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Short communication

Induction of CYP1A and ABC transporters in European sea bass (*Dicentrarchus labrax*) upon 2,3,7,8-TCDD waterborne exposure

Camilla Della Torre*, Michela Mariottini, Maria Luisa Vannuccini, Anna Trisciani, Davide Marchi, Ilaria Corsi

Department of Physical Earth and Environmental Sciences, University of Siena, Via Mattioli 4, 53100 Siena, Italy

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ABSTRACT

The aim of this study was to characterize the responsiveness of CYP1A and ABC transport proteins in European Sea bass (*Dicentrarchus labrax*) waterborne exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (2,3,7,8-TCDD) (46 pg/L) for 24 h and 7 days. Genes modulation (abcb1, abcc1-*2, cyp1a*), EROD activity were investigated in liver and 2,3,7,8-TCDD bioconcentration in liver and muscle. TCDD induced significantly *cyp1a* gene expression and EROD activity at 24 h and 7 d. A significant up-regulation of abcb1 was also observed but only after 7 days. No modulation of abcc1 and abcc2 genes was observed. Waterborne TCDD exposure was able to induce CYP1A and abcb1 encoding for P-glycoprotein in juvenile of European sea bass.

1. Introduction

Among organochlorines, the 2,3,7,8-tetrachlorodibenzo-para-dioxin (TCDD) is the most potent carcinogenic toxicant able to induce a wide spectrum of biological defects (Mandal, 2005; White and Birnbaum, 2009). Due to the high lipophilicity and low biodegradability, TCDD is persistent in the marine environment and bioaccumulate in food chains (up to pg/g in fish Nunes et al., 2011; Skinner, 2011) and be transferred to humans through diet (Domingo and Bocio, 2007).

The accumulation and consequent toxicity of noxious chemicals including TCDD in fish not only depends on the partition-equilibrium between the organisms and the surrounding aquatic environment but it is also strongly influenced by route of exposure (water and food) as well as by the organism's clearance/detoxifying capabilities (Focardi et al., 2005). In fact species-specific sensitivity to dioxin-like compounds has been documented, making difficult to predict the actual risk related to exposure to such highly toxic compound for fish species (Doering et al., 2013). In this view the characterization of the defence mechanism employed in response to toxic compounds exposure in fish will contribute to the development of appropriate practises for protection of endangered natural populations as well as the production of healthy farmed seafood.

The primary biological system for the first phase of detoxification of organic pollutants is the cytochrome P450s (CYP450) (van der Oost et al., 2003). Response of CYP450 and -CYP1A in particular- has been extensively studied in fish and is regulated by the aryl hydrocarbon receptor (AhR) (Hahn, 1998). A complementary mechanism involved in the cellular detoxification process is the multixenobiotic resistance mechanism (MXR) (Kurelec, 1992). The MXR activity is mediated by transport proteins, called ABC transporters, which hydrolyse ATP to pump out of cell a wide range of pollutants limiting their accumulation and toxicity (Xu et al., 2005). The most toxicological relevant ABC transport proteins are the P-glycoprotein (P-gp, encoded by abcb1) and multidrug resistance associated proteins (MRP1-5, encoded by abcc1-*5*) (Leslie et al., 2005). The P-gp is actively involved in the efflux of unmodified compounds while substrates of MRPs are products of phase I and II metabolism in the form of glutathione, glucuronic or sulphate conjugates, thus working as phase III (Haimeur et al., 2004).

The involvement of ABC transporters in the protective response against heavy metals (Miller et al., 2007; Zucchi et al., 2009; Della Torre et al., 2012a; Long et al., 2011) organic hydrocarbons (Doi et al., 2001; Costa et al., 2012; Diaz de Cerio et al., 2012; Yuan et al., 2014), pharmaceuticals (Caminada et al., 2008) and pesticides (Zaja et al., 2011) has been suggested in fish. Up to date no information are currently available on the involvement of these

* Corresponding author. Tel.: +39 (0)577232830; fax: +39 (0)577232806.
E-mail address: dellatorre2@unisi.it (C. Della Torre).

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proteins in 2,3,7,8-TCDD disposition and toxicity. Moreover the mechanisms of regulation of ABC transporters in fish are still unclear, and significant interest is now summoned by the possibility to link phases 0, I, II and III starting from gene transcription profile to get a clearer picture of cell detoxification pathways occurring in fish (Paetzold et al., 2009; Della Torre et al., 2010; Costa et al., 2013; Yuan et al., 2014).

The European sea bass (*Dicentrarchus labrax*) is considered a species of high commercial value in the Mediterranean area (FAO, 2003). It is also a relevant ecotoxicological model representative of estuarine and coastal environments (Almeida et al., 2012; Della Torre et al., 2012b). Significant levels of organochlorine compounds have been detected in both farmed and wild specimen of European sea bass (Corsi et al., 2003; Trocino et al., 2009; Carubelli et al., 2007; Greco et al., 2010; Nunes et al., 2011) highlighting the susceptibility of the species to bioconcentrate toxic environmental pollutants. Several ABC transporters (abcb1, abcc1-*2* and abcg2) have been identified in the European sea bass hepatocytes and the MXR sensitivity to exposure planar toxic hydrocarbons recently reported with a significant up-regulation of abcc1-*2* in response to benzo(a)pyrene (Ferreira et al., 2014).

The aim of the present study was thus to characterize the responsiveness of CYP1A, and ABC transport proteins towards waterborne exposure to 2,3,7,8-TCDD in juveniles of European sea bass.

