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ScienceDirect
Ecotoxicology and Environmental Safety 71 (2008) 446–453
Ecotoxicology and Environmental Safety
www.elsevier.com/locate/ecoenv

Hydroxyl radical generation and oxidative stress in *Carassius auratus* liver, exposed to pyrene ☆

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Received 6 April 2007; received in revised form 5 December 2007; accepted 25 December 2007
Available online 15 February 2008

Abstract

This paper studied the hydroxy radical generation and oxidative stress in the liver of goldfish *Carassius auratus* under the effect of pyrene. Fish were exposed to different concentrations (0.001, 0.005, 0.01, 0.05 and 0.1 mg/L) of pyrene for 10 days, with one group assigned as control. Based on the hyperfine splitting constants and shape of the electron paramagnetic resonance (EPR) spectrum, the free radical which was generated in fish liver was identified as hydroxyl radical ($^{\bullet}OH$). The $^{\bullet}OH$ signal intensity showed a significant increase compared with the control. The changes of the activities of catalase (CAT), superoxide dismutase (SOD), and glutathione-S-transferase (GST) were detected. The reduced glutathione (GSH) level decreased significantly while oxidized glutathione (GSSG) level was increased at higher concentration (0.005–0.1 mg/L), resulting in a decreased GSH/GSSG ratio, and the malondialdehyde (MDA) content increased significantly at 0.005–0.1 mg/L pyrene . The results clearly showed that *C. auratus* was subjected to oxidative stress and damage when exposed to pyrene.

Keywords: *Carassius auratus*; Pyrene; Electron paramagnetic resonance (EPR); Hydroxyl radical ($^{\bullet}OH$); Antioxidant defenses; Malondialdehyde (MDA)

1. Introduction

Reactive oxygen species (ROS), including superoxide anion radical ($O_2^{\bullet -}$), singlet oxygen ($^1O_2$), hydrogen peroxide (H_2O_2) and the highly 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. Of the ROS, hydroxyl radical is the most reactive oxygen radical formed via Fenton reaction in living systems. In general, this radical is considered to be a harmful byproduct of oxidative metabolism, which can cause molecular damage in living system, and also, play a critical role in initiating and catalyzing a variety of radical reactions in the presence of oxygen (Livingstone, 2001; Cheng et al., 2002).

Under physiological conditions, the normal production of ROS is matched by several cellular mechanisms. These mechanisms mainly consist of antioxidant molecules and scavenger enzymes, which is an important reactive oxygen removal system in the body of aerobic organisms. However, when ROS generation exceeds the capacity of the cellular antioxidants, it will cause oxidative stress and significant oxidative damage (Matés, 2000). The oxidative stress produced may even lead to lipid peroxidation (LPO), enzyme inactivation, DNA strand breaks, and covalent binding to protein and nucleic acid. Xenobiotic agents through redox cycling have the potential to produce ROS that overcome the protection afforded by antioxidant defense mechanisms, thereby leading to oxidative damage which is manifested by damage to tissue macromolecules including DNA, proteins and lipids. Recently, it has been found that the ROS production and resulted oxidative damage may be an important mechanism of toxicity in

☆Any studies in our experiments were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare.

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0147-6513/$ - see front matter © 2007 Elsevier Inc. All rights reserved.
doi:10.1016/j.ecoenv.2007.12.016

organisms exposed to a wide range of xenobiotics (Livingstone, 2001; Oakes and Van Der Kraak, 2003; Shi et al., 2005a, b). However, endogenous ROS produced in vivo has extremely short half-lives and is present at low concentrations, and thus, is hard to measure directly by conventional analytical techniques. Electron paramagnetic resonance (EPR) spectroscopy allows both characterization and quantification of free radicals in organisms. Recently, EPR combined spin trapping has been shown to be an appropriate technique to identify free radicals formed in organism tissues under normal as well as stressful conditions (Cheng et al., 2002; Takeshita et al., 2004).

