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Differential transcription of genes involved in peroxisome proliferation in thicklip grey mullets *Chelon labrosus* injected with benzo(*a*)pyrene

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ABSTRACT

Benzo(a)pyrene (B(*a*)P) is a mutagenic polycyclic aromatic hydrocarbon (PAH) commonly released into the environment. B(*a*)P induces phase I biotransformation metabolism and peroxisome proliferation which is characterised in rodents by increased peroxisomal volume density, accompanied by the transcriptional induction of peroxisomal proteins. The aim of the present work was to study peroxisome proliferation at the transcriptional level, in comparison to the transcription of the well-characterised *cytochrome P450 1A* gene (*cyp1a*) in the thicklip grey mullet *Chelon labrosus*. For this purpose, genes coding for the major peroxisomal membrane protein PMP70 and CYP1A were cloned using degenerate primers. Then juvenile mullets were single injected with B(*a*)P (5 mg/kg) and transcription of *palmitoyl-CoA oxidase (aox1), multifunctional protein (mfp1), 3-ketoacyl-CoA thiolase (thio), Δ^2,Δ^4 dienoyl-CoA reductase 2, pmp70, catalase* and *cyp1a* was semi-quantified in liver and gills 1 and 7 days after the injection. Transcription of *aox1* and *cyp1a* was induced in gills 1 day after B(*a*)P injection. *Cyp1a* transcription was also induced in mullet liver one day after injection, indicating that B(*a*)P was available for the liver. This was further proved by the significant accumulation of B(*a*)P-like metabolites in bile 7 days after the injection. In liver, *aox1, mfp1* and *thio* transcription was induced at day 1 followed by the induction of catalase transcription at day 7 that may reflect a response to an oxidative stress caused by B(*a*)P itself or by oxyradicals produced through the biotransformation metabolism and the peroxisomal β-oxidation. These hepatic responses were not reflected at AOX1 activity level. In conclusion, it has been shown for the first time that the three enzymes in the fish peroxisomal β-oxidation pathway are transcriptionally induced by B(*a*)P. It remains to be tested whether this enhanced transcription is reflected in an increase in the volume of peroxisomes.

1. Introduction

Organic toxic pollutants such as the model mutagenic polycyclic aromatic hydrocarbon (PAH) benzo(*a*)pyrene (B(*a*)P) are widespread in aquatic environments and upon uptake by biota they exert a wide variety of biological effects (Michel et al., 1992; Gunawickrama et al., 2008). Induction of biotransformation metabolism (Jönsson et al., 2006) and peroxisome proliferation (Au et al., 1999) are two examples of such effects that may be employed in sentinel fish as biomarkers of exposure to these compounds in pollution biomonitoring programmes. Mullets, such as thicklip grey mullets *Chelon labrosus*, may be an appropriate sentinel species for such monitoring programmes (Orbea et al., 1999, 2002; Bilbao et al., 2006; Ferreira et al., 2006) since they are abundant in coastal and estuarine habitats and are able to live in highly polluted environments where they have been described to accumulate high concentrations of PAHs and organochlorine contaminants (Ferreira et al., 2004, 2006).

Peroxisome proliferation is defined as a pleiotropic cellular response characterised by increased peroxisomal volume density (Fahimi and Cajaraville, 1995; Cancio and Cajaraville, 2000; Cajaraville et al., 2003a; Cajaraville and Ortiz-Zarragoitia, 2006) and is usually accompanied by the transcriptional induction of at least a subset of peroxisomal enzymes, consisting mainly of those enzymes involved in lipid homeostasis such as the three enzymes of the peroxisomal β-oxidation pathway; palmitoyl-CoA oxidase (AOX1), multifunctional protein (MFP) and 3-ketoacyl-CoA thiolase (THIO) (Qi et al., 2000) . Peroxisome proliferation has been reported in terms of AOX1 activity induction in laboratory experiments in fish such as *Sparus aurata* (Pedrajas et al., 1996), *Ictalurus punctatus* (Mather-Mihaich and Di Giulio, 1991), *Oncorhynchus mykiss* (Yang et al., 1990; Scarano et al., 1994; Oakes et al., 2005), *Danio rerio* (Ortiz-Zarragoitia

