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Diethyl phthalate enhances apoptosis induced by serum deprivation in PC12 cells

Yongkun Sun¹, Kumiko Takahashi¹, Toshiyuki Hosokawa², Takeshi Saito³ and Masaaki Kurasaki^{1, 4,*}

¹: Environmental Adaptation Science, Division of Environmental Science Development, Graduate School of Environmental Science, Hokkaido University, 060-0810 Sapporo JAPAN

²: Higher Education Research and Development Division, Institute for the Advancement of Higher Education, Hokkaido University, 060-0817 Sapporo JAPAN

³: Laboratory of Environmental Health Sciences, Faculty of Health Sciences, Hokkaido University, 060-0812 Sapporo JAPAN

⁴: Group of Environmental Adaptation Science, Faculty of Environmental Earth Science, Hokkaido University, 060-0810 Sapporo JAPAN

*: Address correspondence to:

Environmental Earth Science, Hokkaido University
Sapporo 060-0810, Japan;

Running Title: Diethyl phthalate enhances apoptosis

Abstract: In this study, to examine the mechanism of diethyl phthalate toxicity to cells, the effects of diethyl phthalate on apoptosis in a PC12 cell system were investigated by assaying apoptotic factors such as caspase-3, Bax, cytochrome c, and DNA damage. Diethyl phthalate was shown to enhance the apoptosis induced by serum deprivation according to the results of DNA electrophoresis and TUNEL signal assays, although it could not induce apoptosis itself in the cells. This enhancement was thought to due to an increase of caspase-3-like activity. In addition, the expression of bax and contents of cytochrome c in the cytosol showed a tendency to increase in the cells exposed to diethyl phthalate. These results indicated that diethyl phthalate, a potential endocrine disrupter, affects the apoptotic system in PC12 cells. Diethyl phthalate may enhance oxidative stress such as that induced by reactive oxygen species in PC12 cells.

Key words: Diethyl phthalate; Apoptosis; Caspase; Cytochrome C; Bax; PC12 cell

Phthalates (PAEs), phthalic acid esters, are used widely in industrial production of plastics and daily consumable products as plasticizers to produce polymeric materials. Recently many studies have reported that some of them are endocrine-disrupting chemicals [1-3], and they exhibit toxicity and bioaccumulation [4-6]. PAEs are of interest because of their potential toxicity to humans since animal toxicity studies suggest that some PAEs affect male reproductive development, apparently via inhibition of androgen biosynthesis [7]. PAEs, diesters of phthalic acid, are manufactured by reacting phthalic anhydride with alcohols of desired carbon-chain lengths. One of the phthalates, diethyl phthalate (DEP), is widely used in personal care products, plastics and medical devices at various concentrations. However, it is toxic at high exposure levels as well as at low doses for a prolonged period. Treatment with higher concentrations of DEP results in mitochondrial proliferation as well as accumulation of glycogen, cholesterol and triglycerides within the liver, but exposure to lower concentrations for a longer period result in an increase in the number of peroxisomes numbers leading to severe hepatocellular changes [8].

The potential for exposure is, to a certain extent, a consequence of the physical and chemical properties of each phthalate. As molecular weight increases, vapor pressure, water solubility, and dermal uptake are reduced [9,10]. The major route of human exposure to most PAEs is ingestion. Exposure by inhalation, through drinking water, and via dermal contact tends to be limited [11]. After ingestion, PAEs are metabolized to their corresponding hydrolytic monoesters and may further metabolize to more hydrophilic oxidative products. These metabolites can be excreted unchanged or can undergo phase II biotransformation to glucuronide conjugates [12].

Bioanalysis for toxicity of DEP is considered important for clarification of its effects

on future generations. Continuous exposure to DEP through food, gestation and lactation over three generations, in spite of dose reduction, leads to an enhanced toxic effect in the latter generations. However, the effects of DEP on development and differentiation during pregnancy are still unclear [13].

Recently, in our laboratory, we developed a new risk assessment method using a PC12 cell system based on apoptotic reaction as an indicator of risk [14], because apoptosis is known to have a close relation to differentiation and development. In addition, apoptosis is induced in rat pheochromocytoma PC12 cells when the cells are cultured in serum-free medium. The apoptotic PC12 cells exhibit DNA fragmentation [15]. Thus, PC12 cells have proven to be a useful system for studying the effects of trace amounts of chemical substances on apoptosis. Using this system, we have reported that 2,4,5-trichlorophenoxyacetic (2,4,5-T), nonylphenol and copper at low concentrations affect the apoptotic reaction [16-18].

Apoptosis is a morphological and biochemical description of a physiological cell death mechanism that is commonly associated with programmed events necessary for the differentiation and development of individuals and organs [19]. Apoptotic cell death is characterized by chromatin condensation, DNA fragmentation, cellular shrinkage, and membrane blebbing [20]. Apoptosis caused by oxidative and chemical stresses is regulated and executed by two major protein families, the Bcl-2 and caspase families, which are highly conserved from worms to humans [21,22].

