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Toxicogenomic analysis in the combined effect of tributyltin and benzo[*a*]pyrene on the development of zebrafish embryos

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ABSTRACT

There is a growing recognition that the toxic effects of chemical mixtures are been an important issue in toxicological sciences. Tributyltin (TBT) and benzo[*a*]pyrene (BaP) are widespread pollutants that occur simultaneously in the aquatic environments. This study was designed to examine comprehensively the combined effects of TBT and BaP on zebrafish (*Danio rerio*) embryos using toxicogenomic approach combined with biochemical detection and morphological analysis, and tried to gain insight into the mechanisms underlying the combined effects of TBT and BaP. The results of toxicogenomic data indicated that: (1) TBT cotreatment rescued the embryos from decreased hatching ratio caused by BaP alone, while the alteration of gene expression (in this article the phrase gene expression is used as a synonym to gene transcription, although in is acknowledged that gene expression can also be regulated by, e.g., translation and mRNA or protein stability) relative to zebrafish hatching in the BaP groups was resumed by the cotreatment with TBT; (2) BaP cotreatment decreased TBT-mediated dorsal curvature, and alleviated the perturbation of Notch pathway caused by TBT alone; (3) cotreatment with TBT decreased BaP-mediated bradycardia, which might be due to that TBT cotreatment alleviated the perturbation in expression of genes related to cardiac muscle cell development and calcium handling caused by BaP alone; 4) TBT cotreatment brought an antagonistic effect on the BaP-mediated oxidative stress and DNA damage. These results suggested that toxicogenomic approach was available for analyzing combined toxicity with high sensitivity and accuracy, which might improve our understanding and predictability for the combined effects of chemicals.

1. Introduction

The combined effects of exposure to chemical mixtures are a longstanding research topic in toxicological sciences and environmental risk assessment (Schwarzenbach et al., 2006), since the presence of contaminant mixtures is inevitably found in the environment. Although scientists generally have a good understanding about the toxicity of individual chemical pollutants and toxicity data are often available, reliable and relevant data on interactions are lacking for most chemical combinations (Borgert et al., 2001; Hertzberg and Teuschler, 2002). There is a great need to bridge the gap between our understanding of the toxic effects of exposure to individual xenobiotics and the effects from exposure to chemical mixtures (Olmstead and LeBlanc, 2005). There have been a series of studies in environmental toxicology addressing various scientific questions in mixture toxicology (Yang et al., 2004; Kortenkamp, 2007; Mumatz et al., 2011). Importantly, research should be undertaken to develop feasible methods for determining the toxic effects of exposure to chemical mixtures.

Toxicogenomics provides a tool to improve our understanding and predictability of combined effects (Altenburger et al., 2012), which allows observing the interplay between impacted environmental conditions and dynamic responses of organisms on genes. Toxicogenomic techniques are multivariate and nontargeted with respect to gene transcript. The potential for toxicogenomic methods to provide knowledge of modes of action of chemicals has been anticipated (Ankley et al., 2006). There is a hope that toxicogenomic methods will lead to an improved selection of end points suitable for specific risk assessment (Altenburger et al., 2012). The perspective of the 'omics' techniques to provide tools for advanced biomarkers have gained experimental support (Yang et al., 2007; Hook et al., 2010). For example, the applications of toxicogenomic approaches for understanding the combined effects of environmental mixtures, with respect to toxicokinetic and toxicodynamic

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processes, have been summarized (Spurgeon et al., 2010). The investigation of the effects of mixture exposure in organisms using novel toxicogenomic methodology has become a major research activity (Altenburger et al., 2012), and microarray technology is currently evolved as the most widely applied transcriptome analysis tool in ecotoxicology. In this study, we performed a microarray analysis to investigate the combined effects of pollutants as well as their underlying toxicological mechanisms by global transcriptional patterns using tributyltin (TBT) and benzo[*a*]pyrene (BaP) as targeted chemicals.

TBT and BaP are widespread pollutants that occur simultaneously in many aquatic environments. TBT is one of the organotin compounds which have been worldwide used in agriculture and industry as biocides, heat stabilizers and chemical catalysators. BaP is a high molecular weight (5-ring) polycyclic aromatic hydrocarbon (PAH) which is present worldwide due to anthropogenic activities such as vehicle exhausts, oil shipping and refining. Some researches on adult fishes have been performed to elucidate the toxicity of their mixture and the interactions between them. TBT inhibits cytochrome P450 1A isozyme and the metabolism and bioactivation of co-administered BaP in brook trout (*Salvelinus fontinalis*); BaP, in turn, stimulates TBT metabolism (Padrós and Pelletier, 2000). TBT cotreatment inhibits the BaP-mediated induction of hepatic glutathione S-transferase (GST) activity while BaP cotreatment inhibits the TBT-mediated decreases in GST activity and glutathione content (Padrós et al., 2003). Cotreatment with BaP causes a significant reduction of TBT-mediated malondialdehyde contents elevation; Cotreatment with TBT decreases BaP-mediated glutathione peroxidase (GPx) activity induction; Cotreatment with TBT and BaP does not significantly alter the reduced glutathione levels caused by TBT or BaP alone (Wang et al., 2006). However, only one paper reports the histological evidence of antagonistic effects of TBT on BaP toxicity in the Arctic charr (*Salvelinus alpinus*) (Ribeiro et al., 2007). The combined effects of TBT and BaP, and the molecular mechanisms involved have not been inextenso investigated.

