Antioxidant defences role during post anoxic recovery in bivalve mollusc *Scapharca inaequivalvis*

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Abstract

Euryoxic organisms are able to withstand hypoxic conditions until the restoration of normal O_2 levels, when the re-introduction of oxygen causes a decisive burst in reactive oxygen species (ROS). The consequent oxidative damage is countered by defence systems constituted by antioxidant enzymes, such as SOD, CAT, Se-GPx and G6PDH. Specimens of* Scapharca inaequivalvis *were exposed to anoxia for 3, 6 and 24 hours and subsequent reoxygenation for 1, 3, 5 and 24 hours. It has been investigated SOD, CAT, Se-GPx and G6PDH activity and MnSOD and CuZnSOD expression in the digestive gland. The results showed SODs activity to be high during both anoxia and reoxygenation. CAT and Se-GPx increase significantly overall during recovery. Only CuZnSOD expression was greater after 6 and 24 hours of anoxia. The presence of high levels of antioxidant enzymes during anoxia suggests that the response of* S. inaequivalvis *is typical of euryoxic animals and represents an interesting model for studying the processes of ischaemia/reperfusion in mammals.

Introduction

Invertebrates and the lesser-developed vertebrates have developed a good tolerance of anoxia and ischaemic episodes that allows them to survive [1], by lowering their basal metabolic rate and using their glycogen reserves [2]. Mammals, on the contrary, have a limited tolerance to low oxygen levels and are less able to withstand the oxidative burst caused by reactive oxygen species (ROS), characteristic of reoxygenation. Reoxygenation triggers lipid peroxidation, protein oxidation [3], an increase in Ca^{++} responsible for converting xanthine dehydrogenase (XDH) into xanthine oxidase (XOD) [4].

The extent of the biological damage caused by the oxyradicals depends on the efficacy of the antioxidant defences [5] such as superoxide dismutase and its manganese and copper-zinc isoforms (Mn/CuZnSOD), which dismutes $O_2^{\bullet-}$ into H_2O_2 and O_2; catalase (CAT) which dismutes H_2O_2 into water and oxygen; glutathione peroxidase (Se-GPx) which reduces H_2O_2 to water using reduced glutathione (GSH) as a substrate, glucose 6 phosphate dehydrogenase (G6PDH) which helps to maintain the activity of Se-GPx [6].
In this study, specimens of *Scapharca inaequivalvis* (a euryoxic mollusc of the Adriatic Sea) [7], underwent anoxia and subsequent reoxygenation to evaluate the alterations of the main antioxidant systems mentioned above.

Materials and methods

Experimental procedure

Specimens of *Scapharca inaequivalvis* (Mollusca, Bivalvia) from the north Adriatic, kept in tanks containing sea water (salinity: 28‰, temperature: 9.0±0.5°C, 14 days).
The animals were kept in conditions of anoxia for 3, 6 and 24 hours and then reoxygenated for 1, 3, 5 and 24 hours. The controls were taken from the normoxic tanks.
The water was deoxygenated using a continuous flow of gaseous nitrogen until a percentage of about 10% of oxygen was obtained. The tests were conducted in pooled digestive glands (3 pools of 7 animals for each condition).

Extraction

Digestive gland subcellular fractions were prepared essentially as described in Livingstone [8]. All procedures were carried out at 4 °C. Frozen pooled digestive gland was homogenized with an Ultraturrax homogenizer in 1:4 (w/v) homogenization buffer (20mM Tris–HCl pH 7.6, 0.5M sucrose, 0.15M KCl, 1mM EDTA). The homogenate and resulting supernatants were progressively centrifuged at 500g for 30 min, 12,000g for 45 min and 100,000g for 90 min to obtain the different cellular fractions. The mitochondrial and cytosolic fractions were combined together in the same ratio as in the original homogenate and used for enzymes activity (U/g wet/weight) and expression determinations (arbitrary units).

