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Interactive effects of nickel and chlorpyrifos on Mediterranean mussel cAMP-mediated cell signaling and MXR-related gene expressions

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ABSTRACT

The aim of this study was to infer putative interactive effects of a binary mixture between nickel (Ni) and chlorpyrifos (CHP) on mussel cell signaling, and also to unravel downstream effects on transcriptional regulation mediating cytoprotective responses. Mussels were exposed for 4 days to Ni (0.77 mg/L), CHP (4.5 mg/L), or the mixture Ni/CHP (0.135 mg/L Ni and 0.61 mg/L CHP). Cyclic AMP content and PKA activity in gills were evaluated as biological endpoints related to cell signaling. Expression of the *MgPgp* (ABCB1) and *MgMvp* genes was also assessed as involved in the mussel MXR mechanism. Levels of cAMP and PKA activities were not modified in mussels exposed to Ni or CHP, whereas they significantly increased in organisms exposed to the mixture. Similar responses were also detected for MgPgp expression, which is thought to be under cAMP/PKA-mediated regulation. Expression of *MgMvp* was unaffected by CHP or Ni/CHP exposure, and increased by Ni. The differential regulation of *MgPgp* and *MgMvp* expressions could be ascribed to the different intracellular localization and function of the two transporters. On the whole, present data indicated that Ni and CHP elicited interactive effects on mussel physiology, both at the signal transduction and at the gene expression levels.

1. Introduction

Several anthropogenic activities lead to a gradual increase of aquatic contaminants, not only in quantity, but also in diversity. Thus, gaining relevant information about the mode of action of single compounds has been proven a challenging task. However, unpredictable interactions of chemical mixtures can affect different biochemical pathways in natural environments. From a toxicological point of view, these interactions can strongly influence the overall impact of chemical stressors on living organisms. Although an increasing number of studies invested large efforts to understand mixture toxicity (Evrard et al., 2010; Banni et al., 2011; Dondero et al., 2011; Maria and Bebianno, 2011), the complex interplay of different toxicants among several biological pathways remains difficult to unravel.

Pesticides and metals are largely used in several human activities, and are widely detected in the different aquatic compartments, including marine environments (Bayen et al., 2004; Labrada-Martagón et al., 2011). Among them, this study was focused on the metal nickel and the pesticide chlorpyrifos. Nickel (Ni) is found in coastal environments as a result of industrial discharges from electroplating, smelting, mining, and refining operations, as well as other industrial emissions (Brix et al., 2004). Chlorpyrifos (CHP) is a broad-spectrum organophosphate compound employed as the active ingredient in several insecticides, being among the most widely used insect control products (Lemus and Abdelghani, 2000).

Both Ni and CHP are categorized as priority substances within the European Water Framework Directive for the protection of aquatic ecosystems (Directive, 2008/105/EC). Moreover, both compounds are detected in marine environments, where they tend to partition from the water column into benthic sediments due to their hydrophobicity and affinity for the organic matter. Therefore, sediments frequently become a long-term repository for such compounds, implying that benthic organisms including sessile bivalves can be particularly subjected to their putative joint toxicity. Nevertheless, scarce information is currently available about their putative sublethal effects and mode of action in marine species.

Mytilus galloprovincialis and other marine mussels are extensively used as sentinel organisms in environmental monitoring programs due to their wide distribution, sedentary lifestyle, tolerance to a wide range of environmental conditions, and because they are filter feeders with a very low metabolism, which allows the bioaccumulation of many chemicals in their tissues (Viarengo et al., 2007). Recent studies indicated that mussels are suitable organisms to evaluate contaminant effects on physiological mechanisms involved in cell signaling and in

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the control of stress-related gene expression (Martin-Diaz et al., 2009; Barmo et al., 2011; Franzellitti et al., 2011).

Pollutants may act as endocrine disrupters and interfere with the signal transduction pathway by binding to membrane receptors and mimicking the action of natural hormones (Amaral Mendes, 2002). Earlier studies indicated an increase of cyclic AMP (cAMP) levels in tissues of various organisms exposed to pollutants, including metals and organic compounds (reviewed in Fabbri and Capuzzo, 2010). Cyclic AMP acts as a second messenger and is involved in signal transduction pathway mediating physiological responses to extracellular signals. Transduction of signals by a number of hormones and neurotransmitters is known to occur via the activation of adenylyl cyclase which increases intracellular cAMP levels and activates protein kinase A (PKA) (Rich and Karpen, 2002). In mussels, cAMP binds to the regulatory subunits of PKA and mediates a plethora of cellular and physiological responses, including regulation of transcription for genes engaged in the environmental stress response (Fabbri and Capuzzo, 2010).

Multixenobiotic resistance (MXR)-related gene regulation was identified as a target for several environmental pollutants in bivalve species (Franzellitti and Fabbri, 2006; Luckenbach and Epel, 2008; Martin-Diaz et al., 2009; Paetzold et al., 2009). The MXR response allows aquatic organisms to survive in their habitat despite high pollution levels by over-expressing membrane and intracellular transporters, such as the P-glycoprotein (Pgp) and the Major vault protein (Mvp) respectively. These proteins act by actively transporting parental or metabolized forms of potentially harmful xenobiotics out of the cell thus preventing their cellular accumulation.

