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Bioaccumulation and ROS generation in liver of *Carassius auratus*, exposed to phenanthrene

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Abstract

In the present study, the bioaccumulation and reactive oxygen species (ROS) generation were studied after fish (*Carassius auratus*) were exposed to different concentrations (0.01, 0.02, 0.05, 0.07 and 0.1 mg/L) of phenanthrene for 4 days. The accumulation of phenanthrene in liver increased with the exposure concentration ($R^2=0.88$). A secondary spin trapping technique was used followed by electron paramagnetic resonance (EPR) analysis, to study the ROS production. The ROS generated in fish liver after exposure to phenanthrene was identified as hydroxyl radical ($^\bullet$OH). The $^\bullet$OH signal intensity of the EPR spectrum showed a significant increase ($p<0.05$) compared to the control when the phenanthrene concentration was as low as 0.05 mg/L. A good positive relationship ($R^2=0.97$) was found between the $^\bullet$OH formation and exposure concentrations. The changes of the activities of catalase (CAT), superoxide dismutase (SOD), glutathione-*S*-transferase (GST), and contents of reduced glutathione (GSH) also were detected. The results clearly indicated that phenanthrene could induce $^\bullet$OH generation and result in oxidative stress in liver of fish.
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Keywords: Antioxidant defenses; *Carassius auratus*; Phenanthrene; Electron paramagnetic resonance (EPR); Hydroxyl radical ($^\bullet$OH); Oxidative stress; Reactive oxygen species (ROS)

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a wide spread class of environmental chemical pollutants and known to exert acutely toxic effects as well as to have mutagenic or properties (Swartz et al., 1990; Kanaly and Harayama, 2000; Yang, 2000; Maskaoui et al., 2002; Wu et al., 2003; Pérez-Cadahía et al., 2004; Zhang et al., 2004a,b). These hydrophobic compounds can be easily taken up by aquatic organisms due to their ability to interact with cellular molecules following binding to lipophilic sites. Many researchers are performing risk assessments and toxicological studies of PAHs (Aas et al., 2000; Alcha et al., 2000; Cheung et al., 2001; Pérez-Cadahía et al., 2004; Pan et al., 2006). Exposure to PAHs increases the expression of cytochrome P450 and some CYP1A-produced PAH metabolites may produce reactive oxygen species (ROS) and oxidative stress (Pacheco and Santos, 1998; Livingstone, 2001; Meyer et al., 2002). It is known that oxidative stress is an important mechanism of toxicity induced by PAHs (Di Giulio et al., 1993; Livingstone, 2001; Shi et al., 2005a). A short-term naphthalene exposure over-activated the fish phagocytes could result in lipid peroxidation (LPO) induction (Ahmad et al., 2003). Shi et al. (2005a) showed that naphthalene could induce ROS generation and result in oxidative damage in liver of *Carassius auratus.*

ROS, including superoxide anion radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the very reactive hydroxyl radical ($^\bullet$OH) are generated during normal metabolism in aerobic organisms. $O_2^{\bullet -}$ and $^\bullet$OH are usually referred to as free radicals because of their unpaired electrons. Under normal physiological conditions, ROS generated from metabolism of extraneous chemicals in the body can be removed well

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by the antioxidant defense system. However, when ROS generation exceeds the capacity of the cellular antioxidants, it will cause oxidative stress and significant oxidative damage (Mate's, 2000). However, endogenous ROS produced in vivo has extremely short half-lives and is present in low concentrations. Little direct evidence is available to prove ROS generation and oxidative stress in aquatic organisms exposed to PAHs.

Phenanthrene, a tricyclic PAH, which is considered the most abundant hydrocarbons in the aquatic environment (Mai et al., 2002; Maskaoui et al., 2002; Chen et al., 2004) was chosen as a test compound. Fishes are particularly vulnerable to PAH and other persistent contaminants due to their high position in the aquatic food web. In this study, freshwater fish, goldfish *C. auratus* that are commonly found in China were chosen. The objective of this study was to investigate the potential oxidative stress induced by ROS in vivo. The antioxidant defense system, including activities of catalase (CAT), superoxide dismutase (SOD), glutathione *S*-transferase (GST), and contents in reduced glutathione (GSH) were also studied.

2. Materials and methods

2.1. Chemicals

Phenanthrene (purity > 99%), α-phenyl-*N*-*tert*-butylnitrone (PBN, purity > 98%), acetone (HPLC grade), dichloromethane (HPLC grade), hexane (HPLC grade), dimethylsulfoxide (DMSO, purity > 99.9%), Florisil, and bovine serum albumin (BSA) were purchased from Sigma-Aldrich Company (USA), Other reagents with analytical grades were obtained domestically.