Antioxidant enzymes determination

SOD activity was measured at 550 nm and determined with the xanthine oxidase–cytochrome c method according to Crapo *et al.* [9]. The CuZn-containing form of SOD was assayed by the inhibitory effect of KCN on SOD activity [10].
CAT activity was measured by decreasing H_2O_2 concentration at 240 nm [11]. Decays in absorbance were recorded in 50mM sodium phosphate buffer (pH 7.0) containing 20mM H_2O_2 and the enzyme sample.
Se-GPx activity was evaluated according to Paglia and Valentine [12] at 340 nm. Se-GPx activity was monitored through NADPH absorbance diminution using H_2O_2. Activity was calculated from the difference between the presence and absence of the sample.
G6PDH activity was assayed using the modified method of Löhr and Waller [13] by spectrophotometrically monitoring of the variation in $NADP^+$ absorbance at 340 nm using glucose 6 phosphate (G6P).

Protein content determination

Total soluble protein concentration was measured according to Lowry *et al.* [14] by a Bio-Rad DC Protein Assay kit using a spectrophotometrical method (750 nm), with microassay procedure and bovine serum albumin as the standard. Values reported as mg protein/ml were used to normalize enzyme expression.

Immunological assay

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of different subcellular fractions, was performed in an Invitrogen Xcell SureLock Mini-Cell. Samples were added with LDS (lithium dodecyl sulphate) buffer and reducing agent (0.5M dithiothreitol) and heated for 10 min at 70°C. Aliquots of partially purified MnSOD (obtained by gel chromatography using a Sephadex G-75 column, 1x90 cm) from the bivalve mollusc *S. inaequivalvis*, and CuZnSOD from bovine erythrocytes, were loaded in the gels as standards to identify the two SOD isoforms. Samples were loaded with a protein content range of 10 to 15 µg. After electrophoresis, proteins were transferred for 1 h to polyvinylidene difluoride (PVDF) membranes in an Invitrogen Xcell SureLock Blot Modul. PVDF membranes were treated with specific chromogenic western blot immunodetection kits. Blots were blocked for 45 min and incubated with the diluted primary specific antibody for 1 h: rabbit polyclonal anti-CuZnSOD and anti-MnSOD diluited 1: 10,000 and 1:7,000, respectively, were used.
Membranes were then incubated with alkaline phosphatase-conjugated anti-rabbit IgG for 45 min (secondary antibody). Immunopositive bands obtained after incubation with chromogenic solution (BCIP/NBT) were semi-quantified by Quantity One Software (Bio-Rad).

Statistical analysis

Descriptive statistics (means and standard deviations) were performed on all considered parameters. All data were checked for normal distribution before statistical analysis. Results were statistically analyzed using one-way analysis of variance (ANOVA), followed by T test and Bonferroni test, using STAT Statistical Package Software; values of $p<0.05$ were considered significantly different.

Results

Antioxidant enzymes

During the anoxic phase, total SOD activity (Fig. 1) increases after 6 hours (significantly different than to 3, 24 hours of anoxia, $p<0.01$). In reoxygenation, a significant increase in SOD activity was registered after 24 hours respect to almost all conditions except recovery (Rec) 1h ($p<0.01$).

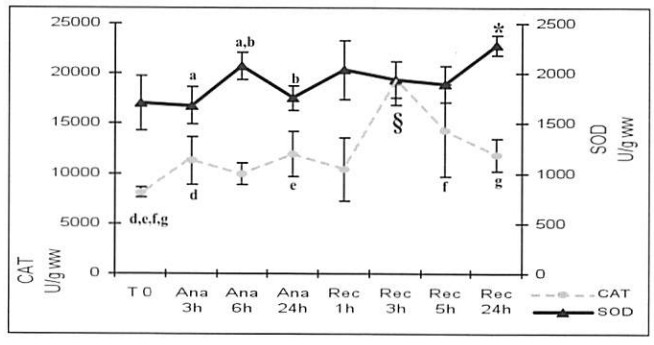

*Figure 1. SOD and CAT activities (U/g ww) in digestive gland during anoxia (Ana) and recovery (Rec); (n=3-5 ± d.s). Same letters at the top of the bars indicate significant differences: a-g=p<0.01; SOD: *condition significantly different from other; p<0.01, except Rec 1h. § CAT: condition significantly different from other, p<0.01.*

