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Bisphenol A inhibits proliferation and induces apoptosis in micromass cultures of rat embryonic midbrain cells through the JNK, CREB and p53 signaling pathways

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ABSTRACT

Bisphenol A (BPA) has been widely used in the manufacture of polycarbonate plastic, water bottles and food containers. Previous studies have established that BPA could cause developmental toxicity by inhibiting the proliferation and differentiation of rat embryonic midbrain (MB) cells in vitro. However, the underlying mechanisms have not been well studied yet. In the current study, we examined the proliferation and differentiation of MB cells treated with 10^{-12}–10^{-4} M BPA and found that only 10^{-4} M BPA inhibited proliferation and differentiation. Then, we investigated the cell cycle progression and apoptosis of MB cells; 10^{-4} M BPA caused an explicit S phase and G2/M phase arrest in the cell cycle and increased the percentage of apoptotic cells. Moreover, the phosphorylation of mitogen-activated protein kinase (MAPK) and cyclic-AMP response binding protein (CREB) and the expression of some apoptotic regulatory genes were investigated. BPA (10^{-4} M) reduced the phosphorylation of C-Jun N-terminal kinase (JNK) and CREB, and increased the mRNA expression level of Bax and p53. Our findings demonstrated that BPA could cause developmental toxicity through anti-proliferation and pro-apoptosis in MB cells. Multiple signaling pathways, such as the JNK, CREB and p53-mitochondrial apoptosis pathways, participate in these effects.

1. Introduction

Bisphenol A (BPA) is a high production volume chemical used primarily in the manufacture of polycarbonate plastic, epoxy resins and dental sealants. Moreover, BPA can migrate into food and beverages from products made of polycarbonate plastic, such as baby bottles, tableware, food containers and water bottles (Vandenberg et al., 2007; NTP-CERHR, 2007). Therefore, human exposure to BPA is widespread. The U.S. Centers for Disease Control have estimated that 95% of Americans have detectable levels of BPA in their urine (Calafat et al., 2005). Additionally, BPA can be detected in the blood of pregnant women, amniotic fluid, placental tissue and umbilical cord blood, indicating some degree of fetal exposure (Engel et al., 2006; Padmanabhan et al., 2008; Tan and Mohd, 2003).

Many studies indicate that the developing fetus is more sensitive to xenobiotics than the adult (Vandenberg et al., 2007). Although there is no direct evidence that human exposure to BPA adversely affects development, studies with laboratory rodents show that exposure to BPA during pregnancy and/or lactation can reduce survival, birth weight, and growth of offspring early in life. In an extensive review by the National Toxicology Program (NTP) Center in the US, the developmental toxicity of BPA for fetuses, infants and children, especially the effects on the brain and behavior were deemed worthy of "some" concern (Chapin et al., 2008). Our previous studies indicated that BPA induced brain and neural tube abnormalities in rat embryos cultured in vitro (Xing et al., 2010). BPA could also inhibit the proliferation and differentiation of rat embryonic midbrain cells (Xiao et al., 2011). However, the underlying mechanism is still unknown.

During embryonic development, cells proliferate rapidly and are vulnerable to chemicals. In addition, apoptosis functions to clear abnormal or redundant cells in developing embryos (Hardy et al., 2003). Apoptosis plays an important role in embryogenesis; several studies have confirmed the function of apoptosis in normal embryonic development (Chan and Shiao, 2008; Lotz et al., 2006). However, excessive apoptosis caused by chemicals could lead to developmental defects (Chan, 2011).

Mitogen-activated protein kinase (MAPK) cascades have been shown to play a key role in the transduction of extracellular signals to cellular responses. In mammalian cells, three MAPK families have been clearly characterized: extracellular signal-regulated kinase (ERK), C-Jun N-terminal kinase (JNK), and p38 kinase. The MAPK families play important roles in complex cellular programs

Abbreviations: BPA, bisphenol A; MB, midbrain; NR, neutral red; MAPK, mitogen-activated protein kinase; JNK, C-Jun N-terminal kinase; ERK, extracellular signal-regulated kinase; CREB, cyclic-AMP response binding protein.

