA; 150mM of NaCl; 0.5% SDS; 0.1 mg/ml of proteinase K, pH 8.0) at 37°C. The control cells were treated with 0.1% DMSO for 24 or 48 h as a solvent control. The lysate was extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ice-cold 100% ethanol and sodium acetate (3M, pH 5.2) at −20°C. The sample was then centrifuged at 12,000 × g at 4°C, washed with 70% ethanol, and incubated with RNase (50 μg/ml). DNA samples and standard DNA markers were analyzed by electrophoresis with 2.0% agarose gel at 2 V/cm for 6 h, and the bands were stained with EB (0.5 mg/l) in preparation for UV light visualization using the BioSpectrum Imaging System (UVP, CA).

Measurement of caspase activity. Caspase activity (caspase-3, -8, and -9) was measured using a Caspase assay kit from Keygen in accordance with the manufacturer's instructions. Briefly, the SK-N-SH cells were exposed to DE-71 (25.6μM) or the solvent control (0.1% DMSO) for 24 h, after which they were collected, washed twice with PBS, and lysed for 20 min on ice with 50mM of N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) buffer (pH 7.4) that was made up of 100mM of NaCl, 0.1mM of EDTA, 1mM of dithiothreitol (DTT), and 0.1% CHAPs. The lysates were centrifuged at 10,000 × g for 3 min at 4°C, and then 50 μl of reaction buffer (50mM of HEPES/NaOH, pH 7.4; 100mM of NaCl; 0.1mM of EDTA; 1mM of DTT; and 10% glycerol) and 5 μl of caspase-3, -8, or -9 substrate were added to each sample. The samples were then incubated at 37°C for 5 h. The absorbance was recorded at 405 nm using a microplate reader (Molecular Device, M_2). The activity of the various caspase substrates was expressed as a percentage of enzyme activity compared with the controls. The samples were analyzed in triplicate.

Quantification of ROS. To evaluate whether DE-71 induces ROS generation that may in turn be involved in cell apoptosis, the ROS levels were determined using DCFH-DA. This was de-esterified to 2′,7′-dichlorodihydrofluorescein by cellular esterases and then oxidized by ROS to form fluorescent 2′,7′-dichlorofluorescein. An increase in fluorescence intensity was used to quantify the generation of net intracellular ROS. The SK-N-SH cells were exposed to three concentrations of DE-71 (6.4, 12.8, and 25.6μM), 0.1% DMSO (solvent control), and 10μM of H_2O_2 (positive control). After exposure for 0.5, 1, 2, 4, and 24 h, 25 μl of DCFH-DA stock solution (200μM) was added to each well. The cells were then incubated for 30 min in the dark. The culture medium was removed, and 100 μl of DMSO was added to each well. After incubation for another 20 min, the fluorescence intensity was measured immediately using a microplate reader (Molecular Device, M_2). The excitation and emission wavelengths used were 490 and 530 nm, respectively. Five replicates of the samples were analyzed.

Intracellular calcium measurement. To investigate whether Ca^{2+} signaling involves cell apoptosis, we measured the intracellular calcium dynamics in SK-N-SH cells by flow cytometry using the Ca^{2+}-sensitive fluorescent dye Fluo-3/AM. Briefly, SK-N-SH cells were exposed to 0.1% DMSO or 25.6μM of DE-71 for 0.5, 1.5, 3, 6, 12, 18, and 24 h. Following exposure, the cells were washed three times with phenol red-free and serum-free MEM, and 4μM of Fluo-3/AM was loaded for 30 min at 37°C in the presence of 0.06% pluronic F-127. The cells were diluted five times in phenol red-free MEM that contained 1% FBS and were incubated for another 30 min at 37°C. The cells were then trypsinized, washed three times with HEPES buffer saline (10mM of HEPES; 1mM of Na_2HPO_4; 137mM of NaCl; 5mM of KCl; 1mM of $CaCl_2$; 0.5mM of $MgCl_2$; 5mM of glucose; 0.1% bovine serum albumin [BSA], pH 7.4), pelleted by centrifugation, and resuspended in HEPES buffer saline at 37°C. The intracellular calcium was analyzed immediately for Fluo-3/AM fluorescence intensity by flow cytometry (excitation at 488 nm and emission at 525 nm) (Beckman-Coulter Epic Altra). Approximately 10,000 cells were analyzed from each treatment.

Preparation of the cytoplasmic and mitochondrial fractions. Cytoplasma and mitochondria proteins were collected using a cell mitochondrial kit from Beyotime in accordance with the manufacturer's instructions. Briefly, the cells were exposed to 0.1% DMSO and DE-71 (at concentrations of 4.3, 12.8, or 25.6μM) for 24 h, after which 9×10^7 cells were harvested and washed twice with ice-cold PBS. They were then incubated in cell lysis buffer (250mM of sucrose; 1mM of DTT; 10mM of KCl; 1mM of EDTA; 1mM of ethyleneglycol-bis(aminoethylether)-tetraacetic acid [EGTA]; 1.5mM of $MgCl_2$; phenylmethylsulfonyl fluoride; 20mM of HEPES, pH 7.4) at 4°C and homogenized with a glass homogenizer. The cell lysate was centrifuged at 800 × g for 10 min to remove any unbroken cells, and the supernatant was further centrifuged at 15,000 × g for 10 min at 4°C. The resulting supernatant contained the cytoplasmic fraction and the pellet contained the mitochondrial fraction. The mitochondrial pellet was further resuspended in a mitochondrial lysis buffer at 4°C, and both the resuspended mitochondrial pellet solution and the supernatant were further centrifuged at 24,000 × g at 4°C to remove the nuclei. The protein concentration of the cytoplasma and mitochondria was measured by the Bradford method using BSA as a standard.

