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Bisphenol-A suppresses neurite extension due to inhibition of phosphorylation of mitogen-activated protein kinase in PC12 cells

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Abstract

An endocrine disrupter, bisphenol-A is widely used in the production of plastics and coatings. Recently, it was reported that bisphenol-A affected neurotransmitters in the mammalian brain. On the basis of these reports, it was considered that bisphenol-A affected neuronal differentiation. In this study, the morphological changes in nerve growth factor (NGF)-induced differentiation caused by bisphenol-A were confirmed using a PC12 cell system. When a low concentration of bisphenol-A was added to medium containing NGF, it inhibited neurite extension. In addition, to clarify whether bisphenol-A affects the early and late stages of the NGF-signaling pathway in cell differentiation, changes of phosphorylation of MAP kinases and cAMP-response element binding protein (CREB) in PC12 cells treated with and without BPA in medium containing NGF were investigated using western blot analysis. As results, bisphenol-A significantly inhibited phosphorylation of CREB and ERK1/2 MAPK.

Keywords: bisphenol-A, PC12 cell, MAPK, ERK, neurite, nerve growth factor

1. Introduction

Some pesticides and industrial chemicals can affect animal physiology by mimicking the effects of endogenous hormones. Several of these compounds have been shown to have estrogenic activity by *in vitro* and *in vivo* bioassays [1,2]. One such endocrine disrupter, bisphenol-A (BPA) is widely used as a monomer for the production of plastics, resins and coatings that are extensively used in the food-packaging industry and dentistry [3]. Mariscal-Arcas *et al.* [4] reported that BPA remained of concern, given the proven undesirable effects of low-level exposure and higher susceptibility of pregnant women. Le *et al.* [5] reported that BPA was found to migrate from polycarbonate water bottles at rates ranging from 0.20 ng/h to 0.79 ng/h. They described that exposure to boiling water increased the rate of BPA migration by up to 55-fold. Miyamoto and Kotake [6] estimated that 95% confidence intervals for the daily intake for high-exposure populations were estimated to be 0.037-0.064 µg/kg/day for males and 0.043-0.075 µg /kg/day for females. The relatively low affinity of BPA for nuclear estrogen receptors (ERs) and its weak bioactivity in standard tests of estrogenicity [7] led to the consideration that BPA had negligible effects in human. However, exposure to BPA could have adverse effects on humans because of their ubiquitous presence in the environment and potential for accumulation in brown adipose tissues [8].

Lee *et al.* [9] reported that BPA could be detected in umbilical cords, suggesting its transfer to fetuses. Kubo *et al.* [10] also reported that the current methods to determine the no observable adverse effect level (50 mg/kg per day) of artificial industrial chemicals may not be sufficient to detect disruption of sexual differentiation in the fetal brain. Durando *et al.* [11] reported that prenatal exposure to a low dose of BPA perturbed mammary gland histoarchitecture and increased the carcinogenic susceptibility to a chemical challenge administered 50 days after the end of exposure. Dekant and Volkel [12] reported human exposure to BPA by

biomonitoring including the ways of exposure to it and assessment of environmental exposures. Even at doses below the supposedly safe daily limit for human exposure (US Environmental Protection Agency) BPA impairs the synaptogenic response to 17β -oestradiol (E2) in the hippocampus of the ovariectomised rat [13]. Palanza *et al.* [14] summarized that during fetal life the intrauterine environment is critical for the normal development, and even small changes in the levels of hormones, such as estradiol or estrogen-mimicking chemicals, can lead to changes in brain function and consequently in behavior. In addition, a low dose of BPA prevented the synaptogenic response to testosterone in the adult rat brain [15]. Yoneda *et al.* [16] found that prenatal exposure to BPA led to a reduction of dopamine content in the mouse brain. They used a PC12 cell system and concluded that the reduction of dopamine depended on an increase of dopamine release in neuronal cells exposed to BPA. Lee *et al.* [17] reported an estrogen receptor-independent neurotoxic mechanism of BPA, because the cell vulnerability to BPA was not significantly different in PC12 cells overexpressing estrogen receptor (ER) α and ER β . In addition, these reports suggest that BPA affects the development, function, and morphology of the brain.

Rat pheochromocytoma PC12 cell line have been extensively used as model systems for the study of tumorigenesis, apoptosis and neurodegenerative diseases. Nerve growth factor (NGF) activation of the Ras/ERK pathway leads to the phosphorylation and activation of RSK2, which then phosphorylates cAMP-response element-binding protein (CREB) at Ser-133 [18]. In addition, NGF was found to activate p38/MAPK and its downstream effector MSK1 or MAPKAP kinase 2, which may then catalyze CREB phosphorylation. Thus, a variety of signaling pathways have evolved that can trigger CREB phosphorylation and thereby activate immediate-early gene (IEG) transcription [19].

On the basis of these reports concerning PC12 cells and neuronal

differentiation, it was considered that BPA affected neurite outgrowth in PC12 cells. The neurite outgrowth in PC12 cells has been confirmed to be regulated by the mitogen-activated protein kinases (MAP kinases) and CREB [20,21].

In this study, to investigate whether BPA is harmful to organism, the morphological changes in NGF-induced differentiation caused by BPA were examined using a PC12 cell system. In addition, changes of phosphorylation of MAP kinases and CREB in PC12 cells treated with and without BPA in medium containing NGF were investigated using western blot analysis to clarify whether BPA affected the early and late stages of the NGF-signaling pathway.

2. Materials and methods

2.1. Materials

PC12 cells were purchased from the American Type Culture Collection (USA and Canada). Rabbit polyclonal antibodies against phosphorylated and unphosphorylated forms of transcription factor CREB, p44/42 MAP kinases (extracellular signal-regulated kinases; ERK1/2), the high affinity NGF receptor TrkA and MEK/MAP kinase were obtained from Upstate Biotechnology (Lake Placid, NY), Promega (Madison, WI) and/or Cell Signaling Technology (Beverly, MA). Antibodies to phosphorylated forms of p38 and JNK/MAP kinases were from Promega, and those to unphosphorylated forms of p38, and JNK/MAP kinases were from Sigma (St. Louis, MO). A blocking reagent was bought from Roche Diagnostics (Mannheim, Germany). Neurite Outgrowth Assay Plus Kit, anti-tubulin beta III isoform were bought from Millipore (Billerica, MA). The secondary antibody, anti-rabbit immunoglobulin, chemiluminescent detection reagents and fluorescein isothiocyanate (FITC)-labeled avidin were obtained from Amersham Pharmacia Biotechnology (Buckinghamshire, UK). Dulbecco's modified Eagle's medium (DMEM),

BPA, 17 β -estradiol (E2), 17 α -ethynylestradiol, an agonist of ER and 2.5S NGF were from Sigma. Fetal bovine serum (FBS) was from HyClone (Rockville, MD). The BPA ELISA kit was bought from Takeda (Japan). The cytotoxicity detection kit was purchased from Promega. Other chemicals were of analytical reagent grade. BPA and E2 were dissolved in ethanol, and used as a vehicle.

2.2. Cell culture

PC12 cells in 25 and 75 cm² flasks (Nunc, USA) were maintained in a humidified incubator with 5% CO₂ at 37°C, and cultured in DMEM supplemented with 10% FBS.