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such as proliferation, differentiation, development, transformation and apoptosis (Zhang and Liu, 2002). Many studies have indicated that the cyclic-AMP response binding protein (CREB) is upstream of Bcl-2 and that CREB phosphorylation could positively regulate the anti-apoptotic factor Bcl-2 and inhibit apoptosis (Lebesque et al., 2009; Kwak et al., 2008). In many cell types, the anti-oncogene p53 has the ability to activate transcription of various proapoptotic genes, including those encoding members of the Bcl-2 family such as Bax, Noxa and Puma, and finally regulate the induction of apoptosis (Amaral et al., 2010).

Therefore, we hypothesize that the developmental toxicity of BPA may be due to the inhibition of cell proliferation and promotion of apoptosis, and multiple signaling pathways may participate in the toxicity. In the present study, we employed micromass cultures of rat embryonic midbrain cells to investigate the effects of BPA on cell proliferation and apoptosis and then examined whether MAPK, CREB and p53 are involved in the effects of BPA.

2. Materials and methods

2.1. Chemicals and antibodies

BPA (97% pure) was obtained from Sigma (St. Louis, MO, USA). BPA was dissolved in dimethyl sulfoxide (DMSO; Amresco Company, Solon, Ohio, USA) and added to the culture medium. The final volume of DMSO in medium was 0.1%. Polyclonal rabbit anti-rat ERK1/2, ERK1/2 phosphorylation, JNK, JNK phosphorylation, p38, p38 phosphorylation, CREB, CREB phosphorylation antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). The β-actin antibody was purchased from Santa Cruz (Santa Cruz Biotechnology, CA, USA).

2.2. Animals

Primiparous female Sprague–Dawley rats and adult males from the same strain were supplied by Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China). Rats were housed in a barrier system (temperature: 20–26 °C; relative humidity: 40–70%) with a 12-h light/dark cycle, and given free access to food and water. The cages were made of polycarbonate with stainless steel wire lids and corncob granules as bedding. Timed-pregnant animals were acquired by making groups of one male and two females during the dark cycle. The day that sperm was detected in the vaginal smear was regarded as day 0 of gestation. All the procedures were approved by the Animal Care Committee of Peking University.

2.3. MB cell cultures

MB cells were cultured according to INVITTOX Protocols No.114 (INVITTOX Protocols No. 114). On day 13 of gestation, timed-pregnant rats were sacrificed by cervical dislocation after carbon dioxide narcosis. Embryos were removed from the uterus and transferred to sterile warm (37 °C) Hank's balanced salt solution. MB were isolated, washed three times and incubated in sterile warm (37 °C) calcium- and magnesium-free phosphate buffered saline (PBS) for 20 min. PBS was then replaced with 0.125% trypsin in PBS for 10 min at 37 °C, and trypsin activity was terminated by adding medium (Ham's F12 nutrient mixture: fetal bovine serum: L-glutamine: Pen/Strep: 88:10:1:1). Cells were dissociated by repetitive flushing through a pipette with a 200 μl tip. A single cell suspension was ensured by passing the suspension through a sterile stainless steel 200-mesh filter. Cells were counted in a hemocytometer and adjusted to 5×10^6 cells per ml. For the assessment of proliferation and foci differentiation, a 5 μl drop of the cell suspension was plated in the center of each well of a 96-well microplate. For the analysis of the cell cycle, western blot and real-time PCR, a 300 μl drop of the cell suspension was plated in the center of each well of a 6-well microplate. Then, the plates were placed in an incubator for 2–3 h. After that, 200 μl or 2 ml of medium with or without test chemicals was added into each well. The culture medium contained the following concentrations of BPA: 0, 10^{-12}, 10^{-10}, 10^{-8}, 10^{-6}, or 10^{-4} M. The concentrations of BPA were selected according to the previous study (Xiao et al., 2011) and the definition of low-dose for BPA effects ($\leqslant 1 \times 10^{-7}$ M) (Vandenberg et al., 2007; Wetherill et al., 2007). The cultures were again placed in an incubator for 5 days at 37 °C, with 5% CO_2 and 100% humidity.

2.4. Assessment of cell proliferation and differentiation

Cell proliferation was measured using the neutral red (NR) uptake bioassay. On day 5, the MB cell cultures were fixed with a 4.5% glutaraldehyde solution, rinsed with saline and stained with 0.05% NR in saline for 30 min at room temperature. Then, the cultures were rinsed with saline, and acetic acid–ethanol was added. The absorbance was measured at 540 nm after at least 2 h.