Western blotting analysis. In total, 100 μg of cytoplasmic protein or 50 μg of mitochondrial protein was boiled for 5 min in denaturing sample buffer (SDS buffer), electrophoresed on a 15% SDS polyacrylamide gel, and transferred onto a polyvinylidene difluoride membrane. The membrane was further probed with antibodies against Bax (2 or 3× concentration: 1 or 1.5 μg/ml), Cyt c (1× concentration: 0.5 μg/ml), or Cox II (1:200 dilution) or β-actin (1:1000 dilution) overnight at 4°C and washed six times with Tris buffer saline (containing 0.5% Tween-20, pH 7.5). After a secondary reaction with horseradish peroxidase–conjugated anti-rabbit (1:10,000 dilution), anti-mouse (1:10,000 dilution), or anti-goat antibody (1:1000 dilution), the immunoblot analysis was evaluated using supersignal west pico chemiluminescent substrate.

Statistical analysis. The normality of the data was verified using the Kolmogorov-Smirnov test, and the homogeneity of the variance was checked using Levene's test. One-way ANOVA and Tukey's multiple range tests were used to determine the significant differences between the control and exposure groups. The student's *t*-test was used to compare the caspase activity in the control and exposure groups. The criterion for statistical difference was set at $p < 0.05$. All the statistical analyses were performed using SPSS 13.0 (SPSS, Chicago, IL). Nonlinear regressions were run with Graph prism software, and the LC_{50} value (defined as the concentration that resulted in the death of half of the cells) was calculated.

RESULTS

Cell Viability

In the MTT assay, a dose-dependent decrease in cell viability was observed at values of 90.1 ± 0.1%, 84.1 ± 1.4%, 76.3 ± 1.6%, 34.2 ± 2.8%, and 22.0 ± 0.7% at 8.9, 22.1, 44.3, 66.4, and 88.5μM, respectively, following treatment with DE-71 (Fig. 1A). The calculated LC_{50} was about 53.2μM. In the LDH assay, no significant LDH leakage was found at DE-71 concentrations of 8.9μM, but significant LDH leakage was observed at DE-71 concentrations of 22.1μM (Fig. 1B).

Apoptosis

Using the fluorescence probe (AO and EB), typical apoptotic cells were observed when SK-N-SH cells were exposed to 25.6μM of DE-71 for 48 h (Fig. 2). This was demonstrated by the presence of hypoploid cells and cell shrinkage in the

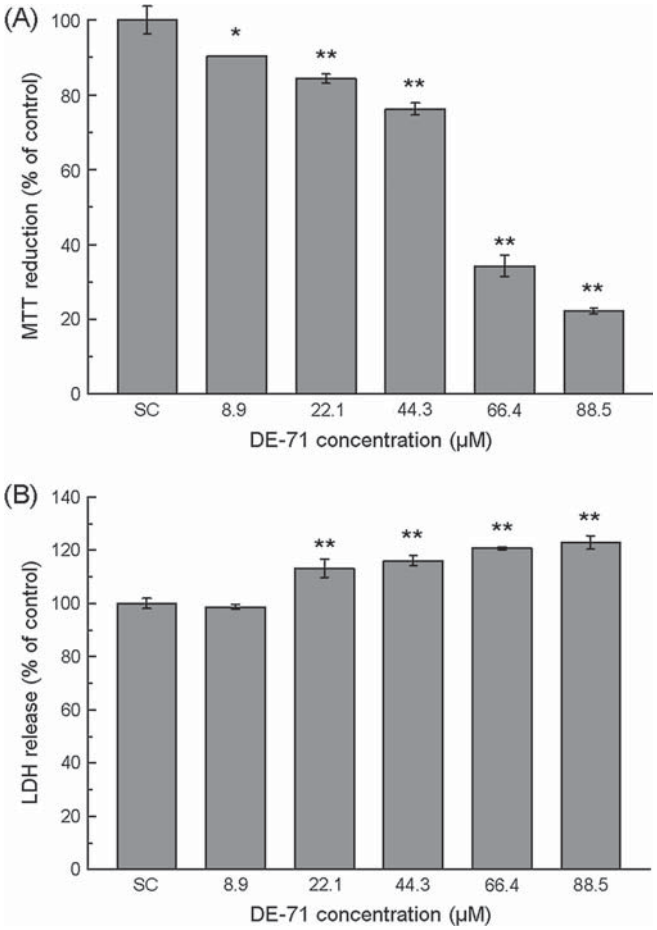


FIG. 1. Cell viability was determined by (A) MTT and (B) LDH assay after exposure of the cells to various concentrations of DE-71 (8.9, 22.1, 44.3, 66.4, and 88.5μM) and 0.1% DMSO (solvent control, SC). The data are expressed as the mean ± SEM of five replicates. The significance of the difference between the control and exposure groups is indicated by $*p < 0.05$ and $**p < 0.01$.

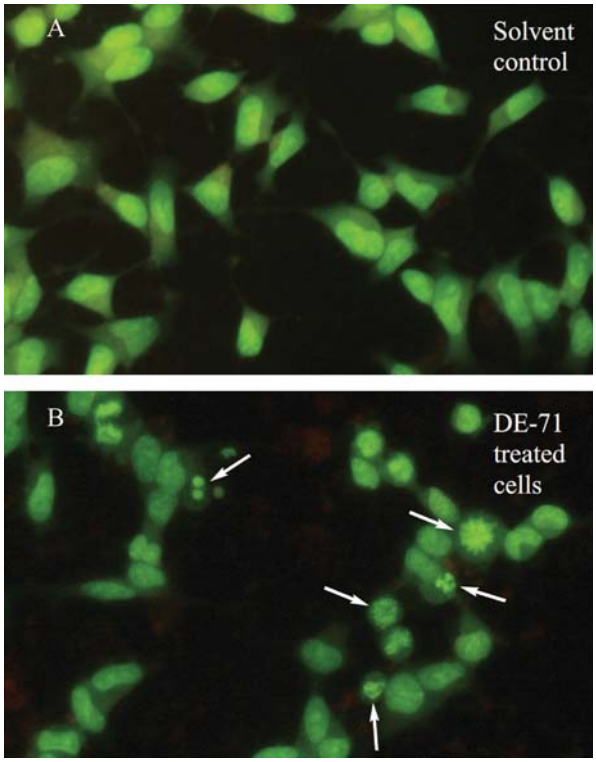


FIG. 2. Cell apoptosis as demonstrated by morphological examination in SK-N-SH cells treated with DMSO control (A) and DE-71 (25.6μM) for 48 h (B). The cells were stained using AO and EB. The arrows indicate the condensed and fragmented nuclei in the cells (200×).

