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Metal detoxification in the marine teleost fish *Sparus aurata* L. and *Dicentrarchus labrax* L.

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ABSTRACT

Transcription of ATP-binding cassette (ABC) transporters has been evaluated in cell lines and primary cultures from gilthead seabream and European sea bass teleost fish exposed to methylmercury (MeHg), arsenic, cadmium or lead. The mRNA expression levels showed *abcb1*, *abcc2* and *abcc5* constitutive gene expression in all seabream tissues analyzed; however, we were unable to detect any constitutive transcription of *abcb1* in many of the sea bass tissues. Furthermore, ABC mRNA expression levels were all affected by metal exposure, especially in the case of fish cell lines and erythrocytes, and greatly depended on cell type and fish species. Thus, while ABC transcription was up-regulated in the seabream cell line it was down-regulated in the sea bass cell line, while the opposite occurred in the primary cultures. All these data point to the importance of ABC transporters in metal detoxification and in the differential regulation in seabream and sea bass cells.

1. Introduction

In recent years, the contamination of aquatic systems by metals has triggered public concern about possible health risks and ecological damage. Increased human exposure to metals has been recognized as being related to the ever-increasing use of these substances in industrial, agricultural, domestic and technological products (Bradl, 2005; Duffus, 2002). The risks exist because some metals may bioaccumulate and biomagnify as they pass through the aquatic food web (Monroy et al., 2014). The EPA (Environmental Protection Agency) recommends minimizing the intake of certain fish species due to the high levels of Hg (Morcillo et al., 2017b). Therefore, it is important for fisheries and the aquaculture industry to know the extent of metal accumulation in fish, the body/tissue distribution pattern and biological effects, as well as the levels that may be transferred to humans. To help in this task, the use of cell lines or primary cell cultures has been successfully applied in fish for toxicological purposes, as they have in mammals (Bols et al., 2005; Yin et al., 2007). Indeed, many cytotoxicity studies have positively related *in vitro* and *in vivo* EC_{50} values in fish exposed to different contaminants, demonstrating the value of using fish cell lines or primary cell cultures in this field of research (Fent, 2001; Segner, 2004). Thus, our previous findings showed that *in vitro* metal (Pb, As, Hg and Cd) exposure induced cytotoxicity, oxidative stress, immunotoxicity and finally cell death, mainly through apoptosis, in primary cultures and cell lines from gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) (Morcillo et al., 2015a, b, 2016a, b, c, 2017a), the most widely farmed fish species in the Mediterranean area. However, apart from metallothioneins (MT), little attention has been given to the detoxification mechanisms that exist in fish species in order to reduce and survive the toxic effects of metal pollutants.

Such detoxification mechanisms include the activity of the ATP-binding cassette (ABC) superfamily of highly conserved transport proteins, which have been described in an increasing number of fish species (reviewed by Ferreira et al., 2014). These proteins include multidrug resistance-associated proteins (MRPs, ABCC) and P-glycoprotein (P-gp, ABCB) (Ayrton and Morgan, 2001). Of these, ABCB1 (P-gp, MDR1) is the most relevant protein in the efflux of xenobiotics in mammals (Doyle and Ross, 2003) and it has been detected in several fish species (Ferreira et al., 2014). ABCB1 is located in the apical membrane of polarized cells, where it facilitates the movement of substances and promotes the excretion of xenobiotics (Nornberg et al., 2015). However, ABCC transporters in cells vary, and may be localized in the apical or basolateral membranes, depending on the cell types (Luckenbach et al., 2014). ABCC1 and ABCC2 are the best characterized transporters, the evidence from animal models suggesting they play a role in organ defense, while other members like ABCC3, 4 and 5, have been far less studied (Keppler and Konig, 2000). ABC transporters have

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Table 1
Values of EC_{50} (mM) for gilthead seabream and European sea bass primary cultures of head-kidney leucocytes (HKLs), peripheral blood leucocytes (PBLs), erythrocytes and cell lines exposed to metals for 24 h.

Metal	HKLs		PBLs		Erythrocytes		Cell lines	
	Gilthead seabream	European sea bass	Gilthead seabream	European sea bass	Gilthead seabream	European sea bass	Gilthead seabream SAF-1	European sea bass DLB-1
Cd	0.082	1.5	0.179	2.6	0.022	0.080	0.1	0.004
MeHg	0.012	0.3	0.014	0.542	0.021	0.0028	0.015	0.013
Pb	2.8	3.4	2.6	4.2	0.523	1.1	3.2	1.6
As	2.7	3.2	1.5	4.2	0.134	0.190	0.082	0.030
Reference	Morcillo et al. (2015a)	Morcillo et al. (2015b)	Morcillo et al. (2016b)	Morcillo et al. (2016b)	Morcillo et al. (2016c)	Morcillo et al. (2016c)	Morcillo et al. (2016a)	Morcillo et al. (2017a)

been reported to promote resistance against metals in some mammals and fish species. Thus, the piscine cell line, PLHC-1 exposed to Hg for 72 h induced MRP gene expression and efflux activity (Della Torre et al., 2012). Cd-resistant zebrafish ZF4 cells exhibited an accelerated rate of Cd excretion, enhanced activity of MRP-like transport and the elevated expression of ABCC2 and ABCC4 (Long et al., 2011).