The Bcl-2 family, which is involved in the regulation of caspase activity, is subdivided into pro-apoptotic (for example Bax and Bad) and anti-apoptotic (for example Bcl-2) members, which share one or more similar regions [23,24]. In this study, to examine the multigenerational effects of DEP, its effects on apoptosis were

investigated in our PC12 cell system.

Materials and Methods

Materials. PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Dulbecco's modified Eagle's medium (DMEM), streptavidin-conjugated peroxidase, o-phenyldiamine dihydrochloride (OPD), DEP was obtained from Sigma-Aldrich (St. Louis, MO USA). Fetal bovine serum (FBS) was bought from HyClone (Rockville, MD USA). Caspase assay kits and benzyloxycarbonyl-Val-Ala-Asp-CH₂F (Z-VAD) were from Promega (Madison, WI). Trypan blue stain solution (0.5%) was from Nacalai Tesque (Kyoto, Japan). Biotin-16-2'-deoxy-uridine-5'-triphosphate, proteinase K and the blocking reagent were from Roche Diagnostics (Mannheim, Germany). Terminal deoxynucleotidyl transferase (TdT) was from Toyobo (Osaka, Japan). Monoclonal antibodies against Bax, and cytochrome c were from Oncogene Research Products (Boston, MA, USA) and Santa Cruz Biotechnology (Santa Cruz, USA), respectively. Biotinylated donkey anti-mouse immunoglobulin was from Amersham Pharmacia Biotech (Buckinghamshire, UK). Other chemicals were of analytical reagent grade.

Cell culture. PC12 cells were maintained in DMEM supplemented with 10% FBS in a humidified incubator at 37°C and 5% CO₂. The cells were preincubated in 25 cm² flasks for over night, and then the medium was replaced with serum/serum-free DMEM with or without DEP. When the medium was changed to medium without serum, cells in the flask were washed twice with serum-free DMEM.

Cell viability. Cell viability was measured by trypan blue exclusion assay. PC12 cells were incubated in the DMEM containing serum with 0 to 10,000 ng/ml of DEP for 72 hr. After the incubation, cells were stained in 0.25% trypan blue solution in phosphate-buffered saline. Total cells and trypan blue-stained cells were counted using a hemocytometer. Cell viability was expressed as a percentage against the total cell number in each experiment. Each experiment was repeated 4 times.

Genomic DNA isolation from PC12 cells. After treatment with DEP under the various conditions for 72 hr in PC12 cells, genomic DNA was isolated by the method of Yamanoshita et al. [14]. The cells were washed with 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, and harvested using a scraper. They were then centrifuged at 2,000 rpm for 10 min and the supernatants were removed. The cells were incubated with 10 mM Tris-5 mM HCl, 0.1 M EDTA, 0.5% SDS, and 20 µg/ml of pancreatic RNase for 1 hr at 37°C. Proteinase K was added to the suspension up to 100 µg/ml, and the enzyme reaction was carried out for 3 hr at 50°C. The genomic DNA was extracted with phenol and chloroform. The obtained DNA was resuspended in 89 mM Tris-borate buffer, pH 8.1, containing 1 mM EDTA (1×TBE).

The ladder pattern of DNA was analyzed by agarose gel electrophoresis. From 3 to 5 µg of DNA was subjected to electrophoresis on 1.5% of agarose gel. After the electrophoresis, DNA was visualized by staining with ethidium bromide under UV illumination.

Quantification of DNA fragmentation in PC12 cells by the TdT mediated dUTP-biotin nick end labeling (TUNEL) method. After cultivation of PC12 cells in the

serum/serum-free medium with 0 to 10,000 ng/ml DEP for 72 hr, genomic DNA was isolated from PC12 cells. The obtained DNA was resuspended in 1×TBE buffer, and equal amounts of DNA were put into 96-well plate wells. Quantification of DNA fragmentation in PC12 cells was measured by the method previously described [25,26]. The plate was incubated at 4°C for overnight. After the incubation, the plate was washed with the 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl. Nonspecific binding sites were blocked with 2% blocking reagent in the same buffer for 30 min. Then the plate was washed with same buffer twice. TdT reaction was performed in a reaction mixture consisting of 30 mM Tris-HCl buffer, pH 7.4, containing 140 mM sodium cacodylate, 1 mM cobalt chloride, 0.1 units of TdT and 2 nM biotinylated-dUTP at 37°C for 1.5 hr. After the reaction, the plate was washed three times with the same buffer, and the plate was incubated with streptavidin-conjugated peroxidase (1:400 diluted) for 1 hr. Following this, the plate was washed five times with the same buffer. After washing, 0.1% OPD in 50 mM phosphate-citrate buffer, pH 5.0, containing 0.03 % sodium perborate was added to each well and incubated for 0.5 hr at 37°C. Then, HCl was added to stop the enzyme reaction. Absorbance at 495 nm was measured with a Microplate Reader model 450 (BIO-RAD).

