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Presence and inducibility by β-naphthoflavone of CYP1A1, CYP1B1 and phase II enzymes in *Trematomus bernacchii*, an Antarctic fish

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Abstract

This study investigated some aspects of xenobiotic metabolism in the Nototheniidae *Trematomus bernacchii*, a key sentinel species for monitoring Antarctic ecosystems. After laboratory exposure to β-naphthoflavone (βNF), basal levels and time-course induction of CYP1A, CYP1B and CYP3A were measured as enzymatic activities, immunoreactive protein content and mRNA expression in liver, gills, intestine and heart. Additional analyses in the liver included enzymatic activities of testosterone hydroxylase, (ω)- and (ω − 1)-lauric acid hydroxylase and some phase II enzymes related to the AhR battery genes, DT-diaphorase, glutathione *S*-transferases and UDP-glucuronyl transferases. Responsiveness of hepatic CYP1A1 after exposure to βNF demonstrated an higher sensitivity of MEROD than EROD activity and long lasting expression of mRNA still induced after 20 days from the treatment. Testosterone metabolism, oxidation of lauric acid and activities of phase II enzymes were not affected by βNF indicating that their modulation is not mediated by Ah receptor. Induction of CYP1A was more limited in gills and absent in intestine and heart. The first nucleotide sequence for CYP1B1 in an Antarctic fish has been obtained, revealing a homology of 89% and 72% respectively to CYP1B1 of plaice and CYP1B2 of carp. Constitutive expression of CYP1B1 was restricted to gills where it was also induced by βNF. Obtained results represent an additional contribution to the ecotoxicological characterization of *T. bernacchii* and further support the use of biomarkers for early detection of chemical pollution in Antarctica.

Keywords: β-Naphthoflavone; CYP1A1; CYP1B1; UDP-GT; GST; DT-diaphorase; *Trematomus bernacchii*; Antarctic fish

1. Introduction

Cytochrome P450 (CYP) enzymes constitute a unique, widespread superfamily of heme-containing proteins metabolising endogenous and exogenous compounds like drugs, carcinogens, steroids and fatty acids (Nelson et al., 1996). Some of these enzymes are specifically inducible by their substrates (Nelson et al., 1996) and CYP1A is recognized as an important biomarker of exposure to polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and halogenated dibenzo-*p*-dioxins (Goksoyr, 1995). In most fish species CYP1A is expressed at very low level and it is effectively induced by many planar hydrocarbons through their binding to a cytosolic aryl hydrocarbon receptor (AhR).

This receptor, after ligand binding and dimerization with the AhR nuclear translocator (ARNT), acts as a transcriptional activator at the responsive elements (XREs) in the promotor of CYP1A1 and other responsive genes (Hankinson, 1995; Handschin and Meyer, 2003).

In mammals, the battery of AhR-responsive genes include CYP1A1, CYP1A2, CYP1B1 and some phase II enzymes such as uridine 5′-diphosphate-glucuronyl transferase (UDP-GT), glutathione *S*-transferases (GST) and NAD(P)H: quinone oxidoreductase (DT-diaphorase) (Nebert et al., 1990). Although considerable progress has been made in the characterization of AhR system in fish (Hahn, 2002), the genes activated through this receptor by PAHs remain largely unknown in several marine species. It has been demonstrated that β-naphthoflavone (βNF)

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and 3-methylcholanthrene (3MC) induce CYP1A1, UDP-GT and GST in rainbow trout *Oncorhynchys mykiss* (Zhang et al., 1990), βNF induces CYP1A1 and UDP-GT in sea bass *Dicentrarchus labrax* (Novi et al., 1998), and CYP1A1 in dab *Limanda limanda* (Lemaire et al., 1996) and in gilthead seabream *Sparus aurata* (Pretti et al., 2001).

Besides rainbow trout where CYP1A1 and 1A3 have been found (Ragergh et al., 2000), teleosts possess a single gene of CYP1A subfamily and possibly at least one member of the CYP1B subfamily (cytochrome P450 Home page, http://drnelson.utmemm.edu/Cytochrome P450.html). In this respect, CYP1B1 was first cloned in plaice *Pleuronectes platessa*; it was found expressed in gills and not inducible by βNF (Leaver and George, 2000). More recently, CYP1B1 and 1B2 were characterized in the carp *Cyprinus carpio* where they are constitutively expressed and induced by PAHs in the gills and, for CYP1B1, also in liver and intestine (El-Kady et al., 2004a,b). These findings indicate an elevated complexity about the presence and regulation of CYP1B in fish, highlighting the importance of additional studies on this system which in mammals is involved in the metabolism of hormones such as 17β-estradiol and in the activation of several PAHs to carcinogenic metabolites (Shimada et al., 1996). In several fish species CYP3A-like proteins are not inducible by βNF and constitutively expressed, showing the oxidation of testosterone and lauric acid (Miranda et al., 1989; Pretti et al., 2001).

