Environmental Toxicology and Pharmacology 28 (2009) 439–447

Contents lists available at ScienceDirect

Environmental Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/etap

Metallothioneins and heat shock proteins 70 in marine mussels as sensors of environmental pollution in Northern Adriatic Sea

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ARTICLE INFO

Article history:
Received 25 May 2009
Received in revised form 17 July 2009
Accepted 28 August 2009
Available online 6 September 2009

Keywords:
Biomonitoring
Ecotoxicity
Mussel *Mytilus galloprovincialis*
Heavy metal contamination
Metallothioneins
Heat shock protein 70

ABSTRACT

In an attempt to assess the intensity of environmental pollution in industrial zones of Kvarnerian Bay in Northern Adriatic Sea and the reactivity of *Mytilus galloprovincialis* to these changes, in this study we estimated the concentration of heavy metals at four locations in both sea-sediment and in the mussels. Further we tried to correlate these changes with seasonal variations in environmental temperature, pH and salinity, as well as with the expression of metallothioneins (MTs) and heat shock proteins (HSPs) in the digestive tract of the mussels. Sampling *in vivo* was performed monthly, during the year 2008, while under the laboratory conditions the reactivity of acclimated mussels were tested to increasing concentrations of $CdCl_2$ and to thermal stress. The data have shown that the induction of MTs and HSP isoforms of the 70-kDa size class were highly affected by model agents treatment including contamination of sea-sediment by Pb, Hg and Cd, implying that these stress proteins might be power biomarkers of marine pollution.

1. Introduction

In order to protect and preserve the natural environment and quality of the water in the Adriatic Sea, which is a narrow part of Mediterranean Sea, constant monitoring of the environmental pollution of the coastal industrial area is performed in accordance with the strictest criteria. Mediterranean marine mussels *Mytilus galloprovincialis* are widely used as sentinel organisms, which according to the recent studies promptly react to various pollutants by synthesis of evolutionary highly conserved stress proteins, such as metallothioneins (MTs) and heat shoch proteins (HSPs) that play also a key role in cellular protection against environmental stress. Thus, in aquatic invertebrates MTs might be transcriptionally induced by the essential and non-essential metals, such as Cu and Zn or Cd, Pb, Ag and Hg, respectively (Isani et al., 2000; Ivanković et al., 2005; Ng and Wang, 2005; Wang and Rainbow, 2005), as well as by oxidative stress and inflammation (Amiard et al., 2006; Coyle et al., 2002; Lynes et al., 2007; Sarkar et al., 2006), while the different forms of HSPs might be induced by elevation of temperatures (Castelli et al., 2004), as well as by a great variety of chemical stressors, that might be often found in seawater or in the sediment (Choi et al., 2008; Ivanina et al., 2009; Kinder et al., 2007; Pantzartzi et al., 2009; Rosen et al., 2008). There is, however, the wide variation among and within species in the constitutive expression of MTs and HSPs, as well as in the threshold of their induction, showing that the expression of these stress proteins depends on a variety of physiological and environmental conditions.

In an attempt to assess the intensity of pollution of Kvarnerian Bay in Northern Adriatic Sea, which is still not well studied on an environmental point of view, in this study we analyzed the concentration of heavy metals (Pb, Hg and Cd) in sea-sediment and in local mussels. Further, trying to assess the reactivity of *M. galloprovincialis* to these changes, tissue expression of MT I+II protein and HSP70 was analyzed both *in situ* and in laboratory conditions.

2. Material and methods

2.1. Mussels collection and sample preparation

Mussels *M. galloprovincialis* Lamarck, 1819 (Mollusca – Bivalvia) were collected at four different regions in Northern Adriatic Sea (Fig. 1): S1 ("Rijeka"-port; N: 45°19.316′); S2 (shipyard "3. Maj"; N: 45°20.093′); S3 ("Kantrida"-periurban zone; N: 45°20.333′) and S4 ("Limski kanal"-mariculture area; N: 45°07.906′).

To check the seasonal variations the collection of wild mussels was performed monthly, during the 1-year period (2008). Bivalves were transported to the laboratory in the ambient seawater, where the gills and digestive glands were dissected, immediately frozen in liquid nitrogen and stored at −80 °C before determination of heavy metals and MTs.

For *in vitro* studies wild population of mussels were collected at mariculture area S4 ("Limski kanal"). The transportation in the laboratory was performed in aerated aquaria with ambient seawater. Their reactivity to heat shock and $CdCl_2$ was then tested after the period of acclimatization for 3 days on 18 °C (room temperature).