Measurement of caspase-3-like activity. The activity of caspase-3-like protease was detected using the caspase assay system kit according to the instruction manual. This kit uses DEVD-pNA as the substrate and this chemical is cleaved by DEVDases such as caspase-3 protease. The activity of caspase-3-like protease in the PC12 cells was indirectly measured as absorbance at 405 nm depending on released pNA from the cleaved substrate. PC12 cells were incubated in serum-free medium with 0 to 1000

ng/ml DEP for 72 hr. After the incubation, the cells were washed with phosphate-buffered saline, harvested, centrifuged and lysed. Each 30 µg of protein in the cell lysate was divided into two tubes for the assay. After adding of caspase inhibitor, Z-VAD, to one tube at 400 µM, the both tubes were adjusted to equal volumes using distilled water. Following this, assay buffer and DEVD-pNA substrate were added to all tubes, and the reaction mixtures were incubated overnight. A caspase-3-like activity was expressed as a relative content against that in the cells incubated in the medium containing serum without DEP.

Western blot analysis using antibodies against Bax and cytochrome c. The PC12 cells were cultured in the DMEM containing 10% FBS with 0 to 10,000 ng/ml DEP for 72 hr. After the treatment, the cells were washed with 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, and harvested using a scraper. The obtained cells were centrifuged at 2,000 rpm for 3 min to remove the supernatants.

For analysis of cytochrome c, leaked cytochrome c in cytosol was isolated using cytochrome c Release Apoptosis Assay kit (Calbiochem, Germany). After adding of 10 ml ice-cold PBS, the mixture was centrifuged again at 1,800 rpm for 5 min. After removing the supernatant, the cells were resuspended in 500 µl of Cytosol Extraction Buffer Mix containing protease inhibitor cocktail and 1mM DTT. The mixture was allowed to stand on ice for 10 min.

For analysis of bax, the cells were resuspended in the lysis buffer consisting of 2 mM HEPES, 100 mM NaCl, 10 mM EGTA, 1 mM PMSF, 1 mM Na₃VO₄, 0.1 mM Na₂MoO₄, 5 mM β-glycerophosphoric acid disodium salt, 50 mM NaF, 1 mM MgCl₂, 2 mM DTT and 1% TritonX-100.

The cells obtained from different preparation were disrupted by sonication for 1 min with a Sonifier 250 (Branson). To remove the unbroken cells, the lysed cells were centrifuged at 3,000 rpm for 5 min. The supernatant was transferred into a 1.5 ml tube and centrifuged at 11,000 rpm for 30 min. The treated lysate (20 μ g of proteins) was separated by polyacrylamide gel electrophoresis (12.5% polyacrylamide for separation gel and 3% for stacking gel) and the electrophoresed proteins were transferred to nitrocellulose membranes with a semidry blotting system, type-AE6678 (ATTO, Japan). The membranes were incubated for overnight at 4°C in 40 mM Tris-HCl buffer, pH 7.4, containing 150mM NaCl and 5% blocking reagent. The membranes were incubated for 90 min at room temperature with primary antibodies (dilution; 1: 1,000) in 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 1% blocking reagent. The membranes were washed five times for each 3 min with 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 0.3% Tween 20, and incubated at room temperature for 90 min using anti-mouse IgG biotinylated as secondary antibody (dilution; 1: 1,000) in the 1% blocking buffer. The membranes were washed five times for each 3 min in the same washing buffer. Finally, streptavidin-conjugated peroxidase (1:2,000 diluted with the same blocking buffer) was added and the membrane was incubated for 1 hr at room temperature. Protein bands that responded to antibodies were detected with an enhanced chemiluminescence system after washing of the membrane with washing buffer.

Statistical analysis. Each value is expressed as the mean \pm SEM. Statistical analyses were performed by one-way analysis of variance (ANOVA), followed by the Fisher's test.

Results

Cell viability.

To confirm whether DEP exhibited cell toxicity, the cell viability was measured by trypan blue staining after the PC12 cells were exposed to 1 to 10,000 ng/ml DEP for 72 hr to. As shown in fig. 1, no significant differences of viability were observed in the cells exposed to 1 to 10,000 ng/ml DEP in comparison with those of cells cultured in serum-medium without DEP. A significant decrease in viability was observed in the cells cultured in the serum-free medium without DEP. This decrease was considered to be due to apoptosis induced by serum deprivation. These results indicated that DEP was not toxic in this cell system.

Detection of DNA fragmentation by agarose gel electrophoresis.

Apoptosis has been reported to be induced by serum deprivation in PC12 cells [19]. To clarify whether DEP induced apoptosis and/or affected the apoptosis induced by serum-deprivation, DNA fragmentation of the PC12 cells cultured in medium containing serum and serum-free medium including DEP was investigated (fig. 2).