Similar investigations are of particular interest for Antarctic organisms due to the limited knowledge on their responsiveness to pollutants and the need of biomonitoring the constant increase of human activities. Several factors have been suggested to modulate sensitivity of Antarctic organisms and specific metabolic responses to xenobiotics, including the extreme environmental conditions of the Southern Ocean, the marked seasonality of food supply and local characteristics associated with naturally elevated bioavailability of cadmium (Regoli et al., 1998, 2000, 2002, 2005). Previous studies on the fish *Chionodraco hamatus* and *Trematomus bernacchii* exposed to PAHs demonstrated a significant induction of CYP1A as both EROD activity and protein content (Focardi et al., 1995; Regoli et al., 2005) and a different mRNA expression of CYP1A has been demonstrated in *T. bernacchii* from a polluted and a clean site (Miller et al., 1999).

T. bernacchii is a benthic opportunistic feeder, nearly ubiquitous in Antarctic ecosystems, and considered a key indicator species for monitoring anthropogenic impact. In this respect, metabolism and interactions between different classes of pollutants have been investigated in this species through a wide panel of biological responses, i.e. inducibility of CYP1A and metallothioneins, oxyradical metabolism and susceptibility to oxidative stress, onset of DNA damages and vitellogenin gene expression (Regoli et al., 2005; Canapa et al., 2007). The aim of this work was to further characterize the time-course of responsiveness to chemicals in *T. bernacchii* after laboratory exposures to βNF. Basal levels and induction of CYP1A, CYP1B and CYP3A were measured in liver, gills, intestine and heart, as enzymatic activities, immunoreactive protein content and mRNA expression. Hepatic responses were also analyzed as enzymatic activities of testosterone hydroxylase, (ω)- and (ω − 1)-lauric acid hydroxylase as markers of CYP3A; DT-diaphorase, glutathione *S*-transferases and UDP-glucuronyl transferases were measured as phase II enzymes related to the AhR battery genes. The overall results were expected to extend our knowledge of the drug metabolising system of *T. bernacchii* and to improve the use of biomarkers in this sentinel species for early detection of biological disturbance in Antarctic marine ecosystem.

2. Materials and methods

2.1. Chemicals

β-Naphthoflavone (βNF), methoxyresorufin, resorufin, ethoxyresorufin, and ethoxycoumarin were obtained from Sigma Chemical (St. Louis, USA). Testosterone and its metabolites were obtained as previously reported (Longo et al., 1991). Rabbit polyclonal antibody against rat CYP1A1 was purchased from Gentest (Woburn, USA), while the antibody anti-trout CYP3A27 was kindly supplied by Dr. M. Celander (Göteborg, Sweden). All chemicals and reagents were of analytical grade.

2.2. Experimental design and preparation of microsomes

Sexually mature *T. bernacchii* (approximate length range 18–24 cm, weight range 80–180 g) were sampled during the XIX Italian Antarctic Expedition (austral summer 2003–2004) from Tethys bay, a pristine area near the Italian base at Terra Nova Bay. Fish were acclimatized for 1 week in aquaria with running seawater at a controlled temperature of $-1 \pm 0.5\,^{\circ}C$. Fish were randomly selected in the study groups with various sizes similarly distributed and without consideration of sex differences: a previous study demonstrated that activities of cytochrome P450 did not differ between males and females of *T. bernacchii* sampled in different points of reproductive cycle and with different seasonal food availability (Canapa et al., 2007). Organisms were intraperitoneally injected with a single dose of βNF (10 mg/kg dissolved in corn oil); injected volumes were 100 μl per 100 g fish weight in all the experiments and control organisms were treated with corn oil only. Fish were sacrificed after 1, 3, 7 and 20 days from injection; liver, gills, intestine and heart were rapidly excised from five specimens for each group, frozen in liquid nitrogen and maintained at $-80\,^{\circ}C$ until analyses. Microsomes and cytosolic fractions were prepared after $100{,}000 \times g$ centrifugation as previously described (Longo et al., 1991). Protein concentrations were measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.3. Enzyme assay

Cytochrome P450 (CYP) content was measured in liver by the method of Omura and Sato (1964). Ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MEROD) activities were determined in liver, gills, intestine and heart by measuring the production of resorufin, as previously reported (Lubet et al., 1985), with a Perkin-Elmer