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The biological importance of glutathione peroxidase and peroxiredoxin backup systems in bivalves during peroxide exposure

Rafael Trevisan*, Danielle Ferraz Mello, Marcela Uliano-Silva [1], Gabriel Delapedra, Miriam Arl, Alcir Luiz Dafre

Biochemistry Department, Federal University of Santa Catarina, Florianópolis 88040-900, Brazil

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ABSTRACT

Organic peroxide elimination in eukaryotes essentially depends on glutathione peroxidase (GPx) and peroxiredoxin (Prx) enzymes, which are supported by their respective electron donors, glutathione (GSH) and thioredoxin (Trx). This system depends on the ancillary enzymes glutathione reductase (GR) and thioredoxin reductase (TrxR) to maintain GSH and Trx in their reduced state. This study discusses the biological importance of GR and TrxR in supporting GPx and Prx during cumene hydroperoxide (CHP) exposure in brown mussel *Perna perna*. $ZnCl_2$ or 1-chloro-2,4-dinitrobenze (CDNB) was used to decrease GR and TrxR activities in gills, as already reported with mammals and bivalves. $ZnCl_2$ exposure lowered GR activity (28%), impaired the *in vivo* CHP decomposition and decreased the survival rates under CHP exposure. CDNB decreased GR (54%) and TrxR (73%) activities and induced glutathione depletion (99%), promoting diminished peroxide elimination and survival rates at a greater extent than $ZnCl_2$. CDNB also increased the susceptibility of hemocytes to CHP toxicity. Despite being toxic and causing mortality at longer exposures, short (2 h) exposure to CHP promoted an up regulation of GSH (50 and 100 μM CHP) and protein-thiol (100 μM CHP) levels, which was blocked by $ZnCl_2$ or CDNB pre-exposure. Results highlight the biological importance of GSH, GR and TrxR in supporting GPx and Prx activities, contributing to organic peroxides elimination and mussel survival under oxidative challenges. To our knowledge, this is the first work that demonstrates, albeit indirectly, the biological importance of GPx/GR/GSH and Prx/TrxR/Trx systems on *in vivo* organic peroxide elimination in bivalves.

1. Introduction

The result of cellular energy production mediated by aerobic metabolism occurs simultaneously with the generation of potential toxic oxygen intermediates, known as reactive oxygen species (ROS), which in excess may pose a considerable threat to cellular homeostasis (Cadenas and Davies, 2000). Among ROS, peroxides (*e.g.* H_2O_2) can be generated by diverse metabolic processes, including dismutation of superoxide anion, or as byproduct of oxidases such as monoamine oxidases (Dringen et al., 2005). Organic peroxides are also generated by cells, for example trough the action of cyclooxygenases and lipoxygenases or by oxidation of polyunsaturated fatty acids (Dringen et al., 2005). At physiological levels, or close to that, ROS play an important role activating/deactivating cellular signaling pathways, allowing adaptive responses during stressful situations (Schieber and Chandel, 2014). Excessive ROS production is harmful to the cell, and toxicity is potentiated by its high reactivity. Reaction with biomolecules such as proteins, nucleic acids and lipids, can affect cellular metabolism (Imlay, 2003). The cellular defense against ROS is mediated by enzymatic and non-enzymatic antioxidants, responsible for maintaining the cytosol reductive environment and cellular function (Valko et al., 2007).

Intracellular peroxide metabolism is mainly mediated by three different antioxidant enzymes: catalase (Cat), glutathione peroxidase (GPx) and peroxiredoxin (Prx). While the catalytic cycle of Cat is independent of reducing agents and catalyzes only H_2O_2, GPx and Prx use intracellular electron donors for their peroxidase activity against H_2O_2 and organic peroxides. GPx uses glutathione (GSH) as reducing agent, generating oxidized glutathione (GSSG). A high GSH/GSSG ratio is maintained by glutathione reductase (GR), using NADPH as a final electron donor (Bindoli et al., 2008). On the other hand, 2-Cys Prxs

* Corresponding author.
E-mail address: rafael.trevisan@gmail.com (R. Trevisan).
[1] Present address: Institute of Biofísica Carlos Chaga Filho, Federal University of Rio de Janeiro, 21044-020 Rio de Janeiro, RJ, Brazil.

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reduce peroxides with electrons provided by thioredoxin (Trx) (Rhee and Woo, 2011). Oxidized thioredoxin is converted back to its reduced state by thioredoxin reductase (TrxR), which also uses NADPH as final electron donor (Rhee and Woo, 2011). Therefore, peroxide elimination relies on peroxidase enzymes themselves and also on their backup systems composed by the reductase enzymes, such as GR and TrxR, and their ability to reduce their respective substrates, GSSG and oxidized Trx (Flohé et al., 2011). The reduced forms of GSH and Trx are considered important cellular thiol pools closely associated to ROS detoxification processes. Decreases in the GSH/GSSG or reduced/oxidized Trx ratios are usually associated with the adverse effects of oxidative compounds, triggering death/survival responses (Circu and Aw, 2010). Therefore, thiol/disulphide ratios can be used as markers of oxidative stress, since the maintenance of GSH and Trx in their reduced forms is essential for a number of cellular functions, including those dependent on GPx and Prx activities (Toledano et al., 2013).