The morphological characteristics of apoptosis are frequently accompanied by multiple cleavage of DNA into 180-200 base pairs. The oligonucleosomal-sized fragments can be visualized as a characteristic DNA ladder following agarose gel electrophoresis [27]. When apoptosis was induced in the cells, DNA ladder was observed (fig. 2B). As shown in fig. 2A, the DNA ladder pattern was hardly observed in the cells cultured in the serum medium containing 0 to 10,000 ng/ml DEP. From the results, it was indicated that DEP itself could not induce apoptosis in PC12 cells. On the other hand, when apoptosis is induced by serum-deprivation, DNA ladder patterns were observed.

observed in the cells incubated in the serum-free medium containing 0 to 10,000 ng/ml DEP (fig. 2B). Furthermore, it was suggested that the DNA patterns increased with exposure of more than 1 ng/ml DEP. These results meant that DEP enhanced the apoptosis induced by serum deprivation in the PC12 cells.

Determination of DNA fragmentation by quantified by the TUNEL method.

DNA fragmentation occurs via the action of endonucleases. The TUNEL method specifically labels DNA ends generated by endonuclease activity [28]. To confirm the results of DNA electrophoresis, quantification of DNA ladders was carried out by the TUNEL methods in the cells treated with 0 to 10,000 ng/ml DEP for 72 hr.

As shown in fig. 3A, the TUNEL signals did not show any changes among the cells cultured in the serum-containing medium with DEP. The results were in good agreement with data for DNA electrophoresis from the cells cultured in the serum-containing medium with DEP (fig. 2A). It meant that DEP could not affect DNA and induce apoptosis.

On the other hand, the TUNEL signals in the cells cultured in the serum-free medium including 100 and 1,000 ng/ml DEP were significantly increased as compared with those in the cell cultured in the serum-free medium without DEP as shown in fig. 3B. These results also coincided with the results of DNA ladder pattern (fig. 2B). In addition, TUNEL signal in the cells cultured in the serum-free medium was significantly higher than that in serum-containing medium. It was indicated that TUNEL signal reflected the situation of apoptosis induced by serum deprivation in the cells. In summary, the enhancement of apoptosis induced by serum deprivation due to treatment of DEP was verified from the TUNEL assay.

Measurement of caspase-3-like activity.

To investigate whether caspase activity under the condition of apoptosis induced by serum deprivation were changed by addition of DEP, the activity of caspase-3 was measured in PC12 cells cultured in serum-free medium containing 0 to 1,000 ng/ml DEP for 72 hr. The obtained results are shown in fig. 4. To confirm that the apoptosis of the PC12 cells was mediated by the caspase pathway, Z-VAD, an inhibitor of the caspase family, was used for analysis. The presented activity was obviously inhibited by addition of the caspase inhibitor Z-VAD. The results indicated that the increased activity was due to caspase-3. In addition, caspase-3-like activity in the cell cultured in the serum-free medium showed significantly higher than that in the cell cultured in serum-containing medium. It was showed that the apoptosis induced by serum deprivation was mediated by caspase-3. Furthermore, when DEP was added to the serum-free medium, caspase activity in the cells was significantly increased as compared with that in the cells cultured in the serum-free medium without DEP. Furthermore caspase-inhibitor Z-VAD strongly inhibited also this increased activity in the cells. These results indicated that the enhancement of apoptosis caused by DEP was mediated by caspase-3 depending pathway.

Contents of Bax and cytochrome c detected by Western blot analyses.

From the results of TUNEL and caspase assay, it was supposed that DEP enhanced apoptosis induced by serum deprivation, although DEP itself could not induce apoptosis. Usually apoptosis induced by serum deprivation was mediated by Bcl-2 family [19]. To confirm whether DEP affected the pathway of apoptosis via the Bcl-2 family, Western

blot analyses using the cell lysis from PC12 cells treated with 0 to 10,000 ng/ml DEP were performed using the monoclonal antibodies against Bax and cytochrome c.

As shown in fig. 5A, the detected Bax contents in the cells cultured in the serum-free medium with DEP (10 ng/ml) showed significantly higher than that in the cells cultured in the serum-free medium without DEP. As unexpected, there is no significant difference of Bax contents between cells cultured in the medium with and without serum in ANOVA analysis, but if student-t test (paired) is applied, significant increased of Bax contents was observed by serum deprivation ($P < 0.05$).

In the case of cytochrome c, there is no significant difference among the cell cultured in the medium with and without serum, and treated with and without DEP in ANOVA analysis (fig. 5B). However when student-t test (paired) is used for statistical analysis, the significant differences of cytochrome c contents between in the cells cultured in the medium with and without serum ($P < 0.05$) and between in the cells cultured in the serum-free medium with and without DEP (100 ng/ml) ($P < 0.05$).

Totally, from the results, it was suggested that Bax and cytochrome c showed a tendency of increase due to serum deprivation, and this increase was enhanced by treatment of DEP.

