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ORIGINAL PAPER

Roles of catalase and glutathione peroxidase in the tolerance of a pulmonate gastropod to anoxia and reoxygenation

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Abstract Humans and most mammals suffer severe damage when exposed to ischemia and reperfusion episodes due to an overproduction of reactive oxygen species (ROS). In contrast, several hypoxia/anoxia-tolerant animals survive very similar situations. We evaluated herein the redox metabolism in the anoxia-tolerant land snail *Helix aspersa* after catalase inhibition by 3-amino-1,2,4-triazole (ATZ) injection during a cycle of wide and abrupt change in oxygen availability. The exposure to anoxia for 5 h caused a change of only one of several parameters related to free radical metabolism: a rise in selenium-dependent glutathione peroxidase (Se-GPX) activity in muscle of both saline- and ATZ-injected animals (by 1.9- and 1.8-fold, respectively). Catalase suppression had no effect in animals under normoxia or anoxia. However, during reoxygenation catalase suppression kept high levels of muscle Se-GPX activity (twofold higher than in saline-injected snails up to 30 min reoxygenation) and induced the increase in hepatopancreas SOD activity (by 22 %), indicating higher levels of ROS in both organs than in saline-injected animals. Additionally, catalase-suppressed snails showed 12 % higher levels of carbonyl protein—a sign of mild oxidative stress—in muscle during reoxygenation than those animals with intact catalase. No changes were observed in glutathione parameters (GSH, GSSG and GSSG:GSH ratio), TBARS, and GST activity in any of the experimental groups, in both organs. These results indicate that catalase inhibition inflicts changes in the free radical metabolism during reoxygenation, prompting a stress-response that is a reorganization in other enzymatic antioxidant defenses to minimize alterations in the redox homeostasis in land snails.

Keywords Free radicals · Glutathione · Hypoxia · Ischemia · Oxidative damage

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Introduction

The occurrence of ischemia and reperfusion in mammalian tissues is associated with extensive oxidative damage and it is well known that oxidative injury is caused by overproduction of reactive oxygen species (ROS) (Zweier et al. 1987; Serviddio et al. 2005). However, many hypoxia/anoxia-tolerant animals are able to survive situations analogous to ischemia/reperfusion events, in which higher ROS production is expected, such as during post-hypoxia reoxygenation. The ability of these animals to manage such putative elevated ROS production has been associated with an efficient endogenous antioxidant system, which, in many cases, becomes enhanced by the activation of individual or few antioxidants during hypoxia/anoxia (Welker et al. 2013). The activation of antioxidants under oxygen restriction in many hypoxia-tolerant species has been regarded as a relevant mechanism to control the effects of ROS overproduction that may happen at hypoxia and/or reoxygenation—this general mechanism is known as "preparation for

oxidative stress" (Hermes-Lima and Zenteno-Savín 2002; Welker et al. 2013).

Catalase is one of the endogenous antioxidants whose activity or expression increases in response to hypoxia/anoxia or analogous low oxygen stress situations. Elevated catalase levels indicate that this enzyme could play an important role in the management of ROS levels. This is observed in aquatic species, such as goldfish *Carassius auratus* (Lushchak et al. 2001), common carps *Cyprinus carpio* (Lushchak et al. 2005), barnacles *Balanus amphitrite* (Desai and Prakash 2009) and corals *Veretillum cynomorium* (Teixeira et al. 2013), and in terrestrial animals, such as garter snakes *Thamnophis sirtalis parietalis* (Hermes-Lima and Storey 1993), leopard frogs *Rana pipiens* (Hermes-Lima and Storey 1996), and red-eared turtles *Trachemys scripta elegans* (Krivoruchko and Storey 2013). On the other hand, some animals submitted to anoxia/hypoxia do not show the activation or even show a decrease of catalase levels, for example, the marine gastropod *Littorina littorea* (Pannunzio and Storey 1998), the pacu fish *Piaractus mesopotamicus* (Sampaio et al. 2008) and the aquatic salamander *Proteus anguinus* (Issartel et al. 2009). In these cases, other antioxidants may act to manage ROS levels. For example, although catalase activity decreased, total glutathione levels increased in the hepatopancreas of *L. littorea* exposed to anoxia (Pannunzio and Storey 1998). Therefore, the individual relevance of each component of the intricate antioxidant system to the resistance to oxidative stress remains to be determined (Gorr et al. 2010).

To identify the relative importance of one particular component of the antioxidant defense system many researchers have employed the use of specific inhibitors, including drugs, RNA interference or knockout animals. In mammals, the lack (knockout) of catalase lowers the capacity to decompose H_2O_2 (Ho et al. 2004), while catalase overexpression prevents ischemia–reperfusion injury in liver (Ushitora et al. 2010) and heart (Li et al. 1997), and increases lifespan (Schriner et al. 2005; Schriner and Linford 2006). In a previous study, we analyzed the relevance of catalase in a hypoxia-tolerant species by injecting 3-amino-1,2,4-triazole (ATZ), an irreversible catalase inhibitor, in Nile tilapia (*Oreochromis niloticus*) prior to a cycle of hypoxia-reoxygenation. There were no significant differences in several antioxidant and oxidative stress markers between saline- and ATZ-injected fish. However, the disulfide/reduced glutathione ratio was increased during reoxygenation only in ATZ-injected fish, demonstrating a redox imbalance caused by catalase suppression (Welker et al. 2012). This result indicates that catalase is relevant for the control of hepatic ROS formation in that fish species exposed to hypoxia/reoxygenation.

Considering that catalase contributes to maintain the redox balance in animals under natural challenges, we hypothesized that its importance would be more evident in a situation of a much wider change in oxygen availability, such as reoxygenation after anoxia instead of hypoxia. A more severe level of oxidative stress may be observed when reoxygenation is made with a wider difference in oxygen concentration (Zinchuk et al. 2003; Serviddio et al. 2005). For example, air-breathing animals naturally experience instantaneous reoxygenation during emersion after a period of aquatic submersion (Ponganis et al. 2009; Meir et al. 2009; Hermes-Lima et al. 2012). In this context, the pulmonate gastropod *Helix aspersa* is a suitable model to investigate wide and abrupt changes in oxygen availability since it withstands anoxia for 15 h (Ramos-Vasconcelos 2005). The aim of the present study was to evaluate the role of catalase, through its in vivo inhibition by ATZ injection, on the maintenance of redox balance in *H. aspersa* during a cycle of anoxia and reoxygenation.

Materials and methods

Animals and drug injection

Land snails *Helix aspersa* (Muller 1774) weighing around 15 g were purchased from a commercial supplier (Funcia Escargots) in São Paulo state, Brazil. Animals were kept in the laboratory at 23 ± 1 °C inside clear plastic 34 L cages under a 12 L:12 D cycle. Cages were inspected and washed everyday with neutral detergent, kept with sodium hypochlorite 2–2.5 % for 15 min, and thoroughly rinsed with water. During inspection any dead or abnormal animals were removed from the cages. Animals were daily fed ad libitum with a balanced diet made in the laboratory and had daily renewed water. The diet was composed of cornmeal (41 %), fat-free soybean bran (20 %), wheat (15 %), calcium carbonate (20 %), dicalcium phosphate (2.5 %), sodium chloride (0.5 %), animal mineral supplement (0.5 %) and micronutrients supplements (0.5 %). Before any experimental procedure, animals were maintained in the laboratory during an acclimation period for 3 weeks. Only adult snails, showing the development of the apertural lip, were used in this study. In order to calculate the volume of drug to be injected, animals were weighted and the weight of the shell (23 %) was discounted from the total weight.

Preliminary tests were made in order to assess the ATZ concentration that is able to induce significant inhibition of catalase activity in *H. aspersa* without the side effect of increased mortality. The concentration of 1 mg ATZ/g wet weight, commonly used in in vivo studies in animals (Bagnyukova et al. 2005; Gorr et al. 2010; Welker et al. 2012) proved to be adequate. Animals were injected with 1 mg/g of net wet weight ATZ solution made in 0.9 % (w/v) NaCl.

Net wet weight was calculated by subtracting the estimated weight of the shell (23 %) from the total weight of the animal (Ramos-Vasconcelos 2005). Injections were performed with a Hamilton syringe by perforating the central part of the foot muscle and dispensing the volume in the hemocele. After injection animals were maintained under normoxia for 6 h. This procedure resulted in a 90 % suppression of catalase activity. Thus the concentration of 1 mg/gww was further used in the experiments. The experimental procedure was approved by the Animal Ethics Committee of the Universidade de Brasília and performed during summer (November and December).