treatment populations but the absence of significant apoptosis in the control cells. Flow cytometric analysis revealed a dose-dependent increase in the percentage of apoptotic cells (Fig. 3A). After 24 h of exposure, the proportion of apoptotic cells (sub-G_1 phase cells) increased from 2.7 to 5.6, 6.1, and 9.7% compared with the control group in the 6.4, 12.8, and 25.6μM of DE-71 treatment groups, respectively (Fig. 3B). Likewise, the sub-G_1 phase population increased from 3.8 to 5.8, 8.3, and 11.6% compared with the control group in the 6.4, 12.8, and 25.6μM of DE-71 treatment groups after 48 h of exposure (Fig. 3B). In the DNA-laddering analysis, ~180 bp DNA fragments were found to form in groups of cells exposed to DE-71 for 24 and 48 h, but no DNA ladders were observed in the control cells (Fig. 4).

Caspase Activity

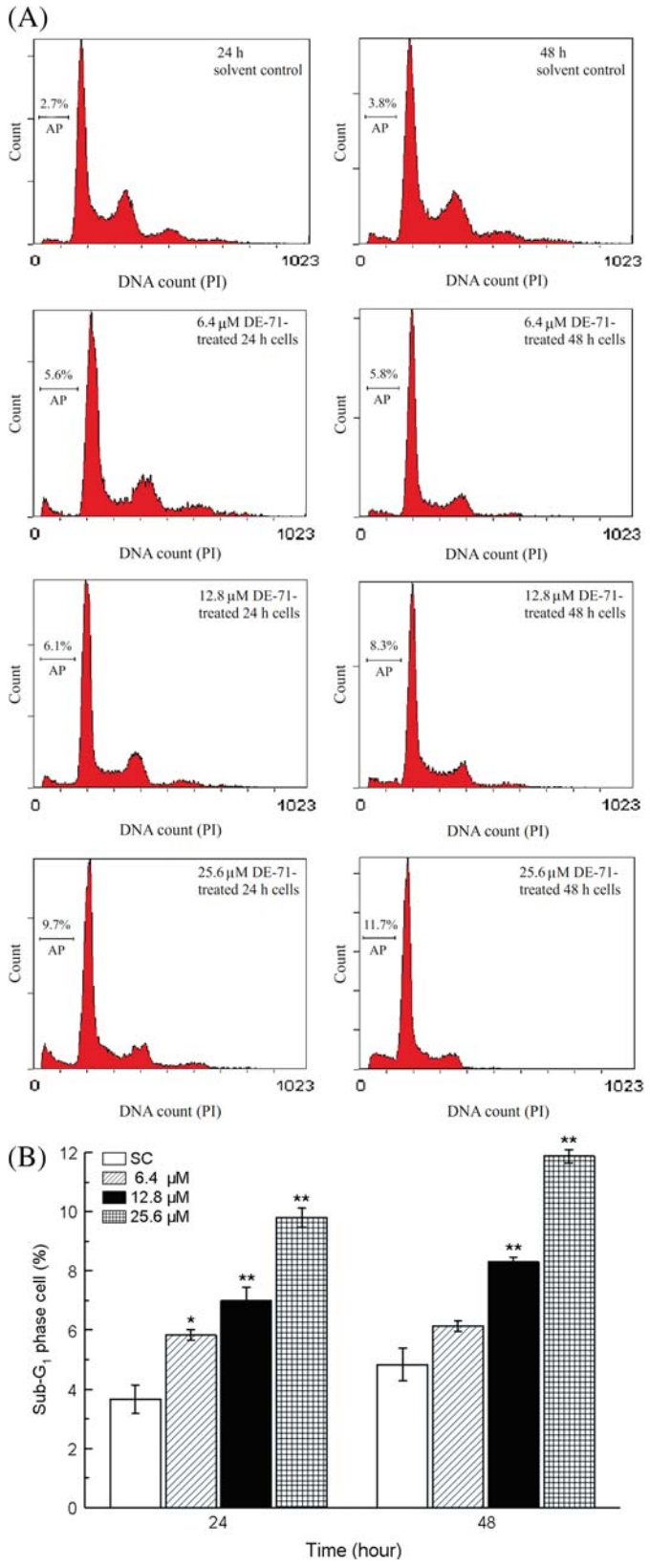
To investigate whether caspase is involved in the apoptosis of SK-N-SH cells, we measured the caspase-3, -8, and -9 activity. The activity of caspase-3 was markedly activated at 3.4 times the intensity of the activation in the control cells after treatment with 25.6μM of DE-71 (Fig. 5). Likewise, 1.6-fold and 1.7-fold increases in the activation of caspase-8 and -9, respectively, were also observed (Fig. 5).

ROS Assay

Both time course and dose-dependent ROS generation were measured, but no ROS generation was observed in any of the exposure groups or in the 0.1% DMSO control (Table 1). However, H_2O_2 (10μM) exposure resulted in a significant increase in ROS generation (Table 1). The result suggests that ROS is not involved in inducing apoptosis in SK-N-SH cells during DE-71 exposure.