2.3. Effects of BPA in PC12 cells that responded to NGF

Cells were incubated in DMEM containing 1% FBS, 10 ng/ml BPA and 50 ng/ml NGF (2.5S) for 5 days. Gunning *et al.* [22] reported that neurite outgrowth was clearly observed more than 3 days after treatment of PC12 cells with 50 ng/ml NGF. The medium containing NGF and BPA was changed every 2 days. Cell differentiation was observed using a phase-contrast microscope (Olympus IMT-2, Japan). To clarify whether any morphological changes relate to ER reaction, 10 ng/ml E2 or 100 ng/ml 17 α -ethynylestradiol was added into the medium with NGF and BPA. In addition, the growth of neurite was evaluated by Neurite Outgrowth Assay Plus Kit (Millipore) using anti-tubulin antibody according to their instruction manual. In this study, the BPA concentration in the medium chosen was 10 ng/ml because the released BPA in the baby bottles ranged 2.4-14.3 μ g/kg when filled with boiled water and left at ambient temperature for 45 min [23]. In addition, Jeng *et al.* described that 17.52 nM BPA (equivalent to 4 ng/ml) detected in serum affected extracellular-regulated kinases [24].

2.4. Quantification of BPA concentration in the cells treated with BPA

BPA contents in the cells were measured using a BPA ELISA kit (minimum sensitivity: 10 pg/ml, linear range: 50 pg/ml to 100 ng/ml) after they were treated with 10 and 500 ng/ml BPA. According to the time course from 24 to 72 hr, the medium in the flask was discarded and the cells were rinsed once with 40 mM Tris-HCl buffer, pH 7.4, containing 0.9% NaCl to remove the excess BPA. Then 200 µl of fresh buffer was added and the cells were sonicated for 10 to 30 sec with a type 250 Branson Sonifier (USA). The sonicated cells were centrifuged to divide the soluble and insoluble fractions. The insoluble fraction was dissolved using 0.1% Triton X-100. The obtained soluble and insoluble fractions were used for the quantification of BPA contents. Usually in neurotoxicological research BPA dose to rats was employed as 0.5 to 5 mg/kg [25,26]. Then in this study, high exposure of BPA was selected as 500 ng/ml.

2.5. Cytotoxicity assays

The cytotoxicity of BPA was evaluated using a cytotoxicity detection kit based on the detection of lactate dehydrogenase (LDH) activity released from dead cells [27]. The cell-free supernatants of the culture media containing 1% FBS after centrifugation of the media for the PC12 cells treated with 0.001 to 50,000 ng/ml BPA were collected and then transferred to multititer plates. A substrate mixture containing tetrazolium salts was added to the wells, followed by incubation for 0.5 hr. The formazan dye formed was quantitated by measuring the absorbance at 450 nm.

Western blot analysis using antibodies against signal transduction factors

Cells were treated with 50 ng/ml NGF for various times after treatment with BPA in medium containing 1% FBS for 24 hr. The extraction was

performed according to the method of Numata *et al.* [28]. The sample solution was heated for 10 min at 100°C and centrifuged for 5 min at 4°C before analysis by western blotting. The treated lysate was separated by polyacrylamide gel electrophoresis [29], and transferred to nitrocellulose membranes with a type-AE6678 semidry blotting system (ATTO, Japan). The membranes were incubated for 1 hr at room temperature in 40 mM Tris-HCl buffer, pH 7.4, containing 150mM NaCl and 2% blocking reagent. Then they were incubated for 2 hr at room temperature with primary antibodies in 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 1% blocking reagent. Following this, the membranes were washed three times for 3 min each time in TBTS consisting of 40 mM Tris-HCl buffer, pH 7.4, 150 mM NaCl and 0.3% Tween 20, and then incubated at room temperature for 1 hr with horseradish peroxidase-conjugated secondary antibodies (dilution; 1:400) or an anti-biotin antibody (1:500) to detect biotinylated protein markers in the 1% blocking buffer. The membranes were next washed three times for 3 min each time in TBTS and then washed twice for 3 min each time in Tris-HCl buffer consisting of 40 mM Tris-HCl, pH 7.4, and 150mM NaCl. Protein bands that responded to antibodies were detected with an enhanced chemiluminescence system using ECL-plus kit (GE Health Care Bioscience, UK). Each experiment was repeated at least three times.

2.6. Microscopic detection of CREB and phospho-CREB in PC12 cells treated with BPA and NGF

Cells were cultured on sterilized coverslips in 6-well plates and treated with 50 ng/ml NGF for various times after treatment with BPA in medium containing 1% FBS. The coverslips were washed with 10 mM Tris-HCl buffer, pH 7.4, containing 0.01% Tween 20. Nonspecific binding sites were blocked with 2% BSA for 30 min at room temperature and reacted with anti-phospho-CREB (1:1,000) in PBS with 0.01% Tween 20 and 2% BSA

for 2 hr at room temperature. After washing with the same buffer, the coverslips were incubated with the biotin-tagged secondary antibody for 2 hr at room temperature. To visualize the phospho-CREB, streptavidin-conjugated FITC (diluted 1:100 in PBS) was used. The fluorescent signals of FITC depending on phospho-CREB in the cells were observed using a Bio Rad MRC-1024 confocal imaging system (Bio Rad Microscience, UK).

2.7. Statistical analysis

In all figures, each value is expressed as mean $\pm$ SEM. Statistical analyses were performed using one-way analysis of variance (ANOVA) with the Tukey-Kramer post hoc test. The test was performed only when the results of ANOVA were $p < 0.05$. An InStat for Macintosh (GraphPad Software, U.S.A.) was employed for the statistical analysis.

3. Results

3.1. Effects of BPA and E2 on NGF-induced differentiation of PC12 cells

To examine whether BPA and E2 affected cell differentiation, morphological changes of PC12 cells treated with BPA were representatively observed, as shown in Fig. 1A. It is well documented that PC12 cells differentiate into neuronal cells with the addition of NGF. In growth medium in the absence of NGF for 5 days, PC12 cells are round or polygonal and tend to grow in small clumps (Fig. 1A-a). The cells are sensitive to NGF. Morphologically, the fibers that extended from PC12 cells in the presence of NGF for 5 days resembled those produced by cultured primary sympathetic neurons [30]. PC12 cells branched profusely, had numerous varicosities and formed fascicles (Fig. 1A-b). However, when 10 ng/ml BPA was added to the medium containing

NGF, PC12 cells scarcely exhibited these morphological features (Fig. 1A-c). On the other hand, when 10 ng/ml E2 was added to the medium containing NGF, NGF-induced neurite extension in the cells was hardly changed compared with those treated with NGF alone (Fig. 1A-e). The same tendency was confirmed in the experiments of neurite outgrowth measurement in the same condition (Fig. 1B). Although neurite outgrowth in PC12 cells was significantly increased by addition of NGF, this increasing was hardly observed by the treatment of BPA. In addition, there was no effect of E2 on decreasing of neurite outgrowth like as BPA.

To confirm whether ER relates to inhibition of the NGF-induced neurite extension, morphological change was observed in the cells treated with 17 α -ethynylestradiol, an agonist of ER as shown in Fig. 2. PC12 cells differentiate like as neuronal cells with treatment of NGF (Fig. 2A-a). When 10 ng/ml BPA was added to the medium containing NGF, PC12 cells scarcely exhibited these morphological features (Figs. 2A-b and 2B) as same as Fig 1A-c. However these morphological changes are independent from presence of 17 α -ethynylestradiol (Figs. 2A-b and 2B). Based on these results BPA was considered to inhibit the NGF-induced differentiation in PC12 cells. In addition, the inhibitory effects of BPA were suggested not to be due to hormonal effects mediated by ERs.