Abbreviations: AOX1, palmitoyl-CoA oxidase; MFP, multifunctional protein; THIO, 3-ketoacyl-CoA thiolase; DECR, Δ^2,Δ^4-dienoyl-CoA reductase 2; PMP70, peroxisomal membrane protein of 70 kDa; CAT, catalase; CYP1A, cytochrome P450 1A; PPAR, peroxisome proliferator activated receptor; PPRE, peroxisome proliferator response elements; RXR, retinoid X receptor; AhR, aryl hydrocarbon receptor; PAH, polycyclic aromatic hydrocarbon; B(*a*)P, benzo(a)pyrene; ROS, reactive oxygen species.

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and Cajaraville, 2005; Ortiz-Zarragoitia et al., 2006) and *Pimephales promelas*, *Semotilus atromaculatus, Notropis hudsonius* or *Catastomus commersoni* (Oakes et al., 2005) exposed to different chemical compounds. Fibrate hypolipidemic drugs, phthalates, PAHs, polychlorinated biphenyls, bleached kraft pulp and paper mill effluents, alkylphenols, estrogens and various pesticides have been reported to be peroxisome proliferating compounds in fish (reviewed by Cajaraville et al., 2003a). However, other studies have reported low or no sensitivity towards peroxisome proliferators in *Cyprinus carpio* and *Oryzias latipes* (Scarano et al., 1994; Hoff et al., 2003). In vertebrate responsive species, peroxisome proliferation is under the regulation of the peroxisome proliferator activated receptor α (PPARα), a transcription factor that belongs to the nuclear receptor superfamily (Isseman and Green, 1990), whose expression has been already reported in liver and gills of mullets (Ibabe et al., 2004; Raingeard et al., 2006, 2009). However, peroxisome proliferation has not yet been studied in mullets after treatment with any toxic organic compound. Moreover, peroxisome proliferation has never been studied at the transcriptional level in any fish species.

Similarly, PAHs can be biotransformed effectively in fish liver by phase I biotransformation metabolism, where cytochrome P450 1A (CYP1A) catalyses the conversion of parent compounds into easily excretable metabolites. *Cyp1a* gene expression is regulated in fish by the aryl hydrocarbon receptor (AhR) whose agonists are xenobiotics such as planar PAHs and polychlorinated biphenyls (PCBs) and dioxins (Hahn, 2002). Transcriptional induction of *cyp1a* is a sensitive and specific adaptive response of fish exposed to xenobiotics such as B(*a*)P (Hahn and Stegeman, 1994; Woodin et al., 1997; Jönsson et al., 2006), which is considered a potent AhR inducer (Hahn and Stegeman, 1994). Thus, regulation of gene expression by AhR plays a crucial role in the metabolism and clearance of xenobiotics that reach the organism from the environment (Hahn, 2002) Therefore, the transcriptional activation of *cyp1a* in fish exposed to organic compounds is commonly used as biomarker of exposure to planar organic compounds (Hahn and Stegeman, 1994). In addition, since some PAHs are excreted as polar metabolites in bile, the levels of biliary PAH metabolites are also sensitive biomarkers to assess recent exposure to PAHs (Van der Oost et al., 2003).

However, by catalysing the oxidative metabolism of lipophilic compounds through phase I biotransformation metabolism, many compounds such as B(*a*)P and other PAHs, are transformed to mutagenic/carcinogenic intermediates (Willett et al., 2000). In addition, highly reactive oxygen species (ROS), including superoxide anion, hydrogen peroxide and hydroxyl radicals are produced in this process as by-products (Cajaraville et al., 2003b; Jifa et al., 2006) causing oxidative stress. The same applies to the peroxisome proliferation process where an increased β-oxidation activity results in increased H_2O_2 production (Cajaraville et al., 2003b; Schrader and Fahimi, 2006). Different enzymatic and non-enzymatic mechanisms within the cell are involved in avoiding oxidative damage and among them, the main peroxisomal enzyme catalase (CAT) participates in the elimination of H_2O_2.