$[Ca^{2+}]_i$ Measurement

To verify whether a calcium signal is involved in the apoptosis pathway, we used the Ca^{2+}-sensitive dye Fluo-3/AM to investigate whether exposure to DE-71 would induce $[Ca^{2+}]_i$ in SK-N-SH cells. The cells displayed an increase in Fluo-3/AM fluorescence intensity after exposure to 25.6μM of DE-71, a level that was two to three times greater than the intensity to which the DMSO-treated controls were exposed (Fig. 6A). The time course of the Fluo-3/AM fluorescence intensity in the



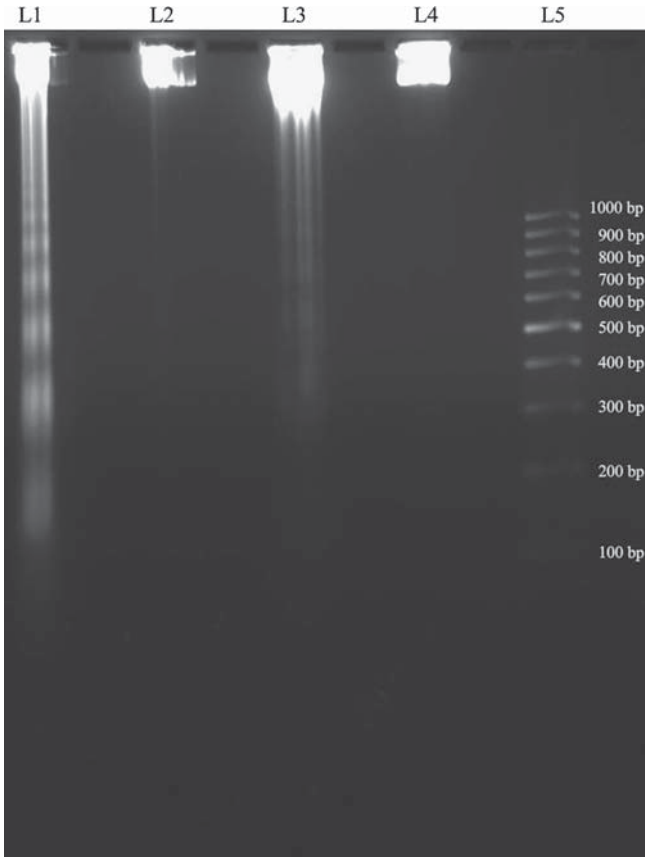


FIG. 4. DNA laddering in SK-N-SH cells induced by exposure to 25.6μM of DE-71 for 24 and 48 h as determined by agarose gel electrophoresis. L1: Cells treated with DE-71 for 48 h; L2: 48-h control cells; L3: cells treated with DE-71 for 24 h; L4: 24-h control cells; L5: DNA marker.

DE-71-treated cells is shown in Figure 6B. The intensity increased to a maximal level of 25.6μM of DE-71 after 12 h of exposure, which suggests that DE-71 induces $[Ca^{2+}]_i$ change in a time-dependent manner.

To verify the source of $[Ca^{2+}]_i$, we measured the change of $[Ca^{2+}]_i$ using flow cytometry. As shown in Figure 7A, $[Ca^{2+}]_i$ was significantly increased after the cells exposure to 25.6μM of DE-71. However, there was no significant change in the $[Ca^{2+}]_i$ measured using a calcium-free medium containing 1mM EGTA (Fig. 7A). This experimental protocol can effectively blunt available calcium influx. The results indicate that the increased intracellular calcium level by DE-71 is due to calcium influx from the extracellular sites without calcium release from internal stores.

FIG. 3. Flow cytometry measurement of the DNA degradation (percentage of hypoploid cells) characteristics of apoptosis after 24 and 48 h of exposure to 0.1% DMSO (solvent control, SC) or 6.4, 12.8, and 25.6μM of DE-71 using hypotonic PI staining. The decreased retention of PI by the hypoploid cells shifts their fluorescence intensity leftward on the linear *x*-axis of the representative histograms (A). The results are presented as the mean ± SEM from three independent experiments (B).

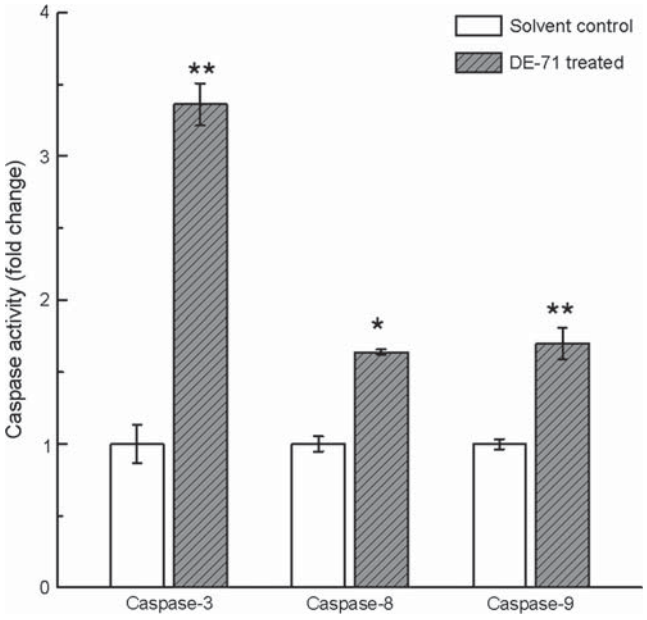


FIG. 5. Caspase-3, -8, and -9 activity in SK-N-SH cells exposed to 0.1% DMSO (solvent control, SC) or 25.6μM of DE-71 for 24 h. The values represent the mean ± SEM of three replicates. The significance of the difference between the control and exposure groups is indicated by $*p < 0.05$ and $**p < 0.01$.

To determine whether the NMDA receptor mediates calcium entry and cell apoptosis in DE-71-induced SK-N-SH cell death, an NMDA receptor antagonist was added in the form of 3μM of MK801. We observed a significant decrease in the number of apoptotic cells (Fig. 7B), with the percentage of apoptosis decreasing significantly after the cells were treated with a culture containing 25.6μM of DE-71 and 3μM of MK801 for 24 or 48 h (Fig. 7B).

