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Inhibition by polycyclic aromatic hydrocarbons of ATPase activities in *Sebastiscus marmoratus* larvae : Relationship with the development of early life stages

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ABSTRACT

Sebastiscus marmoratus larvae were exposed to waterborne polycyclic aromatic hydrocarbons (PAHs) containing 3–5 rings, benzo[a]pyrene (BaP), pyrene (Py) or phenanthrene (Phe), respectively at 0.01, 0.1 and 1 μg L^{-1}. Cumulative mortality, frequency of dorsal curvature and rate of pericardial and yolk sac edema in larvae treated for 8 days were significantly increased in a dose-dependent manner. All three PAHs resulted in reduction of the lower jaw, and the extent of reduction increased with increasing concentrations of the PAHs. Na^+/K^+-ATPase and Ca^{2+}-ATPase activity in larvae exposed to the three PAHs were all significantly inhibited in a dose-dependent manner. Analysis using the Pearson correlation coefficient indicated a significant correlation between the rate of the dorsal curvature and edema and the inhibition of Na^+/K^+-ATPase and Ca^{2+}-ATPase activity, suggesting that the developmental defects caused by PAHs were related to their inhibition of Na^+/K^+-ATPase and Ca^{2+}-ATPase activity.
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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widespread contaminants. They are produced during the incomplete combustion of coal, gas, oil, and wood. The largest fraction enters marine waters as land-based runoff or atmospheric deposition or oil spills (National Research Council, 2003). Recent increases in the aquatic accumulation of PAHs over the last decade have been detected and are associated with increased use of motor vehicles (Van Metre et al., 2000; Lima et al., 2002). It is reported that concentrations of benzo[a]pyrene (BaP) vary from 1.0 to 23.4 ng L^{-1} in the surface seawater of Maluan Bay in Xiamen, China, whereas the concentration of total PAHs are 5.118 μg g^{-1} in the sediments of Xiamen western harbor, China (Tian et al., 2004). The levels of BaP, pyrene (Py) and phenanthrene (Phe) in the surface water from the Jiulong River Estuary and Western Xiamen Sea were 0.56–3.32, 0.22–2.19 and 0.16–1.37 μg L^{-1}, respectively (Maskaoui et al., 2002).

There is a wide range of studies which indicate that fish embryos and larvae are highly sensitive to PAHs (Carls et al., 1999; Heintz et al., 1999, 2000; Colavecchia et al., 2004; Couillard, 2002; Sundberg et al., 2005). Gross malformations resulting from exposure to PAHs include pericardial and yolk sac edema, jaw reductions, presumptive skeletal defects described as spinal malformations such as lordosis or scoliosis (dorsal curvature), and craniofacial skeleton disorders. Reductions in larval heart rate (bradycardia) and cardiac arrhythmia have also been observed (Incardona et al., 2004, 2009). Increased weathering of crude oil, which shifts the composition from predominantly two-ring (e.g., naphthalenes) to three-ring PAHs (e.g., Phe), result in a greater toxic potency and a higher frequency of malformations (Carls et al., 1999; Heintz et al., 1999). It is indicated that the key developmental defects induced by weathered crude oil exposure are mediated by low-molecular-weight tricyclic AHs through aryl hydrocarbon receptor (AhR)-independent pathway (Incardona et al., 2005). Tricyclic AHs and weathered crude oil cause early cardiac dysfunction during key stages of cardiac morphogenesis. These effects are independent of the AHR and are most likely secondary to impacts on cardiac ion channels or other targets involved in the cardiac conduction system (Incardona et al., 2004, 2005). Another study indicates that some tetracyclic PAHs (pyrene and benz[a]anthracene) produce developmental toxicity through the AHR pathway (Incardona et al., 2006). Previous studies suggest that there could be other common mechanism, since edema and dorsal curvature are common symptoms in fish embryos treated with chemicals such as dioxins (Bello et al., 2004), cadmium

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(Blechinger et al., 2002) and ethanol (Reimers et al., 2004). Adenosine triphosphatase (ATPase) belongs to a group of enzymes that play an important role in intracellular functions and are considered to be a sensitive indicator of toxicity (Yadwad et al., 1990). Na^+/K^+-ATPase is involved in ionic balance and osmotic homeostasis in fish (Richards et al., 2003), and the link between gill Na^+/K^+-ATPase activity and osmoregulatory capacity is well described (Glover et al., 2007). Ca^{2+}-transport depends on a high-affinity plasma membrane Ca^{2+}-ATPase (Wong and Wong, 2000). This type of localization corresponds to cartilage with Ca^{2+}-Mg^{2+} ATPase activity in the plasma membrane of the chondrocytes, and suggests a role for the plasma membrane Ca^{2+} pump in the calcification of cartilage (Akisaka and Gay, 1985). The amount of Ca^{2+}-Mg^{2+} ATPase (Ca^{2+} pump) in growing cartilage is more than twice that in adult cartilage (Sharawy et al., 2000). This Ca^{2+} pump may act synergistically with the Na^+/Ca^{2+} exchanger, which is also reported to be present on the osteoidal aspects of osteoblasts (Stains and Gay, 1998, 2002). It has been shown that lower molecular weight PAHs can cause a decrease in the activity of ionspecific ATPases in rainbow trout (*Salmo gairdneri*) (Englehardt et al., 1981), and anthracene can cause a decrease in the activity of Na^+/ K^+-ATPase and Mg^{2+}-ATPase in gill tissue homogenates of bluegill sunfish (*Lepomis macrochirus*) using in vitro enzyme analysis (McCloskey and Oris, 1993). However, the relationship between developmental defects and inhibition in ATPase activities in fishes exposed to PAHs is still unclear.