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doi:10.1016/j.etap.2009.08.005

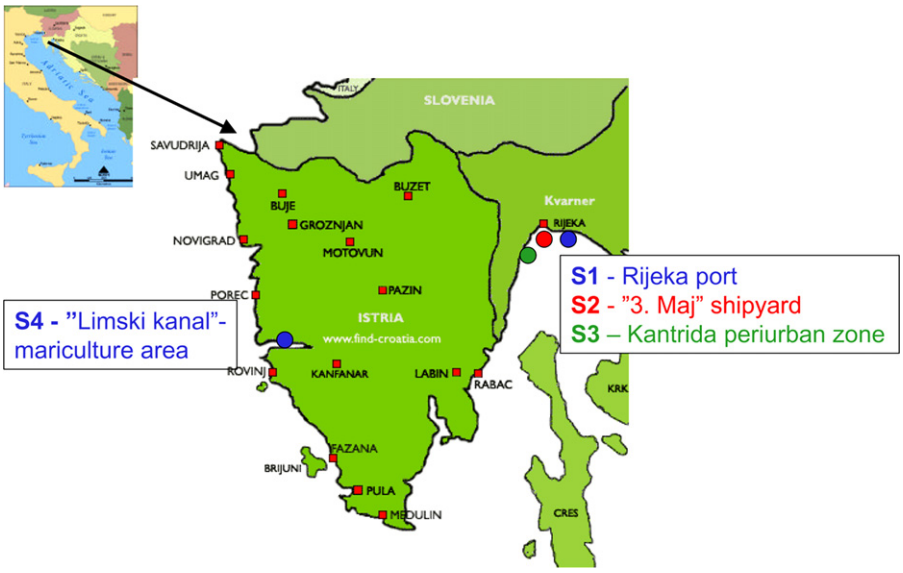

Fig. 1. A map showing mussels sampling stations in the Kvarnerian bay of North Adriatic Sea in Croatia.

Digestive gland tissues from five mussels was pooled and homogenized in 0.2 ml buffer (0.5 M NaCl, 0.1 M Tris, 0.01 M EDTA, pH 7.5) containing 10 μl 40 mM PMSF (Sigma; P7626) in acetone. Secondary homogenization was done after dilution with 0.3 ml of the same buffer. After centrifugation (12,000 × *g*/4 °C/10 min), supernatants were stored at −20 °C.

2.2. Sea-sediment collections

The surface layer of sediment (0.5 cm) was collected by SCUBA diving as previously described (Traven et al., 2008). The sea depth varied between 5 and 39 m. After sampling the sediments were frozen in polyethylene bags and kept at −20 °C until further processing.

2.3. Environmental temperature, salinity and pH

Surface seawater temperature and salinity (0.5 m) was recorded at regular intervals using reversing Amarell GmbH&Com KG Toluene thermometer and a Yeo-Kal MKII high precision salinometer. pH was measured by MP220 basic pH/mV^0/C Meter (Mettler Toledo; USA).

2.4. Heavy metal analyses

Heavy metals (Pb, Hg and Cd) were analyzed in the sea-sediment and in the soft tissue of mussels. Samples ($N = 120$) were dried in an oven (Memmert – UNE 500; Germany) at 105 °C overnight or through several hours, respectively, until the constant dry weight was achieved. Tissue water content, calculated as a percentage from wet and dry tissue mass of mussels was between 0.2 and 0.3%.

Specimens (1.0 or 0.5 g) were then heated with HNO_3 (65%; Suprapur; Merck) in a microwave oven (High performance microwave unit; Milestone 1200 mega). Heavy metal content was determined with Atomic Absorption Spectroscopy (PerkinElmer; Analyst 600; USA) with autosampler (PerkinElmer; AS 800; USA). Hg was determined with the Flow Injection Mercury System (PerkinElmer; FIAS 400; USA), whereas Pb and Cd were determined with Graphite Furnace Atomic Absorption Spectroscopy (GFAAS) method. As heavy metal standards IAEA 407 and IAEA 436 (International Atomic Energy Agency; Reference material; Austria) were used.

Concentration standards were 0.216–0.228 mg/kg (for Pb), 4.04–4.34 mg/kg (for Hg) and 0.050–0.054 mg/kg (for Cd). Measurements were performed at a fixed wavelength of 283.3 nm (for Pb), 253.7 nm (for Hg) and 228.8 nm (for Cd).

