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Toxic responses of *Perna viridis* hepatopancreas exposed to DDT, benzo(a) pyrene and their mixture uncovered by iTRAQ-based proteomics and NMR-based metabolomics

Qinqin Song[a,b,c], Hailong Zhou[a,b,*], Qian Han[a,b], Xiaoping Diao[a,b]

[a] State Key Laboratory of Marine Resource Utilization in South China Sea, Hainan University, Haikou 570228, China
[b] Institute of Tropical Agriculture and Forestry, Hainan University, Haikou 570228, China
[c] Key Laboratory of Coastal Zone Environment Processes, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China

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ABSTRACT

Dichlorodiphenyltrichloroethane (DDT) and benzo(a)pyrene (BaP) are environmental estrogens (EEs) that are ubiquitous in the marine environment. In the present study, we integrated isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic and nuclear magnetic resonance (NMR)-based metabolomic approaches to explore the toxic responses of green mussel hepatopancreas exposed to DDT (10 μg/L), BaP (10 μg/L) and their mixture. The metabolic responses indicated that BaP primarily disturbed energy metabolism and osmotic regulation in the hepatopancreas of the male green mussel *P. viridis*. Both DDT and the mixture of DDT and BaP perturbed the energy metabolism and osmotic regulation in *P. viridis*. The proteomic responses revealed that BaP affected the proteins involved in energy metabolism, material transformation, cytoskeleton, stress responses, reproduction and development in green mussels. DDT exposure could change the proteins involved in primary metabolism, stress responses, cytoskeleton and signal transduction. However, the mixture of DDT and BaP altered proteins associated with material and energy metabolism, stress responses, signal transduction, reproduction and development, cytoskeleton and apoptosis. This study showed that iTRAQ-based proteomic and NMR-based metabolomic approaches could effectively elucidate the essential molecular mechanism of disturbances in hepatopancreas function of green mussels exposed to environmental estrogens.

1. Introduction

Marine environmental contaminants are very complicated and include polycyclic aromatic hydrocarbons (PAHs), pesticides, benzo(a) pyrene (BaP) and dichlorodiphenyltrichloroethane (DDT), which derive from different sources and accumulate in the tropical marine ecosystem (Bussolaro et al., 2012; da Silva Rabitto et al., 2011; Miranda et al., 2008). For BaP and DDT, some areas in the China Sea have reached serious degrees of pollution (Hu et al., 2009), and the results of Yang et al. (2008) showed no apparent decrease in DDT concentration in the sea water of the China Sea over the past ten years (Yang et al., 2008). Moreover, profiles in mangrove sediments showed a continuing local scale increase in ΣPAH (29–438 ng g^{-1}) and ΣDDT (0.2–41.0 ng g^{-1}) (Kaiser et al., 2016). Because of their hydrophobic characteristics, persistent organic pollutants (POPs) are widespread and have highest biomagnification in the fat tissue among the tissues of marine animals (De Wit et al., 2010) such as Atlantic bluefin tuna (Maisano et al., 2016). Additionally, the level of free sex hormones was lower in BaP-exposed than in control marine fish *Sebastiscus marmoratus* (Zheng et al., 2006). In 2006, the Council of the European Union asked the academic circles to consider exposures to chemical cocktails in risk assessment.

This study focused on the effects of DDT, BaP and the mixture of DDT and BaP on the hepatopancreas of *Perna viridis*. Both DDT and BaP are environmental estrogens (EEs). BaP is a typical PAH that is highly lipophilic and commonly detected in the marine environment. It is also a confirmed reproductive and developmental toxicant to wildlife (Corrales et al., 2014), reducing gonad weights, damaging ovarian follicles, and leading to infertility (Mohamed et al., 2010), as well as delaying gametogenesis and progesterone levels in soft-shell clams (Siah et al., 2003). It is produced in the partial combustion of various organic matters, and the International Agency for Research on Cancer (IARC) has classified it as a human carcinogenic substance (Macor and Pavanello, 2009). DDT is a well-known pesticide and as persistent and harmful organochlorine mixture has negative effects on reproductive development via disruption of multiple endocrine pathways (Holm

* Corresponding author at: Institute of Tropical Agriculture and Forestry, Hainan University, 58 Renmin Road, Haikou 570228, Hainan, China.
E-mail address: zhouhl@hainu.edu.cn (H. Zhou).

