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Acute effects of benzo[a]pyrene on liver phase I and II enzymes, and DNA damage on sea bream *Sparus aurata*

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Abstract In the present study biotransformation and detoxification responses to acute exposure to the polycyclic aromatic hydrocarbons benzo[a]pyrene (B[a]P) were investigated in the liver of *Sparus aurata* (sea bream). Sexually immature gilthead sea bream were treated by intraperitoneal injection of B[a]P (20 mg kg^{-1}) for 6, 12, 24, and 48 h. B[a]P accumulation was quantified in sea bream liver by mean of gas phase chromatography (GPC-MS) after the various exposure periods. The following biological responses were measured: (1) ethoxyresorufin-*O*-deethylase (EROD) activity, as a phase I biotransformation parameter; (2) liver glutathione *S*-transferase (GST) activity as a phase II conjugation enzyme. DNA damage was assessed over time using the single-cell gel electrophoresis comet assay. B[a]P bioaccumulation in the liver resulted in a biphasic curve with an increasing uptake up to 5.55 ± 0.67 μg g^{-1} dry weight after only 6 h exposure and 4.67 ± 0.68 μg g^{-1} dry weight after 48 h exposure. EROD activity showed a nonsymmetrical bell-shaped kinetic with a maximum at 24 h and lower but significant activities at 12 and 48 h with respect to control animals. Hepatic GST activities were only significant after 48 h exposure. Comet assay showed an increase in liver cells DNA damage with a maximum after 48 h exposure reaching up to 12.17 %DNA in the tail.

Keywords Acute exposure · Benzo[a]pyrene · EROD activity · GST activity · DNA damage

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are produced by any incomplete combustion of organic material and are therefore present worldwide due to anthropogenic activities. Benzo[a]pyrene (B[a]P), a model PAH compound, is classified as a potent carcinogen and/or mutagen (Shaw and Connell 1994). Biotransformation of lipophilic chemicals is a requisite for detoxification and excretion (Rodriguez-Ariza et al. 1999). The first step is usually catalyzed by cytochrome P450-dependent monooxygenases (phase I) and their products are subsequently coupled to endogenous metabolites (phase II) (Buhler and Williams 1988; Landis and Yu 1995). Following

metabolism by phase I and II enzymes, the major route of metabolites excretion is biliary (Klaassen and Rozman 1991). However, certain biotransformation steps are responsible for the activation of foreign chemicals to reactive intermediates that ultimately result in toxicity, mutagenicity, or carcinogenicity (Nebert and Gonzalez 1987).

In recent years, attention has focused on the induction of cytochrome P-450 (P450) as a sensitive biomarker of fish exposure to certain contaminants in the marine environment (Gravato and Santos 2002). Activation of the isoenzyme cytochrome P4501A (CYP1A) can be measured by determining 7-ethoxyresorufin-*O*-deethylase (EROD) activity, which is frequently used as biomarker of exposure to PAH (Van der Oost et al. 2003). Glutathione *S*-transferases (GSTs) are essential components of the cellular antioxidant defence system, since they catalyze the conjugation of GSH to several xenobiotic and endogenous electrophilic chemical compounds (Arrigo 1999; Rahaman et al. 1999). The single-cell gel electrophoresis assay (SCGE) or alkaline comet assay, due to its simplicity, sensitivity, and need for few cells, is ideal as a short-term genotoxicity test (Singh et al. 1988; Tice 1995; Fairbairn et al. 1995).

The economic importance of juvenile sea bream as an estuarine representative species as well as its importance in Tunisian fish farming justifies the relevance of this research concerning the effect of B[a]P. *Sparus aurata* was demonstrated to be a very sensitive species concerning liver biotransformation responses (Rodriguez-Ariza et al. 1999). Tunisian coastal waters neighboring industrial areas are reported to be contaminated with organic compounds, including PAHs, which thus affect aquaculture activities (Banni et al. 2005; Louati et al. 2001; Mzoughia et al. 2002; Trabelsi and Driss 2005; Zaghden et al. 2007).

This work aims to study the acute effect of B[a]P on detoxification enzymes and DNA damage in juvenile sea bream *Sparus aurata*.