Discussion

In this study, it was confirmed that DEP enhanced apoptosis induced by serum deprivation. This finding was supported by the results of electrophoresis (fig. 2), measurements of TUNEL signals (fig. 3) and caspase-3-like activity (fig. 4), and Western blot analyses (fig. 5). As shown in figs. 2 and 3, enhancement of apoptosis was observed when 10 to 100 ng/ml DEP was added to the medium for PC 12 cells.

However, judging from the results for cell viability, DEP itself showed no cell toxicity (fig. 1), although it enhanced the apoptosis induced by serum deprivation.

Matsunaga et al. [29] reported that apoptosis was induced by chemicals which enhanced oxidative stress in the cells. In addition, it was reported that di(2-ethylhexyl) phthalate (DEHP) induced apoptosis of Neuro-2a cells due to activation of Trim17 [30]. Apoptosis induced by DEHP was also reported to depend on an increase of Bax content [31]. In addition, mono-(2-ethyl hexyl) phthalate induces spermatogenic cell apoptosis in guinea pig testes at the prepubertal stage in vitro [32]. On the other hand, as shown in fig. 5, the Bax content was also increased by treatment with DEP. However, DEP itself could not induce apoptosis (figs. 2 and 3). The reason for this discrepancy between DEP and DEHP is still unclear. DEP itself could not enhance the Bax contents in the cell (data not shown). This suggested that the mechanism of toxicity of each DEP derivative differed in the apoptotic pathway.

Recently Al-Saleh et al. [33] reported on the PAE residues in plastic water bottles kept at low and high temperatures. They reported that the highest concentrations of DEP and DEHP detected in the bottles were about 1.5 and 1.0 ppb (ng/ml), respectively. Of course, in this study, the detected concentration in DEP had no effects on any apoptotic factor. However, water from plastic bottles is drunk daily by many persons throughout the world. Pereira and Rao [34] reported that LDH activity in the serum and liver in males were significantly increased and four times higher in DEP- and PCB+DEP-treated groups than in a control, no-dose group. It can be concluded from the reports that continuous exposure to DEP in combined form leads to a synergistic interactive toxic effect. In addition, judging from the findings that DEP and its derivative affected apoptosis or apoptotic factors, if infants or neonates are exposed to

DEP simultaneously through mother's milk and through the transplacental route, it would be detrimental to growing infants and neonates.

The influence of some endocrine disruptors on apoptosis induced by serum deprivation was investigated using PC12 cells [14,16]. It was reported that tributyl tin and 2,4,5-T completely inhibited the apoptosis induced by serum deprivation. On the other hand, DEP enhanced apoptosis induced by serum deprivation. This enhanced effect was also observed in the case of nonylphenol, an endocrine disrupter [17]. It has been reported that apoptosis or programmed cell death is a highly regulated process and there are a number of pro-apoptotic (e.g., Bax, Bad) and anti-apoptotic proteins (e.g., Bcl-2) [35,36]. Apoptosis can be triggered by signals arising from within the cell or by extrinsic death activators. There are two major apoptotic pathways. The intrinsic apoptotic pathway involves the release of cytochrome c from mitochondria. The extrinsic apoptotic pathway is mediated by the TNF receptor (TNF-R) superfamily of death receptors, and one of the early steps committing a cell to apoptosis is the activation of caspases [37].

As shown in fig. 5 in this study, Western blot analyses using the cell lysis from PC12 cells after treatment with DEP revealed that the contents of Bax and cytochrome c showed a tendency to increase as compared with those in cells not treated with DEP. Bax is a pro-apoptotic Bcl-2 family member that is thought to bind Bcl-2 and thus to block its anti-apoptotic function. From these results, it was thought that the expression of Bax was activated by addition of DEP, and Bax enhanced membrane permeability and promoted cytochrome c release. There is a hypothesis that, in PC12 cells, synthesis of reactive oxygen species (ROS) catalyzed by chemical substances such as DEP give damages DNA [34]. ROS trigger the transactivation of p53, resulting in expression of

Bax. These reports suggested that the DEP-enhanced apoptotic pathway was involved in the release cytochrome c from mitochondria, but not the pathway mediated by the TNF receptor. This hypothesis was supported by Matsunaga et al. [29]. They reported that crude extracts of quinone compounds induced mitochondrial dysfunction via reduced membrane potential. In addition, it was indicated that the release of cytochrome c from mitochondria might in turn activate caspase-9 and caspase-3. In our study, caspase-3-like activity was increased by treatment with DEP (fig. 4). This increase might be related to the increase of oxidative status in the cells exposed to DEP.

In conclusion, DEP enhanced the apoptosis induced by serum deprivation. This enhancement was due to increases of caspase-3-like activity in the cells exposed to DEP. In addition, the Bax content and cytochrome c released into the cytosol showed a tendency to increase in the cells. These results suggest that DEP may enhance oxidative stress such as that induced by ROS in PC12 cells. Further investigation is needed to clarify the detailed mechanism of enhancement of apoptosis by treatment with DEP, and the effects of DEP on differentiation and development.

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Declaration of interest

The authors declare that there are no conflicts of interest.