3.2. BPA accumulated in the nuclei and cytosol of PC12 cells in cultured BPA-treated-medium

To evaluate the contents of BPA taken into the PC12 cells cultured in 10% FBS-medium containing 10 or 500 ng/ml BPA, intracellular contents of BPA in the PC12 cells were measured using a BPA ELISA kit. As shown in Fig. 3A, BPA was detected in the cytosol and nuclei in PC12 cells

treated with 10 and 500 ng/ml BPA. In the case of treatment with 500 ng/ml BPA, BPA contents in cytosol were significantly higher than those in nuclei. As shown in Fig. 3B, the detected BPA contents in nuclei were almost constant during the time course. On the other hand, BPA contents in cytosol slightly decreased from 24 to 72 hr. These results indicated that BPA accumulated in the cells, but was thought to be degraded gradually.

3.3. Analysis of cytotoxicity of BPA to PC12 cells

To examine whether the observed inhibitory effects on cell differentiation (Fig. 1) were caused by cytotoxicity of BPA in the medium with a low content of FBS, the cytotoxicity of BPA and E2 to PC12 cells was estimated using a non-radioactive cytotoxicity assay. BPA or E2 in concentrations ranging from 1 pg/ml to 50 µg/ml was added to the medium for the PC12 cell culture, and the LDH activity in the medium was measured at 72 hr after the addition of BPA or E2. Although LDH activity in the medium of PC12 cells treated with a high concentration of BPA (>50 µg/ml) increased significantly, the activity did not change significantly in the medium of PC12 cells treated with 10 pg/ml to 1 µg/ml BPA (Fig. 4A). Similar results were obtained in the case of PC12 cells treated with E2 (Fig. 4B). These results indicated that the inhibition of cell differentiation was not due to the cytotoxicity of less than 1000 ng/ml BPA or E2 .

3.4. BPA inhibits the MAP kinases, but not TrkA in NGF-induced neuronal differentiation of PC12 cells

To confirm where BPA affected the signaling pathway of neuronal differentiation, the effect of BPA on activated CREB was investigated using western blotting and immunohistochemistry (Fig. 5). NGF stimulated phosphorylation of CREB (Fig. 5A). After treatment with BPA, phosphorylation of CREB in PC12 cells upon NGF-induced differentiation was significantly decreased in comparison with that in NGF-induced

control cells (Figs. 5A and B), although the contents of CREB protein were hardly changed by BPA treatment (Figs. 5B and C). Signals of phosphorylated CREB in the western blot analysis for PC12 cells cultured in the medium containing NGF and/or E2 were almost the same as for control cells (data not shown). In histochemical analysis the same tendency was observed (Fig. 5D). FITC signals depending on anti-phospho-CREB appeared with addition of NGF to PC12 cells (Fig. 5D-c). These signals disappeared after treatment with BPA (Fig. 5D-d).

To observe the phosphorylation of the ERK1/2 MAPK in the upstream of the CREB signaling pathway in PC12 cells treated with BPA and E2, an antibody against phosphorylated ERK1/2 was used. The phosphorylation of ERK1/2 in PC12 cells treated with 10 ng/ml BPA was significantly inhibited (Figs. 6A and C), although the contents of ERK1/2 protein were not changed by BPA treatment (Figs. 6B and C). Phosphorylation in response to NGF is known to reach a maximum 5-10 min after NGF treatment [31]. Independently of the BPA incubation time, the phosphorylation was significantly inhibited by the BPA treatment (data not shown). These results suggested that the reaction to BPA occurred immediately. In contrast to the Erk subgroup of the MAPK superfamily, which is activated by mitogens and growth factors, the p38 and JNK subgroups of the MAPK family are believed to be stimulated primarily by proinflammatory cytokines and cellular stress [32,33]. An examination of whether PC12 cell treatment with NGF led to p38 or JNK activity in the copresence of BPA was carried out. Signals of the phosphorylation of p38 (Fig. 7A) and JNK (Fig. 7B) did not change as compared with those from PC12 cells treated with NGF alone. Nor did BPA affect NGF-induced phosphorylation of MEK1/2, which is a MAPK upstream of ERK1/2 (Figs. 7C and D), or the NGF-induced phosphorylation of TrkA, which is a receptor for NGF (Fig. 7E). These results suggested that BPA inhibited the phosphorylation of ERK1/2, which plays a key role in the cell signaling

pathway of NGF-induced differentiation, and did not affect the NGF receptor. On the other hand, E2 did not inhibit phosphorylation of ERK1/2 (data not shown).

4. Discussion

The present study demonstrated that BPA at a low concentration inhibited morphological changes due to cell differentiation. To study the cell differentiation, an *in vitro* model system using rat PC12 cells was utilized, since PC12 cells treated with NGF have been extensively used as model cells for studying the nervous system and neuronal differentiation [34,35]. As shown in Figs. 1 and 2, when BPA was added to the medium containing NGF, it dramatically inhibited neurite extension. The inhibitory effect of BPA was considered to be suppressed phosphorylation of ERK1/2 MAPK in the signaling pathway (Fig. 6), which involves protein kinases that are important in regulating the growth and differentiation of PC12 cells [36]. Similar studies of inhibitory or inductive effects through phosphorylation of the MAPK signaling pathway have also been demonstrated using PC12 cells [34, 37]. For example, the enzymatic activity of phospholipase C- γ 1 is at least partially involved in the blockage of neuronal differentiation via abrogation of MAPK activation in PC12 and PLC- γ 1 cells [37]. Previously, it was reported that PC12 cells exposed to chronic cocaine treatment had prolonged c-fos expression, which was linked to the cells' inability to develop and maintain neurite-like processes [34].

Although BPA was present in PC12 cells treated with it (Fig. 3), BPA itself did not show cytotoxicity to the cells (Fig. 4). Thus it was supposed that BPA-inhibited differentiation in PC12 cells treated with NGF was independent of its cytotoxicity.

There are three distinct MAPK cascades; ERK1/2 MAPK, p38/MAPK, and c-Jun kinases (JNK/MAPK), also called stress-activated protein

kinases [38]. It is known that binding of NGF to its receptor tyrosine kinase (TrkA) activates the Ras/Raf/MEK/Erk/RSK/CREB pathway, which is also called the Erk1/2 MAPK pathway. JNK/MAPK and p38/MAPK are believed to be stimulated primarily by proinflammatory cytokines and cellular stress [32,33,39]. However, certain growth factors have also been reported to activate the JNK/MAPK and p38/MAPK pathways. In addition, it has been demonstrated that exposure of PC12 cells to NGF induces the p38/MAPK pathway, which then leads to CREB [18,40], although it is revitalized by oxidative stress [32]. JNK activity was not significantly enhanced by NGF treatment, although it was strongly stimulated by UV irradiation. On the basis of those results, BPA inhibition of neuronal differentiation was considered to depend on some changes in the NGF signaling pathway.

In this case, two possibilities were considered: one was that BPA suppressed protein kinase expression, and the other was that BPA inhibited phosphorylation of the protein kinases. As shown in Fig. 6, the ERK1/2 protein contents detected by western blot analysis were hardly changed by BPA and NGF treatment. Second, in this study the activated states of those ERK1/2 kinases were investigated using antibodies against phosphokinases.

It was noted that the phosphorylation of ERK1/2 was markedly inhibited by BPA as compared with that from PC12 cells treated with NGF alone (Fig. 6). Phosphorylated CREB was decreased by BPA treatment (Fig. 5), whereas phosphorylated p38 and JNK were hardly changed by exposure to BPA (Fig. 7). After NGF-induced differentiation of PC12 cells treated with BPA, phosphorylation of TrkA and MEK1/2 was not changed as compared with NGF treatment alone (Figs. 7C-E).

