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Oxidative stress in digestive gland and gill of the brown mussel (*Perna perna*) exposed to air and re-submersed

Eduardo A. Almeida[a,*,1], Afonso Celso Dias Bainy[b], Alcir Luiz Dafre[c], Osmar F. Gomes[a], Marisa H.G. Medeiros[a], Paolo Di Mascio[a,*]

[a]*Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26.077, 05513-970 Sao Paulo, Brasil*
[b]*Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, Brasil*
[c]*Departamento de Ciências Fisiológicas, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, 88040-900 Florianópolis, Brasil*

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Abstract

Mussels *Perna perna* were exposed to air for 24 h showing a clear increase in the levels of lipid peroxidation and oxidative DNA damage, measured as 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo). The levels of lipid peroxidation increased both in the digestive gland and gills, while oxidative DNA damage increased only in the gills. After the 24 h of air exposure, mussels were re-submersed for a period of 3 h, leading values to return to a pre-aerial exposure levels. Control animals were kept immersed during the whole period. Several antioxidant and complementary enzymes (superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glucose-6-phosphate dehydrogenase (G6PDH), glutathione *S*-transferase (GST) and the levels of total glutathione (Total GSH) were assayed in a second set of experiments where one group of mussels were exposed to air for 18 h and other to 1 h re-submersion after 18 h aerial exposure. Only a 52% increase in the glutathione *S*-transferase activity was observed in the digestive gland, which remained elevated to about 40% after 1 h re-submersion, showing that defense systems can be modulated even during oxygen deprivation in *P. perna*. The DNA and lipid oxidative damage observed after aerial exposure indicates that mussels face an oxidative challenge, and are able to counteract such an "insult" as values of lipid peroxidation and DNA damage returned to control values after 3 h re-submersion.

Keywords: Mussel; *Perna perna*; Aerial exposure; Lipid peroxidation; DNA damage; Antioxidant

1. Introduction

Reactive oxygen species (ROS) are continually produced in organisms as by-products of cell respiration, and can cause numerous deleterious effects to cells, including lipid peroxidation, inactivation of

* Corresponding authors. Tel.: +55 11 3091 3815x224; fax: +55 11 3815 5579.
E-mail address: pdmascio@iq.usp.br (P. Di Mascio).
[1] Present address: Departamento de Bioquímica y Biología Molecular, Universidade de Córdoba, Campus de Rabanales (Edificio C-6, planta 2), carretera Madrid-Cádiz km 396[a], 14071 Córdoba, España.

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enzymes, oxidation of DNA bases, and protein degradation (Halliwell, 1993; Lemaire and Livingstone, 1993). The level of lipid peroxidation, which leads to the formation of secondary products such as malondialdehyde (MDA), has been largely measured as indicator of injury caused by ROS in different marine organisms. Also, oxidative damage to DNA producing 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodGuo), has been used as an index of DNA damage due to cellular ROS generation (Storey, 1996; Lemaire and Livingstone, 1993; Ravanat et al., 2000; Livingstone, 2001; Cadet et al., 2002).

To protect against ROS, cells possess specific antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), which decompose superoxide anion radical (SOD) and hydrogen peroxide (CAT and GPx) (Sies et al., 1979; Keeling and Smith, 1982; Sies, 1993). Glutathione *S*-transferases (GST), catalyze the conjugation of glutathione (GSH) to electrophilic xenobiotics and/or oxidized components (Tan et al., 1987). Moreover, complementary enzymes such as glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PDH), produce GSH and NADPH to maintain the cellular antioxidant status (Reed, 1986).

Oxidative stress occurs in organisms when the rate of ROS production exceeds the rate of its decomposition by antioxidant systems, leading to an increase in oxidative damage to different cellular targets. This may occur under various condition, one of the most important being the exposure of the organism to hypoxia or anoxia followed by re-oxygenation (Hermes-Lima and Zenteno-Savín, 2002; Storey, 2004). Such condition can lead to significant changes in cell oxidative metabolism, generally induced by oxygen sensors that are modulated by changes in oxygen concentration.

