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Immunotoxicity of the xenoestrogen 4-nonylphenol to the cockle *Cerastoderma glaucum*

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Abstract

The in vivo effects of 4-nonylphenol (NP) on functional responses of haemocytes from the cockle *Cerastoderma glaucum* were investigated after 7 days exposure to sublethal NP concentrations (0, 0+acetone, 0.0125, 0.025, 0.05 and 0.1 mg/l NP). Haemocytes from both controls and exposed cockles were collected, and the effects of NP on total haemocyte count (THC) and volume of circulating cells, intracellular superoxide anion (O_2^-) levels, acid phosphatase and lysozyme-like activities in both haemocyte lysate (HL) and cell-free haemolymph (CFH) were evaluated. Exposure of cockles to 0.1 mg/l NP significantly increased THC ($p < 0.05$) with respect to controls. Analysis of haemocyte size frequency distribution showed that the haemocyte fraction of about 7–8 μm in diameter and 250 femtolitres in volume increased markedly in cockles exposed to the highest NP concentration tested. Apoptosis resulting in cell volume reduction in NP-exposed animals cannot be excluded. No statistically significant variation in intracellular O_2^- levels was observed. Conversely, significant increases ($p < 0.05$) in acid phosphatase activity were observed in CFH from 0.05 and 0.1 mg/l NP-exposed animals; no significant differences in enzyme activity were recorded in HL. Lysozyme-like activity also increased significantly in CFH from cockles exposed to 0.05 mg/l NP ($p < 0.05$) and 0.1 mg/l NP ($p < 0.001$). Instead, lysozyme-like activity decreased significantly ($p < 0.05$) in the HL of animals exposed to 0.05 mg/l NP. Our results suggest that NP induces variations in the functional responses of haemocytes of *C. glaucum*, mainly by reducing cell membrane stability and promoting cell degranulation.

Keywords: Nonylphenol; Xenoestrogen; Cockles; Haemocytes; Immunotoxicity

1. Introduction

In the last few years, increasing interest has focused on evaluating the adverse effects of endocrine-disrupting chemicals (EDCs) in aquatic organisms. EDCs are a heterogeneous group of substances able to alter many endocrine functions in organisms (Neubert, 1997). Among them, xenoestrogens have been extensively studied owing to their capability to mimic natural estrogens (estrogen mimics) (WHO/IPCS, 2002). One of the best documented effects of xenoestrogens is the induction of vitellogenins (Vg), precursors of the egg-yolk proteins vitellins (Vn), which provide energy reserves for embryo development in oviparous organisms (Suzuki et al., 1992; Denslow et al., 1999). Conversely, only few data are available about xenoestrogen-mediated immunomodulation in aquatic organisms, bivalves in particular.

It is well known that haemocytes are circulating cells involved in internal defences in bivalves (Cheng, 1981). Alterations in immunocompetence have been reported for many bivalve species after exposure, both in vitro and in vivo, to inorganic and organic contaminants, such as heavy metals (Coles et al., 1995; Pipe et al., 1999; Matozzo et al., 2001), organotins (Matozzo et al., 2002), fungicides (Alvarez and Friedl, 1992), polycyclic aromatic hydrocarbons (PAHs) (Grundy et al., 1996; Gómez-Mendikute et al., 2002) and chlorinated phenols (Florence et al., 1997). Among the functional responses of bivalve circulating cells, total haemocyte count (THC), viability, motility, aggregation and adhesion capability, phagocytosis, production of reactive oxygen species (ROS), hydrolytic and

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oxidative enzyme activities, and lysosomal and cell membrane stability are generally recognised as useful biomarkers in immunotoxicity studies.

Both in vitro and in vivo studies have recently been performed to assess xenoestrogen-induced immunomodulation in bivalve molluscs (Canesi et al., 2006; Champeau and Narbonne, 2006; Gauthier-Clerc et al., 2006; Canesi et al., 2007). In a previous study, we demonstrated that in vivo exposure of the clam *Tapes philippinarum* to the xenoestrogen 4-nonlyphenol (NP) induced alterations in immune functions (Matozzo and Marin, 2005). NP is used in the production of nonylphenol ethoxylates (NPEs), NP phosphites and insecticide sprays (Maguire, 1999). NPEs are non-ionic surfactants commonly employed in plastics, latex paints, lubricating oils, emulsifiers, household and industrial detergents, and paper and textiles (Lee, 1999).