Western Blotting Analysis

The level of Bax protein and its translocation from the cytoplasma to the mitochondria and the release of Cyt c from the mitochondria were evaluated using Western blotting to further determine the apoptotic mechanism. The levels of Bax in the cytoplasma decreased in the 25.6μM of DE-71 treatment group but were obviously enhanced in the mitochondria in the cells exposed to 12.8 and 25.6μM of DE-71 (Fig. 8A). Likewise, the protein levels of Cyt c were clearly enhanced in the cytoplasma after exposure to 12.8 and 25.6μM of DE-71 but were lower in the mitochondria (Fig. 8A), indicating the release of Cyt c from the mitochondria. A Bax dimer band (46 kDa) was also detected in the mitochondria when anti-Bax antibody was added at three times the original concentration (1.5 μg/ml) (Fig. 8B), which suggests an increase in the amount of Bax protein translocated to the mitochondria from the cytoplasma.

DISCUSSION

PBDEs are a ubiquitous presence in the environment and have also been found in animal and human tissue. Among the PBDEs, five tetra-, penta-, and hexa-BDE congeners (BDE-47, -99, -100, -153, and -154) predominate in human tissue, typically accounting for 90% of the total concentration found in human bodies (de Wit, 2002). The greatest concern about the potential adverse health effects of PBDEs relates to their developmental neurotoxicity (Costa and Giordano, 2007). Previous studies have shown that exposure to PBDE 209 and other PBDE congeners (including PBDE 47, 99, 153, 183, 203, and 206) during brain growth results in spontaneous deranged behavior, learning and memory defects, and dysfunction in the cholinergic receptors in the hippocampus of adult mice and rats (e.g., Viberg *et al*., 2003a,b, 2006, 2007). Indeed, several *in vitro* studies have demonstrated that PBDEs can induce cell apoptosis in certain cell lines (e.g., He *et al*., 2008; Madia *et al*., 2004; Reistad *et al*., 2006). However, little information exists about the possible cellular mechanisms underlying the neurotoxic effects of PBDEs. In this study, we have therefore attempted to characterize these signaling mechanisms by

TABLE 1

Dose-dependent and Time Course Induction of ROS in the Cultured SK-N-SH Cells after Exposure to Various Concentrations of DE-71. Cells Were Also Treated with 10μM H_2O_2 as Positive Control. The Measured ROS is Expressed as Fluorescence Intensity. Value Represents the Mean ± SEM of Five Replicate Wells from Three Experiments. Student's *t*-Test Was Used to Test Significant Change between Cells in the DMSO-treated Control and H_2O_2 and DE-71 Treatment

Time (h)	Negative control (0.1% DMSO)	6.4μM DE-71	12.8μM DE-71	25.6μM DE-71	Positive control (10.0μM H_2O_2)
0.5	67.6 ± 1.0	66.3 ± 0.8	48.2 ± 10.2	86.7 ± 14.7	1367.3 ± 17.2***
1	79.8 ± 0.7	83.2 ± 2.5	88.4 ± 1.4	82.8 ± 2.0	1014.2 ± 63.2***
2	128.0 ± 2.3	130.6 ± 2.0	122.7 ± 2.0	121.8 ± 3.6	149.5 ± 7.1*
4	177.4 ± 3.5	175.7 ± 2.5	162.5 ± 2.9	165.8 ± 2.8	197.5 ± 3.9*
24	77.5 ± 1.8	82.2 ± 3.7	85.4 ± 7.0	73.5 ± 5.2	154.9 ± 6.6**

$*p < 0.01$; $**p < 0.01$; $***p < 0.001$.