2. Materials and methods

2.1. Animal treatments

Juveniles of European sea bass (*D. labrax*) (7–12 cm TL) were obtained from a fish farm (Agroittica Toscana, S.r.l., Piombino, Italy) and maintained for 96 h before the experiment in flow-through circulating aquarium containing 36‰ artificial sea water (protocol E 724-98 ASTM, 2004) at 16 °C, constant (12:12 light:dark) photoperiod without feeding. Fish husbandry and experimental procedures were conducted within the EU legislation for the protection of animals used for scientific purposes (Directive, 2010/63/EU).

Fish ($N = 15$) were exposed to 46 pg L^{-1} of 2,3,7,8-TCDD dissolved in dimethyl sulfoxide (DMSO 0.001‰). A group ($N = 15$) was exposed only to DMSO (0.001‰) and a control group ($N = 15$) was kept in clean artificial sea water. The chosen 2,3,7,8-TCDD concentration was far below lethal nominal concentration (Technical Report N°11, 2004- Australian Government, Department of Environment and Heritage) but still able to induce a significant biological responses in fish (Ortiz Delgado et al., 2008). The exposure was performed in 35 L aquaria. Animals were not fed during the experiments; pH, T, salinity of sea waters were maintained constant during the experiments. A semi-static experimental condition was chosen and test solution of 2,3,7,8-TCDD and DMSO (0.001‰) were renewed every 24 h in order to maintain relatively consistent levels of chemical exposure. Samples collection was done after 24 h and 7 days of exposure in order to address time-dependent biological response. Fish were anesthetized with clove oil and then sacrificed by blow in the head and liver excited and stored at −80 °C for molecular and biochemical analysis. Aliquots of liver and muscle from each individual were pooled and stored at −20 °C for 2,3,7,8-TCDD residue analysis.

2.2. Gene expression by real-time PCR (q-PCR)

RNA isolation and cDNA generation was performed according to Della Torre et al. (2012b). Specific primers for *cyp1a*, abcb1, abcc1-*2* and the housekeeping *r18S* were designed for q-PCR using IDTDNA www.idtdna.com (Table 1). Q-PCR was performed in Stratagene 3000xP thermal cycler. Each amplification reaction contained 10 μl SYBRGr® Green (Biorad), 0.75 μl of Forward and Reverse primers 10 μM and 1 μl cDNA in 25 μl total volume. The cycling parameters were: 3 min denaturation at 95 °C, 40 cycles at 95 °C for 45 s, annealing at 55 °C for 60 s, elongation at 72 °C for 60 s, followed by the melting curve analysis. The expression of target genes was calculated as relative abundance respect to the housekeeping *r18S* using Q-Gene application (http://www.qgene.org).

2.3. EROD activity

The S9 liver fractions were obtained according to Della Torre et al. (2008). EROD activity was measured according to the fluorimetric methods for 96 well microplate described by Eggens and Galgani (1992). Total proteins were measured according to Bradford (1976) using a Shimadzu UV-160A visible recording spectrometer and BSA as standard.

2.4. TCDD residue analysis

Extraction from liver and muscle was carried out with an Accelerated Solvent Extractor system (ASE 200, Dionex), using toluene (60 ml) (US EPA, 1996). All the extracts were purified on multilayer silica, alumina and carbon columns for the 2,3,7,8-TCDD (Dioxin-prep, Supelco). For determination of 2,3,7,8-TCDD we used isotope dilution according to US EPA 1613 method revision B (US EPA, 1994). Two μl of extract was injected in a gas chromatograph with *Polaris* MS ion trap detector. 2,3,7,8-TCDD was identified and quantified using HRGC-MS (ion Trap mass spectrometer; Trace™ GC 2000 with AS3000 autosampler, ThermoFinnigan). The quality assurance and quality control (QA/QC) of the procedure were tested by analysing two replicates of Certified Reference Material WMF-01 (fish tissue), from Wellington Laboratories Inc. (average error of 10%). 2,3,7,8-TCDD recovery was higher than 62%. Results were blank corrected. The analytical detection limit was used based on a signal-to-noise ratio of 3. Instrumental detection limit (IDL) for 2,3,7,8-TCDD was estimated 0.2 pg/μl. Limit of detection (LOD) for the 2,3,7,8-TCDD was 0.001 ng g^{-1} wet weight. A solvent blank was analysed to check the response of chromatography.

2.5. Statistical analysis

Data were expressed as mean ± standard deviation (s.d.). Data were log transformed and the effects of time and treatments in the expression of *cyp1a* and abc genes and EROD activity were made by the ANOVA test using Bonferroni post-hoc, taking $p = 0.05$ as significant cut-off. All calculations were performed using the STATISTICA 7 software.

3. Results

As no differences in any biological parameter and 2,3,7,8-TCDD accumulation were found between untreated and DMSO-exposed specimens, fish exposed only to artificial sea water were taken as reference group for further analysis.

A significant up-regulation of *cyp1a* gene was observed in 2,3,7,8-TCDD exposed group respect to controls at both 24 h and 7 days of treatment. After 7 days of 2,3,7,8-TCDD exposure *cyp1a* relative abundance was significantly higher than after 24 h.

Concerning abc genes expression a significant up-regulation of abcb1 up to 2-folds respect to controls was measured after 7 days of exposure. *Abcb1* gene relative abundance increased significantly from 24 h to 7 days in fish exposed to 2,3,7,8-TCDD. No modulation was observed for abcc1 and abcc2 genes upon 2,3,7,8-TCDD