As BPA is known to be an endocrine disrupter, whether E2 inhibited phosphorylation of ERK1/2 MAPK was investigated. The phosphorylation of ERK1/2 in PC12 cells treated with NGF and E2 was hardly changed

(data not shown). The difference between BPA and E2 concerning the inhibitory effects on the NGF-induced differentiation in PC12 cells was thought not to depend on the estrogenic action mediated by ERs (Fig. 2). Recently, α - and β -subunits of ERs were confirmed to be expressed in PC12 cells [41,42]. The activity of β -subunits as ERs is not as strong as that of α -subunits. From this viewpoint, it is difficult to consider that the effect of BPA found in this study was mediated by ERs. This idea is in good agreement with a recent report of Lee *et al.* [17], who described an estrogen receptor-independent neurotoxic mechanism of BPA, because the cell vulnerability to BPA was not significantly different in PC12 cells overexpressing the α - and β -subunits of the estrogen receptor compared with PC12 cells expressing a vector alone.

On the other hand, many researchers have reported that BPA affects the development, function and morphology of the brain. For example, MacLusky *et al.* [13] have reported that BPA, even at doses below the safe daily limit for human exposure, impairs the synaptogenic response to E2 in the hippocampus of the ovariectomised rat, and that a low dose of BPA prevents the synaptogenic response to testosterone in the adult rat brain [15]. In addition, prenatal exposure to BPA leads to a reduction of dopamine content in the mouse brain [16]. Ogiue-Ikeda *et al.* [43] also proposed that even a nanomolar dosage of endocrine disruptors such as BPA could induce significant effects on memory processes on the basis of their investigation of the hippocampus. The BPA inhibited the growth of neurite (Figs. 1 and 2). Our study showed that this inhibition was caused by inhibition of phosphorylation of ERK1/2 (Fig. 6). However it does not directly explain the dysfunction of brain caused by BPA because our experiment was done with *in vitro* system. Further investigation will be needed to clarify the relationship between BPA and brain function.

5. Conclusion

The present study revealed the mechanism of the inhibitory effects of BPA on neuronal differentiation induced by NGF in PC12 cells. BPA suppressed neurite outgrowth, and phosphorylation of ERK1/2 MAPK but not phosphorylation of the JNK/MAPK and p38/MAPK pathways under NGF-induced differentiation of PC12 cells. Further investigation will be needed to clarify whether a low dose of BPA affects cell differentiation in the neonatal period.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. Morphology of PC12 cells treated with BPA or E2 under the neurite extension condition (A). PC12 cells treated with ethanol (a) and 50 ng/ml NGF alone (b) were used as negative and positive controls, respectively. PC12 cells were treated with 50 ng/ml NGF for 5 days after treatment with 10 (c) or 500 (d) ng/ml BPA and 10 (e) ng/ml E2. The bar represents 20 μ m. In B, neurite outgrowth in five independent experiments in panel A are measured using neurite outgrowth assay plus kit using anti-tubulin antibody. Mean intensities for neurite outgrowth indicate with SEM (n=5), * denotes $p<0.01$.

Fig. 2. Morphology of PC12 cells treated with BPA or E2 inhibitor under the neurite extension condition (A). PC12 cells treated with 100 ng/ml NGF alone (a) were used as negative and positive controls, respectively. PC12 cells were treated with 100 ng/ml NGF for 5 days after treatment with 10 ng/ml BPA (b), and 10 ng/ml BPA and 100 ng/ml 17 α -ethynylestradiol (c) The bar represents 20 μ m. In B, neurite outgrowth in four independent experiments in panel A are measured using neurite outgrowth assay plus kit using anti-tubulin antibody. Mean intensities for neurite indicate with SEM (n=4). Significant difference ($p<0.01$) is observed between A and B, and A and C.

Fig. 3. Accumulated amounts of BPA in the PC12 cells after treatment with BPA. After preincubation in 10% FBS-containing medium for 24 hr, cells exposed to 10 and 500 ng/ml BPA were cultured for 24 hr (A) and cells exposed to 500 ng/ml BPA were cultured for 24 to 72 hr (B). Results from three separate experiments are expressed as mean $\pm$ S.E.M. (*: $p<0.05$, n=3)

Fig. 4. Cytotoxic effects of BPA and E2 on PC12 cells grown in medium

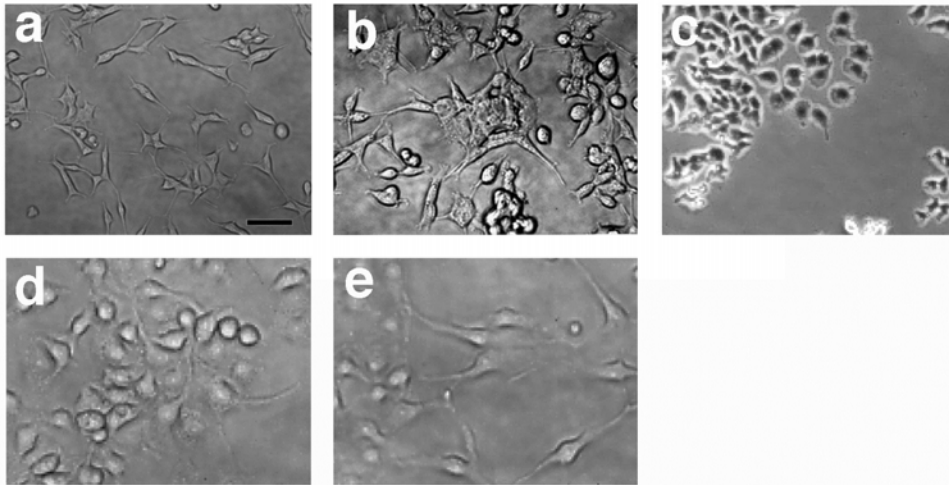
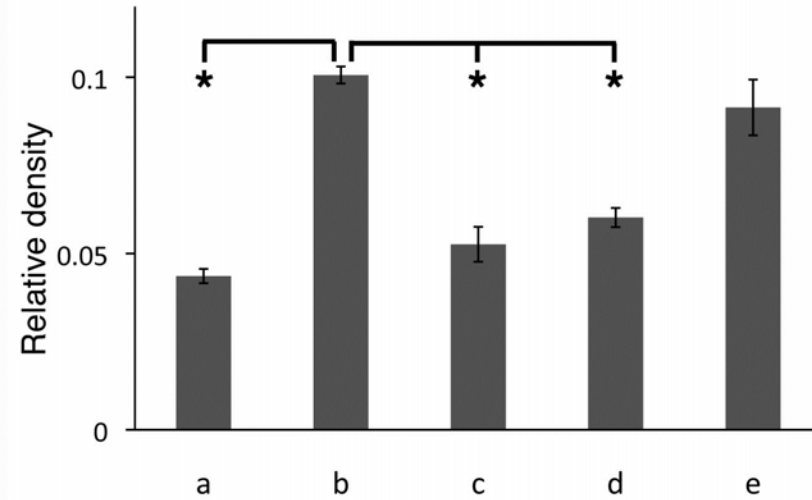
containing 1% FBS (A and B). The cytotoxic effects were evaluated by measuring the LDH released from the cytosol of dead cells. A, cells exposed to 10^{-3} to 5×10^4 ng/ml BPA and B, cells exposed to 10^{-3} to 5×10^4 ng/ml E2. The degree of cytotoxicity is presented as the percentage of LDH activity relative to the nontreated control. Results from three separate experiments are expressed as mean $\pm$ S.E.M. (*: $p < 0.05$, $n = 3$)