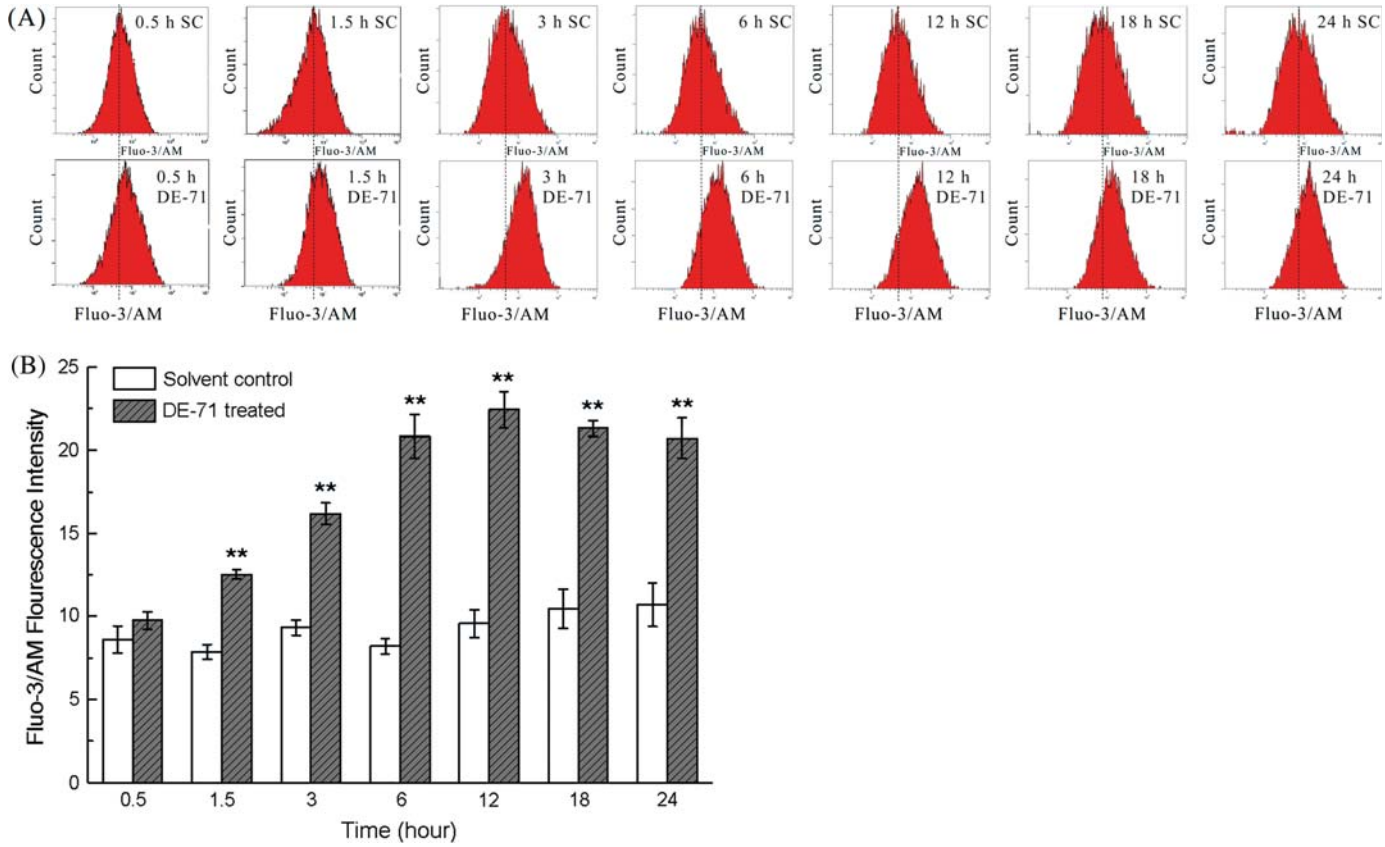


FIG. 6. Intracellular calcium ($[Ca^{2+}]_i$) in SK-N-SH cells measured by flow cytometry. The SK-N-SH cells were treated with 25.6μM of DE-71. $[Ca^{2+}]_i$ was measured by loading the cells with 4μM of Fluo-3/AM and examining their fluorescence intensity. Following treatment with 0.1% DMSO (solvent control, SC) or DE-71 for 24 h, the Fluo-3/AM fluorescence intensity of the cells exposed to DE-71 increased to 21.1, compared with an intensity of 8.2 in the control cells (0.1% DMSO, SC) (A). (B) The time-dependent $[Ca^{2+}]_i$ change. The results are presented as the mean ± SEM from three independent experiments.

employing a simple, fast, and low-cost system (cultured SK-N-SH neuroblastoma cells) to evaluate the potential toxic mechanisms of DE-71 in the nervous system. The results demonstrate that DE-71 can indeed cause cell apoptosis, as demonstrated by morphological examination, flow cytometric analysis, and DNA laddering. The mechanisms of apoptosis that we have identified involve the activation of caspase-3, -8, and -9 and the induction of $[Ca^{2+}]_i$ but not the generation of ROS or oxidative stress. We have also found that the apoptosis pathway involves the translocation of Bax to the mitochondria and the release of Cyt c from the mitochondria.

DE-71 was observed to cause a dose-dependent inhibition of mitochondrial reduction capacity (MTT assay) and LDH leakage. Previous studies have shown that BDE-47 causes a significant increase in LDH release at concentrations of 50μM (Kodavanti *et al*., 2005) but does not cause the release of LDH in cultured rat CGCs (up to 60μM) (Kodavanti and Ward, 2005). Similarly, it has been found that no LDH release occurs in cultured human astrocytoma cells following exposure to BDE-99 (up to 100μM) for 24 h, although the MTT significantly decreases (Madia *et al*., 2004). This discrepancy may be caused by species-specific differences in enzyme activity or signal cascade pathways. However, further investigations are necessary to draw firm conclusions about the varying pathways of cytotoxic effects in different models.

Apoptosis is characterized by cell shrinkage, chromatin condensation, DNA fragmentation, membrane budding, the appearance of membrane-associated apoptotic bodies, and the activation of specific cysteine proteases known as caspases (Lee *et al*., 2005). In this study, exposure to DE-71 caused hypoploid cell shrinkage and DNA fragmentation, which are typical characteristics of apoptosis. The results of the flow cytometry analysis of the cell cycle further indicate that exposure to DE-71 significantly increases the proportion of cells arrested at the sub-G_1 phase (Fig. 2B). The appearance of a hypodiploid sub-G_1 peak also indicates that the cells were undergoing apoptosis. It should be noted that PI staining may show both late apoptotic and necrotic cells (PI positive) when used in flow cytometry. Therefore, the significant level of cell death detected by flow cytometry in the SK-N-SH cells may be a combination of both apoptotic and necrotic components. However, morphological examination did not uncover any obviously necrotic cells, which suggests that necrotic cells should only make up a small proportion of cells showing