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et al., 2006), resulting in cryptorchidism, low infant birth weight, and decreased sperm motility and semen quality in males (Bhatia et al., 2005). Skinner et al. (2013) reported that DDT exposure could promote obesity and associated diseases transgenerationally. Valvi et al. (2012) found that organochlorine compounds, including DDT, might influence obesity in males and that sex and high-fat intake may influence the susceptibility to organochlorine effects.

Tropical marine ecosystems contain an abundant biodiversity of organisms and complicated physical and chemical factors that can impact the bioeffects of various pollutants, making it difficult to evaluate the actual toxic effects of compounds. To obtain basic comprehension of this intricate condition, it is essential to study the toxic responses of marine animals, such as shellfish exposed to mixtures of pollutants (Oliveira et al., 2015). *P. viridis* is a suitable indicator organism for detecting pollution of the marine ecosystem, since it is filter feeding, benthic and sessile organism. It is also quite tolerant to pollutants compared to many other animals (Campos et al., 2012; Wu and Wang, 2011). Additionally, the hepatopancreas is easily affected by many kinds of toxicants (Atli and Grosell, 2016). Thus, in the present study, the hepatopancreas of *P. viridis* was selected as the target tissue to uncover the toxic mechanism of DDT, BaP and their mixture.

Through the emergence and development of systems biology "-omic" methods (Suárez-Ulloa et al., 2013), we can discover the molecular mechanism of EEs from the levels of DNA, RNA, protein and metabolites and screen effective biomarkers at the molecular level (Ji et al., 2013a, 2013c; Wu and Wang, 2011). These methods include iTRAQ, which can quantify differential expression of proteins from several samples in one experiment utilizing isotope-coded covalent tags without gel (Pütz et al., 2012). Because of its high repeatability and sensitivity, iTRAQ has been widely applied in quantitative protein expression research (Han et al., 2013; Martyniuk et al., 2012). Metabolomics approaches generally focus on the global small molecular metabolites in diverse biological processes (Brandao et al., 2015; Cappello et al., 2017a,b, 2015, 2016; Lindon et al., 1999; Liu et al., 2011a; Maisano et al., 2017; Wu et al., 2013a). It can discover toxic responses provoked by EEs via the comparative metabolic profile of marine animals (Cappello et al., 2017a; Ji et al., 2014a; Zhang et al., 2011a,b). A combined proteomics and metabolomics method could reveal the disturbance of proteins and metabolites involved in the same metabolic pathway, which can help to give a better understanding of the molecular mechanism of EE effects in marine bivalves (Ji et al., 2013c; Marrocco et al., 2011). Therefore, we investigated the toxic responses of hepatopancreas in male *P. viridis* exposed to DDT, BaP and their mixture using a combined approach of iTRAQ-based proteomics and nuclear magnetic resonance (NMR)-based metabolomics. NMR data are highly reproducible and quantitative over a wide dynamic range and are unmatched in determining unknown structures; NMR is also non-destructive and can be utilized in vivo (Markley et al., 2017). Thus, NMR-based metabolomics is a very powerful tool for elucidating the toxic responses of *Perna viridis* hepatopancreas exposed to DDT, BaP, and their mixture.