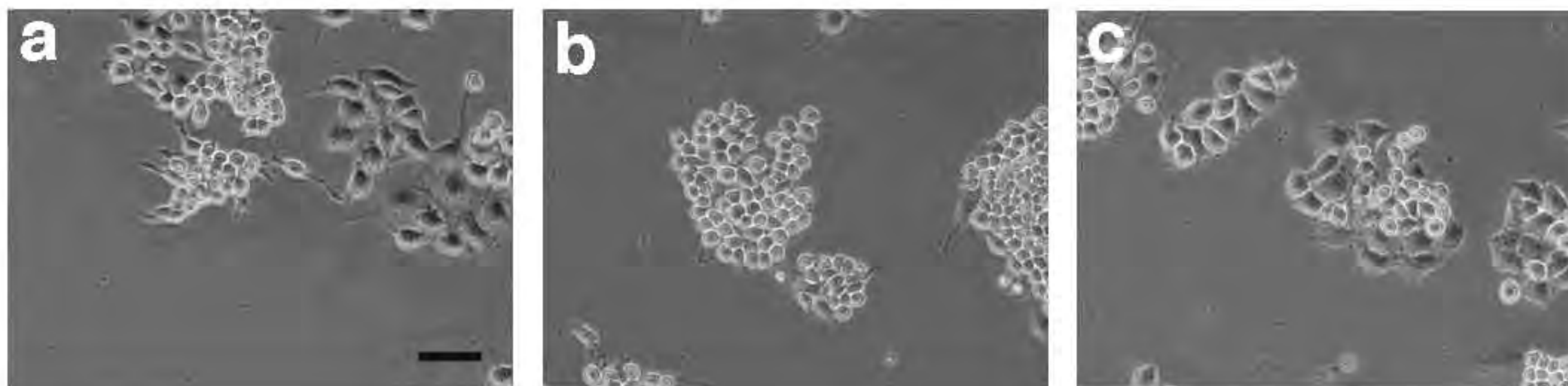
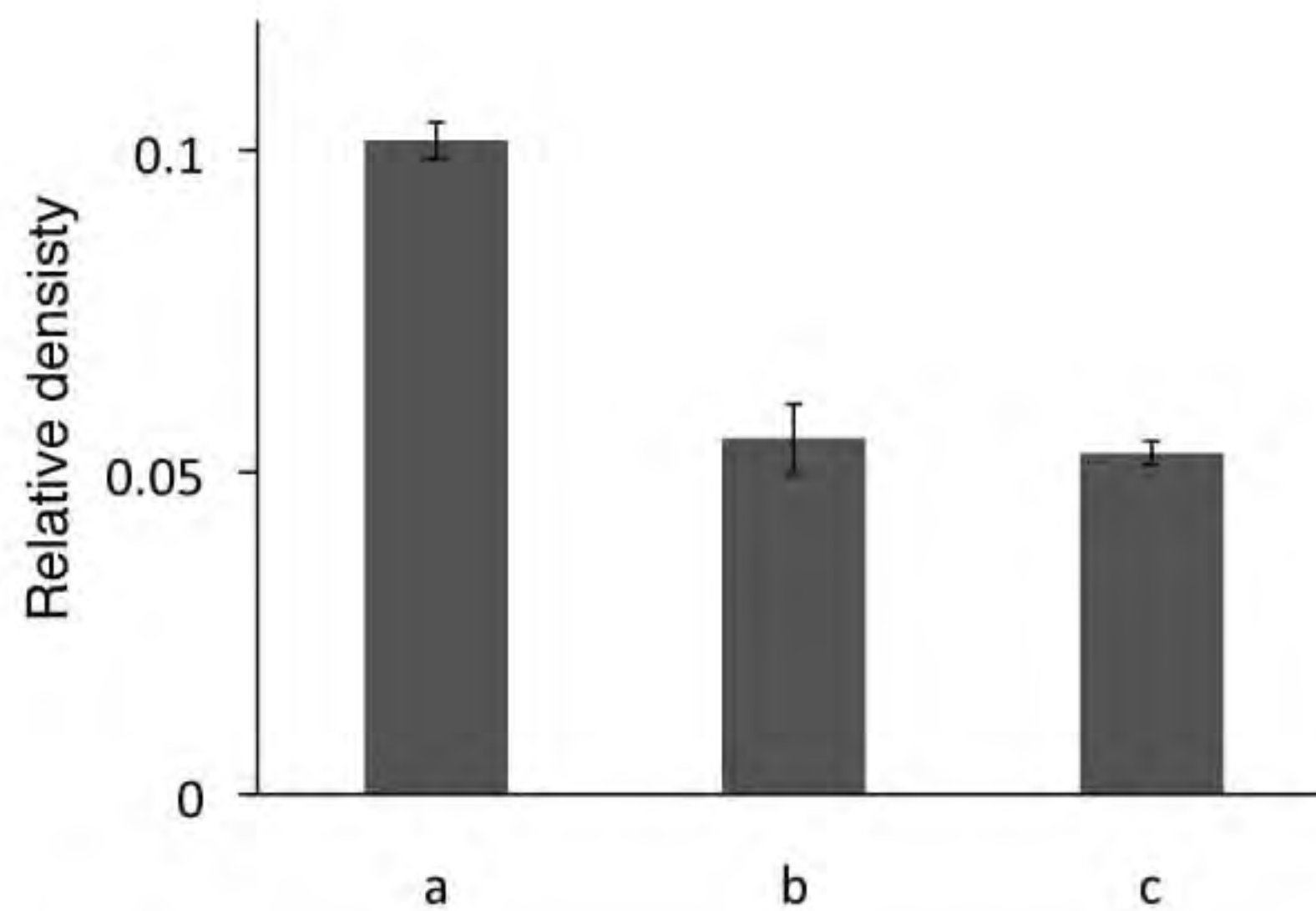
Fig. 5. Effects of BPA on the NGF-induced phosphorylation of CREB. PC12 cells were serum-starved (1%) for 2 hr and then treated with NGF (50 ng/ml) for 10 min after treatment with BPA (10 ng/ml) for 24 hr. Cells treated with only NGF were used as a positive control. Total lysates were immunoblotted with antibodies specific for phosphorylated CREB (A), or the phosphorylation-independent state of the corresponding CREB (B), as indicated on the right. Positions of the protein size markers are indicated on the left. Data from three independent experiments in panels A and B are shown in the graph (C). Mean intensities for phosphorylated CREB indicate with SEM ($n = 3$), * denotes $p < 0.05$. In D, FITC signals depending on anti-phospho-CREB in PC12 cells are shown. PC12 cells were serum-starved for 2 hr (a), and then treated with 10 ng/ml BPA (b), PC12 cells treated with NGF (50 ng/ml) for 10 min (c), PC12 cells treated with NGF (50 ng/ml) for 10 min after treatment with 10 ng/ml BPA for 24 hr (d).

Fig. 6. Effects of BPA and E2 on phospho-ERK1/2 MAPK in the repeated NGF-induced neurite extension of PC12 cells. PC12 cells were serum-starved (1%) for 2 h and then treated with NGF (50 ng/ml) for 0 to 60 min after treatment with 10 ng/ml BPA (A and B). Cells treated with NGF and without BPA were used as a positive control. Total lysates were immunoblotted with antibodies specific for the phosphorylated ERK1/2 (A), and the phosphorylation-independent state of the corresponding ERK1/2

(B). Data from three to five independent experiments in panels A and B are shown in the graph (C). Mean intensities of staining of western blot analysis for phosphorylated ERK1/2 and ERK1/2 indicate with SEM (n=3-5). Significant differences ($p<0.05$) were observed between a and b. In the photographs, positions of the protein size markers are indicated on the left.