Anoxia and reoxygenation exposure

To investigate the effects of ATZ, anoxia, ATZ plus anoxia and ATZ plus reoxygenation, we submitted animals to different conditions consisting of 15 groups: (1) control group was composed by animals that were not injected nor subjected to oxygen deprivation; (2) saline normoxic group was composed by animals injected with a volume of 5 μL/g of wet weight of 0.9 % NaCl solution and kept under normoxia for 12 h; (3) ATZ normoxic group was composed by animals injected with a volume of 5 μL/g of net wet weight (e.g., an animal with a net weight of 8 g would be injected with 40 μL) of ATZ 0.2 g/mL in 0.9 % NaCl and kept under normoxia for 12 h; (4 and 5) saline anoxia group and ATZ anoxia group were composed by animals injected with saline or ATZ, respectively, kept under normoxia for 6 h, and exposed to anoxia for 5 h; and (6–15) reoxygenation saline and ATZ groups animals were composed by animals injected with saline or ATZ, respectively, and exposed to 5 h anoxia followed by normoxic reoxygenation for 15, 30 min, 1, 2 and 12 h.

Anoxia exposure was achieved by transferring animals to 5-L glass cages containing a beaker filled with water followed by an influx of 2.5 L/min N_2 gas. After 30 min of N_2 influx the cage was tightly sealed and animals maintained in this anoxic environment for 5 h. During this period, we observed that animals turned their different behaviors to a standardized one, characterized by an increase in the contact of the foot with the glass walls. After anoxia exposure the cage was opened and maintained under normoxia. Animals were euthanized by decapitation, and foot muscle and hepatopancreas dissected. After dissection tissues were briefly rinsed in 0.9 % NaCl, blotted in paper, frozen in liquid nitrogen and stored at −80 °C until analysis.

3-Amino-1,2,4-triazole (ATZ) concentration

The ATZ concentration was determined in foot muscle and hepatopancreas by reacting acid homogenates with the chromotropic acid 4,5-dihydroxynaphthalene-2,7-disulfonic (Green and Feinstein 1957). This method is based on the reaction between ATZ and chromotropic acid that results in the formation of a compound that can be monitored spectrophotometrically at 525 nm. Briefly, tissues were homogenized in ice-cold 5 % (w/v) trichloroacetic acid (TCA) and centrifuged at 10,000×*g* for 6 min at 4 °C. The supernatant was added to a test tube containing 1.4 mM sodium nitrite and 0.36 mM chromotropic acid. Tubes were maintained in boiling water for 2.5 min, cooled on ice and read at 525 nm. The concentration of ATZ in tissues was calculated by comparing samples absorbance to a standard curve made with ATZ solutions ranging from 0 to 15 μg/mL made in 5 % (w/v) TCA. Since there are chromogenic substances naturally found in tissues [whose amount does not interfere significantly in the method (Green and Feinstein 1957)], tissues from saline-injected animals were also assayed, which allowed to observe the absorbance resulting specifically from ATZ.

Antioxidant enzymes activities

For the determination of antioxidant enzymes activities frozen tissues were homogenized in ice-cold 50 mM potassium phosphate pH 7.2 containing 0.5 mM EDTA. Phenylmethylsulfonyl fluoride (PMSF) was added just prior to homogenization to a final concentration of 0.1 μg/g of tissue wet weight. Homogenates were transferred to tubes and centrifuged at 10,000×*g* for 15 min at 4 °C. Supernatants were collected and kept on ice for further use in the assays.

The enzymatic activities were measured as previously reported in (Welker et al. 2012) with minor modifications, which are briefly described below. Catalase activity was determined by monitoring the decomposition of H_2O_2 at 240 nm during the first 40 s of a reaction mixture containing 50 mM potassium phosphate, pH 7.2, 0.5 mM EDTA and 10 mM H_2O_2 (checked every experiment by a 0.40 absorbance at 240 nm). Tissues were sufficiently diluted to provide a rate of H_2O_2 decomposition of approximately −0.03 abs/min. Glutathione transferase (GST) activity was determined by monitoring the formation of a conjugate between CDNB and glutathione at 340 nm in a reaction mixture containing 50 mM potassium phosphate, pH 7.2, 0.5 mM EDTA, 1 mM CDNB and 1 mM GSH. Reaction conditions were always checked by a rate nearly 0.02 abs/min, due to the spontaneous reaction between GSH and CDNB, in blank cuvettes (without homogenate). Glutathione reductase (GR) activity was determined by monitoring the consumption of NADPH at 340 nm in a reaction mixture containing 50 mM potassium phosphate, pH 7.2, 0.5 mM EDTA, 1 mM GSSG and 0.1 mM NADPH. Glucose 6-phosphate dehydrogenase (G6PDH) activity was determined by monitoring the formation of NADPH at 340 nm in a reaction mixture containing 50 mM potassium

phosphate, pH 7.2, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM glucose 6-phosphate and 0.2 mM $NADP^+$. Selenium-dependent glutathione peroxidase (Se-GPX) activity was determined by monitoring the oxidation of NADPH at 340 nm in a reaction mixture containing 50 mM potassium phosphate, pH 7.2, 0.5 mM EDTA, 4 mM NaN_3, 5 mM GSH, 0.1 U/mL GR, 0.2 mM NADPH and 0.073 mM H_2O_2. Hydrogen peroxide was added to the cuvette to initiate the reaction only after NADPH oxidation became stable (3 min) with all other reagents and homogenate. Total GPX activity was determined in the same way as Se-GPX except for the substitution of H_2O_2 by 0.2 mM cumene hydroperoxide.

One unit of each enzyme activity was defined as the amount of enzyme capable of decompose (H_2O_2, catalase), consume substrate directly (NADPH, GR) or indirectly (NADPH, Se-GPX and total-GPX) or generate product (GSH-CDNB conjugate, GST; NADPH, G6PDH) in a rate of 1 μmol/min. One unit of total superoxide dismutase (SOD) activity was determined by assessing the amount of homogenate that is able to inhibit by 50 % the reduction of cytochrome C by superoxide radicals produced by an enzymatic system (McCord and Fridovich 1969). Final concentration of reagents were 50 mM potassium phosphate, pH 7.2, 0.5 mM EDTA, 0.01 mM oxidized cytochrome C, 0.05 mM hypoxanthine and an amount of xanthine oxidase able to result in a rate of 0.025 abs/min at 550 nm. The enzymatic activities were normalized by the concentration of proteins in the homogenates and are expressed as U/mg of protein. Protein concentration was measured by the reaction of proteins with coomassie brilliant blue G-250 (Bradford 1976), using bovine serum albumin as a standard.

Glutathione parameters, lipid peroxidation, and carbonyl protein

Frozen tissues were homogenized in ice-cold 10 % (w/v) TCA with a hand-held glass homogenizer. Aliquots of the resulting crude homogenates were used for the measurement of glutathione parameters, lipid peroxidation levels and carbonyl protein content. Thiobarbituric acid-reactive substances (TBARS) were quantified as an index of lipid peroxidation. Briefly, the crude homogenate was added to a tube in a reaction medium containing 10 % TCA, 0.25 % thiobarbituric acid (TBA), 0.17 M HCl and 0.3 mM butylated hydroxytoluene (Buege and Aust 1978). As a control in a parallel reaction, all reagents, including the crude homogenate, were added in the absence of TBA. Tubes were heated in boiling water for 15 min, cooled and centrifuged at 10,000×*g* for 6 min at 4 °C. The supernatant was used for the readings at 532 and 600 nm. The absorbance at 600 nm was subtracted from the absorbance at 532 nm. The concentration of TBARS was calculated using the molar absorption coefficient of 156 $mM^{-1}cm^{-1}$.

Oxidative damage to proteins was measured by means of protein carbonyl concentration (Fields and Dixon 1971). To measure carbonyl protein content, the crude homogenates were centrifuged at 10,000×*g* for 6 min at 4 °C and the pellets used in the assay. In this method, the carbonyl group of oxidized proteins reacts with 2,4-dinitrophenylhydrazine (DNPH) leading to the formation of 2,4-dinitrophenylhydrazone, that absorbs light at 370 nm. An aliquot of 10 mM DNPH in 0.5 M HCl was added to the tubes. In a parallel tube containing a pellet from the same sample the DNPH solution was substituted for 0.5 M HCl as a control. Tubes were incubated at room temperature for 1 h and mixed thoroughly every 15 min. At the end of this incubation period, tubes were centrifuged at 10,000×*g* for 6 min at 4 °C and the supernatant discarded. The pellets were washed three times with an ethanol:ethyl acetate (1/1, v/v). Each wash cycle comprises the addition of 1 mL of ethanol:ethyl acetate, vigorous mixing, centrifugation at 10,000×*g* for 6 min at 4 °C, and disposal of supernatants. After the last washing step, pellets were dried by leaving the tubes open. The remaining dry pellets were solubilized in 6 M guanidine hydrochloride in 20 mM potassium phosphate pH 2.3. Tube were again centrifuged at 10,000×*g* for 6 min at 4 °C to remove insoluble particles and the supernatant read at 370 nm.