The presence of molecular sensors for oxygen has been demonstrated in cells of different organisms (Wenger, 2000), although its identity and precise mechanism(s) remain unclear (Semenza, 2000). In marine organisms, the existence of a molecular oxygen sensor within the mitochondrion has been demonstrated in the shrimp *Artemia franciscana* (Kwast and Hand, 1996). Oxygen sensors are heme protein, or a protein that encloses a heme cofactor (Hochachka, 1997; Gong et al., 1998), which are affected by modifications in oxygen concentration. The sensor may also contain a NAD(P)H oxidase, and is able to generate ROS to serve as signaling molecules (Bunn et al., 1988). In accordance to this, there is substantial literature reporting on the modulatory effects of ROS on important molecular and cellular responses during hypoxia (Chandel et al., 1998; Chandel et al., 2000; Waypa et al., 2001; Kulisz et al., 2002; Paddenberg et al., 2003; Smith et al., 2004).

Although the production of ROS by oxygen sensors and other unknown mechanisms during hypoxia has been demonstrated, an elevation in the oxygen consumption during the re-oxygenation period after hypoxia may also increase the generation of ROS in cells due to the simultaneously enhanced flux of reducing equivalents and oxygen (Jones, 1986; Hermes-Lima et al., 1998).

Coastal mussels periodically experience cyclic changes in the available oxygen levels caused by tidal oscillations. During low tides, bivalves are able to withstand periods of shell closure and resultant lack of oxygen. To endure oxygen deprivation, mussels possess adaptive mechanisms such as fermentative metabolism, which produces alternative end products of glycolysis that maximize ATP generation (de Zwaan and Wijsman, 1976; Schick et al., 1986; Zange et al., 1989). However, little is known in respect to the mechanisms involved in the modulation of oxidative stress in mussels during hypoxia and reperfusion, as consequence of tidal oscillations.

Both oxidative damage levels and antioxidant enzymes have been studied in marine bivalves from South America, as indicators of injury caused by oxidative stress (Bainy et al., 2000; Torres et al., 2002; Almeida et al., 2003a,b; Almeida et al., 2004a,b; Wilhelm-Filho et al., 2001; Dafre et al., 2004). However, there is scarce information about the responses of these systems to air exposure and re-submersion in these organisms, particularly in the brown mussel *Perna perna*. Thus, in this work we present results obtained from two sets of experiments that were independently carried out to evaluate different aspects of the oxidative stress response of *P. perna* after air exposure and re-submersion. The goal of the first experiment was to evaluate the levels of lipid peroxidation and 8-oxodGuo in digestive gland and gill of *P. perna* after 24 h of air exposure and 3 h of re-submersion. In the second experiment,

the activities of the enzymes SOD, CAT, GPx, GR, G6PDH, GST as well as the levels of total glutathione (total GSH) were measured in same tissues of mussels exposed to air for 18 h and after 1 h of re-submersion.

2. Materials and methods

2.1. Experiment 1: evaluation of lipid peroxidation and 8-oxodGuo levels in mussels, after 24 h of air exposure and 3 h of re-submersion

Mussel *P. perna*, of similar length (8–10 cm), were purchased from a Aquaculture facility located at Sambaqui beach, Florianópolis, SC, Brazil, and separated in three groups of 6 mussels. Group I was kept immersed on seawater for 27 h. Group II was exposed to air for 24 h and group III was exposed to air for 24 h followed by re-submersion on seawater for 3 h. The water temperature during the experiment was kept constant at 18 °C.

