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Use of toxicogenomics to predict the potential toxic effect of Benzo(a)pyrene on zebrafish embryos: Ocular developmental toxicity

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HIGHLIGHTS

- Zebrafish embryos were exposed to BaP. Differentially expressed genes were identified using microarray analysis.
- Perturbed genes relative to eye development and visual perception were found by ontology assignments and functional cluster.
- The ocular toxicity of BaP was demonstrated by phototactic response and histological analysis.
- Toxicogenomics is effectual for predicting potential toxicity of chemicals with high sensitivity and accuracy.

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ABSTRACT

Benzo(a)pyrene (BaP) is a representative polycyclic aromatic hydrocarbon (PAH), which is ubiquitous in the environment. The toxic effects of BaP on fish embryos have been described in detail, but some potentially toxic effects of BaP might have been neglected owing to the limitations of traditional techniques. In the present research, global transcriptional patterns were used to study the potentially toxic effects of BaP, as well as its underlying toxicological mechanisms. The expression levels of multiple genes were significantly changed by BaP exposure. The results of ontology assignments and cluster analysis showed that BaP could affect the processes of photoreceptor maintenance and phototransduction. We also conducted an experiment on phototactic response and found that larvae exposed to BaP displayed a decreasing response to light. In addition, BaP exposure decreased the cellular density of the ganglion cell layer (GCL) significantly. These results suggested that BaP exposure induced visual system developmental defects and dysfunction by perturbation of photoreceptor development related genes. Our results were helpful for an understanding of the toxicity of BaP. This study also indicated that microarray analysis was effective for predicting the potential toxicity of chemicals with high sensitivity and accuracy.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental contaminants that primarily originate from incomplete combustion such as the burning of fossil fuels, oil, wood, and a wide variety of other anthropogenic sources including municipal waste, industrial effluents, petroleum spills, creosote oil, automobile exhaust, and coal tar production (Fetzer, 2000). The increases in PAHs are associated with automobile use and urban sprawl (Van et al., 2000). There is a long history of research into the carcinogenicity of PAHs, but the teratogenic effects of PAHs were studied only in the recent 20 years. PAHs can influence the development of many organ systems such as the skeleton (Incardona et al., 2005, 2006), cardiac (Incardona et al., 2004), nerve (He et al., 2012a) and thyroid system (He et al., 2012b). However, because developmental defects research is sometimes limited by difficulties in detection using traditional techniques (Ritz, 2010), some potentially toxic effects of PAHs might have been neglected.

Benzo(a)pyrene (BaP), a representative PAH, is commonly found in cigarette smoke, grilled and broiled foods, and as a by-product of many industrial processes (Lee and Shim, 2007). The concentration of BaP in the near shore sea water environment of Mumbai after a large scale oil spill is 20 μg mL^{-1} (79.37 μM) (Ladwani et al., 2013). In the surface water, concentrations of BaP range from 1.0 to 23.4 ng L^{-1} (about 0.004–0.09 nM) in Maluan Bay in Xiamen, China

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(Tian et al., 2004), and reaches 0.56–3.32 μg L^{-1} (about 0.002–0.01 μM) in the Jiulong River Estuary and Western Xiamen Sea (Maskaoui et al., 2002). The level of BaP in sediment taken from Bayou Bonfouca, Louisiana reaches 610 mg g^{-1} (Catallo and Gambrell, 1987). The developmental toxic effects of BaP on fish embryos have been described in detail. Gross malformations resulting from BaP exposure include jaw reductions, skeletal defects (He et al., 2011), pericardial and yolk sac edema and cardiac dysfunction (Huang et al., 2012). However, whether there are any other developmental toxic effects of BaP is still unclear.

To predict, and thus diminish or prevent the harmful effects of pollutants on the environment is the overarching goal in ecotoxicology (Schirmer et al., 2010). Traditional techniques fall short of predicting new toxicity because of their overdependence on phenotype observation. Microarray technology has currently evolved as the most widely applied transcriptome analysis tool in ecotoxicology. In the present study, zebrafish embryos were exposed to BaP at concentrations of 0.02, 0.2 and 2 μM. These concentrations are within the range commonly used in laboratory experiments when testing the effects of PAHs using BaP as a model compound (Canova et al., 1998; Skarphéðinsdóttir et al., 2003; Prevodnik et al., 2007). Then, we tried to study the potentially toxic effects of BaP, as well as its underlying toxicological mechanisms using global transcriptional patterns.