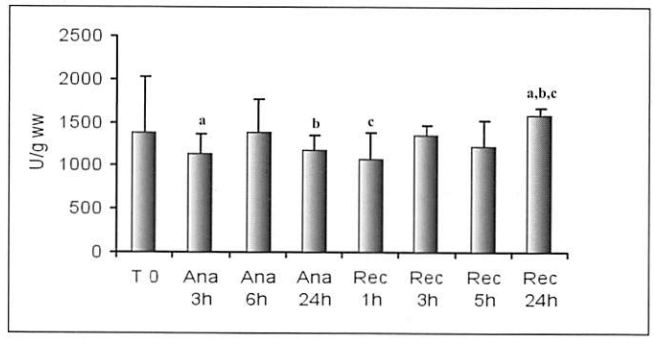

Figure 2. MnSOD activity in digestive gland during anoxia (Ana) and recovery (Rec); (n=3-5 ± d.s). Same letters at the top of the bars indicate significant differences: a-c=p<0.01.

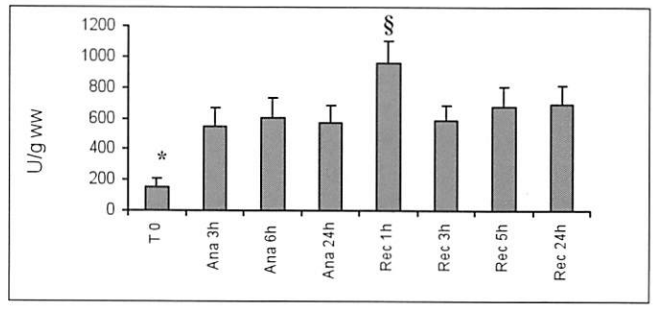

*Figure 3. CuZnSOD activity in digestive gland during anoxia (Ana) and recovery (Rec); (n=3-5 ± d.s). *Condition significantly different from other; p<0.01. § Condition significantly different from other, p<0.01.*

Generally, no significant variations in MnSOD activity were observed (Fig. 2) between T0, anoxia (Ana) and Rec. CuZnSOD activity (Fig. 3) increases considerably after 3 hours of anoxia respect to T0; during recovery it increases considerably after 1 hour of reoxygenation respect to T0 and Ana before dropping again after 3h without any other variations ($p<0.01$).

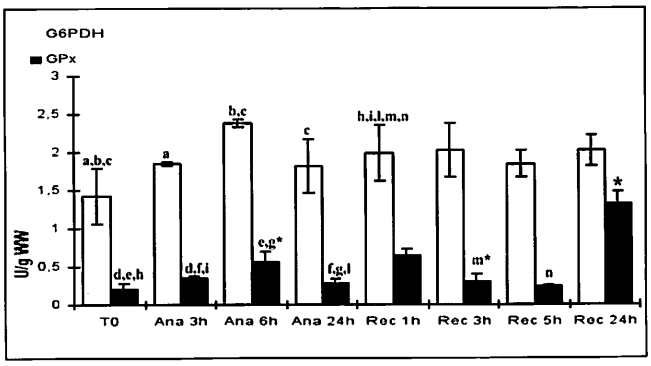

*Figure 4. G6PDH and Se-GPx activities in digestive gland during anoxia (Ana) and recovery (Rec); (n=3-5 ± d.s). Same letters at the top of the bars indicate significant differences: a-n=p<0.01. *Condition significantly different from other; p<0.01.*

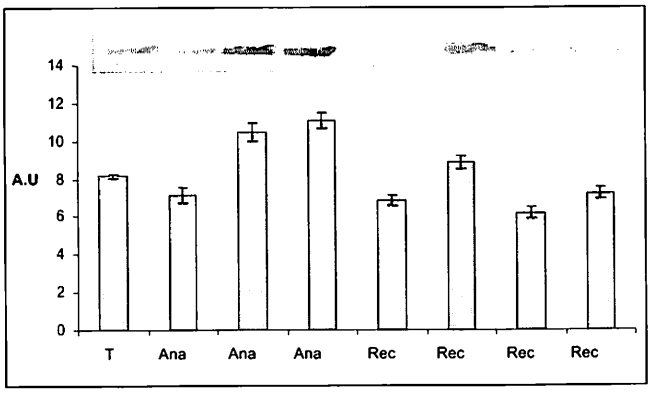

*Figure 5. CuZnSOD expression in digestive gland during anoxia (Ana) and recovery (Rec). Densitometric analysis of blot expressed in arbitrary units (n=3 ± d.s). *Condition significantly different from other; p<0.01, except Rec 3h. In set: Representative immunopositive bands of the corresponding bar are shown above.*