The MB cell cultures were fixed in 4% formaldehyde and stained using Harries hematoxylin. Images of the foci of differentiated cells were captured using a CCD Nikon DXM1200F linked to a Nikon TE2000S microscope. The total area (denoted area) of the foci was analyzed using the computer image analysis software Image Pro Plus 6.0 (Wilk et al., 2006; Wilk and Minta, 2008).

2.5. Flow cytometry analysis

For cell cycle analysis, after 5 days of exposure to BPA, the MB cells were harvested, washed twice with cold PBS, resuspended in 1 ml of DAPI (Beckman Coulter, USA) and incubated at room temperature in the dark for 10 min. The DNA content was then analyzed using a flow cytometer (Cell Lab Quanta SC, Beckman Coulter, USA). The distribution of cells in the cycle phases was represented as the percentages of G0/G1, S, and G2/M phases.

To further determine the presence of apoptosis, MB cells were stained by Annexin V and PI (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions after 5 days of exposure to BPA. Briefly, MB cells were harvested, washed twice with cold PBS and then resuspend in Binding Buffer containing 5 μl Annexin V-FITC and 5 μl PI. The cells were incubated for 15 min at room temperature in the dark then analyzed by flow cytometry within 1 h.

2.6. TUNEL staining

The MB cells were plated on the coverslips coated with poly-D-lysine (Sigma, St. Louis, MO, USA). After 5 days of exposure to BPA, the cells were fixed with 4% paraformaldehyde for 30 min, washed three times with PBS and stained with TUNEL (In Situ Cell Detection Kit, Fluorescein, Roche, Switzerland) following the manufacturer's instructions. The cells were then counterstained with Hoechst 33342 to assess the nuclear morphology. Images of TUNEL-positive cells were digitally captured using a microscope and a CCD camera.

2.7. Western blot analysis

On day 5, the culture medium was aspirated and the cells were quickly rinsed with cold PBS, Buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 5 mM NaF EDTA, 50 mM NaPPi, 1 μM PMSF, 1 mM DTT, 5 μg/ml leupeptin, 2 μg/ml aprotinin, and 1% NP-40 was added, and the cells were placed on ice for 30 min. The lysate were centrifuged at 13,000g for 30 min at 4 °C. The total protein concentration was determined using the BCA (Bio-Rad, Hercules, CA) assay. Samples (50 μg of total protein) were subjected to 10% SDS–PAGE and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech, Buckinghamshire, UK). After blocking with non-fat dry milk (5% w/v), the membrane was then incubated overnight with specific primary antibodies, followed by incubation for 2 h with secondary antibodies. Rabbit polyclonal antibodies against β-actin were used as the internal control. Then, the membranes were detected by chemiluminescence with the ECL-Plus kit (GE Health, USA). Each experiment was repeated three times.

2.8. Reverse transcription, quantitative polymerase chain reaction (RT-qPCR) analysis

Total RNA was extracted from the MB cells using Trizol reagent (Invitrogen, San Diego, CA) according to the product manual. First-strand cDNA was synthesized from 500 ng RNA, using a reverse-transcriptase kit (Takara, Dalian, Liaoning, China). Real-time PCR was performed as described in the SYBR Green PCR Master Mix protocol (Takara, Dalian, Liaoning, China). The primers used for real-time PCR are listed in Table 1. The relative expression of the genes was determined using the $2^{-\triangle\triangle Ct}$ method.

2.9. Statistics

All experiments were repeated at least three times. All values are expressed as the means ± standard deviation when appropriate. Statistical significance was determined by one-way ANOVA, with post hoc correction using the Tukey multiple comparison test. All statistical analyses were carried out using SPSS 13.0. Statistical significance was set at $\alpha = 0.05$.

3. Results

3.1. BPA inhibited the proliferation and differentiation of MB cells

Although 10^{-12}–10^{-6} M BPA showed no significant changes in cell proliferation and differentiation of MB cells, 10^{-4} M BPA decreased cell proliferation to 64%. In addition, the total area of foci containing neurocytes was reduced to 52% (Fig. 1). Microscopic images of micromass cultures are shown in Fig. 2.