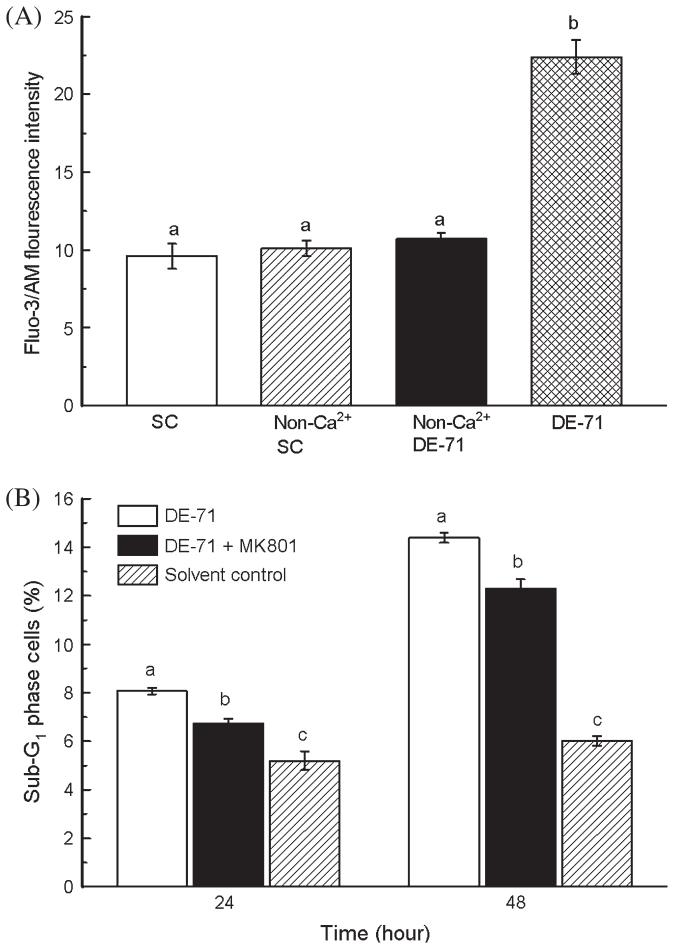


FIG. 7. (A) Intracellular calcium measured in SK-N-SH cells measured by flow cytometry after exposure to DMSO solvent control (SC), DMSO SC without calcium (non-Ca^{2+} SC); DE-71 with calcium (DE-71) and without calcium containing 1mM EGTA (non-Ca^{2+} DE-71). Intracellular calcium was measured by loading the cells with 4μM of Fluo-3/AM and examining their fluorescence intensity. Results are presented as mean ± SEM of three independent experiments. (B) Apoptosis of SK-N-SH cells after exposure to 25.6μM of DE-71 alone and in combination with 3μM of MK801. The percentage of sub-G_1 phase cells (apoptosis) significantly decreased after the cells were treated with a culture containing 25.6μM of DE-71 and 3μM of MK801 compared with cells that were treated with DE-71 alone for 24 or 48 h. The results are presented as the mean ± SEM from three independent experiments. Student's *t*-test was performed to indicate statistical significant differences between each exposure group with DMSO as control. Different letters denote significant difference between data sets.

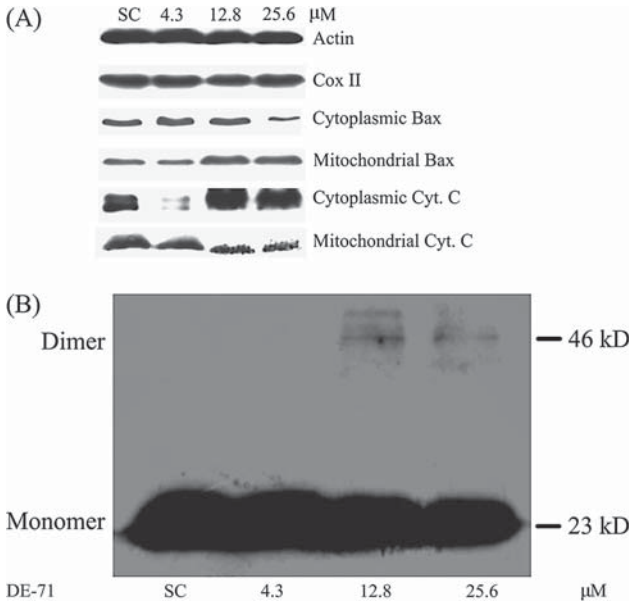


FIG. 8. DE-71-induced Bax insertion and Cyt c release in SK-N-SH cells. The SK-N-SH cells were treated with 0.1% DMSO (solvent control, SC) or 4.3, 12.8, or 25.6μM of DE-71 for 24 h, after which the cytoplasma and mitochondrial fractions were prepared. (A) Both fractions were analyzed for the presence of Bax and Cyt c using Western blotting. (B) Bax oligomeration was detected in the mitochondrial fraction. β-actin and Cox II were used as loading controls for the cytoplasmic and mitochondrial proteins, respectively.

apoptotic-like symptoms. This could be further confirmed by using a combination of PI and annexin V to distinguish between the apoptotic and necrotic cells. Nevertheless, the slight skewing of the results due to this lack of differentiation notwithstanding the combination of morphological examination, DNA laddering, and flow cytometry in this study shows that DE-71 can induce apoptosis in SK-N-SH cells.

It has been reported that exposure to BDE-99 (50μM) causes neuron cell apoptosis in human astrocytoma cells (Madia *et al.*, 2004), and a recent study using nuclear morphology and DNA laddering also indicated that DE-71 causes apoptotic cell death in primary cultured CGCs (Reistad *et al.*, 2006). Taken together, these findings suggest that apoptosis may be one of the mechanisms of PBDE neurotoxicity.

Mitochondrial homeostasis plays a pivotal role in regulating apoptosis and the proapoptotic signals (i.e., ROS or an increase in Ca^{2+} levels) that trigger apoptosis. Many studies have demonstrated that intracellular ROS generation is intimately associated with apoptotic cell death (Ermak and Davies, 2002; Green and Reed, 1998). However, in this study no induction of ROS generation was observed, which suggests that ROS is not involved in apoptosis in SK-N-SH cells. Reistad *et al.* (2006) similarly failed to observe any increase in the level of ROS in primary cultured rat CGCs, although in another study it was found that DE-71 enhances the generation of ROS in human neutrophil granulocytes (Reistad and Mariussen, 2005) and rat cerebellar granule neurons (Reistad *et al.*, 2007). It is worth noting that BDE-47–treated SH-SY5Y cells, which are neuroblast-type subclones of SK-N-SH cells, induce significant ROS generation (He *et al.*, 2008). SK-N-SH cells include both neuroblastic (N) cells and substrate-adherent cells, whereas SH-SY5Y cells include only N cells (Ciccarone *et al.*, 1989). A recent study (Oda *et al.*, 2008) showed that SH-SY5Y and SK-N-SH have different response mechanisms to endoplasmic reticulum stress induced by tunicamycin, and the authors suggested that the difference in predominant cell death

pathways between SK-N-SH and the subclone SH-SY5Y is attributable, at least in part, to the different characteristics of these cell lines. It can thus be inferred that different cell types display different toxic mechanisms. In this study, the intracellular calcium concentration increased significantly. Importantly, the loss of intracellular calcium homeostatic control is associated with cellular apoptosis and is considered to be a common final mechanism of cell death (Kass and Orrenius, 1999). Our results are in agreement with those of a previous study in which BDE-99 was observed to increase the Ca^{2+} concentration in cultured rat astrocytes (Wiegand *et al*., 2001). DE-71 has also been found to inhibit the uptake of Ca^{2+} in microsomal and mitochondrial preparations from rat brains (Kodavanti and Ward, 2005), and BDE-47 (20μM) has been reported to increase Ca^{2+} in nontransformed (chromaffin-like) rat pheochromocytoma (PC-12) cells (Dingemans *et al*., 2007). However, DE-71 (30μM) has not been found to cause $[Ca^{2+}]$ elevation in primary cultured CGCs (Reistad *et al*., 2006). These different responses suggest that there may be different toxic mechanisms operating in different cultured models. No ROS formation was observed in this study, from which it can be concluded that it may be a Ca^{2+}-dependent process that triggers DE-71-induced apoptosis in SK-N-SH cells.