CAT activity (Fig. 1) increases after 3 hours of anoxia ($p<0.01$ in relation to T0) and 24h ($p<0.01$ respect to T0); in the recovery phases, activity increases considerably after 3 hours ($p<0.01$ in relation to the previous conditions), before dropping after 5 and 24 hours of recovery ($p<0.01$ respect to T0) but still remaining higher then T0 ($p<0.01$).
Se-GPx activity (Fig. 4) increases in relation to time zero after 3 and 6 hours of anoxia ($p<0.01$). After 24 hours of anoxia, a reduction in activity ($p<0.01$) is observed as significant in relation to 3 and 6 hours of anoxia. Activity increases after 1 hour of reoxygenation ($p<0.01$ in relation to T0, Ana 3h and Ana 24h), before dropping significantly after 3 and 5 hours of recovery respect to Rec 1h ($p<0.01$). Lastly, there is a rise after 24 hours of recovery significant respect all conditions ($p<0.01$).
G6PDH activity rises (Fig. 4) after 3 and 6 hours of anoxia in relation to T0, then drops after Ana 24h respect to Ana 6h ($p<0.01$). During all recovery period, no significant differences were recorded.

Immunological assay

CuZnSOD expression (Fig. 5) shows well-separated immunoreactive bands because of the enzyme's monomeric form (apparent molecular weight 16 kDa). Band analysis reveals an increase after Ana 6h and Ana 24h respect to T0 ($p<0.01$), followed by a gradual drop in expression for the whole recovery period ($p<0.01$ respect to T0 except for Rec 3h).
MnSOD shows a first band with an apparent weight of about 26 kDa and a second very weak band, with a weight of about 46 kDa after 1, 6 and 12 hours of recovery. The enzyme appear to be equally expressed in all samples, without any differences between anoxic conditions and recovery (data not shown).

Discussion

Antioxidant enzymes

In *S. inaequivalvis*, the trends for the two SOD isoforms activity remain tendentially high in anoxic conditions: this is probably due to the existence of different mechanisms such as a gene component, as well as modulators with positive and/or negative actions.
It is likely that maintaining high SOD levels during the anoxic phase allows a rapid neutralisation of superoxide, with a consequent increase in H_2O_2, which would explain the early drop in the enzyme's activity as well as the consistent increase in CAT for most of the recovery period.
The trend for G6PDH and Se-GPx activity is almost similar, which is probably related to the biochemical synergism of the 2 enzymes, due to the production of NADPH from the pentose pathway. The increase in G6PDH activity probably stabilises CAT since it is needed to maintain the enzyme's structural integrity [15]. CAT activity tends to rise during the early phases of anoxia, probably on account of the high activity of SOD, which continues to produce H_2O_2.
In similar studies, in conditions of anoxia, an increase was observed in catalase activity in the liver of the fish *Carassius auratus* [2] and a drop was observed in *Trachemys scripta elegans* [16] and in *Littorina littorea* [17].
In *S. inaequivalvis*, throughout the entire recovery period, CAT and Se-Gpx show an opposite trend, probably because they compete for the same substrate. The increase in Se-GPx (significant peak at 24 hours of reoxygenation) would suggest high levels of H_2O_2 as well as elevated G6PDH activity; high levels of these enzymes presuppose the existence of lipid perioxidation phenomena.
In *S. inaequivalvis* the activity of all the enzymes studied was constantly higher than in the normal oxygen controls, associated with a clear increase in Se-GPx after 24 hours of normal oxygen conditions, which may be related to GSH's capacity as a potent antioxidant.