Fig. 7. Effects of BPA on the phosphorylation of MAP kinase subgroups. PC12 cells were serum-starved (1%) for 2 h and then treated with NGF (50 ng/ml) for 10 min for detection of p38 (A), and 5 to 15 min for detection of JNK (B) after preincubation with BPA (10 ng/ml) for 24 hr, and then treated with NGF (50 ng/ml) for 10 to 30 min for detection of MEK (C), and 5 to 60 min for detection of Trk (E) after pre-incubation with BPA (10 ng/ml) for 24 hr. Cells treated with NGF only were used as a positive control. Total lysates were immunoblotted with antibodies specific for phosphorylated p38, JNK, MEK and Trk (upper photographs) or the phosphorylation-independent states of the corresponding proteins, p38, JNK, MEK and Trk (lower photographs) as indicated on the right. Positions of the protein size markers are indicated on the left. In panel D, on the basis of data from three independent experiments presented in panel C, intensity p-MEK/MEK is shown. Mean of relative intensities in the western blot analysis indicate with SEM (n=3).

A**B****Fig. 1**

A**B****Fig. 2**

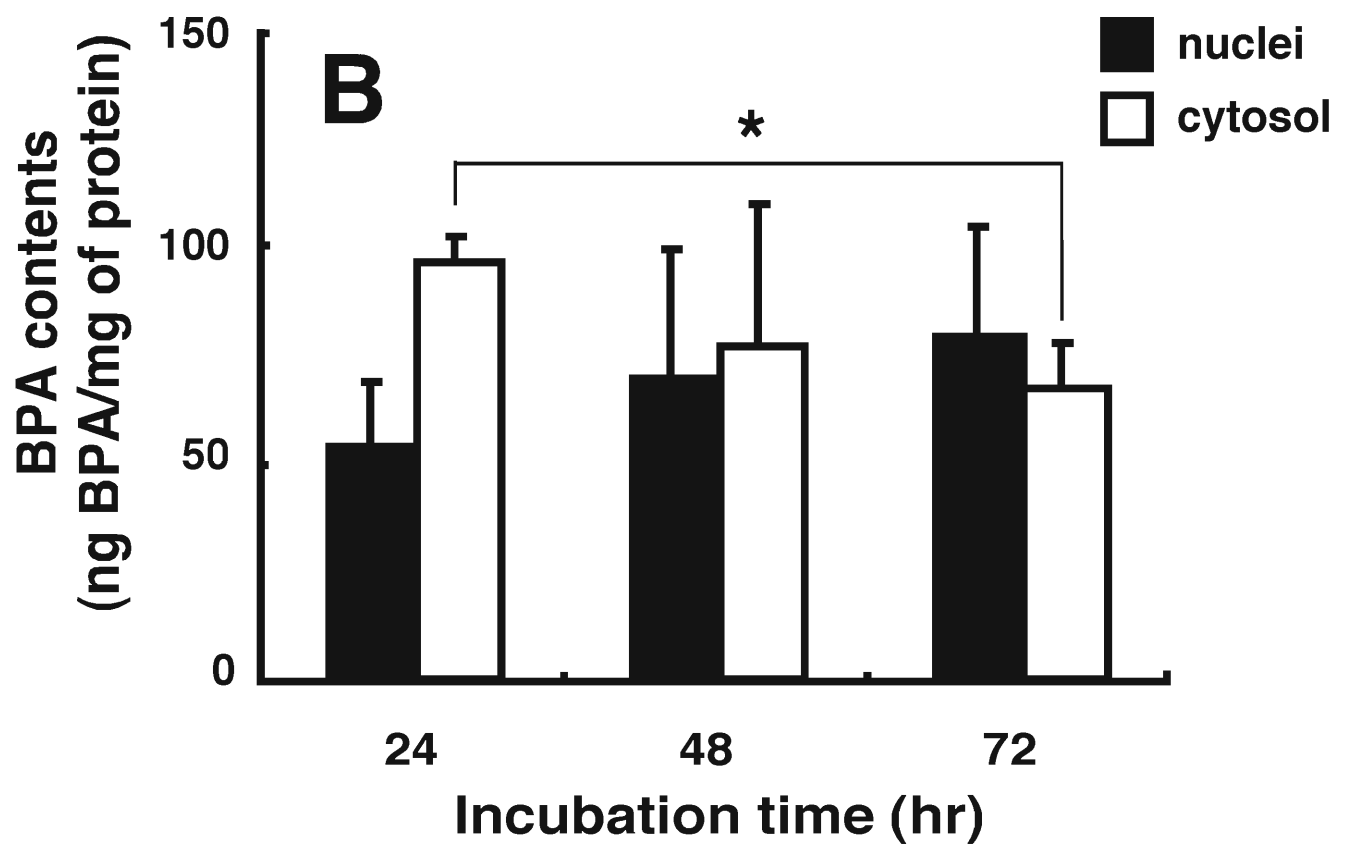
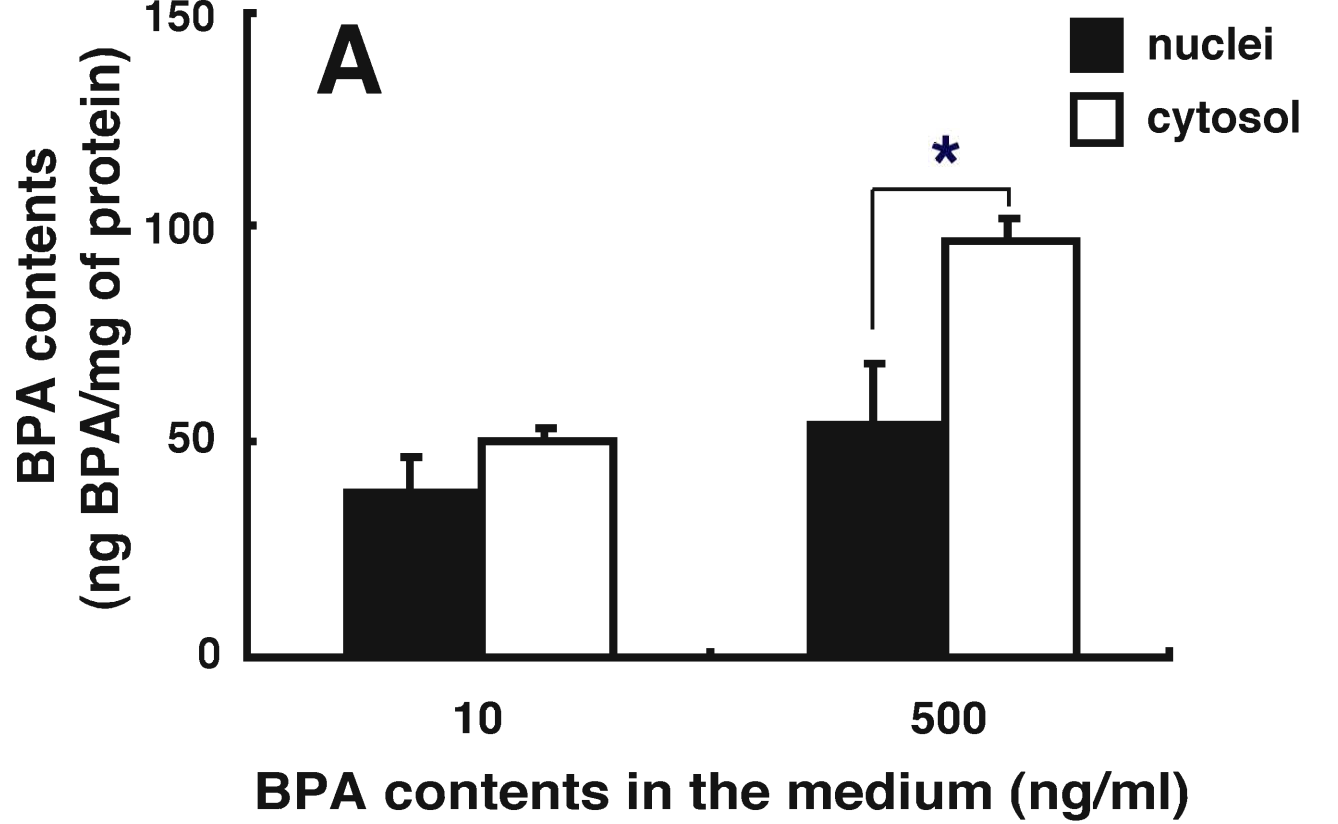