After these treatments, the mussels were killed and their digestive glands and gills were excised and immediately immersed in liquid nitrogen (−180 °C). DNA was then extracted from samples by the chaotropic NaI method, and hydrolyzed for the analysis of the 8-oxodGuo by high performance liquid chromatography coupled to electrochemical detection (HPLC/EC) as previously described (Cadet et al., 2002; Torres et al., 2002; Almeida et al., 2003a,b; Almeida et al., 2004a). The levels of lipid peroxidation were also evaluated, measuring the product malondialdehyde with 2-thiobarbituric acid at 532 nm by high performance liquid chromatography, following methodology previously published (Almeida et al., 2003b; Almeida et al., 2004b).

2.2. Experiment 2: measurement of the enzymes SOD, CAT, GPx, GST, GR, G6PDH and total GSH in mussels, after18 h of air exposure and 1 h of re-submersion

Mussels *P. perna* of similar length (6–7 cm) were manually collected at Joaquina beach (Florianópolis, SC, Brazil), and separated in three groups of 8 mussels. Group I was kept immersed on seawater for 19 h. Group II was exposed to air for 18 h and group III was exposed to air for 18 h followed by re-submersion on seawater for 1 h. The water temperature during the experiment was kept constant at 21 °C.

After these treatments, the mussels were killed and their digestive gland and gill were excised and immediately immersed in liquid nitrogen (−180 °C). The tissues were weighed and homogenized with 1:5 vol. of buffer (Tris–HCl 50 mM, 0.15 M KCl, pH 7.4), and centrifuged at 10,000×*g* for 20 min at 4 °C. The supernatant fraction was centrifuged at 40,000×*g* for 60 min at 4 °C, in order to obtain the cytosolic fraction. This fraction was used to analyze the activity of SOD, CAT, GPx, GR, G6PDH and GST.

SOD activity was evaluated by the inhibition of reduction of cytochrome *c* in the presence of hypoxanthine/xanthine oxidase $O_2^{-\cdot}$ generator system at 550 nm (McCord and Fridovich, 1969). CAT activity was quantified at 240 nm by the H_2O_2 decomposition according to the method of Beutler (1975). GPx activity was assayed by the oxidation of NADPH (linked to GSSG reduction by excess glutathione reductase) at 340 nm, using *t*-butylhydroperoxide as substrate, according to Sies et al. (1979). GR activity was measured by the oxidation rate of NADPH at 340 nm, according to Sies et al. (1979). The activity of G6PDH was analyzed by the increasing absorbance at 340 nm, caused by the reduction of $NADP^+$ to NADPH, according to Glock and McLean (1953). GST activity was determined by increasing in absorbance at 340 nm, using reduced glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) as substrates, according to Keen et al. (1976). The total protein content was determined by method of modified Lowry, using bovine albumin as standard (Peterson, 1977).

Pieces of digestive gland were homogenized in perchloric acid 0.5 M (1:10 vol.), and centrifuged for 2 min at 15,000×*g* at room temperature. After centrifugation, the supernatant fraction was neutralized with K_3PO_4 and the total GSH was determined in this fraction by continuous reduction of 5,5′-dithiobis, 2-nitrobenzoic acid (DTNB) in the presence of oxidized glutathione (GSSG), glutathione reductase (GR) and NADPH, at 412 nm according to method described by Tietze (1969).

2.1. Statistical analyses

Data are presented as mean±standard deviation, and were statistically compared using one-way anal-