To measure glutathione parameters the crude homogenates were centrifuged at 10,000×*g* for 6 min at 4 °C and the supernatant collected for the assay. Reduced glutathione (GSH), glutathione disulfide (GSSG) and glutathione equivalents (GSH-eq = GSH + 2GSSG) were determined by the enzymatic recycling method as in previous publication (Welker et al. 2012). Briefly, supernatant aliquots were added to a reaction mixture containing 100 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.238 % (w/v) TCA, 0.48 % ethanol, 0.1 mM NADPH, 0.1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 0.05–0.4 U/mL GR. This method is based on the GR-catalyzed reaction between GSH and DTNB, leading to the formation of 2-nitro-5-thiobenzoate (TNB), which absorbs light at 412 nm. The rate of TNB formation is proportional to the concentration of GSH in the reaction medium. To quantify only GSSG content, samples were treated with 0.5 M 2-vinylpyridine. Glutathione parameters were calculated by comparison to standard curves ranging from 0.025 to 1.5 μM.

Statistical analysis

The data were evaluated for the assumptions of normality and homogeneity of variance by the Shapiro–Wilk and Levene's tests, respectively, before applying parametric or

nonparametric tests. To analyze the effects of ATZ, anoxia and ATZ plus anoxia, a one-way ANOVA was used to compare five groups (control, saline normoxic, ATZ normoxic, saline anoxia, and ATZ anoxia) followed by the post hoc Tukey comparison test. If the data did not show normal distribution or homogeneity of variance, they were analyzed by the Kruskal–Wallis test followed by Mann–Whitney comparison test. To analyze the effects of reoxygenation and ATZ or the interaction between them, a two-way ANOVA was used followed by post hoc Tukey comparison test. Simple main effects were performed if there was an interaction between the two effects. The effect of catalase suppression was always verified in the 15 and 30 min reoxygenation, independently of interaction, because of the abundance of data showing an overproduction of ROS exceptionally in the first minutes of reoxygenation (Welker et al. 2012). If the data were non-normally distributed or heteroscedastic, the rank transformation approach was used and two-way ANOVA was then applied to the ranked data according to Conover and Iman (1981).

For all analyses, P values below 0.05 were considered to be statistical significant. Data are presented as mean ± standard error of the mean (SEM).

Results

Anoxia and catalase suppression

Catalase activity in hepatopancreas was suppressed by 67–79 % after 6–11 h of ATZ injection under normoxia and anoxia (in comparison to control and saline-injected animals; Fig. 1a). This inhibition was caused by the presence of 0.7–0.8 mg ATZ/gww in hepatopancreas (Fig. 2a). In foot muscle, catalase activity was suppressed by 60–70 % after 6–11 h of ATZ injection under normoxia and anoxia (Fig. 1c). This was caused by the presence of 0.5 mg ATZ/gww in foot muscle during normoxia (Fig. 2c). Except for catalase and ATZ concentration, no other parameter showed changes in response to ATZ injection alone. Anoxia exposure altered only one component of free radical metabolism: it caused a rise in muscle Se-GPX activity in both saline- and ATZ- injected animals in comparison to control (by 1.9 and 1.8 fold, respectively; Fig. 3c). The activities of Se-GPX from hepatopancreas (Fig. 3a) and total-GPX from both organs (Table 1) were not affected by anoxia exposure and/or catalase inhibition. Moreover, anoxia combined or not with catalase suppression had no effect on the activities

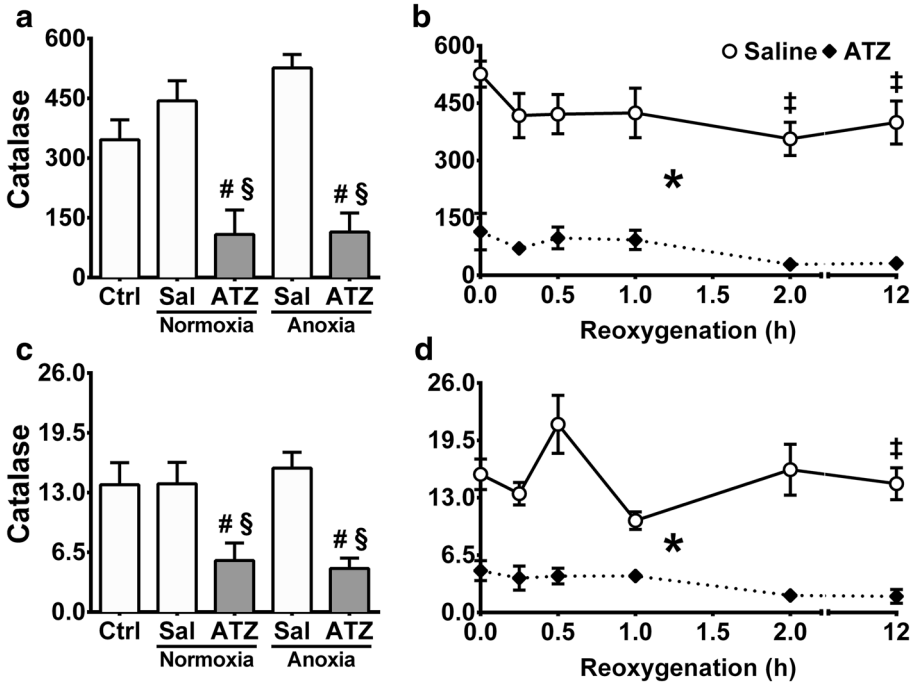

Fig. 1 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on catalase activity (U/mg of protein) of *Helix aspersa* snails. Catalase activity in hepatopancreas **(a)** and foot muscle **(c)** from control (Ctrl, no injection), saline- (Sal, animals injected with 0.9 % (w/v) NaCl) or ATZ-injected (ATZ, animals injected with ATZ at 1 mg/gww solubilized in 0.9 % NaCl) snails maintained in normoxia or exposed to anoxia. Catalase activity in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. *Symbols* denote significant differences from the respective saline group (§) or from the control group (#). Significant ATZ effect is denoted by an *asterisk*. The "‡" symbol represents a significant effect of reoxygenation and differences from the 5 h anoxia group ($n = 6$).

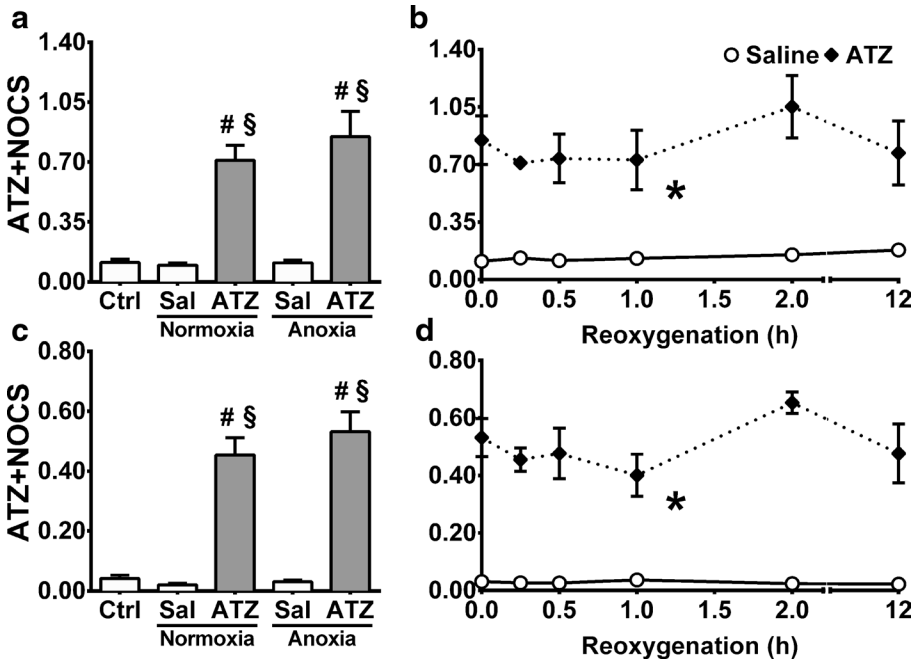

Fig. 2 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on concentration (mg/gww) of ATZ and naturally occurring chromogenic substances (NOCS) of *Helix aspersa* snails. ATZ concentration in hepatopancreas **(a)** and foot muscle **(c)** from control, saline- or ATZ-injected snails maintained in normoxia or exposed to anoxia. ATZ concentration in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. Symbols denote significant differences from the respective saline group (§) or from the control group (#). Significant ATZ effect is denoted by an *asterisk* ($n = 5$–6). See Fig. 1 for information about Ctrl, Sal and ATZ groups

of SOD (Fig. 4), G6PDH (Fig. 5), GR (Fig. 6) and GST (Table 1) and levels of GSH-eq (Table 1), GSH (Sup. Figure 2) and GSSG (Sup. Figure 3), as well as oxidative stress markers (Figs. 7, 8, 9) from either hepatopancreas or foot muscle.