In the present study, we investigated the effects of representative PAHs containing 3–5 rings on the development of a marine teleost. Our objectives were to (1) evaluate and compare the toxicity of these three individual PAHs on larval development and (2) evaluate effects of PAHs on ATPase activities in whole larvae and the relationship between ATPase activities and developmental defects.

2. Materials and methods

2.1. Chemicals

BaP, Py and Phe of >99% purity were obtained from Sigma–Aldrich (St. Louis, MO, USA). These three individual PAHs were dissolved in dimethylsulfoxide [DMSO (>99% purity)] to obtain final concentrations of 1, 10 and 100 μg mL^{-1}.

2.2. Sebastiscus marmoratus embryos exposures

Sebastiscus marmoratus is an ovoviviparous oceanic teleost. Adult *S. marmoratus* were captured from a relatively uncontaminated coastal area in Xiamen, Fujian Province, China. Hatched larvae from one pregnant fish were collected, and exposed to BaP, Py and Phe at nominal concentrations of 0.01, 0.1 and 1 μg L^{-1} in a marine fish saline solution, which was made up with double distilled water as follows: 230 mM NaCl, 8.0 mM KCl, 2.25 mM $CaCl_2$, 1.7 mM $MgCl_2$ and 0.24 mM $NaHCO_3$. The 1 μg L^{-1} BaP, Py and Phe concentrations involve 0.0040, 0.0049, 0.0056 μM respectively, and the other two concentrations were prepared by dilution.

1000 just hatched larvae were kept in 500 mL of the saline solution containing the PAH in a 1 L glass beaker. Three replicate treatments were exposed to each PAH dose. The solutions containing the different concentrations of BaP, Py and Phe were changed twice daily. The control group received an equal volume of the DMSO solvent (100 μL L^{-1}). The larvae were maintained at 18 °C in a relatively dark environment. After exposure for 6 days, the larvae were collected and frozen at −80 °C for biochemical assay.

2.3. Measurement of mortality and malformation rates

30 just hatched larvae were kept in 15 mL of the saline solution in a 10-cm glass button dish. Three replicate treatments were exposed to each concentration. The solutions containing the different concentrations of the three PAHs were changed twice daily. Throughout their development, larvae were examined twice daily under a stereomicroscope (XTB-01, China) to screen for morphological abnormalities and record survival rates within each treatment, and dead larvae were counted and removed. Pericardial edema was defined as any abnormal separation in the pericardium, a portion of coelomic cavity that is mesodermally lined separating visceral organs such as in the heart and body wall (Westerfield, 1995). After exposure for 8 days, cumulative mortality and malformation rate were evaluated.

2.4. Whole-mount alcian-blue staining

S. marmoratus larvae were stained with alcian blue. 10% neutral formalin fixed larvae were stained with 0.1% alcian blue 8GX/80% ethanol/20% acetic acid for 6 h. After a series of washes with 75% and 50% ethanol/PBS (phosphate buffered saline) each for 1 h, larvae were incubated with PBS overnight. For clarification, larvae were treated with 1% KOH/3% H_2O_2 for 20 h, followed by treated with 0.05% trypsin/saturated tetraborate for 1 h. Stained larvae were examined by an Olympus B×41 light microscope.

2.5. Na^+/K^+-ATPase and Ca^{2+}-ATPase activity assay

Na^+/K^+-ATPase and Ca^{2+}-ATPase activities were determined using colorimetric measurement of inorganic phosphate (Pi) production, as previously described (Morgan et al., 1997). Larvae in individual replicate treatments were pooled for analysis. Whole larvae were homogenized in Tris buffer (pH 7.4), the homogenate centrifuged at 10,000 g for 20 min at 4 °C to obtain a post-mitochondrial supernatant, which was used as the enzyme source. The supernatant was incubated at 30 °C for 30 min in the standard reaction mixture containing 50 mM Tris-maleate at pH 7.4, 33 mM KCl, 3.6 mM $MgCl_2$, 80 mM NaCl, 2.5 mM ATP, 0.9 mM ouabain (Ca^{2+}-ATPase only), 0.02 mM $CaCl_2$ (Ca^{2+}-ATPase only), or 1 mM EGTA (Na^+/K^+-ATPase only). The absorbance of the reaction mixture (after terminating the reaction using trichloroacetic acid) was assayed at 660 nm using a Universal Microplate Spectrophotometer. The phosphate liberated was estimated with Malachite Green (Baykov et al., 1988). ATPase activity was expressed as mol Pi liberated per mg protein per hr. Protein concentrations in the supernatants were determined using the Bradford procedure (Bradford, 1976) with bovine serum albumin as the standard.

2.6. Statistical analysis

Results are reported as mean ± standard deviation (SD). Significant differences between means were analyzed with one-way analysis of variance using SPSS 11.0 software, followed by the Duncan post-hoc test. The Pearson correlation coefficient (r) was used to evaluate the correlations among the various endpoints. Statistical significance of difference was set at $p < 0.05$.

3. Results

3.1. Effect of PAH exposure on mortality and rate of abnormal development

After exposure for 8 days, cumulative mortality, frequency of dorsal curvature and rate of pericardial and yolk sac edema of the

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