2.5. Determination and quantification of heat shock protein 70

For detection of HSP 70 proteins the cellular extracts were prepared by homogenization of digestive glands of mussels (10 specimens per group, made in triplicat) in SDS sample buffer containing 150 mM NaCl, 10 mM M Tris–HCl pH 7.6, 1% SDS, 1% sodium deoxycholate and 1 mM phenylmethylsulfonyl fluoride (Sigma; P7626).

HSPs were detected by Western blot analysis, using a slight modification of the previously described method (Hamer et al., 2004; Piano et al., 2004). Briefly, proteins were separated by reducing SDS-PAGE and transferred onto PVDF Western blotting membrane (Roche Diagnostics; 301040) using a transfer buffer containing 150 mM glycine (Sigma) in 20 mM Tris (Sigma; T6687) and 20% methanol (v/v; Merck). Transfers were carried out at 60–70 V for 90 min using Hoefer-miniVE blotter. Membranes were washed in Tris/HCl-buffered saline (TBS) (50 mM, pH 7.5) and blocked overnight at +4 °C in TBS, which contained 0.05% Tween 20 and 2% BSA followed by 1 h incubation with primary monoclonal antibody (mAb) against bovine brain HSP70 (H-5147; Sigma), three cycles of washing (TBS with 0.1% Tween 20; TBS-T buffer) and 45 min incubation with peroxidase-conjugated secondary antibody anti-mouse IgG1 (Sigma). The immune complexes were visualized by using a substrate solution containing 0.013 mM diaminobenzidin (DAB-3.3′-Diaminobenzidine, D5637; Sigma), 0.02% H_2O_2 and 0.03% NiCl/CoCl in PBS. For a negative control, Western blot extract was propped with isotype-matched irrelevant mAbs (IgG1) (Jackson ImmunoResearch Laboratories).

Signals of bands on Western blot membranes were quantified with a calibrated imaging densitometer (Image Station 446CF Kodak; program Kodak ID3.6)

2.6. Exposure to heavy metals in laboratory conditions

Mussels obtained from location S4 (Limski kanal, mariculture) were transported in large containers to the laboratory in Rijeka, where they were kept in well-aerated aquaria (60 lit). Temperature, salinity and pH of seawater in aquarium were maintained constant i.e. in the range of 18 °C ± 0.2 °C, 35.0 ± 0.30 (psu) and 8.10 ± 0.10 (pH). After the acclimatization ten mussels per treatment were then exposed during 24 h to increasing concentrations (from 100 to 500 μg/l) of $CdCl_2$ (PA; Acros Organics; 219141000). Extracted digestive glands were dissected and fixed in 10% buffered formalin solution for immunohistochemical analysis of MTs, or stored at −80 °C for HSPs determination.

2.7. Tissue preparation

The digestive glands dissected from *M. galloprovincialis* were fixed in 10% buffered formalin solution, for a minimum of 24 h. Tissue was then inserted in paraffin wax and sections were cut at 4 μm using HM 340E microtome (Microtom; Germany). Heat induced epitope retrival was done prior to staining procedures by heating tissue slides in boiled citrate buffer pH 6.0 four times, each 5 min, using a microwave steamer.

2.8. Immunohistochemistry

Immunohistochemical studies on digestive gland cells from *M. galloprovincialis* were performed on paraffin embedded tissues using DAKOEnVision+System, Peroxidase (DAB) kit according to the manufacturer's instruction (DAKO Corporation, USA), as previously described (Jakovac et al., 2006). Shortly, endogenous peroxidase activity was eliminated by peroxidase block. Monoclonal anti-MT I+II antibody (cloneE9; Dako Cytomation, USA) diluted 1:50 in phosphate-buffered saline supplemented with bovine serum albumin was added to tissue samples and incubated overnight at 4 °C in a humid environment, followed by 45 min incubation with peroxidase labeled polymer conjugated to goat anti-mouse immunoglobulins containing carrier protein linked to Fc fragments to prevent nonspecific binding. The immunoreaction's product was visualized by adding substrate-chromogen (DAB) solution. Tissues were counterstained with hematoxilin and 37 mM ammonia water, dehydrated in gradient of alcohol and mounted with mounting medium. The specificity of the reaction was confirmed by substitution of anti-MT I+II antibody with mouse irrelevant IgG1 kappa immunoglobulin (clone DAK-GO1; Dako, USA), used in the same conditions and dilutions as a primary antibody.