Materials and methods

Animal treatment

Specimens of sexually immature gilthead sea bream (*Sparus aurata*) weighing 130–150 g (12–15 cm) farmed at the aquaculture farm of the Institut National des Sciences et Technologie de la Mer (INSTM, Monastir, Tunisia) were used. Fishes were acclimated for 15 days to laboratory conditions in a 1,000 l aquarium filled with artificial seawater maintained constantly at 16°C. After the acclimation period, fish were intraperitoneally (ip) injected (0.2 µl/g) with B[a]P. B[a]P was solubilized in dimethyl sulfoxide (DMSO) and then adjusted to the desired concentrations (20 mg kg^{-1}). Control animals were injected with DMSO (0.2 µg l^{-1}). After 6, 12, 24, and 48 h of exposure, fish were killed and livers were removed. One set of samples (ten individual livers) were washed briefly in ice-cold homogenizing buffer and frozen at −80°C until enzymatic analysis. Another set of tissues (ten individual livers) were flash-frozen into liquid nitrogen and stored at −80°C until B[a]P analysis.

For comet assay, liver cells were prepared by mechanical dissociation of liver tissue as follows. Freshly dissected tissue was washed extensively into ice-cold physiological saline containing 500 mM NaCl, 12.5 mM KCl, 5 mM di-sodium EDTA, 20 mM Hepes, pH 7.4. The tissue was minced using scissors and then cells were mechanically dissociated by chopping with a razor blade. The cell mixture was resuspended in 10 ml ice-cold physiological saline and further filtered through a 100 µM sieve. The volume of the elute was adjusted to 50 ml and then cells were washed four times into ice-cold physiological saline by centrifugation at 400 *g*, at 4°C for 5 min. Cells were resuspended at 5×10^5 cells ml^{-1}, in 500 mM NaCl, 12.5 mM KCl, 20 mM Hepes, pH 7.4.

B[a]P analysis

The content of B[a]P in the liver fractions was determined by gas chromatography (GC) coupled to mass spectrometry (MS) based on a protocol described by Baumard and Budzinski (1997). An HP GC (Hewlett-Packard, Palo Alto, CA, USA) equipped with a split/splitless injector was used. The PAHs were quantified relative to perdeuterated PAHs (Quilliam et al. 1994; Baumard and Budzinski 1997). The response factors of the different compounds were measured by injecting a standard reference material [SRM 2260 (24 aromatic hydrocarbons in toluene) (NIST, Gaithersburg, MD, USA) solution spiked with

the same solution containing the perdeuterated PAHs as the one used for spiking the fish liver]. The detection limits for B[a]P was about 50 pg g^{-1} for liver tissues.

Biochemical analyses

EROD activity

Microsomes were obtained according to the methods of Monod and Vindimian (1991) and Lange et al (1993), as adapted by Pacheco and Santos (1997). The supernatant and the resuspended microsomal pellet were frozen in liquid nitrogen and stored at −80°C until further use. Liver EROD activity was measured as described by Burke and Mayer (1974). Microsomal protein concentrations were determined according to the Bradford method (Bradford 1976), using bovine serum albumin as a standard.

Glutathione S-transferase activity

Glutathione *S*-transferase activity was assayed by the method described by Habig et al. (1974), using 10 µg of cytosolic protein, 1-chloro-2,4-dinitrobenzene as substrate, and GSH (1 and 4 mM final concentration, respectively), in 100 mM sodium phosphate buffer, pH 6.5. All GST activity assays were realized in conditions of linearity with respect to incubation time (30 s). Protein concentrations were determined according to the Bradford method (Bradford 1976), using bovine serum albumin as standard.

Comet assay

The Comet assay or single-cell gel electrophoresis on *S. aurata* liver cells was carried out by a procedure based on the well-established method from Singh et al. (1988). The liver cells were resuspended in 50 µl 0.5% low-melting-point agarose (LMA) in Kenny's salt solution (0.4 M NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$) at 37°C. The cells suspended in LMA were transferred onto frosted microscope slides and allowed to gel on a metal tray in the fridge. After gelling the slides were covered with 200 µl of 2% agarose and gelled. Then, the slides were placed in lysing solution [2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris–base, 1% *N*-lauroyl-sarcosine, 1% Triton X-100, pH 13 (LS)] in staining troughs, and incubated for 1 h at 4°C, in the dark to lyse the cells.

