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Investigating appearance and regulation of the MXR phenotype in early embryo stages of the Mediterranean mussel (*Mytilus galloprovincialis*)

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ABSTRACT

Multixenobiotic resistance (MXR) efflux transporters constitute a broad-spectrum physiological defense system allowing marine bivalves to cope with environmental challenges. There is, however, scarce information on the type and role that different MXR transporters may have in embryos, which represent the most sensitive stages of bivalves to environmental stress. In this study regulation of MXR-related transporters was investigated in early developmental stages of the Mediterranean mussel (*Mytilus galloprovincialis*). In vitro fertilization experiments using gametes from naturally-spawning broodstocks were performed to follow embryo development from fertilized eggs (30 min post fertilization, pf) to fully developed D-shape veligers (48 h pf). Quantitative PCR analyses indicated that *ABCB* and *ABCC* transcripts encoding the MXR-related transporters P-glycoproteins (P-gp) and Multidrug resistance proteins (Mrp), respectively, were expressed soon after 30 min pf, with *ABCC* being more expressed than *ABCB*. Copy numbers of both transcripts were increased in trochophorae and D-veligers. MXR efflux activity assessed using the fluorescent substrate rhodamine 123 and selective P-gp or Mrp inhibitors showed that the P-gp mediated efflux was detected only in D-veligers, while a significant Mrp mediated efflux was detected soon after 30 min pf and remained almost unchanged in trochophorae and D-veligers. MXR modulation by propranolol and carbamazepine showed that the pharmaceuticals may act as transcriptional regulators and substrates. Results reported lead to hypothesize that while P-gp aids in xenobiotic efflux performing a prominent protective role, Mrp could be a dual-functioning transporter performing both protective and physiological functions in mussel development.

1. Introduction

In animal life, early stages are considered more sensitive to environmental threats than adult stages, and therefore are addressed to as the critical phases in an organism's lifecycle (Hamdoun and Epel, 2007). The issue of embryo vulnerability is particularly problematic for aquatic organisms that rely their reproductive success on external fecundation and larvae dispersal into the environment, mainly as planktonic forms. This reproductive strategy has the advantage of avoiding competition for resources with adults, temporary reducing benthic mortality while in the plankton, decreasing likelihood of inbreeding in the next generation, increasing ability to withstand local extinction, and colonizing new habitats (Pechenik, 1999). Nevertheless, during their planktonic life larvae face numerous threats: they must survive predators, overcome buoyancy issues, adapt to variations of temperature and light, to sea currents, chemical challenges and feeding issues. Anyway, it is a matter of fact that embryos may develop normally despite the presence of environmental stressors that could disrupt regulatory pathways involved in embryogenesis (Hamdoun and Epel, 2007). This intrinsic robustness of developmental programs is achieved through potent buffering mechanisms that allow fast evolving adaptive behaviors (Bassim et al., 2015; Hamdoun and Epel, 2007).

Marine mussels are exceptionally well-adapted to live in transitional habitats, where they are exposed to fluctuating environmental parameters and elevated levels of natural and anthropogenic pollutants throughout their life-cycle (Franzellitti et al., 2010; Franzellitti and Fabbri, 2005). Detoxification routes from potential harmful chemicals through the Multixenobiotic resistance (MXR) system is considered a general and broad spectrum protective mechanism boosting stress tolerance in these organisms (Bielen et al., 2016; Minier et al., 2000). MXR is constituted by transporters located on outer plasma membrane and internal membranes (such as lysosomal membranes), that act as a first-tier defense against environmental contamination by pumping out from cells both endogenous chemicals and xenobiotics, thus preventing their accumulation and toxic effects (Bard, 2000).

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Amongst the proteins involved in the MXR system the best characterized in an environmental context is the P-glycoprotein (P-gp) (Bard, 2000). In *M. galloprovincialis*, this membrane protein is encoded by the ABCB gene (Franzellitti and Fabbri, 2006), and transports moderately hydrophobic, amphipathic, neutral or positively charged planar organic molecules of high molecular weight with a basic nitrogen atom (Leslie et al., 2005). P-gp mostly transports unmodified xenobiotics so that it is addressed to as a phase 0 transporter, i.e. it mediates the direct extrusion of unmetabolized compounds out of the cell. P-gp induction in mussels has been reported in response to a wide range of chemical and physical stressors, including metals, pesticides, as well as temperature or salinity variations (Bard, 2000; Minier et al., 2000), suggesting that this transporter may be part of a general and broad-spectrum cellular stress response.

Other ABC transporters found in mussels are the Multidrug resistance-related proteins (Mrp) encoded by the ABCC gene (Franzellitti and Fabbri, 2006). Substrates of Mrp are predominantly products of phase I and II metabolism in the form of glutathione, glucuronate or sulphate water soluble conjugates (Leslie et al., 2005). As such, Mrp transporters are addressed to as the phase III of biotransformation processes (Leslie et al., 2005). Furthermore, Mrp can also maintain cellular redox homeostasis via GSH and GSSG transport (Leslie et al., 2005).