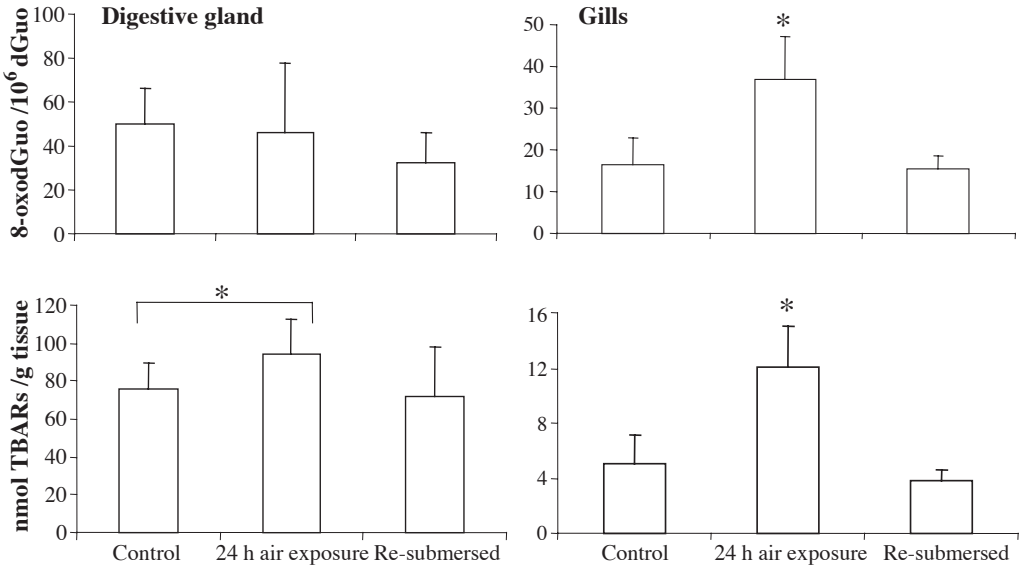

Fig. 1. Levels of lipid peroxidation and 8-oxodGuo in digestive gland and gill of mussel *Perna perna* exposed to air for 24 h, exposed to air for 24 h followed by 3 h of re-submersion, and control mussels. * Indicates statistical differences ($p<0.05$). $N=6$.

ysis of variance, followed by Student–Newman–Keuls test, using the software Statistic for Windows (Zar, 1984). Only $p<0.05$ was accepted as significant.

3. Results

Fig. 1 shows the levels of lipid peroxidation and 8-oxodGuo in gills and digestive glands of mussels. Mussels exposed to air for 24 h had increased levels of lipid peroxidation in both gills and digestive glands, and elevated levels of 8-oxodGuo in gills after 24 h of air exposure, compared to the control group. After 3 h of re-submersion, the levels of both lipid peroxidation and 8-oxodGuo were similar to the control values.

Levels of antioxidant and auxiliary enzymes SOD, CAT, GPx, GR, GST and G6PDH, as well as the levels of total GSH are given in Tables 1 and 2, respectively, for digestive gland and gill of *P. perna*. No differences were observed in such parameters after 18 h of aerial exposure and also after 1 h of re-submersion. The activity of GST was higher after aerial exposure in digestive gland, which remained elevated after the re-submersion period.

Table 1
Level of antioxidant and complementary enzymes (U/mg protein for SOD and CAT, and mU/mg protein for GPx, GR, G6PDH and GST), and of total GSH (μmol/g tissue) in digestive gland of control mussels, in mussels exposed to air for 18 h and exposed to air for 18 h followed by re-submersion on seawater for 1 h

	Control	Exposed	Re-submersed
SOD	178.09±47.87	138.47±32.41	177.32±69.74
CAT	11.44±4.42	10.62±3.80	9.74±3.33
GPx	8.19±3.26	6.85±2.95	7.46±3.80
GR	84.09±15.15	85.87±26.21	59.49±21.46
G6PDH	50.80±13.69	60.02±17.31	48.08±9.95
GST	129.55±33.13	197.40±49.35*	181.49±49.41*
Total GSH	2.57±0.52	2.64±0.88	2.71±0.65

$N=8$.
* Indicates statistical difference compared to the control group ($p<0.05$).

Table 2
Level of antioxidant and complementary enzymes (U/mg protein for SOD and CAT, and mU/mg protein for GPx, GR, G6PDH and GST), and of total GSH (μmol/g tissue) in gill of control mussels, in mussels exposed to air for 18 h and exposed to air for 18 h followed by re-submersion on seawater for 1 h

	Control	Exposed	Re-submersed
SOD	212.65±57.13	168.14±63.90	231.55±49.07
CAT	3.11±0.91	3.50±3.02	3.49±0.76
GPx	9.30±7.56	8.21±6.14	8.28±3.80
GR	84.54±21.00	137.45±56.76	108.71±52.66
G6PDH	116.35±34.56	143.64±31.00	98.77±42.79
GST	630.67±173.51	558.40±203.33	616.10±105.82
Total GSH	1.91±0.49	2.13±0.46	2.14±0.57

$N=8$.