Effects of catalase suppression in reoxygenation

Catalase activity in hepatopancreas remained suppressed throughout the entire reoxygenation period by 77–92 % in relation to saline animals (Fig. 1b). This inhibition was caused by the presence of 0.7–1.1 mg ATZ/gww in hepatopancreas during reoxygenation (Fig. 2b). In foot muscle, catalase activity suppression persisted throughout the reoxygenation period by 60–88 % in relation to saline animals (Fig. 1d). This was caused by the presence of 0.4–0.7 mg ATZ/gww in foot muscle during reoxygenation (Fig. 2d).

Catalase suppression had a significant effect during the reoxygenation period in SOD and G6PDH activities in hepatopancreas (Figs. 4, 5, respectively) and in Se-GPX activity and carbonyl levels in muscle (Figs. 3, 7, respectively; Table 2). In hepatopancreas, the values of SOD activity were 22 % higher (in average) during the reoxygenation period than in saline-injected controls (Fig. 4b). In contrast, hepatopancreas G6PDH activity was 9 % lower (in average) during reoxygenation in ATZ-injected snails (Fig. 5b). In muscle, Se-GPX activity (Fig. 3d) and carbonyl protein levels (Fig. 7d) were increased (in average) by 51 and 12 % in ATZ-injected animals, respectively, in comparison with saline-injected controls.

Reoxygenation alone caused a fall in the activity of three enzymes in comparison to anoxia (Table 2): hepatopancreas catalase (Fig. 1b), muscle GR (Fig. 6d) and muscle Se-GPX (Fig. 3d), by 27, 14 and 33 %, in average, respectively. Reoxygenation also caused a decrease of 10 % of catalase activity in muscle (significant interaction with ATZ; Table 2; Fig. 1d). Immediately after reoxygenation, muscle Se-GPX activity in saline-injected animals became 50 % lower (i.e., returning pre-anoxia values) in comparison to ATZ-injected. This twofold difference between the two groups remained for 30 min of reoxygenation (Fig. 3d).

The other parameters did not change significantly in hepatopancreas in response to reoxygenation with ATZ-induced catalase inhibition or not: activities of Se-GPX (Fig. 3), GR (Fig. 6), total-GPX (Sup. Figure 1) and GST (Sup. Figure 1), levels of carbonyl protein (Fig. 7), TBARS

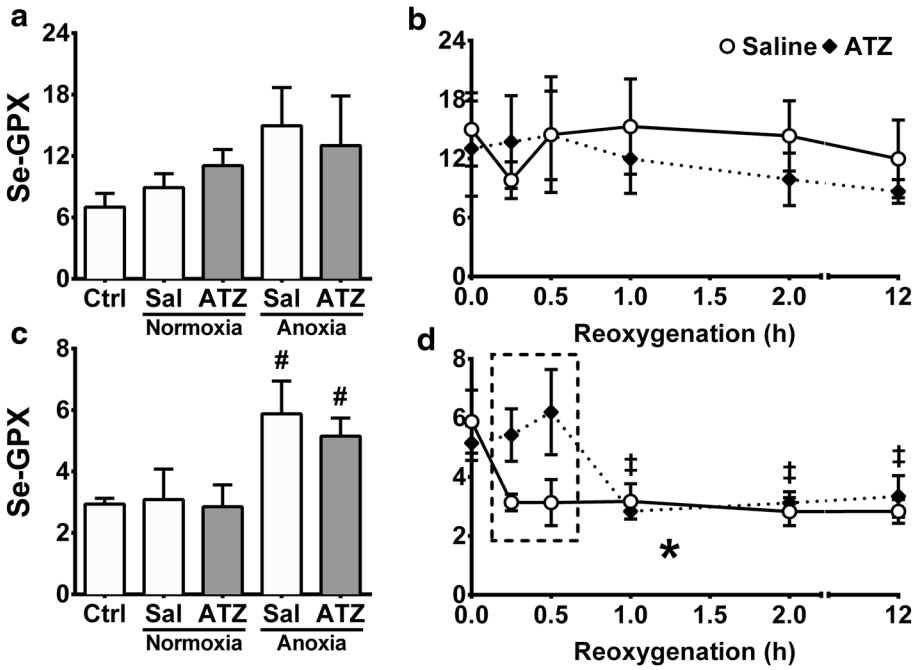

Fig. 3 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on selenium-dependent glutathione peroxidase (Se-GPX) activity (mU/mg of protein) of *Helix aspersa* snails. Se-GPX activity in hepatopancreas **(a)** and foot muscle **(c)** from control (no injection), saline- or ATZ-injected snails maintained in normoxia or exposed to anoxia. Se-GPX activity in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. *Symbols* denote significant differences from the control group (#). Significant ATZ effect is denoted by an *asterisk*. The "‡" symbol represents a significant effect of reoxygenation and differences from the 5 h anoxia group. The *dashed line box* highlights the difference between ATZ and saline-injected groups in the first 30 min of reoxygenation (simple main effect) ($n = 5$–6). See Fig. 1 for information about Ctrl, Sal and ATZ groups

Table 1 Total glutathione peroxidase (total-GPX) activity, glutathione transferase (GST) activity and total glutathione equivalents (GSH-eq) levels in hepatopancreas and foot muscle of control (no injection), ATZ- and saline-injected *Helix aspersa* snails kept under normoxia and exposed to anoxia for 5 h

	Total-GPX (mU/mg prot.)		GST (U/mg prot.)		GSH-eq (μmol/gww)	
	Hepatopancreas	Foot muscle	Hepatopancreas	Foot muscle	Hepatopancreas	Foot muscle
Normoxia						
Control	11.12 ± 1.61	4.44 ± 0.50	1.79 ± 0.18	1.13 ± 0.23	1.62 ± 0.49	0.74 ± 0.17
Saline	10.62 ± 1.54	5.45 ± 1.16	1.62 ± 0.24	0.99 ± 0.28	1.99 ± 0.37	0.81 ± 0.18
ATZ	15.03 ± 2.85	4.37 ± 0.81	1.15 ± 0.15	0.74 ± 0.14	1.14 ± 0.42	0.44 ± 0.12
Anoxia						
Saline	23.36 ± 5.26	8.98 ± 2.14	1.91 ± 0.31	0.94 ± 0.19	1.59 ± 0.45	0.50 ± 0.17
ATZ	22.47 ± 9.24	8.04 ± 0.76	1.62 ± 0.10	0.89 ± 0.16	1.57 ± 0.32	0.47 ± 0.11

There are no statistically significant differences

(Fig. 9), GSH-eq (Sup. Figure 1), GSH (Sup. Figure 2) and GSSG (Sup. Figure 3), and the GSSG/GSH-eq ratio (Fig. 8). In the case of muscle the following parameters were without change in reoxygenation: activities of SOD (Fig. 4), G6PDH (Fig. 5), total-GPX (Sup. Figure 1), GST (Sup. Figure 1), levels of TBARS (Fig. 9), GSH-eq (Sup. Figure 1), GSH (Sup. Figure 2) and GSSG (Sup. Figure 3), and the GSSG/GSH-eq ratio (Fig. 8).

