Chemico-Biological Interactions 194 (2011) 23–30

Contents lists available at SciVerse ScienceDirect

Chemico-Biological Interactions

journal homepage: www.elsevier.com/locate/chembioint

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

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ARTICLE INFO

Article history:
Received 4 April 2011
Received in revised form 12 July 2011
Accepted 1 August 2011
Available online 19 August 2011

Keywords:
Bisphenol-A
PC12 cell
MAPK
ERK
Neurite
Nerve growth factor

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 (NFG)-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.

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 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-estradiol (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

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doi:10.1016/j.cbi.2011.08.001

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 ERβ. 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, E2, 17α-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 regent grade. BPA and E2 were dissolved in ethanol, and used as a vehicle.

2.2. Cell culture

PC12 cells in 25 and 75 cm^2 flasks (Nunc, USA) were maintained in a humidified incubator with 5% CO_2 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 17α-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 μg/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 h, 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 10–30 s with a type 250 Branson Sonifier (USA). The sonicated cells were centrifuged to divide the soluble and unsoluble fractions. The unsoluble fraction was dissolved using 0.1% Triton X-100. The obtained soluble and unsoluble fractions were used for the quantification of BPA contents. Usually in neurotoxicological research BPA dose to rats was employed as 0.5–5 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 0.001–50,000 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 h. The formazan dye formed was quantitated by measuring the absorbance at 450 nm.

2.6. 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 h. 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