In the present study, several ABC genes were identified/analyzed in the gilthead seabream and European sea bass databases and their transcriptional regulation by Cd, As, Hg and Pb exposure was evaluated in primary cultures of seabream and sea bass head-kidney leucocytes (HKLs), peripheral blood leucocytes (PBLs) and erythrocytes, as well as in seabream SAF-1 and sea bass DLB-1cell lines. The results are discussed to throw light on the mechanisms involved in detoxification after metal exposure in these marine fish species.

2. Material and methods

2.1. Animals

Sixty specimens (80 ± 20 g mean weight) of the seawater teleosts gilthead seabream and European sea bass, obtained from a local farm (Murcia, Spain), were kept in seawater aquaria (250 L) in the Marine Fish Facility at the University of Murcia. The water was maintained at 20 ± 2 °C, with a flow rate of 900 L/h, and 28‰ salinity. The photoperiod was 12 h light: 12 h dark and fish were fed with a commercial pellet diet (Skretting) at a rate of 2% body weight per day. The fish were allowed to acclimatise for 15 days before the start of the experimental trial. All experimental protocols were approved by the Bioethical Committee of the University of Murcia.

2.2. Tissue and cell isolation

In order to obtain seabream and sea bass cell suspensions, fish were anaesthetised with 0.21 mM benzocaine (stock dissolved in 4% acetone) (Sigma) and samples were taken under sterile conditions. Briefly, for HKL isolation, head-kidney (HK) tissue was obtained by dissection, transferred to sRPMI [RPMI-1640 culture medium (Life Technologies) supplemented with 0.35% sodium chloride, 100 IU mL^{-1}, penicillin (Life Technologies), 100 mg mL^{-1} streptomycin (Life Technologies) and 5% fetal bovine serum (FBS; Life Technologies)] and passed through a nylon mesh (Morcillo et al., 2015a, 2015b). To isolate PBLs and erythrocytes, blood was withdrawn from the caudal vein with a heparinized syringe and the fish were immediately returned to aquaria. Blood was layered over a 51% Percoll density gradient and centrifuged to separate PBLs and erythrocytes (Morcillo et al., 2016b, 2016c). The cell lines, SAF-1 (ECACC 00122301, Public Health England), derived from the gilthead seabream fin (Béjar et al., 1997), and DLB-1, obtained in our laboratory from the European sea bass brain (Morcillo et al., 2017a), were cultured in 75 cm^2 plastic tissue culture flasks (Nunc) at 25 °C in an incubator using L-15 Leibowitz medium supplemented with penicillin, streptomycin and 10% FBS. Subcultures were made every week with 0.25% (w/v) trypsin (Sigma-Aldrich) plus 0.02% (w/v) ethylendiaminotetracetic acid (EDTA) solution (Sigma-Aldrich). In all cases, cells were washed, counted and adjusted accordingly. Cell viability was determined by the trypan blue exclusion test and was seen to be higher than 98%.

For gene expression and tissue distribution under naïve conditions, brain, gill, liver, skin, gonad, gut, head-kidney, spleen and thymus fragments from three independent fish were removed and immediately frozen in TRIzol Reagent (Life Technologies) at −80 °C until use for RNA isolation.

2.3. Metal exposure and viability

Different metal salts (Sigma-Aldrich) were used: Cadmium chloride ($CdCl_2$), Methylmercury (II) chloride [CH_3HgCl (MeHg)], lead (II) nitrate ($Pb(NO_3)_2$) and trioxide arsenic (As_2O_3). Cell treatments and cytotoxicity assays were carried out as previously described (Morcillo et al., 2015a, b, 2016a, b, c, 2017a), Samples consisted of cells unexposed (controls) or exposed to the EC_{50} of the metals (Table 1) for 24 h at 25 °C. After exposure, cells were collected and TRIzol Reagent was added to extract the total RNA.

2.4. Gene sequences search and bioinformatics analysis

According to the published sequences of ABC transporter orthologues, a BLAST analysis was made through the NCBI (http://www.ncbi.nlm.nih.gov/) and European sea bass genome (http://seabass.mpipz.mpg.de/) databases. Deduced protein sequences from the full or partial gene sequences were obtained (gilthead seabream *abcb1*, *abcc2* and *abcc5*; European sea bass *abcb1*, *abcc1* and *abcc2*) and analyzed for similarity with known orthologue sequences and domain conservation using the BLAST program (Altschul et al., 1990) within the ExPASy Molecular Biology server (http://us.expasy.org). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 6) (Tamura et al., 2013) to confirm that they were expected *bona fide* sequences.

2.5. Gene expression

Total RNA was isolated from frozen samples of TRIzol Reagent following the manufacturer's instructions. The RNA was then treated with DNase I (Promega) to remove genomic DNA contamination. Complementary DNA (cDNA) was synthesized from 1 μg of total RNA using SuperScript III reverse transcriptase (Life Technologies) with an oligo-dT18 primer. The expression of the selected genes was analyzed by real-time PCR, which was performed with an ABI PRISM 7500 instrument (Life Technologies) using SYBR Green PCR Core Reagents (Applied Biosystems). Reaction mixtures (containing 10 μL of 2 × SYBR Green supermix, 5 μL of primers (0.6mMeach) and 5 μL of cDNA template) were incubated for 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C, 1 min at 60 °C, and finally 15 s at 95 °C, 1 min at 60 °C and 15 s