Slides were transferred from LS and rinsed in distilled water three times for 2 min to remove excess salts, then placed in a submarine gel electrophoresis chamber filled with electrophoresis buffer (0.5× Tris–Borate–EDTA Buffer, TBE) and DNA was allowed to unwind under these alkaline conditions for 20 min. Electrophoresis was performed at 300 mA and 20 V for 20 min. The slides were removed from the electrophoresis chamber, and rinsed three times for 5 min with five drops of neutralization buffer (0.4 MTris). The slides were stained with 10 µl of Fluoplus DNA stain 100× (IKZUS Environment) stored in airtight containers in the fridge, and analyzed within 24 h.

All procedures were carried out under low artificial light conditions to minimize ultraviolet (UV)-induced DNA damage. For visualization of DNA damage, slides were examined using epifluorescence microscopy (400× magnification) on a Zeiss photomicroscope. The slides were analyzed using the Scion image software. The parameter used for the scoring was the percentage of DNA in the comet tail (Lee and Steinert 2003).

Statistical analysis

Statistical analyses were performed with SP SS/PC (SP SS, Microsoft, and Redmond, WA). Significant differences between means were determined using one-way analyses of variance (ANOVAs) and the Duncan's test for multiple range comparison with significance level established at $P < 0.05$.

Results

The content of B[a]P in *Sparus aurata* liver after 6, 12, 24, and 48 h is reported in Fig. 1. Data show an uptake of up to 5.55 ± 0.67 µg g^{-1} dry weight after only 6 h exposure. This amount decreases significantly over time to reach 0.68 ± 0.12 µg g^{-1} dry weight after 24 h. A biphasic B[a]P uptake kinetic was observed, with a second increase up to 4.76 ± 0.58 dry weight after 48 h exposure. The B[a]P contents were also measured in control animals; values were under the limit of detection.

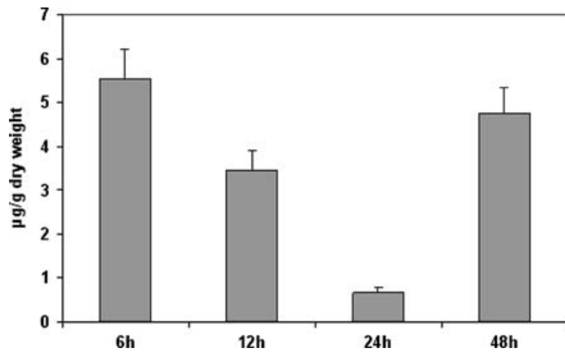

Fig. 1 Benzo[a]pyrene content (µg/g dry weight) in the liver tissues of *S. aurata* exposed to 20 mg/kg of B[a]P. Shown are the various liver uptakes after 6, 12, 24, and 48 h exposure. Analyses were performed by means of gas chromatography (GC) coupled to mass spectrometry (MS). Data are expressed as mean ± standard deviation (SD) ($n = 10$ individual liver). Control liver B[a]P content was under the detection limit

Figure 2 illustrates the EROD activity in *S. aurata* liver after exposure to 20 mg kg^{-1} B[a]P for 6, 12, 24, and 48 h. Liver EROD activity showed a nonsymmetrical bell-shaped kinetic. EROD activity was significantly different in juvenile sea bream after only 6 h exposure (163.15 ± 20.67 pmole min^{-1} mg $proteins^{-1}$) when compared to their controls. The maximum was reach at 24 h with up to 1906.51 ± 132.4 pmole min^{-1} mg $proteins^{-1}$.

The effect of B[a]P exposure on the sea bream liver glutathione-*S*-transferase activity is shown in Fig. 3. No significant effect was observed after 24 h exposure, however a significant response respect to control animals was recorded after 48 h exposure with up to 892.96 ± 53.12 nmole min^{-1} mg $proteins^{-1}$. Although there was no significant differences between control animals and B[a]P exposed animals after 6, 12, and 24 h, an increasing trend was observed for the B[a]P-exposed fishes. The liver GST activity was also assessed in animals injected with NaCl 9‰ (0.2 µg/g) for the same exposure period. No significant differences were observed respect to DMSO-treated animals (data not shown).