2. Materials and methods

2.1. Chemicals

BaP (>99% purity) was purchased from Sigma–Aldrich (St. Louis, MO, USA). It was dissolved in dimethylsulfoxide [DMSO (>99% purity)] to reach stock concentrations of 20, 200 and 2000 μM. 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 BaP exposure were all performed in zebrafish medium (3.5 g L^{-1} NaCl, 0.05 g L^{-1} NaHCO3, 0.05 g L^{-1} KCl, 0.05 g L^{-1} $CaCl_2$). Embryos between 0 and 0.5 h post-fertilization (hpf) were exposed to BaP at concentrations of 0.02, 0.2 and 2 μM. The exposures were carried out in glass petri dishes, and 100 embryos were cultured in 30 mL BaP solution in each petri dish. There were six replicates for each of the four treatments. The BaP 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^{-1}). Parts of the embryos were collected at 72 hpf for histological analysis, RNA extraction and later experiments, while other embryos were cultured to 7 dpf for the phototaxis test.

2.3. BaP detection in embryos

BaP was extracted from embryos at 72 hpf using dichloromethane. Clean up of the extracts was performed on 6% water deactivated aluminum oxide columns with hexane as the eluting solvent. 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. BaP was detected as an ion with a molecular weight of 252 (Patel et al., 2006).

2.4. 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 a Revert Aid Mu-MLV cDNA synthesis kit following the manufacturer's protocol.

2.5. Microarray analysis

Microarray analysis was performed to evaluate the effects of BaP exposure on global transcription. Samples for microarray analysis were independently collected from three petri dishes of the control or 2 μM BaP exposures (50 embryos per petri dish). The total RNA was extracted and sent to Capital-Bio (Beijing, China), where microarray analyses were performed on Affymetrix GeneChip Zebrafish Genome arrays consisting of 14900 transcripts, for a total of six independent arrays: three controls, and three 2 μM BaP exposures. Affymetrix data files [cell intensity (CEL) files] were created from each array using GeneChip Operating Software (Affymetrix).

2.6. Analysis of microarray data

A statistical process similar to the one Gosse et al. (2008) report was implemented. Probe-level data from CEL files were normalized using robust multiarray analysis (Irizarry et al., 2003) as implemented in Bioconductor. Quality control was performed using log-ratio versus log-product (MA) plots and volcano plots (data not shown). Probes with a substantial likelihood of differential expression under treatment conditions were identified using simple *t*-tests combined with mean fold change in accordance with recommendations from the Microarray Quality Control Consortium (Guo et al. 2006; Shi et al. 2006). We selected a *t*-test *p*-value threshold of 0.1 and a minimum absolute fold difference of 2.0 between the controls and exposed data sets. Genes yielded from this approach were then used in pathway analysis.

2.7. Molecular pathway and gene ontology analysis

To identify the molecular pathways and biological processes affected, we computationally analyzed BaP-responsive genes identified using microarray analysis. Gene ontology assignments (Ashburner et al., 2000) and clustering into functional groups were performed using DAVID (Database for Annotation, Visualization, and Integrated Discovery) (Dennis et al., 2003). The results of such ontology assignments and clustering might indicate the toxicity of the BaP. Then, MAS 3.0 was used to build gene networks related to the toxic effect, using a threshold (*p*-value) of 0.1.

2.8. Real-time quantitative PCR

QPCR was performed to validate the results of microarray analysis. Twenty embryos from each replicate of the control and BaP treatments (0.02, 0.2, 2 μM) were collected and RNA was isolated as described above ($n = 6$). QPCR analysis was performed on a Rotor-gene 6000 Real-Time PCR system (ABI, USA) using SYBR green I fluorescent dye. The reactions were performed in a 10 μL volume mix containing 0.2 μL SYBR Green I, 5 pmol L^{-1} specific primers and approximately 50 ng cDNA. The cycling parameters were 95 °C for 10 min, followed by 45 cycles of 95 °C for 20 s, 55 °C for 20 s, and 72 °C for 20 s. Threshold cycles and dissociation curves were determined with Rotor-gene 6000 software, to confirm that only one PCR product was amplified and detected. Genorm and Bestkeeper analyses were performed to elect a proper normalizing gene, and our results suggested that BaP exposure altered the