Fig. 3

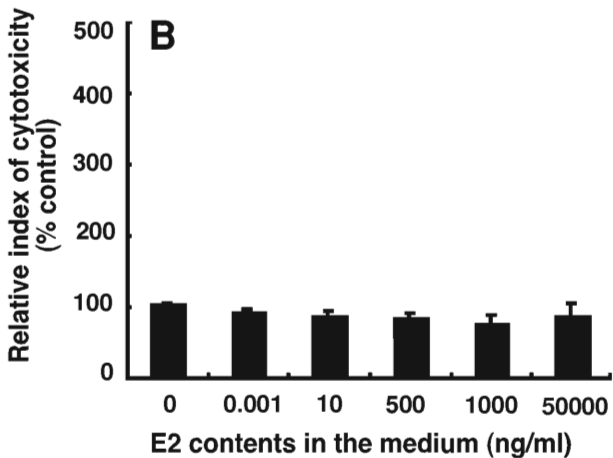
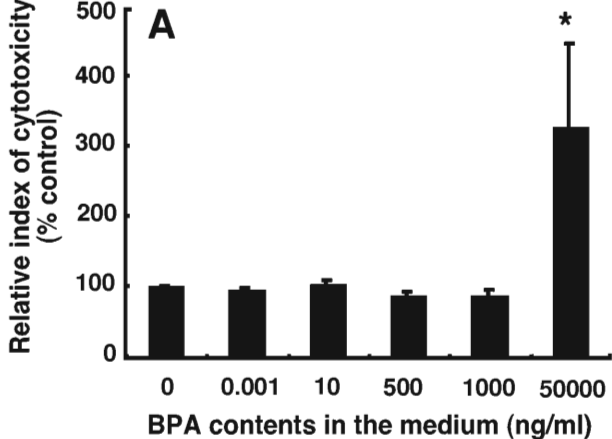
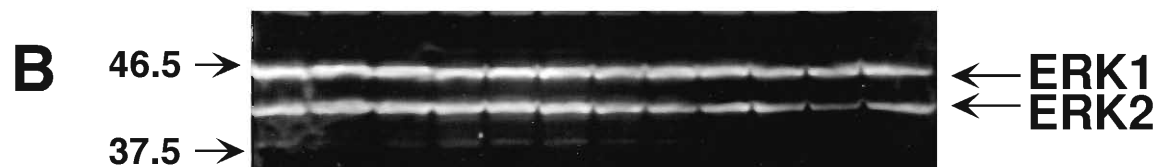


