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How important are glutathione and thiol reductases to oyster hemocyte function?

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ABSTRACT

Bivalves are animals with worldwide distribution. Although they play key roles in economic activities, human feeding and environmental studies, there is a considerable lack of knowledge about the relationship between their immune system and antioxidant defenses. Here, we performed an *in vitro* experiment where *Crassostrea gigas* hemocytes were exposed to the electrophilic compound 1-chloro-2,4-dinitrobenzene (CDNB, 0.1–50 μM) for one hour. CDNB treatment clearly disturbed thiol homeostasis, causing a concentration-dependent decrease in the glutathione (GSH) content and a decrease in the activity of two thiol reductases, glutathione reductase (GR – 2.5 and 50 μM CDNB) and thioredoxin reductase (TrxR – only 50 μM CDNB). The MTT reduction assay showed that none of the CDNB concentrations tested significantly altered cell viability. However, there was a decrease in the hemocyte's ability to uptake the neutral red dye, which indicates lysosomal impairment (≥12.5 μM CDNB). Cellular immunocompetence was further investigated and, despite the lower GSH content, GR activity and impairment in lysosome integrity, hemocyte functions (adhesion capacity, phagocytosis of latex beads and laminarin-induced ROS production) were preserved in the 2.5 and 12.5 μM CDNB treatments. These results suggest a minor importance of thiol pools and GR activity in *C. gigas* hemocyte's immunocompetence, in an *in vitro* acute exposure model. The 50 μM CDNB treatment, however, significantly compromised all the measured hemocyte functions. This response was associated with TrxR inhibition, increased lysosome impairment, decreased GSH content, and lower GR activity. Therefore, it seems that TrxR may be particularly important for the hemocyte function, or, alternatively, it is only affected when a deeply aggravated scenario in thiol homeostasis is set up. Such findings point out the need for further studies towards a better understanding of antioxidant and immune defenses interactions in bivalve cellular systems.

1. Introduction

Bivalves are worldwide distributed animals that arouse the interest of academic community due to its ecological, economic and environmental importance. Bivalves play vital roles in the functioning of aquatic environments and are extensively used as food sources. They also hold many advantages as models for evolutionary studies and as sentinel organisms in monitoring programs [1,2]. Among bivalves, the Pacific oyster *Crassostrea gigas* receives particular attention since is one of the most cultivated aquatic species in the world [3]. In the past few years, Pacific oyster farming has been experiencing disease outbreaks that have resulted in high mortality and considerable decrease in production worldwide [3]. Despite its importance and survival problems that this species has been facing in the aquatic environments, basic knowledge regarding important biological processes such as immune and antioxidant systems are still very limited.

Hemocytes play central roles in the immune system of invertebrates. These cells are responsible for the recognition of foreign microorganisms followed by their engulfment and intra and/or extracellular destruction. Pathogen recognition requires cellular communication events that may involve adhesion receptors such as integrins, which are also involved in hemocyte spreading, degranulation, phagocytosis and encapsulation processes [4]. Therefore, adhesion is an essential mechanism contributing to host defense. Bivalve hemocytes actively phagocyte a range of cell types and particles, including bacteria, algae, yeast, foreign

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blood cells and latex spheres [5]. A series of events occurs during phagocytosis, including attachment, pseudopod formation, engulfment via phagosome and phagosome maturation. Inside a mature phagolysosome, non-self particles are confronted with microbicidal reactions, such as reactive oxygen species (ROS) production, e.g. superoxides and peroxides produced upon NADPH oxidase activation [4]. Although it is still not clear if ROS production by bivalve hemocytes is directly involved in cytotoxicity against ingested foreign organisms [6,7], this does not lessen the importance of oxidative mechanisms in other physiological hemocyte functions. For instance, in *C. gigas* hemocytes, ROS production was shown to be involved in apoptotic induction [8]. Furthermore, in this same species, ROS produced by hemocytes was indispensable for the formation of extracellular DNA traps, which are responsible for bacterial surrounding and entanglement [9]. ROS are also known to be involved in cell signaling, growth regulation, proliferation and death [10], highlighting the importance of a strict intracellular regulation of ROS levels.

ROS scavenging is performed by components of the antioxidant system, which can be enzymatic or non-enzymatic. The tripeptide glutathione (GSH) and the small protein thioredoxin (Trx) can be highlighted as non-enzymatic antioxidants presenting functional thiol groups. GSH is found intracellularly in considerable high concentrations (1–10 mM) [11] and acts in conjunction with enzymes in the elimination of ROS and conjugation to electrophilic compounds [12]. Trx relies on its proximately located cysteine residues acting as an electron donor, which results in the reduction of peroxides and scavenging of free radicals, thus protecting cells against oxidative stress [13]. Many proteins that contain exposed thiols may undergo redox reactions that can be either reversible or irreversible, displaying regulatory actions or leading to functional impairment [14]. GSH and Trx can be used as electron donors for two important enzyme families involved in peroxide elimination: glutathione peroxidases (GPx) and peroxiredoxins (Prx), respectively. GSH and Trx oxidation can be reversed by glutathione reductase (GR) and thioredoxin reductase (TrxR), respectively. The GPx/GSH/GR and Prx/Trx/TrxR systems are therefore crucial for maintaining redox homeostasis inside cells [14].

