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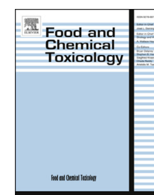

Di-*n*-butyl phthalate exposure negatively influences structural and functional neuroplasticity via Rho-GTPase signaling pathways

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ABSTRACT

Di-*n*-butyl phthalate (DBP) has been reported to cause disruptions in hippocampal plasticity, but its specific mechanism has not yet been ascertained. In this research, a mouse model of chronic DBP exposure was generated by intragastric administration of DBP (10, 50, or 250 mg/kg/d) for 5 weeks. Chronic exposure to high concentrations of DBP (250 mg/kg/d) induced a spatial learning deficit in the Morris water maze in male mice. By determining the activity of Rho-GTPase signaling pathways in the hippocampal tissues, we found that DBP exposure inhibited the activity of Rac1/PAK1/LIMK1 but activated RhoA/ROCK/LIMK2 signaling and eventually suppressed cofilin activity by phosphorylation. Consistent with this, the differential activation was also observed in the acute exposure model of neuronal cells generated by incubation with DBP (100 ng/ml, 1, 10, or 100 μg/ml) for 72 hours. Moreover, acute exposure to high concentrations of DBP (100 μg/ml) altered cell morphology by inhibiting neurite outgrowth. A ROCK inhibitor, but not inhibitors of Rac1 or PAK1, reversed the inhibition of DBP to the activity of cofilin and neurite outgrowth in cells. These findings provide the first evidence that DBP exposure results in impairment of neuroplasticity by differential regulation of Rho-GTPase signaling pathways.

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1. Introduction

Phthalic acid esters (PAEs) are the most widely used plasticizers with the largest production in the world (Kamrin, 2009). They can increase ductility, softness and plasticity of plastic for broad applications in cosmetics, children's toys, industrial dye, food packaging materials and medical products (Johns et al., 2015). Di-*n*-butyl phthalate (DBP) is a commonly used PAE. It connects polymers in plastic through hydrogen bonding and van der Waals forces and easily escapes into the human environment, including the food chain (Mariana et al., 2016). Guo *et al* measured the contents of nine PAEs in eight categories of food samples from two large cities in China and found that more than 94% of food contained DBP (Guo et al., 2012).

Although most of the PAEs in the body can be quickly metabolized, decomposed and discharged from the body through urine and feces, DBP is still likely to accumulate in the body and produce certain types of reproductive and developmental toxicity (Swan,

Abbreviations: ANOVA, analysis of variance; BBP, benzyl butyl phthalate; CREB, cyclic adenosine monophosphate response element binding protein; DBP, di-*n*-butyl phthalate; DiBP, diisobutyl phthalate; DEHP, di(2-ethylhexyl) phthalate; DMSO, dimethyl sulfoxide; DIV, days in vitro; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescent; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, Glutathione-S-transferase; LTP, long-term potentiation; LIMK, LIM kinase; MWM, Morris water maze; PAEs, phthalic acid esters; PAK, p21-activated kinase; PVDF, polyvinylidene fluoride; ROCK, Rho-associated protein kinase; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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2008; Lyche et al., 2009). A single exposure to DBP can significantly reduce sperm count, cause neural tube defects (Drake et al., 2009; Johnson et al., 2012) and induce testicular dysgenesis syndrome (van den Driesche et al., 2015). Recently, studies have demonstrated that DBP also shows neurotoxicity, affecting nervous system function (Miodovnik et al., 2014). For example, Li *et al* discovered reduced numbers of hippocampal neurons and abnormal synapse formation in immature offspring when pregnant rats were given 500 mg/kg body weight DBP via daily intragastric administration from gestational day 6 to postnatal day 21 (Li et al., 2013). They also found that DBP impaired the spatial learning and memory of offspring rats in the Morris water maze (MWM). In humans, the metabolites of DBP are reported to be relevant to rule-breaking behavior and externalization behavior of boys but to have no effect on girls (Kobrosly et al., 2014). The above studies indicate that DBP may cause morphological changes in neurons and thus produce long-term impairments in learning, memory, cognitive ability and other functions. However, the mechanism underlying the effect of DBP on neuroplasticity is unclear. Neuronal apoptosis caused by oxidative damage is insufficient to explain the decline in learning and memory in animals, especially the gender difference.