Discussion

We aimed to evaluate the role of catalase on the maintenance of redox balance in *H. aspersa* during a cycle of anoxia and reoxygenation. Catalase suppression alone (animals under normoxia) did not cause changes in the investigated parameters and anoxia exposure caused only one change, a rise of Se-GPX activity in muscle. However,

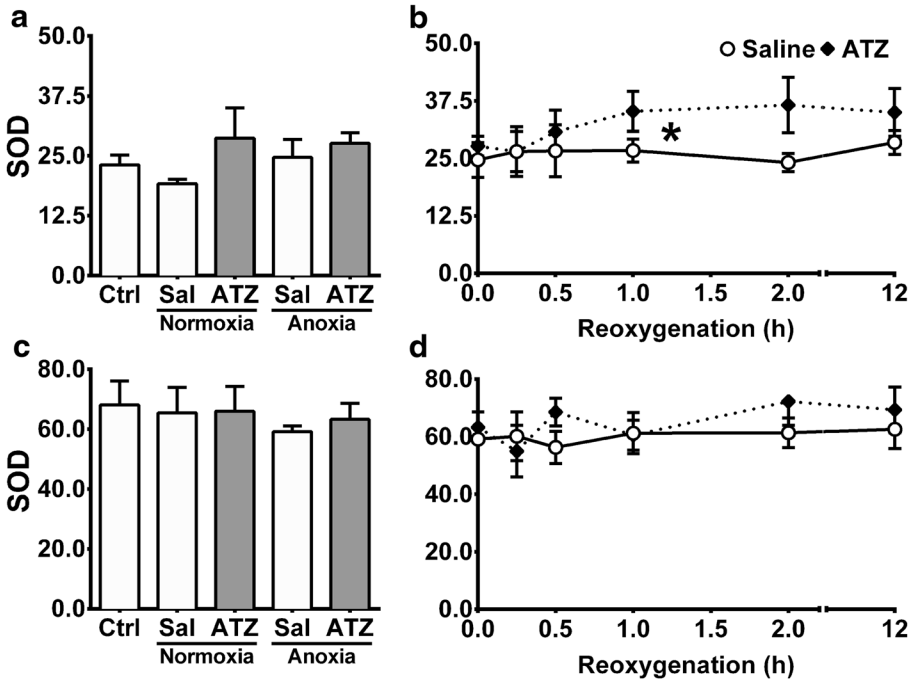

Fig. 4 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on total superoxide dismutase (SOD) activity (U/mg of protein) of *Helix aspersa* snails. SOD activity in hepatopancreas **(a)** and foot muscle **(c)** from control (no injection), saline- or ATZ-injected snails maintained in normoxia or exposed to anoxia. SOD activity in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. Significant ATZ effect is denoted by an *asterisk* ($n = 5$–6). See Fig. 1 for information about Ctrl, Sal and ATZ groups

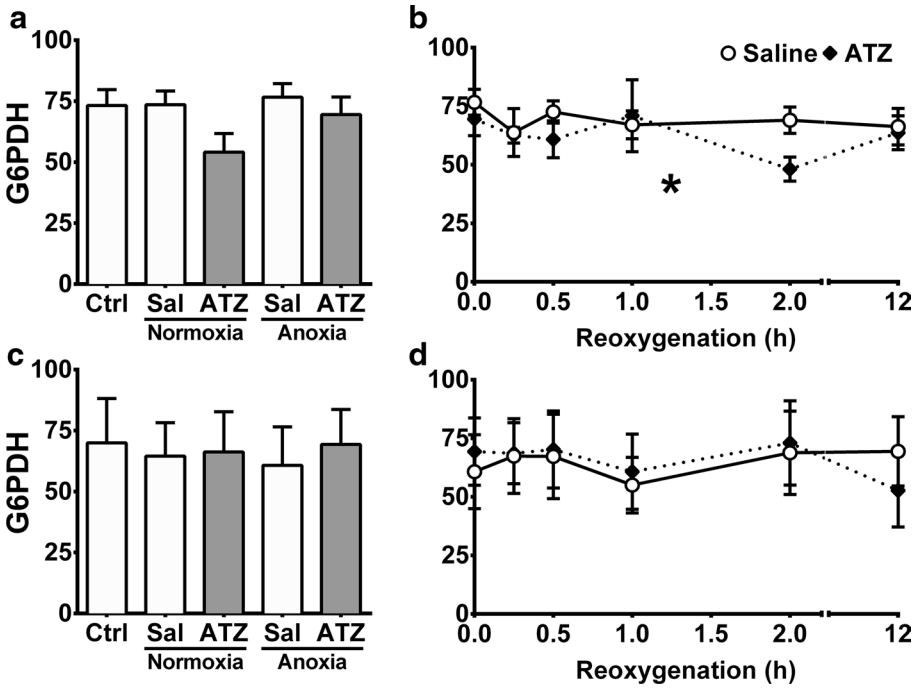

Fig. 5 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on glucose 6-phosphate dehydrogenase (G6PDH) activity (mU/mg of protein) of *Helix aspersa* snails. G6PDH activity in hepatopancreas **(a)** and foot muscle **(c)** from control (no injection), saline- or ATZ-injected snails maintained in normoxia or exposed to anoxia. G6PDH activity in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. Significant ATZ effect is denoted by an *asterisk* ($n = 5$–6). See Fig. 1 for information about Ctrl, Sal and ATZ groups

during the reoxygenation period, catalase suppression caused an increase in SOD activity in hepatopancreas and of Se-GPX activity in muscle in comparison to saline-injected animals, indicating higher levels of ROS in both organs. More than that, during reoxygenation, the inhibition of catalase activity also caused higher levels of carbonyl protein—a marker of oxidative stress—in muscle than in those animals with intact catalase.

Anoxia and reoxygenation stress (not considering ATZ injection)

The maintenance of the markers of redox imbalance (GSSG/GSH-eq ratio) and oxidative stress (TBARS and carbonyl) during anoxia and reoxygenation in saline-injected snails was unexpected since these parameters are often found increased in other animals exposed to similar stressful

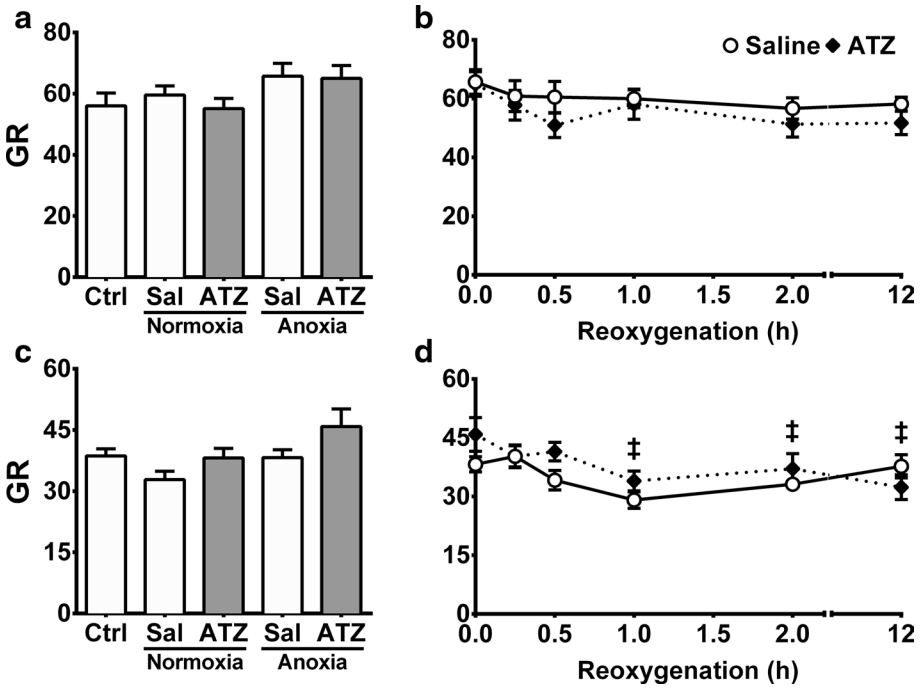

Fig. 6 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on glutathione reductase (GR) activity (mU/mg of protein) of *Helix aspersa* snails. GR activity in hepatopancreas **(a)** and foot muscle **(c)** from control (no injection), saline- or ATZ-injected snails maintained in normoxia or exposed to anoxia. GR activity in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. The "‡" symbol represents a significant effect of reoxygenation and differences from the 5 h anoxia group ($n = 6$). See Fig. 1 for information about Ctrl, Sal and ATZ groups