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 properties (Yang, 2000; Maskaoui et al., 2002; Wu et al., 2003; Pérez-Cadahía et al., 2004; Zhang et al., 2004a, b). Many researchers have performed risk assessments and toxicological studies of PAHs (Aas et al., 2000; Akcha et al., 2000; Cheung et al., 2001; Pérez-Cadahía et al., 2004; Pan et al., 2006). However, the fate of PAHs and the mechanisms of their toxic effect on aquatic organisms are still unclear. 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; Sun et al., 2006; Yin et al., 2007). A short-term naphthalene exposure resulted in lipid peroxides induction of fish phagocytes (Ahmad et al., 2003). Wang et al. (2006) reported that benzo[a]pyrene affected the activities of hepatic antioxidant defense of *Sebastiscus maramoratus*. The recent studies showed that naphthalene and phenanthrene were able to induce ROS generation and resulted in oxidative damage in liver of *Carassius auratus* (Shi et al., 2005a; Sun et al., 2006; Yin et al., 2007).

Pyrene, a PAH with four aromatic rings, has been used as an indictor of PAH pollution (Zhang et al., 2004c). Pyrene constitutes a major portion of PAHs observed in aquatic environment (Kucklick et al., 1997; Zhang et al., 2004a; Luo et al., 2006; Wang et al, 2007; Xu, et al., 2007). The International Agency for Research on Cancer (IARC) classifies pyrene as a group 3 carcinogen (unclassifiable as a human carcinogen). Although being noncarcinogenic and not phototoxic/toxic to freshwater green alga, *Selenastrum capricornutum* (Warshawsky et al., 1995), pyrene was found acutely toxic to marine alga *Phaeodactylum tricornutum* (Okay et al., 2002), and phototoxic to some organisms such as the duckweed *Lemna gibba* L. (Huang et al., 1995) and to fish (Schirmer et al., 1998). Up to date, there are few direct evidences that proved ROS generation and oxidative stress in aquatic organisms exposed to pyrene.

Fishes are particularly vulnerable to persistent contaminants like PAHs due to their high position in the aquatic food web. The freshwater fish, goldfish, *C. auratus* was chosen for this study, since it is commonly found in China. Additionally, this highly tolerant fish can survive under a wide range of stressful conditions, from full anoxia to hyperoxia and temperature alterations from 0 to above 40 °C (Lushchak et al., 2001, 2005; Ford and Beitinger, 2005). The objective of this study was to investigate the potential oxidative stress induced by ROS in vivo after exposure to pyrene. The EPR spin trapping technique coupled with α-phenyl-*N*-*tert*-butylnitrone (PBN) as the spin-trap agent was used to test the hypothesis that pyrene-induced ROS production. Malonaldehyde (MDA), which is a common product of lipid peroxidation and a sensitive diagnostic index of oxidative injury in cells, was used as the indicator of oxidative damage in this study. The antioxidant defense system, including catalase (CAT), superoxide dismutase (SOD), glutathione-S-transferase (GST), and glutathione in the reduced (GSH) and oxidized (GSSG) forms were also studied.

2. Materials and methods

2.1. Fish collection and treatment

Fish (*C. auratus*) of both sexes were obtained from a local aquatic breeding base (aquaculture facility, Nanjing, China) with a mean body length of 9.0 cm and a mean body weight of 25.2 g. 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 0.001, 0.005, 0.01, 0.05 and 0.1 mg/L pyrene, respectively, for 10 days on the basis of the result of preliminary tests. During the experiment, 50% water was replaced daily by adding fresh pyrene solution. Fish were sampled after a 10-day exposure, and then weighed. Then the fish were dissected and some of fresh livers were obtained for EPR. The rest of the livers were homogenized at 4 °C for other experiments.

2.2. 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 dimethylsulfoxide (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, five times; microwave power, 20 mW.

2.3. Tissue preparation

About 100 mg of fish liver was homogenized in 1.0 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 and MDA content. About 50 mg of liver tissue was homogenized in 0.5 mL of 1.0 mM EDTA and 10 μL diluted $HClO_4$ (4000-fold dilution) for measurement of glutathione levels. The