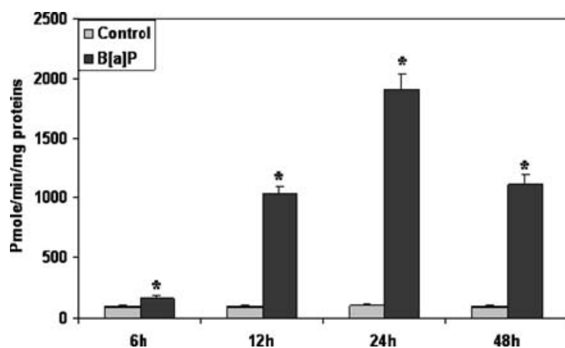

Fig. 2 Liver EROD activity in juvenile sea bream exposed for 6, 12, 24, and 48 h to 20 mg/kg of B[a]P. Control fish were injected with DMSO. EROD assay was carried out using the method developed by Burke and Mayer (1974). Data represent means ± SD. * Significantly different, ANOVA multiple comparison and Duncan's test versus control ($n = 10$; $P < 0.05$)

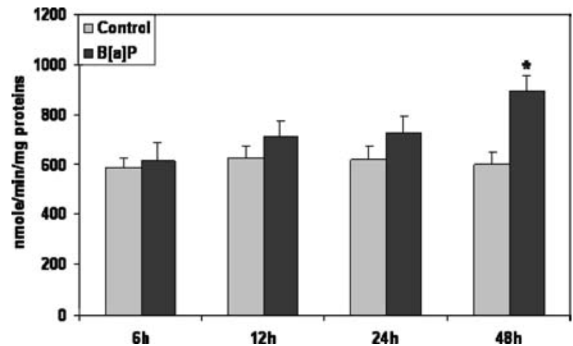

Fig. 3 Liver glutathione *S*-transferase activity (GST) in juvenile sea bream exposed for 6, 12, 24, and 48 h to 20 mg/kg of B[a]P. Control fishes were injected with DMSO. GST assay was carried out using the method developed by Habig et al. (1974). Data represent means ± SD. * Significantly different, ANOVA multiple comparison and Duncan's test versus control ($n = 10$; $P < 0.05$)

Figure 4 illustrates the DNA-damaging effect on the nuclei of *S. aurata* liver cells upon an exposure of 24 h to 20 mg B[a]P. No differences in %DNA in the comet tail was observed after 12 h exposure. A statistically significant (Duncan's test, $P < 0.05$) increase in %DNA in the comet tail of liver cells compared to the control group was reported after 24 h exposure to B[a]P. The maximum was reach after 48 h exposure with up to 12.17 ± 0.38 %DNA in the comet tail.

Discussion

In fish, a number of studies have described occurrence and xenobiotic induction of CYP1A in the liver (Stegeman and Hahn 1994; Sarasquete and Segner 2000; Gravato and Santos 2002; Bouraoui et al. 2008). However, xenobiotic induction of CYP1A and GST in hepatic tissues and their relation with DNA damage after an acute exposure is less studied.

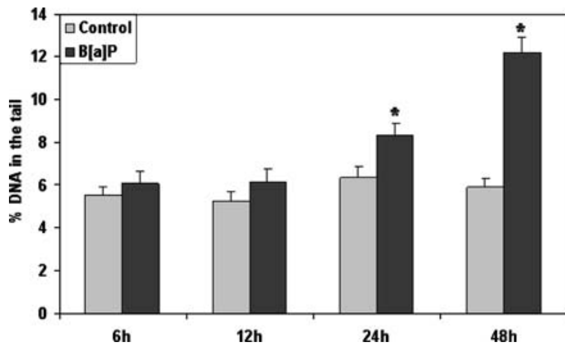

Fig. 4 Percentage of DNA in the comet tail of liver cells of *S. aurata* exposed to B[a]P compared to control. Fifty nuclei were scored per slide, five slides were evaluated per treatment, and each treatment was repeated twice. For visualization of DNA damage, slides were examined using epifluorescence microscopy (100× magnification) on a Zeiss photomicroscope. For data analysis, 1,300 × 1,030 pixel, 8-bit TIFF images files were used employing Scion image software. * Significantly different, ANOVA multiple comparison and Duncan's test versus control ($P < 0.05$)