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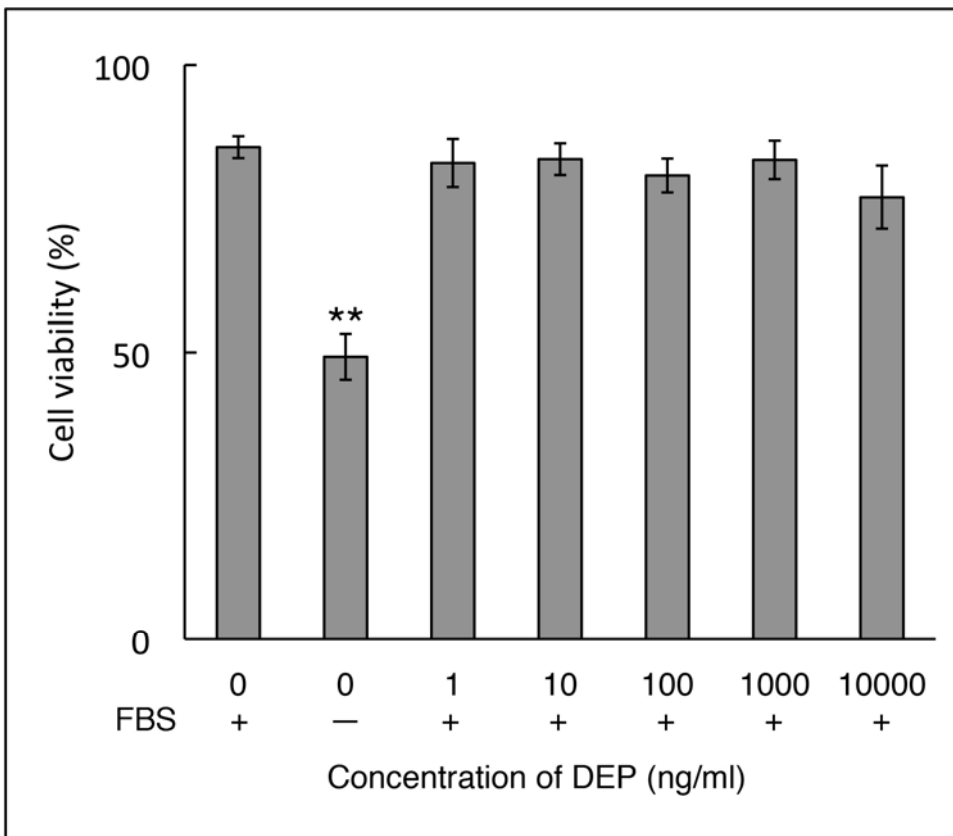


Fig. 1. Cell viability of PC12 cells treated with 0 to 10,000 ng/ml DEP for 72 hr measured by trypan blue staining. PC12 cells cultured in serum-containing medium without DEP for 72 hr were used as a control. Error bars indicate SEM (n=4). ** significantly different from the cells incubated in serum-free medium without DEP (P<0.01).

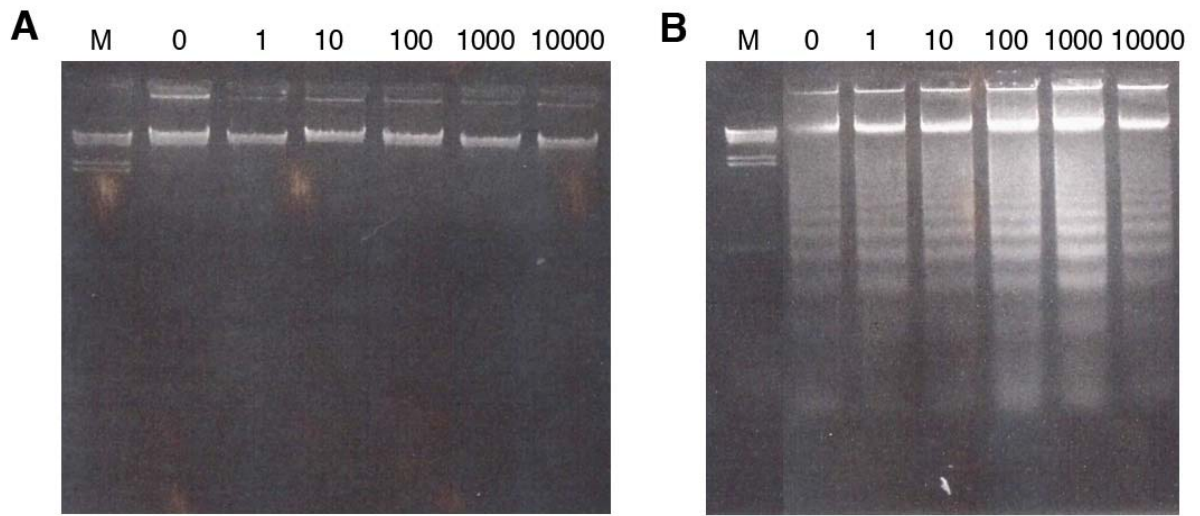


Fig. 2. Agarose gel electrophoresis of DNA extracted from PC12 cells cultured in serum-containing medium (A) and serum-free medium (B) for 3 days after treatment with 0 to 10,000 ng/ml DEP. M indicates λ DNA digested with HindIII as a DNA marker.