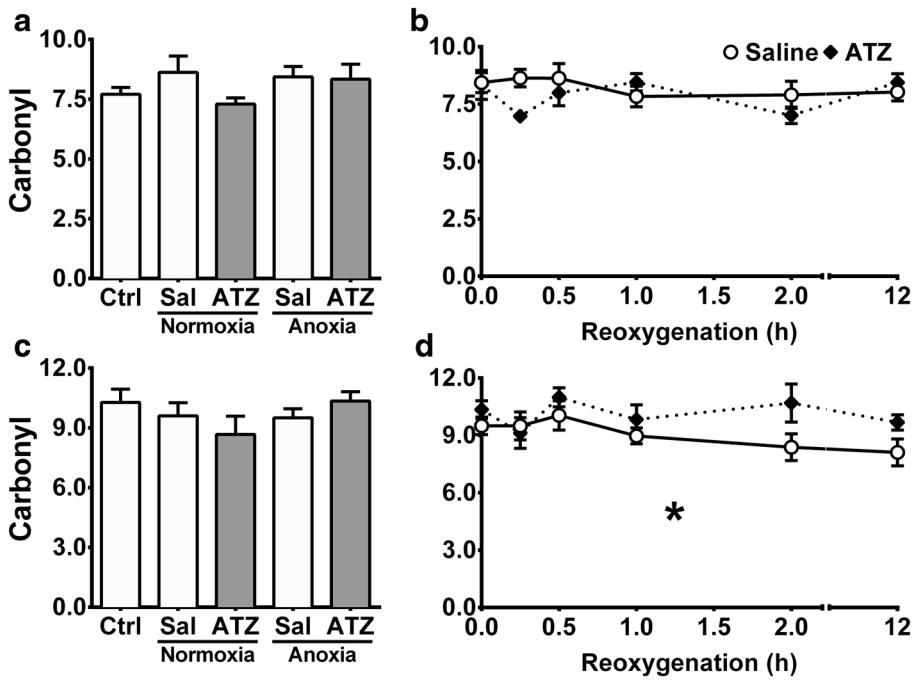

Fig. 7 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on carbonyl protein concentration (nmol/mg of protein) of *Helix aspersa* snails. Carbonyl protein concentration in hepatopancreas **(a)** and foot muscle **(c)** from control (no injection), saline- or ATZ-injected snails maintained in normoxia or exposed to anoxia. Carbonyl protein concentration in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. Significant ATZ effect is denoted by an *asterisk* ($n = 5$–6). See Fig. 1 for information about Ctrl, Sal and ATZ groups

conditions. Therefore, these specific data indicate, at a first glance, that the redox state was not sufficiently disturbed to cause marked oxidative injury in *Helix aspersa*, which seems to endure the putative increase in ROS levels during the anoxia and reoxygenation cycle. This feature could be related to the tolerance of this species to extreme natural conditions. For example, *H. aspersa* survive food and water deprivation (that could cause dehydration) for months in winter (hibernation) and in summer (estivation), and extreme cold temperatures for a few hours (freezing and supercooling) (Ansart et al. 2002; Ramos-Vasconcelos and Hermes-Lima 2003; Ramos-Vasconcelos et al. 2005). However, the absence of changes in the three parameters of oxidative disturbance does not exclude that there was an oxidative burst or changes in redox state (which could be detected by other techniques). In fact, reoxygenation decreased GR and Se-GPX activities in muscle, which may be a consequence of increased ROS levels. There are at least three mechanisms

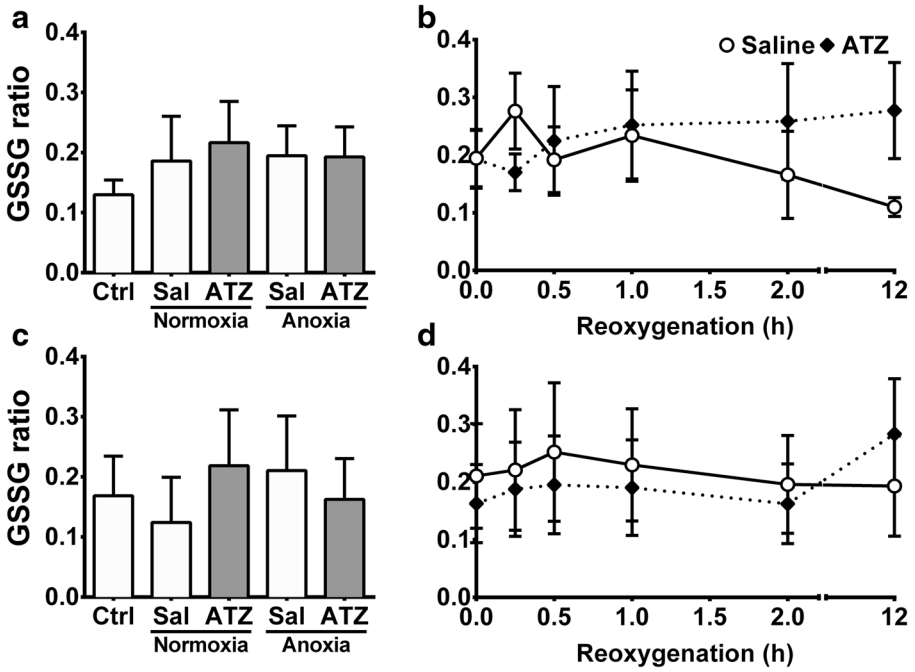

Fig. 8 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on disulfide and total glutathione equivalents ratio (GSSG/GSH-eq) of *Helix aspersa* snails. GSSG/GSH-eq in hepatopancreas **(a)** and foot muscle **(c)** from control (no injection), saline- or ATZ-injected snails maintained in normoxia or exposed to anoxia. GSSG/GSH-eq in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. There were no statistically significant alterations ($n = 5$–6). See Fig. 1 for information about Ctrl, Sal and ATZ groups

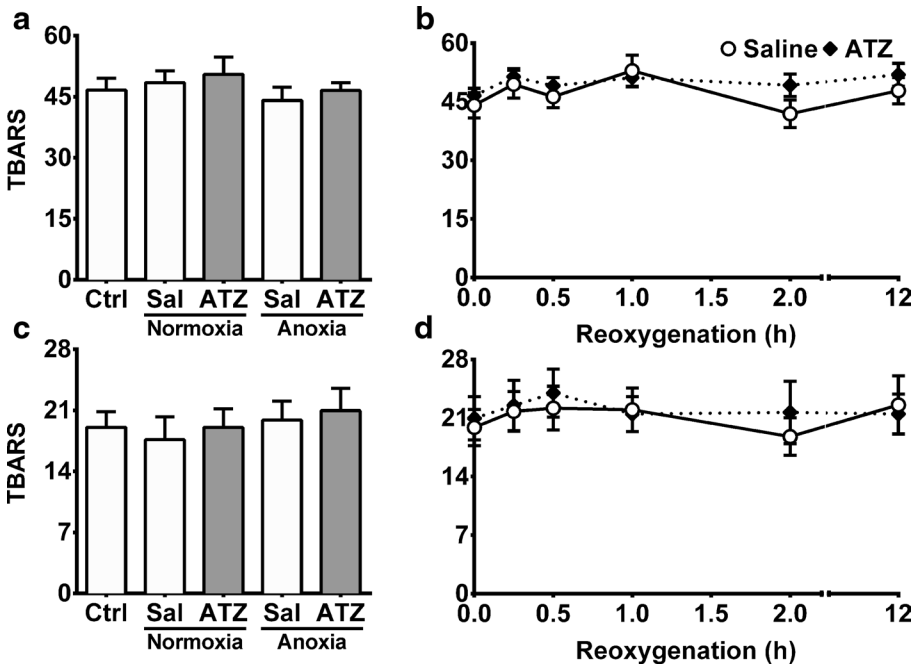

Fig. 9 Effects of anoxia and ATZ injection (**a**, **c**) and of reoxygenation (**b**, **d**) with catalase inhibition or not on thiobarbituric acid-reactive substances (TBARS) concentration (nmol/gww) of *Helix aspersa* snails. TBARS concentration in hepatopancreas **(a)** and foot muscle **(c)** from control (no injection), saline- or ATZ-injected snails maintained in normoxia or exposed to anoxia. TBARS concentration in hepatopancreas **(b)** and foot muscle **(d)** at different reoxygenation times "zero" (5 h anoxia), 0.25, 0.5, 1, 2 and 12 h from saline-injected and ATZ-injected snails. There were no statistically significant alterations ($n = 6$). See Fig. 1 for information about Ctrl, Sal and ATZ groups

by which *H. aspersa* snails could control ROS levels and avoid oxidative stress under wide changes in oxygen availability: (1) managing ROS once they are formed by having high constitutive antioxidant levels or modulating antioxidant defenses during fluctuations of oxygen availability; (2) controlling mitochondrial ROS production itself, avoiding overproduction, or (3) employing both mechanisms.