By depleting extracellular calcium with EGTA, we did not observe an increase in intracellular calcium after exposure to DE-71, suggesting that the increased $[Ca^{2+}]_i$ was from extracellular sites. The underlying process responsible for neuronal cell death is the overstimulation of the glutamate receptor subtype. The NMDA receptor plays an important role in this process by causing an excessive calcium influx, which leads to a series of potentially neurotoxic events (reviewed by Lipton and Nicotera, 1998). MK801 is known to be a potent and selective noncompetitive NMDA receptor antagonist that acts at the NMDA receptor–operated ion channel as an open channel blocker (Wong *et al*., 1986). In our study, the introduction of the NMDA receptor blocker (MK801, 3μM) served to protect against an increase in calcium entry and reduced the percentage of sub-G_1 phase cells after exposure to DE-71, which suggests the involvement of the NMDA receptor in calcium homeostasis. In addition, we did not observe an increase in intracellular calcium when the extracellular calcium was removed by adding EGTA. Taken together, these findings indicate that DE-71 elevates cytosolic-free calcium from the extracellular environment and not through release from the intracellular compartments. In a previous study, Mariussen *et al*. (2002) showed that MK801 serves to protect against CGC death in cells exposed to the PCB mixture Aroclor 1254. It is therefore suggested that the mechanism of PCB neurotoxicity in CGC is characterized by the activation of glutamate receptors, which leads to an increase in intracellular calcium. Reistad and Mariussen (2005) also showed that DE-71 induces excessive calcium influx in human neutrophil granulocytes. Therefore, the complete mechanism of DE-71 neurotoxicity in SK-N-SH cells is likely to be the activation of glutamate receptors and resulting increase in intracellular calcium, followed by the activation of intracellular signaling pathways.

Elevated intracellular Ca^{2+} may either directly activate caspases in neuronal cells (Kass and Orrenius, 1999) or increase Ca^{2+} flux into the mitochondria, which may trigger the release of Cyt c from the mitochondria (Kass and Orrenius, 1999). Caspases have been extensively studied and are thought to be the central executers of apoptosis (Cohen, 1997). Although caspase-3 is considered to be an important player in the apoptotic process, it is activated in the late phase. Rather, it is caspase-8 and -9 that are primarily responsible for initiating caspase activation. In our study, caspase-3, -8, and -9 were activated in cells treated with DE-71 for 24 h, which suggests that DE-71 activates caspases. Although there have been no previous reports of caspase activation caused by PBDEs, one study did find caspase activation by PCBs, with the PCB mixtures Aroclor 1248 and Aroclor 1260 increasing caspase-3 activity in cultured rat neuronal cells (Sánchez-Alonso *et al*., 2004). As PBDEs have very similar chemical and physical characteristics to PCBs, further comparative studies on the toxic mechanisms of PCBs and PBDEs, and especially using congener-specific data, are needed.

Apoptosis is also regulated by the expression of genes of the Bcl-2 family, which includes both proapoptotic (e.g., Bax) and antiapoptotic (e.g., Bcl-2) members (Chaudhary *et al*., 1999). In the nervous system, Bax and Bcl-2 genes dominate in regulating apoptosis (Chaudhary *et al*., 1999) and play vital roles in the regulation of the mitochondrial apoptotic pathway by coordinating caspase activation through the release of apoptogenic factors, such as Cyt c (Green and Reed, 1998). In particular, the proapoptotic proteins (e.g., Bax), which are mainly found in cytosol, are translocated to the mitochondria upon receiving an apoptotic signal, which may initiate homo- or hetero-dimerization and subsequent translocation into the mitochondria. This in turn could play a key role in promoting Cyt c release from the mitochondria (Wolter *et al*., 1997), thus resulting in mitochondrial dysfunction (Gross *et al*., 1998; Oh and Lim, 2006). The cleaved Bax may favorably insert into the inner membrane and form an ion channel, which leads to the release of Cyt c (Schlesinger *et al*., 1997). The dimerization of Bax is an initial event in the integration of Bax into the mitochondrial outer membrane (Gross *et al*., 1998). In our study, DE-71 induced Bax translocation to the mitochondria, as indicated by the enhanced level of Bax in the mitochondria, the detection of 46 kDa of Bax oligomerization, and Cyt c release to the cytoplasma. We are therefore the first to demonstrate that DE71-induced apoptosis in SK-N-SH cells involves the mitochondrial translocation of Bax.

In summary, this study shows that DE-71 can induce apoptosis in SK-N-SH cells and suggests that PBDE toxicity operates via an apoptotic mechanism. We have also found that DE-71 induces apoptotic damage via a calcium-Bax-caspase protease pathway. As PBDEs have been detected in the brains of animals (Basu *et al*., 2007), our results may help to explain

the developmental neurotoxicity of PBDEs as manifested in aberrations in spontaneous behavior and learning and memory defects *in vivo*. Although our findings clearly demonstrate the presence of cell apoptosis, DE-71 contains five BDE congeners, the individual mechanisms of which are not well known. As studies of PCBs have demonstrated that the action of nonplanar PCB congeners on apoptosis depends on their chemical structure (Howard *et al*., 2003), further study on the structure of the individual congeners of PBDEs is recommended.

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