The aim of this work was to investigate the transcriptional regulation of peroxisomal genes in thicklip grey mullets *C. labrosus* in order to understand the peroxisome proliferation process in fish and to explore the possibility of using peroxisomal gene expression profiling as a sensitive biomarker of exposure to organic xenobiotics. For that purpose, the transcription level of several peroxisomal genes (*aox1, mfp1*, *thio*, *Δ^2,Δ^4-dienoyl-CoA reductase 2-decr-*, *peroxisomal membrane protein 70-pmp70-* and *cat*) was determined in liver of thicklip grey mullets injected with the model PAH B(*a*)P, which has been reported to induce AOX1 activity and increased peroxisome abundance in aquatic organisms (Cancio et al., 1998; Au et al., 1999; Cajaraville and Ortiz-Zarragoitia, 2006). Additionally, the transcriptional levels of *aox1*, *thio* and *cat* were measured in gills as indicators of the regulation of the peroxisomal β-oxidation and antioxidant response in this toxicologically relevant tissue. The transcription regulation of the well-characterised and B(*a*)P-responsive *cyp1a* gene in liver and gill and the presence of B(*a*)P bile metabolites were used as a positive control to see whether B(*a*)P injection protocol had been effective in *Chelon labrosus*. In this respect, a single dose of B(*a*)P was selected (5 mg/kg through intraperitoneal injection) because it is known to upregulate *cyp1a* in fish. Sampling was carried out 1 and 7 days after injection, as those are time windows that were expected to be crucial when assessing peroxisome proliferation transcriptionally and functionally.

2. Materials and methods

2.1. Materials

All chemicals were of analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise.

2.2. Animals and experimental procedure

Forty-five immature thicklip grey mullets *Chelon labrosus* (12 ± 5 cm) were collected from Plentzia (43°24′N, 2°56′W), a relatively unpolluted estuary (Orbea and Cajaraville, 2006) in the Biscay Bay in April 2005. Following previous studies, fish were acclimatised to laboratory conditions for 1 week. Water used during the experiment, which came from a clean site (Getaria, 43°18′12″N, 2°12′16″) and is used to keep shellfish for human consumption, had the same conductivity (48.7 mS) and pH (7.84) as water from Plentzia and was previously sterilised with UV light and filtered through active charcoal. Water was renewed and specimens were fed with dried bread daily during the experiment. Animals were maintained at a constant temperature of 20 °C and at a daily photoperiod with 11 h (8:00–19:00 hp) of light.

Mullets were randomly divided into 3 groups prior to the beginning of the experiment. Fish in the first group were intraperitoneally single injected with 5 mg of B(*a*)P/kg, following previous data available in the literature and using corn oil as vehicle. Fish in the second group were injected corn oil (control) and animals in the third group were not treated. One and seven days after the injection 7 fish (per group) were anesthetised by immersion in a saturated solution of 3-aminobenzoic acid ethyl ester before being sacrificed. Liver and gill samples were dissected, placed in RNAlater® and frozen in liquid nitrogen for gene transcription studies. Liver and bile samples were directly frozen in liquid nitrogen for biochemical and immunochemical studies and for the measurement of PAH metabolites, respectively. All the samples were stored at −80 °C.

2.3. Fixed wavelength fluorescence (FF) analysis

At day 7, bile samples of 5 control and 5 B(*a*)P injected organisms were diluted 1:1000 in 50% ethanol. Fixed wavelength fluorescence (FF) was then measured at the excitation/emission wavelength pairs 290/335, 341/383 and 380/430 nm, denoted FF290/335, FF341/383 and FF380/430, respectively. At FF290/335, mainly naphthalene type of low molecular weight PAH metabolites are detected. Benzo(*a*)pyrene (B(*a*)P) type of high molecular weight PAH metabolites are best detected at FF380/430 (Krahn et al., 1987). At FF341/383, mainly pyrene-derived metabolites are detected (Aas et al., 2000). Measurements were performed in quartz cuvettes on a SLM2 Aminco luminescence spectrometer (Spectronic Instruments, Rochester, NY, USA) and slit widths were set at 4 nm for both excitation and emission wavelengths. The FF values were recorded in arbitrary fluorescence intensity units. After subtraction of the signal levels of the solvent, values are represented as relative fluorescence values in comparison to control group.