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Proteomic profile and toxicity pathway analysis in zebrafish embryos exposed to bisphenol A and di-*n*-butyl phthalate at environmentally relevant levels

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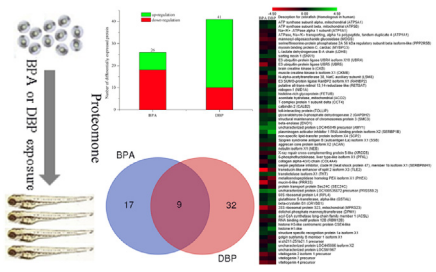
HIGHLIGHTS

- Low concentrations of BPA and DBP caused protein dysregulation in zebrafish embryos.
- BPA perturbs proteins to function in regulatory network of developmental disorder.
- DBP perturbs proteins to function in regulatory network of metabolic disorder.
- Low concentrations of BPA and DBP have potential health risks to zebrafish embryos.

GRAPHICAL ABSTRACT



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ABSTRACT

Bisphenol A (BPA) and di-*n*-butyl phthalate (DBP) are well-known endocrine-disrupting chemicals (EDCs) that have human health risks. Chronic exposure to BPA and DBP increases the occurrence of human disease. Despite the potential for exposure in embryonic development, the mechanism of action of BPA and DBP on vertebrate development and disease still remains unclear. In the present study, we identified proteins and protein networks that are perturbed by BPA and DBP during zebrafish (*Danio rerio*) development. Zebrafish embryos were exposed to environmentally relevant levels of BPA (10 μg/L) and DBP (50 μg/L) for 96 h. By iTRAQ labeling quantitative proteomics, a set of 26 and 41 differentially expressed proteins were identified in BPA- and DBP-treated zebrafish embryos, respectively. Integrated toxicity analysis predicted that these proteins function in common regulatory networks that are significantly associated with developmental and metabolic disorders. Exposure to low concentrations of BPA and DBP has potential health risks in zebrafish embryos. Our results also show that BPA and DBP significantly up-regulate the expression levels of multiple network proteins, providing valuable information about the molecular actions of BPA and DBP on the developmental systems.

1. Introduction

Endocrine-disrupting chemicals (EDCs) represent a global

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environmental health threat and are found in various materials such as additives, plastic products, and building materials or as contaminants in food and personal care products. A large number of studies have shown that EDCs exert their effects by interfering with endogenous hormone action. Exposure to EDCs, even at low levels, can lead to abnormal health outcomes, including male and female reproductive disorders, breast development and cancer, prostate cancer, neuroendocrine issues, thyroid problems, metabolism and obesity, and cardiovascular endocrine issues (Gore et al., 2015).

Bisphenol A (BPA) is a high-volume industrial chemical widely used in manufacturing polycarbonate plastics and epoxy resins, found in everything from plastic utensils to plastic containers, and even dental sealants. The Environmental Protection Agency (EPA) believes that BPA release to the environment exceeds 450 thousands kilograms per year (Bisphenol-A Action Plan, EPA, 2010, https://www.epa.gov/assessing-and-managing-chemicals-under-tsca/bisphenol-bpa-action-plan). Because BPA is a reproductive, developmental, and systemic toxicant in animal studies and also has hormone-like properties, mimicking estrogen (Rochester, 2013), there are questions about its potential impact on public and wildlife health. However, controversy still exists surrounding the risks of BPA contamination, and thus, BPA continues to be a topic of interest to the toxicology and regulatory communities.

Di-*n*-butyl phthalate (DBP) belongs to the class of phthalate esters (PAEs), which are widely used as plasticizers in polyvinyl chloride, polyvinyl acetates, cellulosics and polyurethanes (Parkerton and Konkel, 2000; Staples et al., 1997). Although DBP has been revealed to be a suspected endocrine disruptor and was banned from all use in many countries such as the United States and the European Union, it is still present in water at concentrations sufficient to affect development and reproduction in aquatic organisms. In China, DBP is widely distributed in various aquatic environments at μg/L to even mg/L or mg/kg. For example, the detected DBP concentrations ranged from 0.94 μg/L to 3.60 μg/L in the urban lakes of Guangzhou (Zeng et al., 2008), whereas the concentration of DBP in the Haihe River was estimated between 0.35 μg/L and 40.68 μg/L (Chi, 2009). In Yangtze River, DBP was detected at the highest level of 37.31 μg/L (Wang et al., 2008). Furthermore, increasing evidence indicates that PAEs, especially DBP in the surface water, exhibit strong thyroid hormone activities (Shi et al., 2012) and endocrine disrupting effects (Shi et al., 2009). Therefore, it is of particular importance to evaluate the effects of DBP pollution in aquatic environments and its threat to aquatic organisms and human health.