Fish possess many of the enzymes responsible for xenobiotic metabolism that are seen in mammals (Law 1982; Varanasi 1989). Xenobiotic metabolism in fish occurs mainly in the liver, which displays the highest specific and total enzyme activities of all tissues. The metabolism of B[a]P by the liver in vivo and by hepatic microsomal preparations and in isolated hepatocytes results in the formation of metabolites with increased carcinogenic risk and cytotoxicity (Andersson and Koivusaari 1985; Gill and Walsh 1990).

Our investigation shows that, after an intraperitoneal injection of B[a]P, liver uptake of *Sparus aurata* manifests a biphasic behavior after only 48 h exposure. Indeed a maximum was reached after 6 h, followed by a decreasing phase until 24 h, and then a new increased bioaccumulation of liver B[a]P.

In the present study, B[a]P displayed strong liver EROD induction potency in *S. aurata*. These results are supported by previous studies on immature farmed fish (*D. labrax*), treated by different intraperitoneal PAHs injections, where a liver P450 1A and EROD activity increase was observed (Viarengo et al. 1997). It is interesting to note that data discussed in this work provide information about a very pronounced EROD activity when liver B[a]P uptake was minimal (after 24 h), suggesting a maximum of the metabolization process in liver after only 24 h exposure. The early activation of sea bream liver EROD activity by B[a]P is in accordance with other studies performed in different fish species exposed to the same genotoxic compound even if the exposure method and concentrations used are different (Van der Weiden et al. 1994; Viarengo et al. 1997; Gravato and Santos 2002).

Liver phase II conjugation, measured as GST activity, was significantly increased only after 48 h sea bream exposure to B[a]P, interestingly when a relative decrease of EROD activity respect to 24 h was observed. Therefore, liver EROD activity decrease may be due to increased reactive B[a]P-type metabolites in the cytosol, as reported by Kennedy et al (1991).

Genotoxicity results from metabolism of B[a]P to electrophilic intermediates by phase I mixed function oxidases, followed by binding of metabolites to nucleophilic sites within DNA. B[a]P-diol-epoxide (BPDE), the major ultimate reactive intermediate of B[a]P strongly reacts with the N-2 position of guanine in DNA to form anti-BPDE adducts (Newbold and Brookes 1976; Osborne et al. 1981). Adduct formation causes DNA strand breaks leading either to cell death due to changes in expression of critical survival genes, or transformation due to somatic mutations. Data discussed in this paper report a significant increase of the DNA damage effect of B[a]P on liver cells nucleus of *Sparus aurata* after 24 h exposure. This damage seems to increase over time. The current sea bream results concerning DNA damage induction by B[a]P confirm its genotoxic potential, as observed in previous studies where micronuclei induction was determined in fish erythrocytes (Gravato and Santos 2003) and mussel cells (Venier et al. 1997). Therefore, genotoxicity effects can be expected in fish exposed to xenobiotics, if phase II (conjugation) is saturated and DNA repair mechanisms are inhibited. DNA damage must also be a consequence of phase I metabolites covalent bound with erythrocytic DNA due to the inefficient liver phase II conjugation and the highly reactive B[a]P metabolites redistribution through blood circulation.

Conclusion

Benzo[a]pyrene is one of the most potent liver cytochrome EROD inducers, and considered as the most genotoxic compound. The biochemical and

genotoxic responses of *Sparus aurata* liver cells to acute exposure to B[a]P provide an interesting correlation between detoxification enzymes and DNA alteration in agreement with B[a]P metabolites' pivotal role. Although the route of contamination chosen in this paper, i.e., ip injection, may not necessarily simulate the same biological response as in the field, it was considered as a first step to study the effects of acute exposure on single and mixed pollutions of sea bream.

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