In recent years, it has been reported that, like morphological changes in neurons (structural plasticity), the functional alteration of learning and memory (functional plasticity) based on the mechanism of hippocampal long-term potentiation (LTP) cannot be separated from dynamic changes in the cytoskeleton (Rex et al., 2009), either. The dynamic balance of cytoskeletal actin can be adjusted by Rho-GTPase (Sit and Manser, 2011). The three main members of the Rho family of small G proteins, Rho, Rac and Cdc42, play specific roles in regulating axonal and dendritic morphology (Luo, 2000; Auer et al., 2011). Although the regulatory mechanisms of these three proteins can be much more complex, there is a relatively consistent view: RhoA is mainly involved in growth cone retraction with collapsing guidance cues (Jalink et al., 1994; Thies and Davenport, 2003), while Rac and Cdc42 can activate p21-activated kinase (PAK) (Manser et al., 1994; Burbelo et al., 1995) and its downstream effector LIM kinase (LIMK) (Edwards et al., 1999) and finally inhibit the actin-binding protein cofilin through phosphorylation to promote neurite outgrowth (Ridley et al., 1992; Nobes and Hall, 1995). It should be noted that activities of LIMK and cofilin are also mediated by RhoA and its effector Rho-associated protein kinase (ROCK).

To clarify whether exposure to DBP in the environment affects cytoskeletal reorganization through Rho-GTPase signaling pathways and then interferes with normal neuronal function, finally being reflected in learning and memory functions, in this research, a mouse model of chronic DBP exposure was generated via long-term intragastric administration. Neuronal function of the mice was evaluated using the Morris water maze. In addition, Neuro-2a cells and hippocampal neurons were acutely incubated with different concentrations of DBP, and morphological changes and the migration ability of the cells were observed. Further, activity of Rho-GTPase signaling pathways was evaluated as a possible mechanism through which DBP may affect neuroplasticity.

2. Materials and methods

2.1. Reagents

DBP was purchased from Sigma Chemical Co (catalogue no. 524980, purity ≥99.0%, St. Louis, MO, USA). Neuro-2a cells were obtained from the Shanghai Cell Resource Center. Primary antibodies for p-LIMK1/2 (catalogue no. 3841), LIMK1 (catalogue no. 3842), LIMK2 (catalogue no. 3844), p-cofilin (catalogue no. 3311), cofilin (catalogue no. 3318), ROCK1 (catalogue no. 4035) and Map-2 (catalogue no. 4542) were obtained from Cell Signaling Technology (Beverly, MA, USA). Antibodies for p-PAK1 (catalogue no. sc-393344) and PAK1 (catalogue no. sc-101773) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was obtained from Good Here (Hangzhou, Zhejiang, China). Antibodies for Rac1 (catalogue no. PA1-091) and RhoA (catalogue no. OSR00266W), as well as an active Rho pull-down and detection kit (catalogue no. 16116), were purchased from Thermo Scientific (Waltham, MA, USA). Goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibodies antibody (catalogue no. sc-2004) and goat anti-mouse HRP-conjugated secondary antibody (catalogue no. sc-2005) were obtained from Santa Cruz (Santa Cruz Biotechnology, Inc.) and Cy3-conjugated goat anti-rabbit secondary antibody (catalogue no. 111-165-003) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA, USA).

2.2. Animals and treatment

A total of eighty male and female ICR mice (22 ± 1.3 g body weight) were purchased from the Experimental Animal Center of Zhejiang University (Hangzhou, Zhejiang, China). All mice were housed, six animals per cage, under conditions of controlled temperature (20–23 °C), humidity (40–70%), and a 12 h light/dark cycle, with free access to food and water. All experiments were conducted in accordance with the institutional guidelines established by Zhejiang University for the care and use of laboratory animals. Animals were divided randomly into eight experimental groups of 10 animals each: (1) control group (male); (2) control group (female); (3) low-concentration DBP group (male); (4) low-concentration DBP group (female); (5) middle-concentration group (male); (6) middle-concentration group (female); (7) high-concentration group (male); and (8) high-concentration group (female). Mice in the three different concentration DBP groups received daily intragastric administration of DBP at 10, 50, 250 mg/kg body weight, respectively, for 5 weeks. The mixture was prepared fresh daily before administration by diluting DBP in sunflower cooking oil (Arowana, Shanghai, China). The dose selection was based on the work by Li *et al* (Li et al., 2013). Control mice were given sunflower cooking oil only (5 ml/kg body weight). Body weights were measured once a week. After 5 weeks, all mice were tested in the Morris water maze and then anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg) and sacrificed by decapitation.

2.3. Morris water maze (MWM)

After 5 weeks of DBP treatment, mice were tested in the MWM for 4 days. The MWM was used as previously described with slight modifications (Carey et al., 2014; Katayama et al., 2014). The water maze consists of a circular pool filled water with a hidden platform submerged 1 cm below the water surface. During the first phase of the test (acquisition), each mouse was given 90 s to escape from the water by finding the hidden platform, with a total of four trials per day. During the second phase (retention), the platform was removed, and mice were given 120 s to search for the platform. The swimming speed, the amount of time it took for a mouse to find the hidden platform (escape latency) during the acquisition phase, and the time spent in the target quadrant searching for the platform during the retention phase were recorded and analyzed using EthoVision XT tracking software (Noldus, Netherlands). Investigators who conducted Morris water test were blind to the treatment groups.

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