Fig. 4

NGF - 5 10 15 30 60 - 5 10 15 30 60
 BPA - - - - - - + + + + + +



10 ng/ml BPA

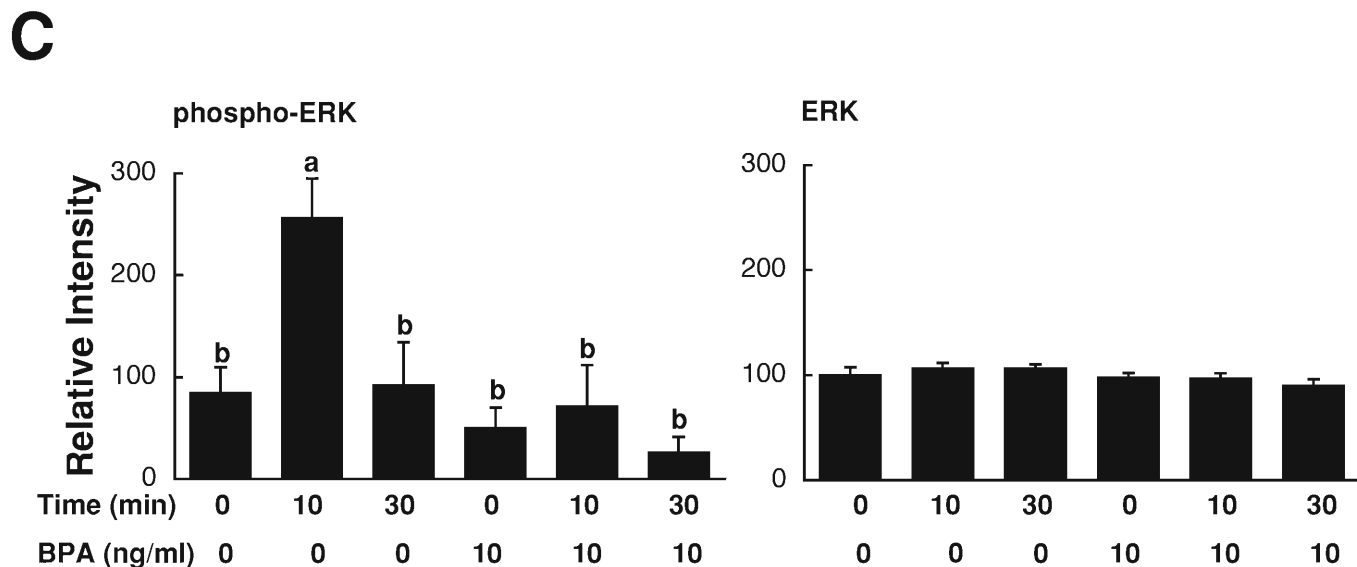


Fig. 6

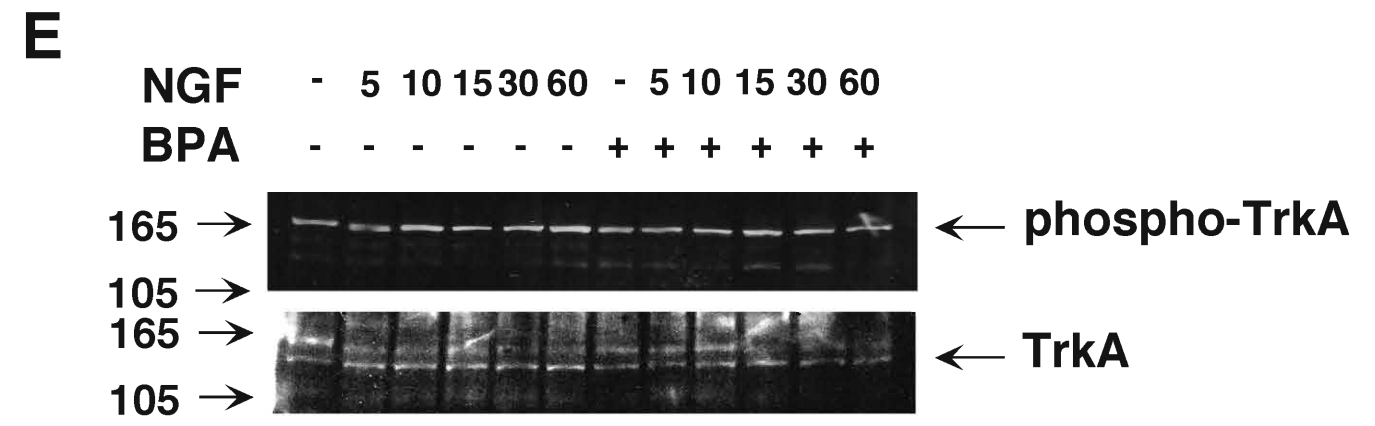
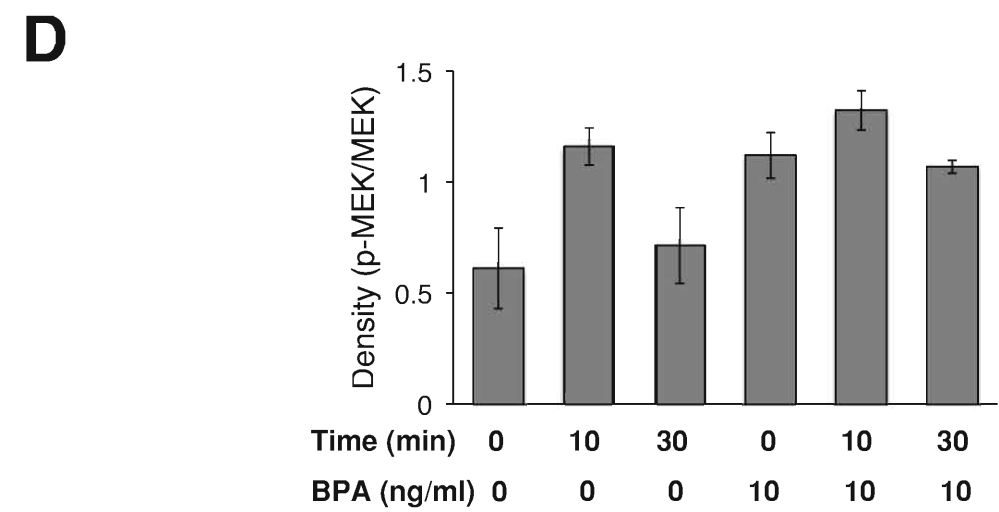
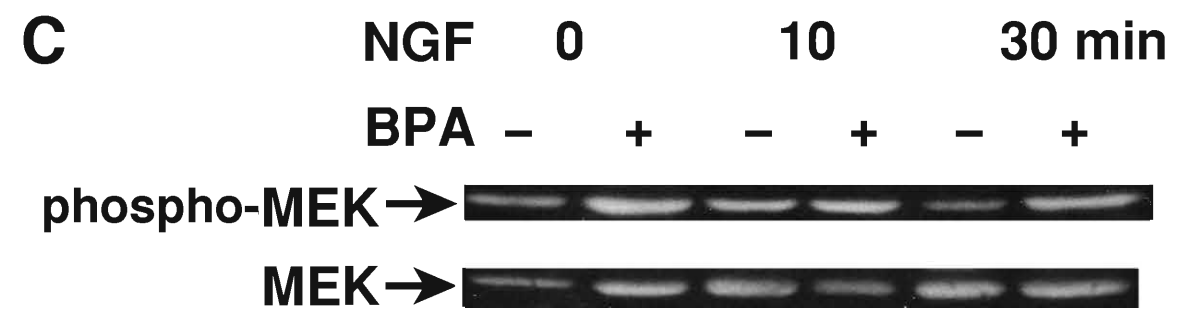
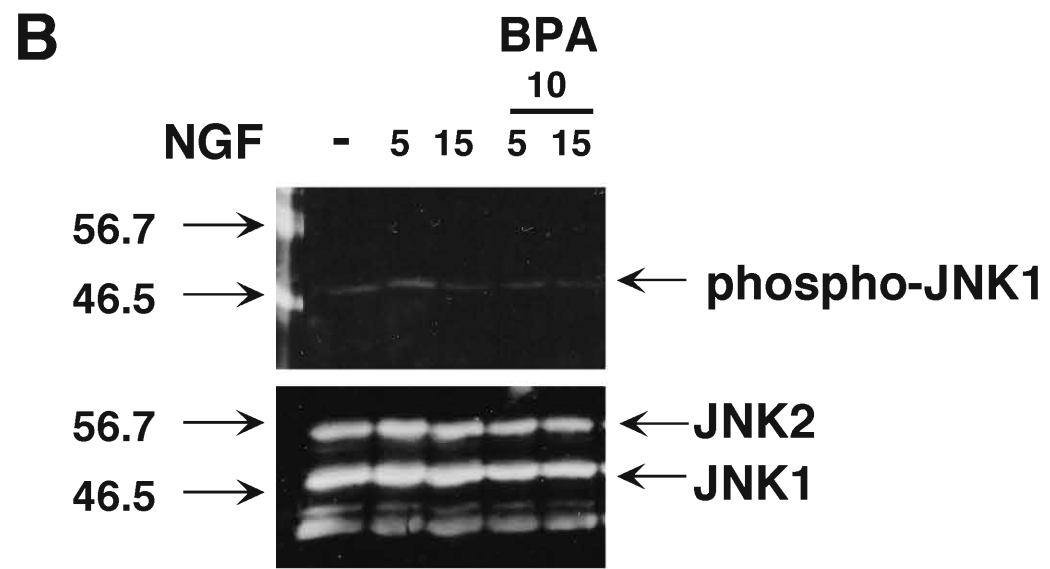
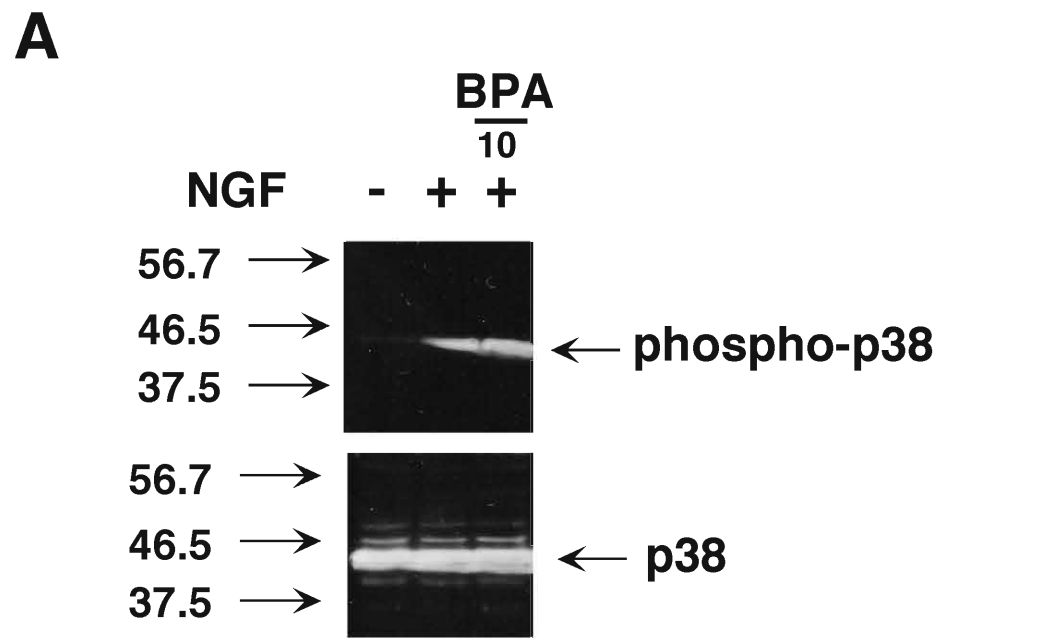


Fig. 7