4. Discussion

Levels of lipid peroxidation and 8-oxodGuo have been evaluated in different tissues of the mussel *P. perna*, as indicative of environmental stress (Almeida et al., 2003a,b, 2004a,b; Wilhelm-Filho et al., 2001), but just some antioxidant defenses were evaluated in *P. perna* mussels submitted to hypoxic conditions, and for a short period (Almeida and Bainy, in press). The exposure of mussels to air for 24 h significantly increased the levels of 8-oxodGuo in gill, and of lipid peroxidation in gill and digestive gland. These results indicate that the gill was more prone to oxidative damage than the digestive gland. Both DNA damage and lipid peroxidation were more than doubled in gills while only lipid peroxidation was increased and to a smaller extension (~20% increase) in the digestive gland. Comparisons between the levels of lipid peroxidation and 8-oxodGuo found in different tissues of *P. perna* and other aquatic species were previously described (Almeida et al., 2003a,b). The reason for this difference is not quite clear, and would be due to structural differences between the two tissues, i.e. regarding the lipid and antioxidant content, efficiency of DNA repair systems, etc., even though gills have direct contact with external environment in a large surface area, which can contribute to its higher susceptibility to oxidative damage.

Previous data have indicated that even when mussels are exposed to air, they can maintain a low oxygen flux through the tissues by periodic opening and closure of the shells (gapping behavior) that ensures a low renewal of oxygen (Kennedy, 1976; Widdows et al., 1979; Guderley et al., 1994). The gapping behavior of mussels would contribute to the enhanced levels of tissue damage once gills could be exposed to atmospheric air during the periods of shell opening. In this case, periodic bursts of oxygen might establish a condition similar to ischemia in mammals followed by reperfusion (Jones, 1986). Anyhow, further experiments need to be addressed to test this hypothesis.

Progress has been made in understanding the transcriptional mechanisms activated to deal with hypoxia, but the underlying mechanism of oxygen sensing needed for this activation is not understood (Wu, 2002). Mechanisms including NADPH oxidase (Acker, 1994; Bunn et al., 1988), cytochrome *P*450 (Fandrey et al., 1990) and an oxygen-binding heme protein (Hochachka, 1997; Gong et al., 1998) have been proposed. Mitochondria have been considered as possible oxygen sensors during hypoxia, through the generation of ROS at the ubisemiquinone site of complex III (Chandel et al., 1998). It has been known that ROS can be produced by incomplete reduction of molecular oxygen on mitochondria electron transport chain. ROS generated in these processes includes the superoxide anion radical and hydrogen peroxide, and the tendency for electron transport chain to generate such ROS through inappropriate electron transfer to molecular oxygen depends on factors that include the availability of molecular oxygen, the reduction state of the electron carriers, and the mithocondrial membrane potential (Turrens and Boveris, 1980; Turrens et al., 1985; Dawson et al., 1993; Korshunov et al., 1997). The decrease in cytochrome oxidase V_{max} during hypoxia is responsible for an increase in mithocondrial redox state (Chandel and Schumacker, 2000), which, in turn, accelerates ROS generation during hypoxia, triggering the activation of different transcriptional factors involved in numerous cellular hypoxia responses. Despite its modulator effect, such increase in ROS production would be also accounted for increases in lipid and DNA damage in cells, as observed in this work. On the other hand, other authors have presented evidence that ROS formation is actually decreased during hypoxic periods, suggesting a different mechanism for HIF-induced metabolic adjustments (Michiels et al., 2002).