The relevant role played by the MXR system as a protective and physiological mechanism in embryo development was deeply investigated in sea urchins (Gokirmak et al., 2014), whereas scarce information is available on other marine species, including mussels. The present study reports the first investigation on the development and regulation of the MXR system in early embryo stages of the Mediterranean mussel, *Mytilus galloprovincialis*. This species plays essential functional roles in coastal ecosystems and also constitutes a commercially important resource in mariculture as well as an ideal sentinel organism in biomonitoring of coastal areas (Viarengo et al., 2007). Investigations were performed on critical stages of early embryos: i) at 30 min post fertilization, ii) at 24 h post fertilization (pf), when the first motile trocophorae larvae is developed, and iii) at 48 h pf, when the first fully developed D-shape shelled veliger is formed and physiological advances towards the establishment of a planktotrophic behavior take place (Bassim et al., 2014). At these stages activities of the main transporters P-gp and Mrp were assessed by using a known fluorescent MXR substrate and selective transporter inhibitors. Moreover, expression profiles of *ABCB* (P-gp) and *ABCC* (Mrp) mRNAs were analysed. Finally, since many pharmaceuticals may act as substrates or regulators of the MXR system (Caminada et al., 2008; Franzellitti et al., 2016), we investigated the potential of the antiepileptic carbamazepine and the β-blocker propranolol to elicit different effects across embryo stages in marine mussels.

2. Methods

2.1. Chemicals

Verapamil (VER), 5-(3-(2-(7-Chloroquinolin-2-yl)ethenyl)phenyl)-8-dimethylcarbamyl-4,6-dithiaoctanoic acid (MK571), rhodamine 123 (Rho123), (±)-propranolol hydrochloride (PROP), carbamazepine (CBZ), were at the molecular biology grade (>99% purity) and were purchased from Sigma Aldrich (Milan, Italy). The DirectZol kit was from Zymo Research (Freiburg, Germany). The Qubit RNA assay and the Qubit protein assay were from Thermo Fisher (Milan, Italy). The iScript supermix and iTaq Universal master mix with ROX were from Biorad Laboratories (Milan, Italy). The Tri-Reagent, dimethyl sulfoxide (DMSO), and any other reagent was from Sigma Aldrich (Milan, Italy).

2.2. Animal holding and larval rearing

Sexually mature Mediterannean mussels (*Mytilus galloprovincialis*) were obtained from a government certified mussel farm (Cooperativa Copr.al.mo, Cesenatico, Italy). They were transferred to the laboratory and acclimated in static tanks containing aerated 35-psu artificial seawater (ASW) at 16 °C (ASTM, 2004). Gamete collection, oocyte fertilization and larvae handling were performed following the procedure described in detail by Fabbri et al. (2014). Briefly, when mussels begun to spontaneously spawn, each individual was immediately placed in a 250 mL beaker containing 200 mL of aerated ASW until complete gamete emission. After spawning, mussels were removed from beakers and sperms and eggs were sieved through 50 μm and 100 μm meshes, respectively, to remove impurities. Egg quality (shape, size) and sperm motility were checked using an inverted light microscope. Eggs were fertilized with an egg:sperm ratio 1:10 in polystyrene 6-well (RNA extraction) or 12-well (MXR activity) cell culture plates. After 30 min, fertilization success (n. fertilized eggs / n. total eggs × 100) was verified microscopically (>85%). Embryos were grown at 16 °C ± 1 °C for 48 h, with a 16 h:18 h light:dark photoperiod. The following steps of the experimental setup are outlined in Fig. 1 and described below.

2.3. Treatments to investigate basal MXR transport activity across early embryo development

Embryos were grown for 48 h under control conditions (Fig. 1). Biological endpoints were assessed at specific developmental stages: fertilized eggs (30 min pf), trocophorae (24 h pf), D-veligers (48 h pf). At each stage, control (unexposed) embryos were employed to assess *ABCB* and *ABCC* copy numbers under basal conditions following procedures for RNA extraction and transcript quantification described below (Fig. 1).

For the evaluation of relative P-gp and Mrp transport activity, embryos at each developmental stage were treated with the selective inhibitors verapamil (VER), specific for P-gp, and MK571, specific for Mrp, at 20 μM and 10 μM concentration, respectively. The compounds were added to the proper wells from stock solutions prepared in DMSO (MK571) or ethanol (VER). A control group was maintained in parallel. After a short pre-incubation period (5 min) with inhibitors, the model fluorescent substrate rhodamine 123 (Rho123, prepared in DMSO) was added to treated samples and controls to reach the assay concentration of 2.5 μM. In any case, solvent concentrations never exceed 0.1% v/v. Rho123 shows low to moderate rates of passive membrane permeation, so that it is effectively extruded by ABC efflux pumps (Luckenbach et al., 2014). Therefore, the amount of Rho123 in a cell is a measure of transporter activity: high activity of transporters is indicated by a weaker Rho123 fluorescence signal, whereas a stronger fluorescence signal corresponds to a lower transporter activity. If the selected transporters are expressed and active, treatment with a specific inhibitor results in accumulation of Rho123 dye in the cells. The duration of exposure and Rho123 concentration were selected during preliminary experimental trials, which showed that these conditions did not significantly affect embryo viability while providing the most stable and repeatable fluorescence readings (data not shown).

Embryos were incubated for 90 min at 16 °C in the dark. At the end of the incubation period, three wells for each condition from the 12-well plates were pooled to obtain approximately 1800 embryos/replicate. Fertilized eggs were collected by centrifugation (800 ×*g* for 10 min at 4 °C), while embryos at 24 h and 48 h pf were collected by a nylon mesh (40 μm pore-filter). All embryos were washed and re-suspended in clean ASW, then centrifuged at 800 ×*g* for 10 min at 4 °C. Pellets were lysed with 1 mL of a 0.1% Triton×100 solution prepared in PBS and using an Ultra Turrax (IKA) homogenizer. Lysates were centrifuged at 3000 ×*g* for 7 min at 4 °C, and the supernatants conveniently diluted for fluorescence measurements performed using a Jasco FP-6200 fluorometer ($\lambda_{excitation}$ = 485 nm; $\lambda_{emission}$ = 530 nm). Due to the possible loss of the Rho123 fluorescence intensity caused by the direct exposure to light, plates and lysates were light-protected with aluminium foils

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