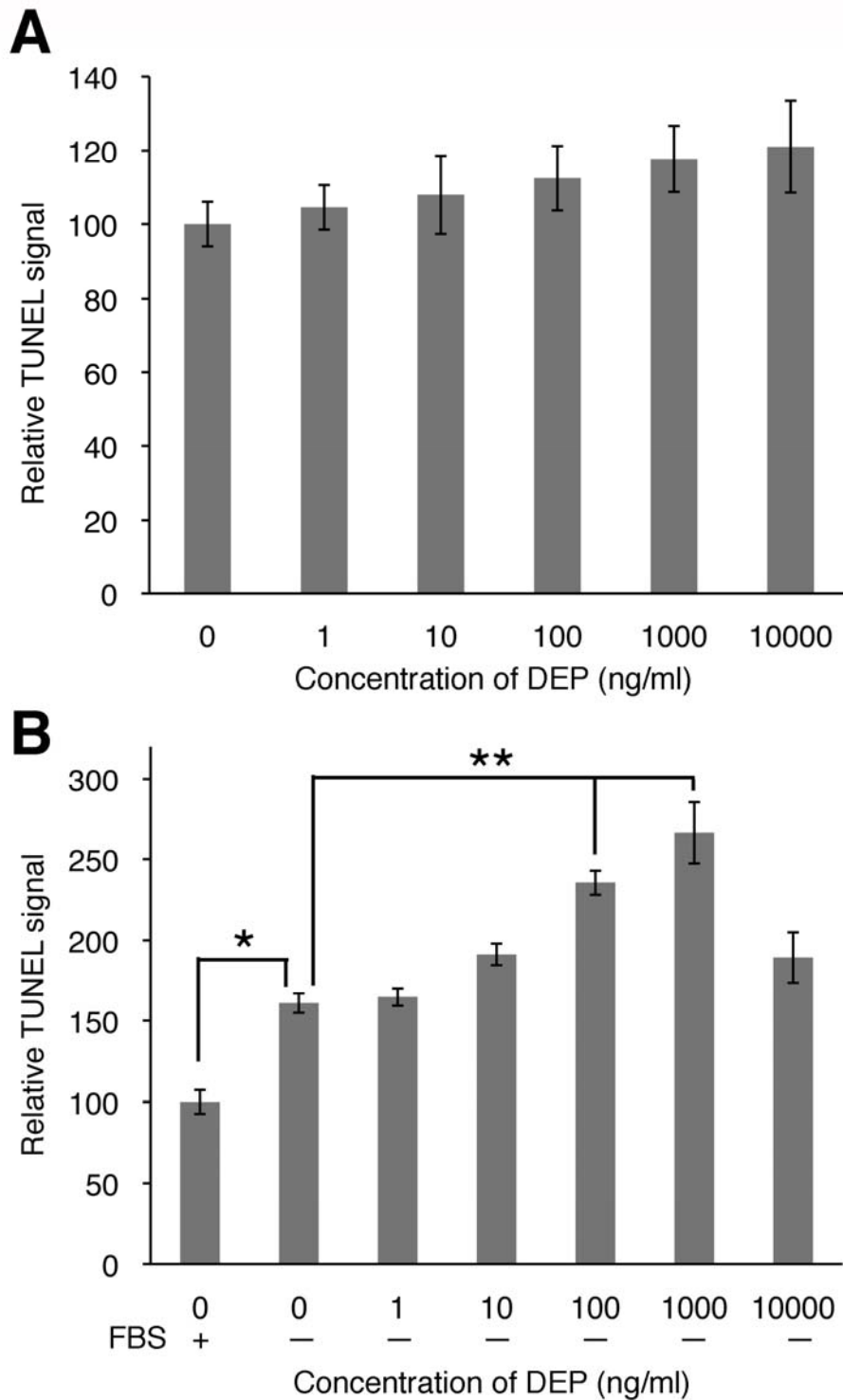


Fig. 3. Quantification of DNA fragmentation in PC12 cells cultured in serum-containing medium with 0 to 10,000 ng/ml DEP (A) and in serum-free medium with 0 to 10,000 ng/ml DEP (B). Error bars indicate SEM (n=4). * and ** significantly different from the cells incubated in serum-free medium without DEP (P<0.05 and P<0.01), respectively.