2. Materials and methods

2.1. Animals

Healthy adult green mussels (n = 200, shell length: 6.0–7.0 cm) were obained from a shellfish culture bed in Li'an Harbor (Hainan, China) in March 2014. Green mussels were acclimatized for one week in tanks containing natural seawater (temperature, 25 ± 2 °C; salinity, 32‰; pH, 8.2 ± 0.1). After one week, they were randomly separated into five groups (DDT, BaP, the mixture of DDT and BaP groups, sea water control and the solvent (0.005% dimethyl sulfoxide (DMSO)) control), each including 40 green mussels. The contaminant concentrations were 10 μg/L DDT, 10 μg/L BaP, also in the mixture of DDT and BaP. The contaminant treatments also contained 0.005% DMSO for dissolving DDT and BaP. The treatments lasted for seven days. The used levels of DDT and BaP were based on our pre-experiments and related studies (Maria et al., 2013). Through the periods of acclimation and exposure, *P. viridis* were fed moderate amounts of *Tetraselmis Chui*. After the 7-day exposures, the gender of each individual was determined by microscopic examination of a tiny amount (1–2 rag) of gonadal tissue based on the gamete size and shape (Lobel et al., 1991). The morphology of the egg is round, the sperm is slim, and the egg is much bigger than the sperm. Then, twelve male *P. viridis* were randomly chosen from each group for sampling. The hepatopancreas was excised and immersed quickly in liquid nitrogen. These samples were preserved at −80 °C before further processing. Because there was no striking difference of proteins between the DMSO and seawater groups in our earlier studies (Song et al., 2016a,b), the seawater control group was not included in iTRAQ-based proteomics. For iTRAQ-based proteomics, each group consisted of two biological replicates, and each replicate was pooled from three green mussels.

2.2. Metabolomic analysis

2.2.1. Extracting metabolites

The metabolite extraction protocol for hepatopancreas was modified from a previously reported method (Fasulo et al., 2012; Wu et al., 2005a). Briefly, after sample homogenization and metabolite extraction, the polar metabolites were transferred to a glass vial, and dried in a centrifugal concentrator. Then, the hepatopancreas extracts were re-suspended in phosphate buffer. The mixture was vortexed and centrifuged (4 °C for 5 min at 3000 g). Finally, the clear supernatant (550 μL) was pipetted into one 5-mm NMR tube before being analyzed.

2.2.2. Analyzing ^{1}H NMR spectroscopy and multivariate data

The hepatopancreas metabolites of male *P. viridis* were resolved with a Bruker NMR spectrometer (Liao et al., 2007; Zhang et al., 2011a), and analysis was performed at 500.18 MHz (25 °C) (Liu et al., 2011b). All ^{1}H NMR spectra were phased, baseline-amended and standardized manually (internal reference: 3-(trimethylsily1)- l-propane-sulfonic acid (TSP) at 0.0 ppm) with TopSpin (version 2.1). The Chenomx Suite was applied to identify the metabolites. Each ^{1}H NMR spectrum was separated into 0.005 ppm bins from 0.2 to 10.0 ppm. To reduce variance, all data were log (glog)-transformed (Parsons et al., 2007; Wang et al., 2013).

SIMCA-P$^+$ was used to analyze the multivariate data, including the supervised multivariate data analysis, the partial least squares discriminant analysis (PLS-DA) and the orthogonal projection of the latent structure with discriminant analysis (O-PLS-DA). We obtained significant responses at the metabolite level in specimens challenged by DDT, BaP and their mixture.

The results were imaged using score plots to display simultaneous categorizations, and the matching loading plots were used to illustrate the contribution of each variable to the categorizations. To strengthen the model's interpretability, the coefficients were considered with variable weight. Coefficient-coded loading plots can thus reflect the metabolic distinctness between the control and treatments (Ji et al., 2014a).

Matlab was used to generate coefficient plots color-coded with the absolute value of coefficients (r). A cool color (i.e., blue) and hot color (i.e., red) correspond to metabolites with highly negative and positive significance, respectively in discriminating between groups. The correlation coefficient was established based on the test of Pearson's product-moment correlation coefficient. The cross-validation parameter (Q^2) was used to assess the validation model. Another validation method for detecting the validity of the PLS-DA models was the permutation test (the permutation number was 200) (Wu et al., 2005b). The metabolites were allocated according to the tabulated chemical shifts.