2.2. Fish collection and treatment

Fish (*C. auratus*) were purchased from a local aquatic breeding base, with average body length and mass about 9.0 cm and 24.2 g, respectively. All fish were acclimated to water dechlorinated with activated carbon for 10 days before the experiment. The total mortality of fish was below 1%. Artificial dry food was provided once a day. During the experiment, the pH was 7.3 ± 0.3, the temperature was 22 ± 1 °C, and the photoperiod was 16 h D/8 h L. The dissolved oxygen levels in the water were kept higher than 5 mg/L by continuous aeration.

After acclimatization, fish were randomly divided into six groups (n=6 for each group) and kept in glass aquaria with a fish/water ratio of 3.4 g/L fish/water. One group was designated for control and the other groups were served as experimental groups that received sublethal concentrations of 0.01, 0.02, 0.05, 0.07 and 0.1 mg/L phenanthrene for 4 days, respectively. During the experiment, 50% water was replaced daily by fresh phenanthrene solution to minimize contamination from metabolic waste. Fish were sampled after a 4-day exposure, and then weighed. Then the fish were dissected and some of fresh livers were obtained for Electron paramagnetic resonance (EPR). The rest of the livers were homogenized at 4 °C for other experiments.

2.3. Analysis of phenanthrene

Homogenized with anhydrous Na_2SO_4, the liver samples were extracted three times by ultrasonic wave method with acetone/hexane (59/41; v/v), 20 min each time. All the extracted solutions were combined. The obtained solvent was concentrated to about 1 mL by rotary evaporator, and then cleaned up with dichloromethane by using an anhydrous Na_2SO_4/Florisil column. The elution was evaporated gently to dryness and the obtained dry residue was dissolved in methanol for high performance liquid chromatography (HPLC).

The level of phenanthrene in methanol was analyzed by a Hewlett Packard (HP) 1100 HPLC equipped with a photodiode array dectection (DAD) (Agilent, Germany), which the absorption wavelength was 252 nm, with an elute of methanol/water (80:20; v/v). The column was Zorbax EclipseXDB-C_8 (4.6 mm i.d. × 150 mm length, Agilent, USA).

The recovery in liver in the analysis was 85.9 ± 2.49% (n=4). Phenanthrene was not detected in the control samples.

2.4. PBN adduct extraction and EPR analysis

PBN adduct extraction was performed according to Shi et al. (2005b). The whole operation was conducted in an incubation system with a continuous purging of N_2. After rinsing with ice-cold physiological salt water, the fish livers were immediately weighed, and a fraction (0.1 g) was removed and homogenized quickly in 1.0 mL 50 mM PBN (dissolved in DMSO) using a Teflon pestle in a Potter homogenizer. Then 0.1 mL supernatants was transferred to a capillary tube with a diameter of 0.9 mm, and frozen by liquid nitrogen for EPR analysis. The EPR spectra were recorded with Bruker EMX 10/12 X-band spectrometer (Bruker, Germany) at room temperature. The operation conditions were: center field, 3470 G; scan range, 200 G; modulation frequency, 100 kHz; modulation amplitude, 0.5 G; receiver gain, 5×10^4 scans, 5 times; microwave power, 20 mW.

2.5. Glutathione and enzymatic activities

About 50 mg of fish liver was homogenized after addition of 0.5 mL of 10.0 mM Tris–HCl buffer (0.01 M Tris, 0.25 M sucrose, 0.1 mM EDTA, pH 7.5) for assessment of enzyme activities. About 50 mg of liver tissue was homogenized after addition of 0.5 mL of 1.0 mM EDTA and 10 μL diluted $HClO_4$ (4000-fold dilution) for measurement of reduced glutathione (GSH) levels. The extracts were centrifuged at 12,000 ×*g* for 10 min at 4 °C (Beckman, Germany). Aliquots of the supernatants obtained were preserved at −80 °C for analysis within 5 days.

GSH levels were measured by the method of Hissin and Hilf (1976). This involved the addition of 4.5 mL phosphate-EDTA buffer (0.1 M potassium phosphate, 1.0 mM EDTA, and pH 8.0) to 0.5 mL original tissue supernatant. The final assay mixture (2.0 mL) contained 100 μL of the diluted tissue supernatant, 1.8 mL phosphate-EDTA buffer (0.1 M potassium phosphate, 1.0 mM EDTA, pH 8.0), and 100 μL of 1 μg/μL *O*-

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