Recently, we observed that mussels *P. perna* exposed to air for 4 h had increased SOD activity, while no difference was found for other antioxidant enzymes (GPx and CAT; Almeida and Bainy, in press). This was interpreted as a preparative response of mussels to ROS production during subsequent arousal, according to the hypothesis of Hermes-Lima et al. (1998), which observed increased levels of SOD and CAT in 30-day aestivating land snails. Considering that air exposure can result in increases in DNA damage and lipid peroxidation, this increase in SOD activity would also be a cellular response to the augmented ROS generation during the hypoxic period, in addition to the preparative response. Although this increase could not protect against oxidative injury during air exposure, it would contribute to decrease damage after ventilation. It

has been postulated that increases in renal SOD in rats protected against oxidative stress during hypoxia (Chen et al., 2003). Indeed, several antioxidant parameters were changed in the marine gastropod *Littorina littorae* during oxygen deprivation, remarkably an increase in oxidized and reduced glutathione (GSSG and GSH), and increase in lipid peroxidation associated to decreases in antioxidant enzymes, which points to the possibility of increased oxidative damage before oxygen replenishment (Pannunzio and Storey, 1998). Also, increased levels of ferritin and metallothionein were observed in anoxia/freezing stress in *L. littorae*, proposed to be a putative protection against metal-mediated ROS formation (English and Storey, 2003; Larade and Storey, 2002). Protein carbonyl and lipid peroxidation were higher during the estivation period in a land snail, compared to the recovery period, related to apneic respiration during estivation and decreased proteolysis during metabolic depression (Ramos-Vasconselos and Hermes-Lima, 2003; Storey and Storey, 2004). Moreover, these organisms also showed increased GPx activity and GSH levels during estivation, and an increase in GSSG/GSH ratio during arousal, which would indicate a preparative mechanism for the re-oxygenation period. In the freshwater snail *Biomphalaria tenagophila*, decreased CAT and SOD activities were observed after 24 h of anoxia and 15 days of estivation, respectively, related to an overall decrease in metabolic rate (Ferreira et al., 2003). On the other hand, GPx activity significantly increased during anoxia and estivation, a clear preparative response strategy to deal with oxidative stress during recovery.

All these data indicate that important changes in antioxidant systems can occur during oxygen deprivation in anoxia/hypoxia tolerant organisms. However, no such changes in antioxidant defenses were observed after 18 h of air exposure in *P. perna* mussels. Considering that increased SOD activity was observed after 4 h of air exposure in a previous work (Almeida and Bainy, in press), it would indicate a time-course response of the antioxidant defenses of *P. perna*, and this remains to be studied in mussels exposed to air for different time intervals, followed by re-submersion.

When mussels were re-immersed in seawater, the levels of damage to lipid and DNA returned to control values. This pattern shows that mussels are able to keep basal levels of oxidative damage when they are ventilating, which may point to the need of oxygen to support metabolic processes related to oxidative damage repair. Despite this possibility, the mechanism underlying such a response remains to be addressed.

As the levels of lipid and DNA damage were higher after air exposure, and turned back to control values after re-submersion, we expected that significant changes in antioxidant defenses would be involved in these results. However, no differences were observed in the antioxidant and auxiliary enzymes in both gill and digestive gland of mussels, after they were exposed to air for 18 h neither after re-submersion. Just GST activity was higher (52%) in digestive gland after the air exposure period, a level maintained high (42%) during at least 1 h after returning to water. This increase may be due to the excretion of end anaerobic products generated and accumulated during the air exposure period. Moreover, there is recent evidences that GST isoforms have also peroxidase activity (Fiander and Schneider, 1999; Veal et al., 2002; Prabhu et al., 2001; Collinson and Grant, 2003; Amicarelli et al., 2004). Then, increases of GST activity during hypoxia would represent the only antioxidant defense activated against oxidative stress, despite such increase could not prevent the oxidative injury during the exposure period. Increase in GST activity of gills was proposed as a response to oxidative stress when the activity of other antioxidant enzymes are lowered (Power and Sheehan, 1996; Sheehan and Power, 1999; Manduzio et al., 2004).