The increase in Se-GPX activity in foot muscle, in conjunction with the maintenance of high activities of other antioxidant enzymes fits in the first hypothesis. The higher levels of this enzyme could confer a better control over H_2O_2 levels under anoxia/reoxygenation. Increased Se-GPX activities or expression were also reported in other situations of low oxygen stress, including freezing and dehydration, as well as aerial exposure of aquatic animals. Examples include: garter snakes *T. sirtalis* (Hermes-Lima and Storey 1993) and wood frogs *Rana sylvatica* (Joanisse and Storey 1996) exposed to freezing; leopard frogs *R.*

Table 2 Hepatopancreas and foot muscle data analysis by the two-way ANOVA test for effects of catalase suppression (ATZ versus saline) and reoxygenation time (anoxia, 0.25, 0.5, 1.0, 2.0, and 12 h) on redox-related parameters in *Helix aspersa*

Parameter	Effect	Hepatopancreas			Foot muscle		
		df	*F*	*p*	*df*	*F*	*p*
CAT	Catalase suppression	1	207.446	**0.000**	1	220.467	**0.000**
	Reoxygenation time	5	4.311	**0.002**	5	2.268	0.059
	Catalase suppression × reoxygenation time	5	0.900	0.487	5	2.466	**0.043**
ATZ	Catalase suppression	1	210.526	**0.000**	1	210.666	**0.000**
	Reoxygenation time	5	1.514	0.199	5	0.527	0.755
	Catalase suppression × reoxygenation time	5	1.173	0.333	5	1.505	0.202
Se-GPX	Catalase suppression	1	0.221	0.640	1	9.537	**0.003**
	Reoxygenation time	5	0.164	0.975	5	3.841	**0.004**
	Catalase suppression × reoxygenation time	5	0.387	0.855	5	1.184	0.328
SOD	Catalase suppression	1	6.285	**0.015**	1	1.564	0.216
	Reoxygenation time	5	1.072	0.385	5	0.544	0.742
	Catalase suppression × reoxygenation time	5	0.446	0.815	5	0.521	0.759
G6PDH	Catalase suppression	1	4.748	**0.033**	1	0.032	0.858
	Reoxygenation time	5	0.997	0.427	5	0.349	0.881
	Catalase suppression × reoxygenation time	5	0.471	0.796	5	0.228	0.949
GR	Catalase suppression	1	3.302	0.074	1	3.523	0.065
	Reoxygenation time	5	1.886	0.110	5	3.688	**0.006**
	Catalase suppression × reoxygenation time	5	0.291	0.916	5	1.532	0.193
Carbonyl prot.	Catalase suppression	1	1.961	0.167	1	7.100	**0.010**
	Reoxygenation time	5	1.180	0.330	5	1.415	0.232
	Catalase suppression × reoxygenation time	5	1.700	0.149	5	0.890	0.493
Total-GPX	Catalase suppression	1	0.268	0.607	5	1.356	0.254
	Reoxygenation time	5	0.137	0.983	1	0.271	0.605
	Catalase suppression × reoxygenation time	5	0.343	0.885	5	0.619	0.686
GST	Catalase suppression	1	0.127	0.723	1	3.691	0.059
	Reoxygenation time	5	0.568	0.724	5	2.017	0.089
	Catalase suppression × reoxygenation time	5	0.471	0.796	5	0.239	0.944
GSH-eq	Catalase suppression	1	0.005	0.945	1	0.004	0.948
	Reoxygenation time	5	0.364	0.871	5	0.757	0.584
	Catalase suppression × reoxygenation time	5	0.270	0.928	5	0.560	0.730
GSH	Catalase suppression	1	0.074	0.786	1	0.016	0.901
	Reoxygenation time	5	0.344	0.884	5	0.524	0.757
	Catalase suppression × reoxygenation time	5	0.438	0.820	5	0.626	0.680
GSSG	Catalase suppression	1	2.713	0.105	1	0.101	0.752
	Reoxygenation time	5	0.838	0.528	5	1.671	0.156
	Catalase suppression × reoxygenation time	5	0.507	0.770	5	1.454	0.219
GSSG/GSH-eq	Catalase suppression	1	1.097	0.299	1	0.154	0.696
	Reoxygenation time	5	0.416	0.836	5	0.174	0.971
	Catalase suppression × reoxygenation time	5	1.252	0.297	5	0.363	0.872
TBARS	Catalase suppression	1	2.682	0.107	1	0.236	0.629
	Reoxygenation time	5	1.716	0.145	5	0.367	0.869
	Catalase suppression × reoxygenation time	5	0.502	0.773	5	0.053	0.998

Significant p values ($p < 0.05$) are in *bold*

pipiens (Hermes-Lima and Storey 1996), aquatic snails *Biomphalaria tenagophila* (Ferreira et al. 2003), and goldfish *C. auratus* (Lushchak et al. 2001) exposed to anoxia; subterranean crustaceans *Niphargus rhenorhodanensis* (Lawniczak et al. 2013), Pacific oysters *Crassostrea gigas* (David et al. 2005), and common carps *Cyprinus carpio* (Lushchak et al. 2005) exposed to hypoxia; and estuarine crabs *Callinectes ornatus* exposed to air (Freire et al.

2011). These observations indicate a particular role of Se-GPX in the control of H_2O_2 in situations of wide changes in oxygen availability. In the case of foot muscle, increased Se-GPX activity observed during anoxia stands as a case of "preparation for oxidative stress" (Moreira et al. 2016).

The observed up-regulation of Se-GPX in anoxia could be via the increased transcription of its mRNA or by covalent modification. The expression of GPX is under control of Nrf2 and NF-κB transcription factors (Banning et al. 2005; Morgan and Liu 2011; Niture et al. 2014). Thus, if ROS production in the hypoxic phase that precedes full anoxia is increased (Welker et al. 2013), this could activate Nrf2 and/or NF-κB and, therefore, induce the expression of Se-GPX. Moreover, in mammalians it is well known that GPX-1 is regulated by the c-Abl and Arg tyrosine kinases (Cao et al. 2003). These kinases are activated in response to oxidative stress, inducing phosphorylation of GPX-1 and activation of the enzyme. Thus, redox-sensitive transcriptional factor activation in hypoxia and the covalent modification of antioxidant enzymes were recently proposed to explain the molecular mechanism of "preparation for oxidative stress" (Hermes-Lima et al. 2015; Moreira et al. 2016).

In the case of hepatopancreas, the maintenance of Se-GPX activity suggests that general maintenance of antioxidant capacity would be of key relevance for oxidative stress avoidance in anoxia/reoxygenation in *H. aspersa*. Under anoxia, the lower metabolic rate is expected to be associated with a general down-regulation of gene transcription, protein translation and function; therefore, the simple conservation of an enzyme activity indicates that it may be particularly relevant (Hermes-Lima and Zenteno-Savín 2002; Welker et al. 2013).

The second hypothesis is related to the action of alternative end oxidases (AOX) and UCPs (type 2 and 3) in restraining ROS formation in mitochondria. AOX are frequently studied in marine invertebrates (Abele et al. 2007). These proteins avoid the over-reduction of ubiquinone and, thus, there are fewer electrons to be transferred to O_2 for superoxide formation (El-Khoury et al. 2013). It was shown that AOX mRNA levels increase in the hepatopancreas of Pacific oysters after a cycle of emersion and re-immersion, a condition that is expected to elicit redox alterations (Sussarellu et al. 2012). In the case of UCPs, there is a correlation between decreased mitochondrial ROS production and over-expression of UCPs 2 and 3 in several animal models [these proteins are also induced by ROS (Mailloux and Harper 2011)]. Accordingly, the lack of changes in TBARS in the liver of the salamander *Proteus anguinus* under post-anoxic reoxygenation was attributed to the high constitutive level of expression of a UCP2 homologue (Issartel et al. 2009). These two groups of proteins, UCPs and AOX, could play a role in *H. aspersa* by attenuating ROS formation. Therefore, the action of AOX and UCPs in restraining ROS formation, plus the action of endogenous antioxidants (enzymes and GSH) in controlling the effects of ROS, could act in concert to minimize oxidative stress in *H. aspersa* during anoxia/reoxygenation.