To our knowledge, there is a considerable lack of studies evaluating the interplay between the immune and antioxidant systems in oysters. Most of the available studies evaluate exposure–responses relationships to pathogens, immune-stimulating agents, pollutants or contaminated environments, and are not able to establish potential direct links between these two systems.

Here, we exposed oyster's hemocytes to the electrophilic agent 1-chloro-2,4-dinitrobenzene (CDNB), classically known as thiol depletor and TrxR inhibitor [15]. Thiol status was assessed by measuring non-protein (namely GSH) and protein (PSH) thiol pools. GR and TrxR activities were also assessed as they represent specific thiol-containing protein targets. Hemocyte viability as well as their adhesion capacity, phagocytosis and ROS production were also measured, in order to evaluate whether impairment in thiol pools could directly affect these important immune functions.

2. Material and methods

2.1. Animals

Adult oysters (*C. gigas*) were purchased from a marine oyster farm located in Florianópolis, Santa Catarina, Brazil. Animals were acclimated for a minimum period of one week and kept in UV treated and filtered seawater at 18–20 °C (1 L/animal) with constant aeration. Seawater renewal and feeding with a commercial plankton diet (Sera® Marin Coraliquid) were done every two days.

2.2. Hemolymph collection and hemocyte treatments

Oyster hemolymph was collected from the adductor muscle using a 0.8 × 30 mm needle attached to a cold 1 mL syringe, and maintained on ice until use. During collection, a droplet of each sample of hemolymph was monitored by a light microscope. Samples with signs of contamination with any microorganisms, gametes, or even with hemocyte aggregation were discarded. Hemolymph was pooled in order to obtain the number of hemocytes needed in each assay, using a minimum of three animals per pool. After hemolymph collection, hemocyte concentration was determined using a Neubauer Improved chamber.

For hemocyte treatments with CDNB (Sigma–Aldrich, Brazil), hemolymph was centrifuged (600 × g, 10 min, 4 °C) and the hemocyte pellet was suspended in filtered sterile seawater (FSW) with or without CDNB (0.1–50 μM) at the final hemocyte density of 1×10^6 cells/mL. CDNB was previously diluted in absolute ethanol and the final concentration of ethanol (0.05%) was equally adjusted in each treatment, including control groups. Hemocytes were then incubated at 20 °C in the dark for 1 h, centrifuged (600 × g, 10 min, 4 °C) and further used to determine their viability, GSH and PSH content, GR and TrxR activities and immunocompetence.

2.3. Cellular viability assays

To determine whether CDNB treatments affect hemocyte viability, the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the neutral red retention (NRR) assays were performed (n = 6). The MTT reduction assay detects viable cells with active metabolism. Viable cells are able to convert MTT into a purple-colored formazan product, whereas dead cells lose this ability. MTT can be reduced not only by mitochondrial dehydrogenases, as classically denoted, but also in other cellular compartments, being an indicative of general metabolic activity [16]. The NRR assay measures lysosome's ability to uptake and retain NR dye, which can be affected when membrane permeability or maintenance of a low intralysosomal pH (which traps the dye) in lysosomes are compromised [17].

The assays used here were slightly modified from Trevisan et al. [18]. In this study, an exact number of cells was used for each assay. After CDNB treatments, a total of 1×10^6 (MTT) and 3×10^5 (NRR) cells was suspended in 300 μL of 0.5 mg/mL MTT or 150 μL of 0.004% NR, respectively. Cells were incubated for 1 h (MTT) or 3 h (NRR) in the dark at 20 °C, and further centrifuged at 800 × *g* for 10 min at 4 °C. Samples incubated with MTT were suspended in 200 μL of DMSO, while samples incubated with NR were washed 2 times with PBS and suspended in 200 μL of acidified ethanol (1% acetic acid, 50% ethanol). Absorbance was read at 550 nm (MTT) and at 560 nm (NRR) and normalized to control group (0 h). Both assays were performed in duplicate.

2.4. Total glutathione and protein thiols content

Total glutathione (GSH-t) and protein thiols (PSH) contents were analyzed in hemocyte pools (n = 4) treated with CDNB. Samples containing 1.5×10^6 cells from each group were suspended in 50 μL of 0.5 M perchloric acid (PCA) and vortexed (cellular disruption and protein denaturation). Homogenates were pH-neutralized with a solution containing 0.3 M 3-(N-morpholino)propansulfonic acid (MOPS) and 2 M KOH, centrifuged at 15,000 × g for 2 min at 4 °C, and supernatants were used to determine GSH-t levels spectrophotometrically at 412 nm, through the enzymatic-coupled method described by Akerboom and Sies (1981). PSH measurements were performed on particulate material (pellet), which was washed with 250 μL of 0.5 M PCA, centrifuged (15,000 × g for 2 min