In the present study, the in vivo immunotoxic effects of sublethal NP concentrations were evaluated for the first time in the cockle *Cerastoderma glaucum*. THC and the volume of haemocytes, intracellular superoxide anion production, acid phosphatase (hydrolytic enzyme) and lysozyme-like activities (bacteriolytic enzyme) in both haemocyte lysate (HL) and cell-free haemolymph (CFH) were chosen as immunomarkers of NP exposure in cockles.

2. Materials and methods

2.1. Cockles

Specimens of *C. glaucum* were collected from a reference site located inside a licensed area for clam culture in the southern basin of the Lagoon of Venice (Italy), and acclimatised in the laboratory for 7 days before exposure to NP. They were kept in large aquaria provided with a sandy bottom and aerated seawater (salinity 35 ± 1‰, temperature 17 ± 0.5 °C) and fed with microalgae (*Isochrysis galbana*).

2.2. Exposure to NP

NP, a mixture of *p*-isomers, was purchased from Fluka Chemika (Cod. 74430, Buchs, Switzerland). A stock solution of NP was prepared in acetone and stored at room temperature for the duration of the experiments. Working solutions were prepared daily by diluting the stock solution in sea water. To evaluate NP effects on functional responses of haemocytes, 15 cockles per concentration were exposed for 7 days to 0, 0+acetone, 0.0125, 0.025, 0.05, and 0.1 mg NP/l. The nominal exposure concentrations were chosen on the basis of the LC_{50} value (0.3 mg NP/l) recorded in *C. glaucum* (Marin et al., 2008). In acetone controls, solvent was added at the highest concentration (8 μl/l) used in NP treatments. Animals were maintained in glass aquaria (without sediment) containing aerated sea water (1 l per animal), in the same thermo-haline conditions used in the acclimatisation period. Water was changed every day, and NP and microalgae added (*I. galbana*, at an initial concentration of about 150,000 cells/l).

2.3. Haemolymph collection

After NP exposure, haemolymph (at least 250 μl per animal) was collected from the anterior adductor muscle in a 1-ml plastic syringe and placed in Eppendorf tubes at 4 °C. Haemolymph from control and NP-exposed cockles was pooled to obtain three replicate samples (five animals per pool) for each experimental condition.

2.4. THC and haemocyte volume determination

THC and haemocyte size frequency distribution were determined on a Model Z2 Coulter Counter electronic particle counter/size analyser (Coulter Corporation, FL, USA). Pooled haemolymph (500 μl) was added to 19.5 ml of 0.45 μm-filtered seawater (FSW). THC and haemocyte volume were expressed as number of haemocytes ($\times 10^6$)/ml haemolymph and femtolitres (fl), respectively.

2.5. Intracellular superoxide anion (O_2^-) assay

Two hundred microlitres of pooled haemolymph was added to 200 μl of 0.2% nitro blue tetrazolium (NBT) in FSW. Haemocyte suspensions were incubated for 60 min in Eppendorf tubes in the dark. Two controls were prepared: the first contained 400 μl NBT only, and the second haemolymph incubated in NBT solution added with 300 Units/ml of superoxide dismutase (SOD; Sigma). After incubation, Eppendorf tubes were centrifuged for 5 min at 780*g*, supernatants removed, and haemocytes suspended in 200 μl phosphate buffered saline, pH 7.2 (PBS: 1.37 M NaCl, 0.03 M KCl, 0.015 M KH_2PO_4, 0.065 M Na_2HPO_4). Haemocytes were then centrifuged for 5 min at 780*g* and supernatants removed. Haemocytes were fixed for 10 min in 100% methanol, centrifuged for 5 min at 780*g* and supernatants removed. Cells were then dried for 3 min at room temperature, added with 200 μl of 50% methanol, centrifuged for 5 min at 780*g*, and suspended in 120 μl of KOH 2 M plus 140 μl DMSO. Two hundred microlitres of haemocyte suspension were put in the wells of a 96-well microplate and absorbance was recorded on a microplate reader (Reader SR400, Techno Genetics) at 620 nm. Results are expressed as optical density per mg protein (OD mg/protein). Haemocyte protein concentrations were quantified according to Bradford (1976) using bovine serum albumin (BSA) as standard.

2.6. Lysozyme-like activity assay

Lysozyme-like activity was quantified in both haemocyte lysate and cell-free haemolymph. To this aim, pooled haemolymph was centrifuged for 10 min at 780*g*. The supernatant, corresponding to cell-free haemolymph (CFH), was collected; the haemocytes were resuspended