Zebrafish embryo-larval assay is a good model for toxic effects research, since the transparency of the chorion enables direct and non-invasive observations during embryogenesis. The development of fish can involve all organic effects and is in favor of mechanism assay using global transcriptional patterns. The aims of this study were: (1) to observe the combined effects of TBT and BaP on zebrafish embryos and investigate the transcriptional mechanisms involved with microarray; (2) to assess the validity of genomics approach for analyzing toxic effects of chemical mixtures.

2. Materials and methods

2.1. Chemicals

BaP (>99% purity) was purchased from Sigma-Aldrich (St. Louis, MO, USA), TBT chloride (>97% purity) was obtained from Fluka AG, Switzerland. They were dissolved in DMSO (>99% purity) to reach stock concentrations of 500 mg/L or 10 μg/L, respectively. All other chemicals were of analytical grade and were obtained from commercial sources.

2.2. Zebrafish embryo exposure

Wild-type TU zebrafish were maintained using routine procedures (Westerfield, 2000). Collection of fertilized eggs, and exposure were all performed in zebrafish medium (3.5 g/L NaCl, 0.05 g/L NaHCO3, 0.05 g/L KCl, 0.05 g/L $CaCl_2$). Embryos between 0 and 0.5 hours post-fertilization (hpf) were exposed to a series of degressive concentrations with a ratio of 10, from 20, 2, 0.2, 0.02, 0.002 to 0.0002 μM of BaP. Median lethal concentrations (LC_{50}) were estimated using the Bliss method (Bliss, 1938). The 95% confidence limits (UCL, LCL) of the LC_{50} values were determined according to a previous report (Sokal and Rohlf, 1995). The (24-h) LC_{50} value for BaP was 26.4 μM. The 95% confidence limits (UCL, LCL) of the LC_{50} values were 21.7–32.1 μM.

Embryos between 0 and 0.5 hpf were exposed to 0.03 nM (10 ng/L) TBT, 2 μM (500 μg/L) BaP, or 0.03 nM TBT + 2 μM BaP. The exposures were carried out in glass petri dishes, and 100 embryos were cultured in 30 mL solution in each petri dish. There were nine replicates for each of the three treatments. The solutions were changed twice daily. Similar criteria were applied to the control group, which received an equal volume of the DMSO solvent (1 μL/L). Embryos were microscopically evaluated with a Nikon TE300 microscope at 72 hpf to quantify the teratogenicity according to another study (Miura et al., 2013). Pericardial edema is defined as any abnormal separation in the pericardium, a portion of coelomic cavity that is mesodermally lined and separates visceral organs such as the heart from the body wall (Westerfield, 2000). Mortality was examined throughout the exposure. Embryos were collected at 72 hpf for BaP detection, total tin analysis, enzyme assays, RNA extraction and later experiments.

2.3. BaP detection in embryos

Zebrafish embryos at 72 hpf were homogenized, and the surrogates d_{10}-perylene were spiked separately into the whole-body homogenate samples and kept at 4 °C overnight for equilibrium before sample extraction. BaP was extracted from the samples using dichloromethane. Clean up of the extracts was performed on 6% water deactivated aluminum oxide columns with hexane as the eluting solvent. Internal standards d_{10}-pyrene for analysis of BaP was added. The processed samples were analyzed for BaP using an Agilent 6890 gas chromatograph linked with an Agilent 5975B mass spectrometer (GC/MS) (Agilent Technologies, Palo Alto, CA, USA), in selected ion monitoring mode. BaP standards (50, 100, 200, and 400 ppb) were prepared in hexane and used to develop a calibration curve, with good to excellent linearity ($r^2 > 0.99$). BaP was detected as an ion with a molecular weight of 252 (Patel et al., 2006). A method blank and a lab blank spike/lab blank spike duplicate were added to ensure that the samples and the analysis process were free of contamination.

2.4. Total tin analysis

The analytical procedure for total tin was based on the method described by Le et al. (1999) with slight modification. Briefly, 10 embryos from each replicate group of each treatment were dried at 80 °C for 12 h and then digested with purified HNO_3 in a Mars5 microwave oven (CEM, Matthews, NC, USA) at 200 W. After digestion, the sample was transferred into a 50 mL measuring flask and diluted with Milli-Q water. Tin concentrations in samples were determined using inductively coupled plasma mass spectrometry (PerkinElmer, ELAN DRC-e, USA). There were three replicates for each treatment ($n = 3$). A method blank and a lab blank spike/lab blank spike duplicate were added to ensure that the samples and the analysis process were free of contamination. The detection limit of tin was 0.0115 ppb.

2.5. RNA extraction and reverse transcription

Total RNA was extracted from the whole embryos using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. First-strand cDNA was synthesized from 2 mg of total RNA using