During particular periods in development, the embryo or the fetus is susceptible to the adverse effects of chemicals. Exposure to environmental chemicals in early life stages can increase the risk of adverse health outcomes later in life in the absence of apparent changes in normal development (Gore et al., 2015; Schug et al., 2011). The developmental origins of health and disease (DOHaD) paradigm provides a framework to assess the effect of environmental chemicals, including EDCs, on long-term health. In mammal studies, perinatal/prenatal exposure to BPA or DBP has been related to the development of obesity, type 2 diabetes mellitus and reproductive problems in offspring (Alonso-Magdalena et al., 2015; Perera and Herbstman, 2011). For example, metabolomics and epigenetic approaches revealed that early-life exposure to DBP significantly increased the level of betaine but decreased the expression of betaine homocysteine S-methyltransferase (BHMT) in SD rat F1 through F3 generation, resulted in transgenerational spermatogenesis failure (Yuan et al., 2017). In zebrafish studies, metabolomics and transcriptomic responses indicated that a specific metabolic disruption by BPA affecting different signaling pathways, such as retinoid and prostaglandin metabolism, in zebrafish embryos (Ortiz-Villanueva et al., 2017). However, the mechanism of the correlations between health and early-life exposure to BPA and DBP is still needed to explore.

Owing to sharing orthologous genes with human, zebrafish (*Danio rerio*) can be considered an attractive model for studying the connection between environmental exposure and human disease (Lieschke and Currie, 2007). In the present study, we aimed to identify proteins and networks targeted by environmentally relevant concentrations of BPA and DBP during vertebrate embryonic development. We evaluated the effects of BPA and DBP on protein changes in zebrafish by proteome analysis and enrichment and pathway analyses. Specifically, we describe the effects of BPA and DBP at the protein level and predict a cohort of protein changes in zebrafish embryos that affect multiple disease functions.

2. Materials and methods

2.1. Test chemicals

BPA (CAS number: 80-05-7, ≥99%) and DBP (CAS number: 84-74-2, 99%) were purchased from Sigma-Aldrich. They were dissolved in dimethyl sulfoxide (DMSO) to obtain stock solutions of 10 g/L each and stored at 4 °C.

2.2. Animals and exposure

The wild-type zebrafish (AB strain) stock used in this study originated from the China Zebrafish Resource Center (Wuhan, China). Adult zebrafish were cultured in a flow-through system in dechlorinated tap water at 28 ± 0.5 °C in a 14 h light: 10 h dark cycle and fed with brine shrimp (*Artemia nauplii*) twice daily in our laboratory. The dissolved oxygen in aquarium water was between 6.5 mg/L and 7.6 mg/L; pH was between 7.2 and 7.4. At 4 h post-fertilization (hpf), embryos were examined under a stereomicroscope, and normal embryos that had reached the blastula stage were selected for subsequent experiments. The experiments were conducted following the National Institute of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011) and were approved by the Animal Care and Use Committee of Jiangsu University (Zhenjiang, China).

Based on our previous work (Xu et al., 2013, 2015) and environmentally relevant concentrations of BPA and DBP (Chi, 2009; Kang et al., 2007), ~300 zebrafish embryos (4 hpf) were randomly distributed into a dish (16 cm in diameter, 5 cm high) as a group and were exposed to nominal 10 μg/L BPA or 50 μg/L DBP (500 mL each) until 96 hpf in triplicate at each treatment condition. The solvent control group received 0.005% DMSO (v/v). All tested embryos were cultured at 28 ± 0.5 °C in a 14 h light: 10 h dark cycle, and fresh solutions were replaced by 50% every 12 h.

2.3. Quantitative proteomic analysis

Quantitative proteomic analysis was performed according to our previous study (Wu et al., 2017b). iTRAQ 8-plex Kit (AB SCIEX) was used for labeling the peptide. There were two or three replicates for each treatments and each replicate included 200 zebrafish larvae. ProteinPilot™ 4.0 software (AB SCIEX) was used for relative quantification of proteins, and differentially expressed proteins were considered if proteins with a statistically significant label ratio of >1.5 (up-regulation, $p < 0.05$) or < 0.67 (down-regulation, $p < 0.05$). Zebrafish SwissProt database (IPI_DANRE_v3.86 database, 40470 sequences) was used for proteins identification.