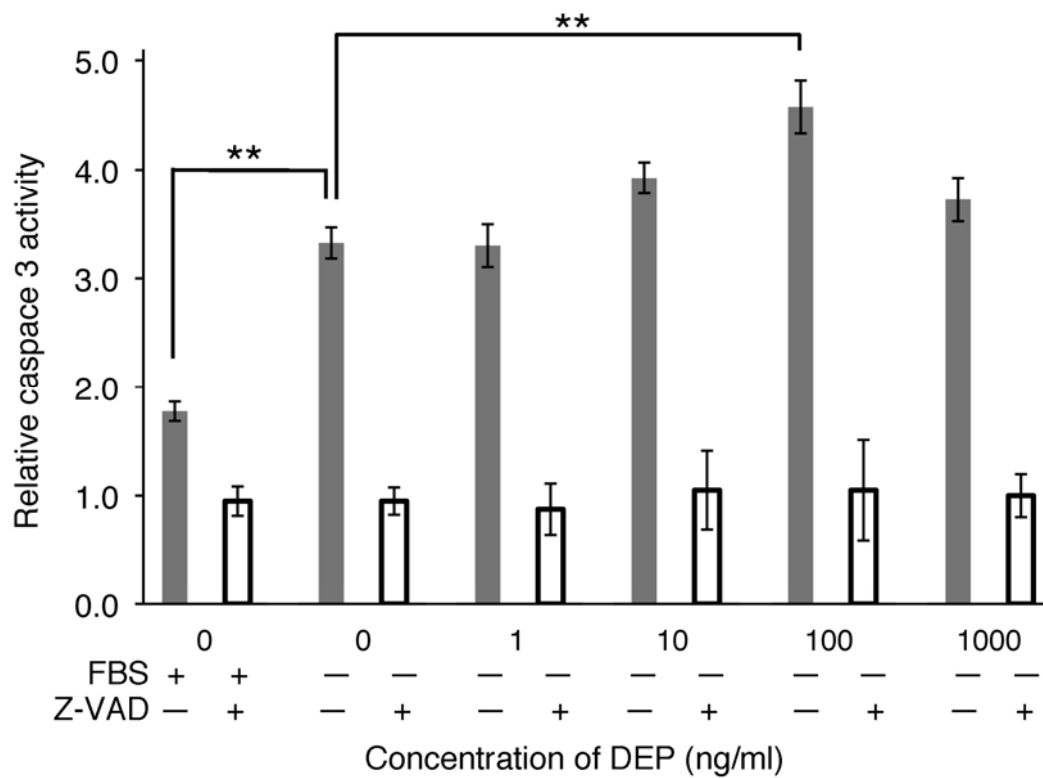


Fig. 4. Caspase-3-like activity of PC 12 cells incubated in serum-free medium containing DEP for 72 hr with (open column) and without Z-VAD (closed column). Z-VAD is a typical inhibitor of caspase-3. Error bar indicates SEM (n=4). ** significantly different from the cells incubated in serum-free medium without DEP (P<0.01).

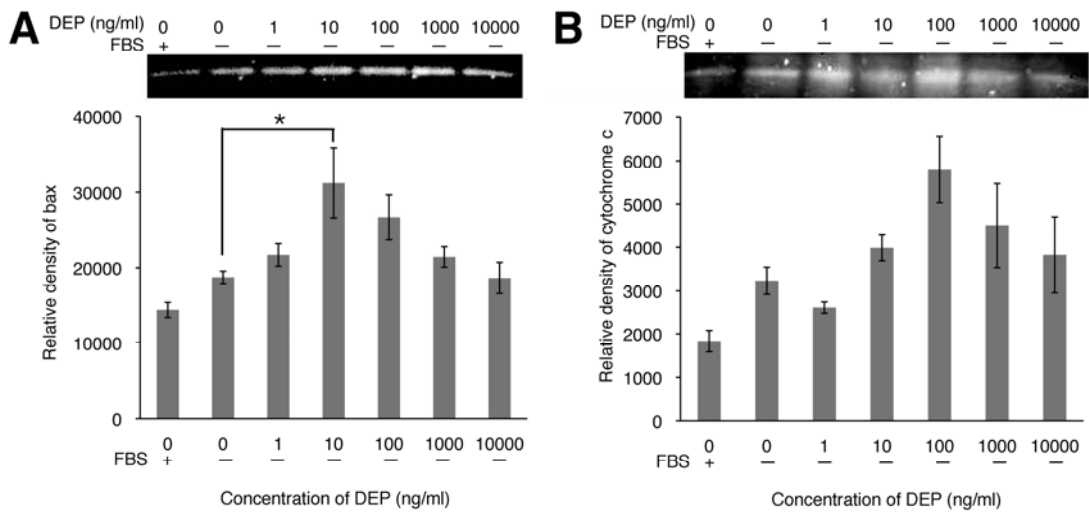


Fig. 5. Representative Western blot analyses for Bax (A) and cytochrome c (B) in cells cultured in serum-free medium with 0 to 10,000 ng/ml DEP for 72 hr. In the figure, the typical pattern and mean intensity of staining of representative Western blot analysis are shown in the upper and lower panels, respectively. Error bars indicate SEM (n=5 for Bax and n=4 for cytochrome c). * significantly different from the cells incubated in serum-free medium without DEP (P<0.05).