The aim of this study was to infer putative interactive effects of a binary mixture between Ni and CHP on mussel cell signaling, and also to unravel potential downstream effects on transcriptional regulation mediating cytoprotective responses in order to obtain a deeper insight into the physiological effects of contaminant mixtures in aquatic systems. Cyclic AMP content and PKA activity were evaluated as biological endpoints related to cell signaling. Expression profiles of the mussel MgPgp (ABCB1) and MgMvp genes encoding the P-glycoprotein and Major vault protein, respectively, were also assessed as involved in the MXR response.

Biological endpoints were evaluated in gills, since this tissue is constantly exposed to dissolved contaminants and it is sensitive to the effects of metals as well as organic compounds (Dailianis and Kaloyianni, 2007; Maria and Bebianno, 2011). Several studies indicated that in gill cells the cAMP-mediated pathway is involved in the regulation of ciliary beat frequency (Fabbri and Capuzzo, 2010; Bardales et al., 2011), which provide the basic mechanism for respiration and feeding in bivalves. Gills also function as a biological barrier between organism and environment. MXR-related transporters are physiologically expressed in gill cells, providing an active defense at the tissue–environment interface, and specific protection against environmental xenobiotics (Luckenbach and Epel, 2008). Transcriptional regulation of the MXR system was identified as a target for several environmental pollutants in bivalve species (Franzellitti and Fabbri, 2006; Martin-Diaz et al., 2009; Franzellitti et al., 2010; Buratti et al., in press).

2. Materials and methods

2.1. Experimental exposure

Mussels (4 to 6 cm in length) were obtained from a government-certified mussel farm (Cooperativa Copr.al.mo, Cesenatico, Italy). They were transferred to the laboratory in seawater tanks and acclimated for 3 days in aquaria. During the acclimatizing period and the experimental exposures, mussels were maintained in 35-psu filtered seawater at 16 °C with continuous aeration (>90% oxygen saturation) under a natural photoperiod. Mussels were fed once a day with a commercial algal slurry (Liquifry, Interpret Ltd., Dorking, Surrey, UK).

At the onset of the exposure experiment, mussels were divided in groups of 30 animals each, and transferred to 30 vessels containing 30 L of water. One liter of seawater for each mussel proved to be the suitable volume of water to avoid overloading and prevent the onset of unfavorable health conditions for mussels. For each experimental condition, 6 vessels containing a total of 180 mussels, represented the 6 replicates for each exposure condition.

The effects of Ni and CHP mixtures were assessed using the "response addition" model of mixture toxicity. This approach employs the toxic unit to model joint toxicity (Matthiessen et al., 1988). Toxic units (TU) express the toxicity of a mixture of compounds as a portion of its threshold effect concentration (Sprague, 1970). The experiments were carried out at a nominal equi-responsive dose. In the single chemical administration, mussels were exposed for 4 days to Ni or CHP at concentrations of 0.77 mg/L and 4.5 mg/L respectively. These concentrations corresponded to the EC50 (1 TU) calculated from a dose-fitting analysis performed on data from lysosomal membrane stability (LMS) assay carried out in a complementary experiment (Dondero et al., 2011). LMS is a sensitive biomarker of animal health status, and provides an early indication of ongoing detrimental effects of chemical exposures (Viarengo et al., 2007). Therefore, it was utilized as the guide biomarker in previous CHP and Ni toxicity assessments (Jones et al., 2008; Dondero et al., 2011). For equitoxic mixture exposures, Ni and CHP were combined at concentrations of 0.135 mg/L and 0.61 mg/L, respectively, corresponding to the EC25 values (0.5 TU) on LMS for each chemical as previously calculated (Dondero et al., 2011). Assuming the response addition model, each chemical give the same contribution to toxicity in the mixture, and the total toxic load for mixture is the same as in the single chemical exposures ($0.5\ TU_{Ni} + 0.5\ TU_{CHP} = 1\ TU_{Ni/CHP}$).

Ni was administered as $NiCl_2$ (Sigma Aldrich, Milan, Italy) from a concentrated stock solution prepared in distilled water, whereas CHP was administered as chlorpyrifos–ethyl (Sigma Aldrich, Milan, Italy) from a concentrated stock solution prepared in dimethylsulphoxide (DMSO). DMSO final concentration of exposure was 0.02% v/v. Chemicals were administered every day together with the algal preparation, and seawater renewed every 2 days. Groups of control (water) and solvent exposed (DMSO) mussels were maintained in parallel to the experiments.

To eliminate potentially confounding effects of sex and age, only adult females screened by microscopic inspection of gonads (Hines et al., 2007) at the end of the experimental exposure and along with tissue dissection were used for subsequent analysis. For each biological endpoint, a pool consisting of gills from 5 different organisms was prepared from each vessel ($n = 6$), immediately frozen in liquid nitrogen and stored at −80 °C until analyzed.

Mussels at zero time were analyzed for the biological parameters to assess their initial health status, and results were not significantly different from control mussels following the 4-day exposure period (data not shown).

2.2. cAMP content

About 200 mg of tissue were homogenized with three volumes of 4 mM EDTA to prevent enzymatic degradation of cAMP (Dailianis et al., 2003). The homogenate was boiled for 5 min and centrifuged (5 min at 16,000 ×*g*, 4 °C). The concentration of cAMP was estimated in the supernatant fraction by a radiochemical assay (Fabbri and Capuzzo, 2006) using [^{3}H]-cyclic AMP (GE Healthcare, Milan, Italy). Results are expressed as pmol cAMP/g wet weight.

2.3. PKA activity

About 200 mg of tissue was homogenized in cold PKA extraction buffer (25 mM Tris–HCl, pH 7.4 containing 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, and 50-fold diluted proteinase