During recovery, the massive introduction of oxygen is thought to cause higher production of the radicals responsible for the increase in antioxidant defences, especially during the early hours, before settling at constant levels. Similar results were reported in *Carassius auratus* [2]. In addition to metabolic depression, the maintenance of high constituent levels of antioxidant enzymes even during anoxia would guarantee a better resistance to the overproduction of ROS that accompanies the early phases of reoxygenation, as a preparatory event able to compensate episodes of oxidative stress [2, 18].

Immunoblotting

SOD is constitutively expressed in most organisms in which it appears to be highly preserved and it is considered to be essential due to the control role it exercises on the cell [19]. The CuZnSOD in eukaryotes is a dimeric protein constituted by two identical subunits weighing 16 kDa [20]. MnSOD on the other hand, is a tetrameric protein formed by four identical subunits weighing 24 kDa [20].
Although CuZnSOD is expressed constitutively, its mRNA levels may alter dramatically in response to physical, chemical, biological and mechanical messengers [21] and hypoxia is one of the main stimuli that cause it to drop [22]. On the contrary, in our study the expression of the enzyme rose: the absence of O_2 does not appear to have an inhibitory effect on the enzyme, except after 3 hour of anoxia. This could be due to the more efficient mechanisms of adaptation in *S. inaequivalvis* in order to survive the oxygen changes typical of the intertidal zone (our data not published).
MnSOD is expressed in many types of cell and tissues at relatively high levels and in the same way it is greatly regulated by a number of intracellular and environmental stimuli [21]. A number of studies have reported MnSOD overexpression as a mechanism that protects against ischaemic/reperfusion damage [23], whereas it would appear that a reduction contributes to damaging the tissues involved. However, no significant variations were observed in this study. During the experiment, the variations in activity in both SOD isoforms show a certain inconsistency with expression. This can be attributed to the changes and/or associations with other proteins that these enzymes undergo in the active form [24]. In this sense, Hermes-Lima *et al.* [18] suggest transcriptional, translational and post-translational regulations may be one of the main causes of the increase in antioxidant enzymes in the adaptation mechanisms employed when oxygen is lacking.
Resistance to anoxia/hypoxia is usually related to the environment in which the animal lives and can be encountered in animals that have evolved in environments characterised by significant fluctuations in oxygen and that do not appear to have suffered consequences regarding their ability to recover once normal oxygen conditions have been reinstated [7]. These adaptations are thought to derive mainly from the activation of anaerobic metabolism with metabolic arrest and maintenance of the cell's redox balance. Conversely, in mammals, anoxia caused by ischaemia, causes consumption and non-production of ATP, with the intracellular degeneration that appears with the reintroduction of O_2 into the ischemic tissue.
The study of the different and greater tolerance of euryoxic invertebrates to oxygen lack than stenoxic species (most vertebrates) could be useful to research on postischemic damage and to understanding the problems connected with ischaemia and subsequent reperfusion in humans.