The H_2O_2 steady state levels are considerably low in marine invertebrates when compared to mammals, with mitochondria being taken as a major site of ROS production (see review of Abele and Puntarulo, 2004). During non-stressful situations, these lower ROS generation rates found in invertebrates (including bivalves) are related to their lower metabolic rate (Buttemer et al., 2010). In bivalves, the rate of ROS production, as well as the antioxidant defences, can be modulated by diverse biotic and abiotic factors, including age, size, temperature, salinity and metabolic rate (Frenzilli et al., 2004; Lau and Wong, 2003; Regoli et al., 2000; Sheehan and Power, 1999; Sukhotin et al., 2002). For instance, the spawning season of *Mytilus galloprovincialis* is marked by an increase in ROS production, such as H_2O_2 and organic hydroperoxides, generating oxidative stress in gills and digestive gland (Soldatov et al., 2008). Thermal stress in summer season and during reproductive activity is also responsible for an increase in oxygen consumption and ROS production in mussels (Arthur, 2001; Verlecar et al., 2008; Wilhelm Filho et al., 2001). Therefore, elimination of peroxides by redundant peroxidase systems can have a major contribution in the protection of marine invertebrates during both physiological and stressful conditions.

It is long known that zinc is able to inhibit GR activity *in vitro* at the micromolar range (Mize and Langdon, 1962). This effect was also detected *in vivo* in mussels *Perna perna* acutely exposed to $ZnCl_2$ (Franco et al., 2006), without alterations on GR protein content (Trevisan et al., 2014). Similarly, it has been shown that 1-chloro-2,4-dinitrobenze (CDNB) inhibits TrxR activity *in vitro* (Arnér et al., 1995). Our group demonstrated that CDNB causes a marked decrease in both GR and TrxR activities *in vivo* in oysters *Crassostrea gigas* (Trevisan et al., 2012). Given that GPx and Prx activities rely on a constant GSH and Trx recycling, the aim of the present study was to evaluate the effects of peroxidase systems impairment of brown mussels during peroxide exposure by previously inhibiting GR and TrxR. Animals were acutely exposed to $ZnCl_2$ or CDNB as an approach to decrease GR and TrxR activities. The role of the peroxidase systems on animal survival, detoxification and cellular viability was investigated in animals subsequently exposed to cumene hydroperoxide (CHP), a model GPx and Prx substrate. Our study discusses the importance of the peroxidase systems and thiol homeostasis in brown mussels under an exogenous pro-oxidant challenge.

2. Material and methods

2.1. Chemicals

CDNB, MTT, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), bovine serum albumin, CHP, ethylenediamine tetraacetic acid (EDTA), GSH, GSSG, GR, N-ethylmaleimide (NEM), neutral red, NADPH, perchloric acid (PCA), tris(hydroxymethyl)aminomethane (TRIS), sodium dodecyl sulfate (SDS), xylenol orange and zinc chloride were obtained from Sigma–Aldrich (Brazil). All other reagents used were of analytical grade.

2.2. Animals and acclimation conditions

Brown mussels *P. perna* (adults, 8–10 cm shell length) were obtained from a commercial mussel farm at Ribeirão da Ilha beach, Florianópolis (Brazil). This area is an important Brazilian bivalve farming site, with constant water quality analysis and metal levels within legislation limits (Sáenz et al., 2010). Mussels were collected during different seasons along the experiments, acclimated over 7 days under laboratory conditions (22–25 °C, 12 h light/dark) in plastic aquaria (1 L clean seawater/animal) and fed every two days with a commercial food for marine filter-feeding invertebrates (Sera Marin Coraliquid). Experimental procedures were followed in accordance to the Federal University of Santa Catarina ethical policy on the use of animals.

2.3. Acute exposure to $ZnCl_2$ and CDNB

Mussels were exposed to different concentrations of $ZnCl_2$ (0; 30; 40; 50 and 100 μM, equivalent to 0, 2.0, 2.6, 3.3 and 6.5 μg Zn^{2+}/L) and CDNB (0; 3; 6; 10 and 25 μM) for 18 h in glass aquaria with 1 L of seawater per animal. These concentrations were based on previous studies of our group with mussels and oysters (Franco et al., 2006; Trevisan et al., 2012, 2014). After exposure, animals were sacrificed and gills were collected for biochemical analyses.

Tissues were homogenized (1:4 w:v) in 20 mM HEPES buffer pH 7.0 and centrifuged at 20,000 *g* for 30 min at 4 °C. Supernatant was collected and activities of enzymes were analyzed spectrophotometrically. GR activity was measured at 340 nm through the NADPH consumption rate in the presence of GSSG (Carlberg and Mannervik, 1985). TrxR activity was measured at 412 nm through the DTNB reduction rate, in the presence of NADPH (Arnér et al., 1999). Total GPx activity was measured at 340 nm through the NADPH consumption rate in the presence of GR, GSH and CHP (Wendel, 1981). All assays were performed using a Cary 50 UV–VIS instrument (Agilent Technologies®, USA).

2.4. Survival of brown mussels to CHP exposure

Mussels were exposed to CHP (0; 100; 300; 1000 and 3000 μM, prepared in seawater, 1 L/animal) over 96 h and mortality was checked daily. After establishment of the LC_{50} (96 h), an additional set of experiments was performed using 100 or 300 μM CHP. Based on the results obtained in experiments outlined in section 2.3, a concentration of $ZnCl_2$ (40 μM) and CDNB (10 μM) was chosen for further studies using 18 h as a pre-exposure period. After the pre-exposure to $ZnCl_2$ or CDNB, mussels were further exposed to CHP 100 or 300 μM for 96 h and the mortality rate was checked daily. An additional group of mussels previously exposed to $ZnCl_2$ or CDNB were also maintained in clean seawater for 96 h, and the mortality rate related only to $ZnCl_2$ or CDNB pre-exposure was assessed. During experiments, water was changed each 24 h and CHP was replaced. Animals were not fed during exposure periods.

2.5. Analysis of in vivo CHP decomposition rate

CHP decomposition rate was analyzed in seawater in the presence or absence of brown mussels, according to our previously published method (Trevisan et al., 2012). To verify whether pre-exposure to $ZnCl_2$ (40 μM) or CDNB (10 μM) for 18 h could