Effects of catalase suppression

The injection of ATZ strongly suppressed catalase activity in both hepatopancreas and foot muscle. The loss of catalase alone under normoxia and even during anoxia had no effect on the activity of other enzymes or GSH levels. During the reoxygenation period, catalase suppression also caused no changes in the parameters of redox imbalance (GSSG/GSH-eq ratio and GSSG levels) in both organs. These results are in accordance with previous studies in which this species showed a high resistance to ROS (Ramos-Vasconcelos et al. 2005). However, some relevant effects of catalase inhibition occurred during reoxygenation. After oxygen was reintroduced, the increase in the activity of SOD in hepatopancreas and the maintenance of high Se-GPX levels in muscle in catalase-suppressed animals—but not in saline-injected snails—indicate that catalase inhibition led to an increase in steady-state ROS levels in reoxygenation. Considering that SOD activity is positively related to superoxide generation in several animal species (Welker et al. 2013), the increase in SOD activity in ATZ-injected animals suggests that catalase suppression was accompanied by an elevation in ROS levels during reoxygenation. Similarly, tilapia fish also show an increase in liver SOD activity during reoxygenation after hypoxia (Welker et al. 2012). In foot muscle, Se-GPX activity of saline-injected snails, which was significantly elevated during anoxia, was back to baseline right after anoxia exposure. In contrast, Se-GPX activity remained elevated in ATZ-injected animals in early reoxygenation. This difference could be explained by the ATZ-induced suppression of catalase, which may have caused an increase in peroxide tonus during reoxygenation. Such fast changes in enzyme activity may occur as a result of reversible covalent modification. As explained above, phosphorylation of GPX has been observed in mammalian cells under oxidative stress. Despite the increase in the activities of antioxidant enzymes, catalase-suppressed snails showed 12 % higher carbonyl protein levels in muscle (Fig. 7) during reoxygenation in comparison to saline-injected animals, although there were no changes in redox balance markers (GSSG/GSH-eq ratio and GSSG) and TBARS. This sign of mild oxidative stress (Lushchak 2014) indicates that catalase plays an important role in coping with the reoxygenation-expected rise in ROS production.

The differences between hepatopancreas and muscle observed in the present study may have a relation to the

lower constitutive level of Se-GPX activity in muscle in comparison to hepatopancreas. As a consequence, during an episode of increased ROS production, muscle Se-GPX may work nearer to its maximal H_2O_2-decomposing capacity than hepatopancreas Se-GPX and this could translate into an increased sensitivity of muscle to rises in ROS levels than hepatopancreas. It is interesting the observation from summer-estivating *H. aspersa*, where hepatopancreas Se-GPX activity was much greater than in winter-estivating animals (120–200 versus 20–30 mU mg protein). This higher Se-GPX activity in summer was related to a lack of hepatic oxidative stress in these summer-animals, contrary to winter-snails (Ramos-Vasconcelos et al. 2005).

There are many cases in which the loss of catalase activity induced by ATZ, RNAi or knockout strategies, were able to alter the cellular redox homeostasis. In frogs under normoxia, ATZ injection increased renal lipid peroxidation levels (Barja de Quiroga et al. 1989). In goldfish under normoxia, ATZ injection prompted an increase in TBARS in both liver and kidney within 24 h (Bagnyukova et al. 2005). In goldfish brain, increased protein carbonyl and TBARS levels were observed within 24–48 h of ATZ injection, as well as activation of GST and GPX activities (Bagnyukova et al. 2005). Administration of ATZ in houseflies induced an increase in peroxide levels (up to 9 days) and in GSSG concentration (Allen et al. 1983). ATZ injection resulted in a sharp rise in H_2O_2 levels in the midgut of a blood-sucking insect (Paes et al. 2001). In another study, suppression of catalase induced by RNAi was responsible for increased mortality in a dipteran after blood ingestion, which is a prooxidant meal rich in iron (Magalhaes et al. 2008). In Nile tilapia, ATZ injection was able to induce hepatic post-hypoxia redox imbalance (Welker et al. 2012). In rat astrocyte cultures exposed to H_2O_2, pre-treatment with ATZ induced a greater level of cell death than cultures will full catalase activity (Liddell et al. 2004). In H_2O_2 challenged rat hepatocytes, ATZ induced a sharp increase in necrotic nuclei (la Rosa et al. 2006). Moreover, catalase knockout was able to accelerate diabetic renal injury in mice via peroxisomal damage (Hwang et al. 2012). Thus, in several studies, the single suppression of catalase was enough to induce redox alterations, while in others catalase inhibition and the exposure to oxidative stressful situations also had deleterious effects. However, an absence of changes in markers of oxidative stress—determined as lipid hydroperoxides—was observed in response to ATZ-derived catalase suppression in isolated hearts of rats submitted to ischemia–reperfusion (Konorev et al. 1993). In the present study, the inhibition of catalase in *H. aspersa* also caused no changes in the levels of indicators of oxidative stress in hepatopancreas (Figs. 7, 8, 9), which could lead to a precipitate interpretation that catalase in this organ is not directly relevant in the management of ROS levels. However, as explained above, the increased hepatopancreas SOD activity in response to catalase suppression during reoxygenation (in comparison to saline-injected animals) indicates (1) a rise in ROS levels and (2) that catalase is actually relevant to control redox imbalance and oxidative stress in this organ. In muscle, the key role of catalase in minimizing oxidative stress is meaningfully corroborated by the higher levels of carbonyl protein and Se-GPX activity during reoxygenation in ATZ-injected animals in comparison to saline-injected ones.

As indicated above, the suppression of catalase in *H. aspersa* snails coincided with an extended higher Se-GPX activity during reoxygenation (up to 30 min). This maintenance of increased Se-GPX activity could be important to manage any augmented H_2O_2 formation during anoxia/reoxygenation prompted by the suppression of catalase activity. This increased activity of Se-GPX may have contributed to uphold higher increases in the level of oxidative stress. Therefore, the results of carbonyl protein together with Se-GPX indicate that catalase is relevant for the control of reoxygenation stress in foot muscle. Considering the existence of different antioxidants and that each of them contribute partially to the control of redox balance, it would be not surprising that the strong suppression of a single antioxidant enzyme could cause drastic changes. Although several enzymes were assessed in the present study, others could have been altered by catalase inhibition, such as peroxiredoxins. In *Biomphalaria glabrata* snails, infection by *Schistosoma mansoni* increases the transcript levels of the BgPrx4 that resemble a homolog of human peroxiredoxin PRDX4 (Knight et al. 2009). Therefore, in *Helix aspersa*, a putative increased antioxidant activity could also be exerted by peroxiredoxins.

Considerations about the measured parameters

The parameters chosen to be measured in the present study are the most commonly assessed in investigations on comparative redox metabolism, allowing the direct comparison of our results to data of several works. Other important antioxidant proteins and oxidative stress markers, which were not measured in the present study, could complement the understanding of the biochemical adaptation to anoxia and reoxygenation cycles: the expression/activity of peroxiredoxins, thioredoxin and glutamate cysteine ligase (involved in GSH biosynthesis), and levels of lipid hydroperoxides (Hermes-Lima et al. 1995), 8-oxo-7,8-dihydro-2′-deoxyguanosine [marker of DNA oxidative damage (Almeida et al. 2007)] and H_2O_2. However, the markers of oxidative stress determined herein—GSSG/GSH-eq ratio, TBARS and carbonyl protein—have been shown to be affected by oxygen deprivation and reoxygenation, as well as estivation, in several animal species, including land

snails (Welker et al. 2013; Hermes-Lima et al. 2015). Our findings in hepatopancreas are supported by a previous study in which the injection of a threefold higher dose of ATZ (3 mg/gww) in *H. aspersa* (causing 90 % loss of catalase activity in hepatopancreas, the only tissue analyzed) induced no relevant changes in TBARS and carbonyl protein levels in that tissue during reoxygenation (15 min–36 h) (Ramos-Vasconcelos 2005). On the other hand, the analysis of muscle in the present study revealed an increase in carbonyl protein levels (Fig. 7) in catalase-suppressed animals during reoxygenation.

Conclusion

The single exposure to anoxia (5 h) caused a change of only one of several parameters related to free radical metabolism, a rise in Se-GPX activity in muscle of both saline- and ATZ-injected animals (by 1.9 and 1.8 fold, respectively). Catalase suppression induced by ATZ had no effect in animals under normoxia or anoxia. However, it caused significant changes during reoxygenation. In ATZ-injected animals, the activities of SOD in hepatopancreas and Se-GPX in muscle became higher than saline-injected controls (by 22 and 51 %, respectively), indicating that catalase suppression caused higher steady-state levels of ROS in both organs. In agreement, catalase-suppressed snails also showed higher levels of carbonyl protein (just a 12 % increase) in muscle during reoxygenation than in those animals with intact catalase. This sign of mild oxidative stress corroborates the view that catalase plays a pivotal role in coping with situations of augmented ROS levels, which is the case of post-hypoxia reoxygenation in tilapia fish (Welker et al. 2012) and post-anoxia reoxygenation in snails (this work). Moreover, the higher activity of muscle Se-GPX observed in catalase-suppressed snails during the initial phase of reoxygenation possibly contributed to avoid a severe increase in oxidative stress, compensating for the very low catalase activity that is still remaining in this tissue.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval All procedures performed in the present study involving animals were in accordance with the ethical standards of the Animal Ethics Committee of the University of Brasília, as well as applicable international guidelines for the care and use of animals.

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