Acknowledgments

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References

[1] Lutz P.L., Storey K.B. 1996. Adaptations to variations in oxygen tension by vertebrates and invertebrates. In: *Handbook of Comparative Physiology*. (Lutz P.L, Storey K.B., Eds.) CRC Press, Boca Raton, USA, C896.
[2] Lushchak V.I., Lushchak L.P., Mota A.A., Hermes-Lima M. 2001. Oxidative stress and antioxidant defenses in goldfish *Carassius auratus* during anoxia and reoxygenation. Am. J. Physiol. Reg. I. Comp. Physiol., 280: R100-R107.
[3] Lefer D.J., Granger D.N. 2000. Oxidative stress and cardiac disease. Am. J. Med., 109: 315-323.
[4] McCord J.M. 1983. The superoxide free radical, its biochemistry and pathophysiology. Surgery, 94: 412-414.
[5] Michiels C., Remacle J. 1988. Use of the inhibition of enzymatic antioxidant systems in order to evaluate their physiological importance. Eur. J. Biochem., 177: 435-441.
[6] Sies H. 1993. Strategies of antioxidant defense. Eur. J. Biochem., 215: 213-219.
[7] De Zwaan A., Cortesi P., Van der Thillart G., Roos J., Storey K.B. 1991. Differential sensitivity to hypoxia by two tolerant marine molluscs: a biochemical analysis. Mar. Biol., 111: 343-351.
[8] Livingstone D.R. 1988. Response of microsomal NADPH-cytocrome c reductase activity and cytocrome P450 in digestive glands of *Mytilus edulis* and *Littorina littorea* to environmental and experimental exposure to pollutants. Mar. Ecol. Prog. Ser., 46: 37-43.
[9] Crapo C., McCord A., Fridovich I. 1978. Preparation and assay of superoxide dismutases. Method. Enzymol., 53: 382-393.
[10] Fridovich I. 1982. Measuring the activity of superoxide dismutases: an embarassment of riches. In: *Superoxide dismutase* (Oberley L.W. Ed.), Vol. 1, CRC Press, Boca Raton, Florida, USA, 69-77.
[11] Aebi H. 1974. Catalase. In: *Methods of Enzymatic Analysis* (Bergmayer H.U. Ed.), Academic Press, New York, USA, 2, 673-683.
[12] Paglia D.E., Valentine W.N. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med., 70: 158-169.
[13] Löhr G.W., Waller D. 1974. Glucose-6-phosphate Dehydrogenase. In: *Methods of Enzymatic Analysis* (Bergmayer H.U. Ed.), Academic Press, New York, USA, 2, 636-643.
[14] Lowry O.H., Rosebrough N.J., Farr A.L., Randall J. 1951. Protein measure with the folin phenol reagent. J. Biol. Chem., 193: 265-275.
[15] Scott M.D., Zuo L., Lubin B.H., Chiu T.Y. 1991. NADPH, not glutathione, status modulates oxidant sensivity in normal and glucose-6-phosphate dehydrogenase-deficient crythrocytes. Blood, 77: 2059-2064.

[16] Willmore W.G., Storey K.B. 1997. Antioxidant systems and anoxia tolerance in a freshwater turtle *Trachemys scripta elegans*. Mol. Cell. Biochem., 170: 177-185.
[17] Pannunzio T.M., Storey K.B. 1998. Antioxidant defenses and lipid peroxidation during anoxia stress and aerobic recovery in the marine gastropod *Littorina littorea*. J. Exp. Mar. Biol. Ecol., 221: 277-292.
[18] Hermes-Lima M., Storey J.M., Storey K.B. 1998. Antioxidant defenses and metabolic depression. The hypothesis of preparation for oxidative stress in land snails. Comp. Biochem. Physiol. B, Biochem. Mol. Biol., 120: 437-448.
[19] Harris E.D. 1992. Regulation of antioxidant enzymes. FASEB J., 6: 2675-2681.
[20] Fridovich I. 1995. Superoxide radical and superoxide dismutases. Annu. Rev. Biochem., 64: 97-112.
[21] Zelko I.N., Mariani T.J., Folz R.J. 2002. Superoxide dismutase multigene family: a comparison of the CuZnSOD (SOD1), MnSOD (SOD2) and EC-SOD (SOD 3) gene structures, evolution and expression. Free Rad. Biol. Med., 33 (3): 337-349.
[22] Jackson R.M., Parish G., Ho J.S. 1996. Transcriptional activation of the Cu,Zn-superoxide dismutase gene through the AP2 site by ginsenoside Rb2 extracted from a medicinal plant, *Panax ginseng*. J. Biol. Chem., 271: 24539-24543.
[23] Cruthirds D.L., Novak L., Akhi K.M., Sanders P.W., Thompson J.A., MacMillan-Crow L.A. 2003. Mitochondrial targets of oxidative stress during renal ischemia/reperfusion. Arch. Biochem. Biophys., 412: 27-33.
[24] Csar X.F., Wilson N.J., Strike P., Sparrow L., MacMahon K.A., Ward A.C., Hamilton J.A. 2001. Copper/zinc superoxide dismutase is phosphorylated and modulated specifically by granulocyte-colony stimulating factor in myeloid cells. Proteomics, 1 (3): 435-443.