The values obtained for antioxidant defenses of *P. perna* in this work are in agreement with those previously described in the literature for this species (Bainy et al., 2000; Almeida et al., 2004a,b; Wilhelm-Filho et al., 2001; Dafre et al., 2004; Almeida and Bainy, in press). *P. perna* also presented antioxidant values similar to those reported for other bivalve species (Viarengo et al., 1991; Solé et al., 1995; Power and Sheehan, 1996; Manduzio et al., 2004; Frenzilli et al., 2004), but some discrepancies were observed. For example, *P. perna* presented higher GR in digestive gland than values reported for *Mytilus edulis* and *Mytilus galloprovincialis* (Manduzio et al., 2004; Frenzilli et al., 2004). The activities of G6PDH, SOD and GR were very higher in *P. perna* than the activities observed in the bivalve *Chamaelea galina*, despite the fact that the activities of CAT and GPx

were lower (Rodríguez-Ortega et al., 2002; Romero-Ruiz et al., 2003). Also, the activity of SOD was much higher than the values observed for the horse mussel, *Modiolus modiolus* (Lesser and Kruse, 2004). These differences are probably related to the influence of different environmental conditions (i.e. water temperature or oxygen availability) as well as due to interspecific differences related to biological condition, such as the biological rhythms and the reproductive cycles.

The activity of the antioxidant enzymes GPx, GR and SOD, as well as the levels of total GSH, were similar in both digestive glands and gills of *P. perna*, contrasting with results presented by Manduzio et al. (2004) for the mussel *M. edulis*, presenting higher GR, GPx and SOD in gills. Compared with digestive gland, CAT activity was ~3 times lower in gills, while the activities of G6PDH (~2 times) and GST (~4 times) were higher in gills. Despite the fact that the identification of the major enzyme responsible for the decomposition of hydroperoxides remains a matter of debate in the literature, the lower CAT activity in the gills could be due to the sufficient GPx activity to metabolize peroxides. Another possible, or even complementary, explanation for the lower CAT activity in gills may be accounted for the postulated ability of gills to excrete hydrogen peroxide directly to the water (Wilhelm-Filho et al., 1994). This would restrain hydroperoxides to reach elevated concentrations in the gills, leading GPx in better position to decompose low levels of hydroperoxides due to its lower K_m.

The higher GST activity in gill compared to digestive gland is probably related to the fact that gill is expected to be a more active site of excretion than digestive gland, due to its direct contact with the environment, providing the removal of undesirable compounds from the organism. Also, the low activity of CAT in gills compared to digestive gland would be compensated by the higher GST activity in this tissue, considering that this enzyme would also have peroxidase activity. Increased GST in gill compared to digestive gland was also found for the mussel *M. edulis* (Manduzio et al., 2004). Increased G6PDH in gills compared to digestive gland can maintain reducing equivalents for the higher GST activity in this tissue, despite no such increase in total GSH and GR activity were observed for this tissue.

5. Conclusions

The results obtained in these two experiments indicate that during air exposure (hypoxia) mussels clearly experiment a pro-oxidative condition. Two further information were found. One was the oxidative damage caused by aerial exposure in *P. perna*, which may be due to gapping behavior plus the generation of ROS as messengers of hypoxic cellular responses. The second finding was the ability of mussels to return to basal levels of lipoperoxides derivatives and DNA oxidative damage 8-oxodGuo, indicating that mussels are able to handle the oxidative stress caused by aerial exposure, given that ventilation and, possibly, oxygen levels are recovered. This recovery ability may be present during tidal oscillations, even though tidal oscillation has shorter duration. Also, the maintenance of antioxidant and complementary enzymes at similar levels during air exposure and re-submersion would contribute to the return of lipid and DNA damage to control values after ventilation. To our knowledge, this was the first report on the levels of 8-oxodGuo in an organism exposed to hypoxia/reoxygenation stress.

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