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RESPONSES OF SUPEROXIDE DISMUTASE, GLUTATHIONE PEROXIDASE AND REDUCED GLUTATHIONE ANTIOXIDANT DEFENSES IN GILLS OF THE FRESHWATER CATFISH (*HETEROPNEUSTES FOSSILIS*) TO SHORT-TERM ELEVATED TEMPERATURE

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Abstract—Superoxide anion radical (O_2^-) has been hypothesized as one of the possible factors involved in the oxidative stress in aquatic animals. The antioxidant enzymes superoxide dismutase (SOD; EC 1.15.1.1) catalyzes the conversion of O_2^- to hydrogen peroxide (H_2O_2) whilst glutathione peroxidase (GSH-Px; EC 1.11.1.9) metabolizes the H_2O_2 to H_2O in part of the cell (cytosol and endoplasmic reticulum). Reduced glutathione (GSH) is an important antioxidant which serves, in part, to set the redox status in tissues. In the present study the patterns of SOD and GSH-Px activities and GSH content were investigated, in an experimental model of elevated temperature, in gills of the fish *Heteropneustes fossilis* in order to investigate whether temperature elevation for 1–4 h may alter the antioxidant system. After an elevated temperature exposure from 25 (control) to 37°C, the SOD activity increased while GSH-Px activity and GSH content decreased significantly ($p < 0.05$) at 32 and 37°C at 1–4 h in comparison to control. Nevertheless a transient increase in GSH-Px activity was observed at 1 and 2 h at 32°C. The results at 27°C temperature were non-significant ($P < 0.05$) in comparison to control. During the extended hours (1 to 4 h) of each elevated temperature, a general trend of increase in SOD activity at 32 and 37°C was observed. However, GSH-Px activity and GSH content were not changed significantly for most of the extended period of elevated temperature. In conclusion, the elevated temperature caused changes in SOD and GSH-Px activities and GSH (thiol) content in gills. © 1997 Published by Elsevier Science Ltd

Key Word Index: Antioxidant, gill, reduced glutathione, glutathione peroxidase, *Heteropneustes fossilis*, superoxide dismutase, temperature

INTRODUCTION

Molecular oxygen (O_2) poses a potential threat in all organisms inhabiting aerobic environments by forming reactive oxygen species (ROS) such as superoxide anion radical (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2). Their increased formation in the body is likely to increase damage referred to as oxidative stress (Sies, 1991). Changes in antioxidant enzymes viz superoxide dismutase (SOD; EC 1. 15. 1.1), catalase (CAT; EC 1. 11. 1.6) and glutathione peroxidase (GSH-Px; EC 1. 11. 1.9) and their protective mechanisms have been identified as early indicators of cellular susceptibility to oxidant injury caused by O_2^- and other ROS such as OH in mammals (Ardelt *et al.*, 1989). An important feature of these enzymes is their inducibility under conditions of oxidative stress; such conditions can serve as an important adaptation to these conditions.

SOD is an oxidoreductase, which exists in several isoforms and greatly accelerates the conversion of O_2^- to H_2O_2 and O_2 ($2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), thereby negating the direct toxic effects of the radical as well as preventing its interaction with metal ions that could culminate in increased OH generation (Fridovich, 1986a, 1989). The efficacy of SOD as an antioxidant relies on its cooperation with other enzymes i.e. CAT and GSH-Px, which metabolize the dismutation product of O_2^-, H_2O_2, by oxidizing the reduced tripeptide glutathione (GSH) into its oxidized form (GSSG) ($2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$). GSH (L-glutamyl-L-cysteinyl glycine) is important in the prevention of cellular oxidative

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stress, detoxification of electrophiles and maintenance of intracellular thiol redox status (Meister and Anderson, 1983).

An extensive review of the literature related to antioxidant activities in aquatic animals has recently appeared (Di Giulio *et al.*, 1989; Lemaire and Livingstone, 1993; Winston, 1991; Winston and Di Giulio, 1991). Antioxidant enzyme activities in aquatic animals can vary seasonally (Palace and Klaverkamp, 1993; Roberts *et al.*, 1987; Viarengo *et al.*, 1991). These aquatic animals show seasonal metabolic variations related both to the fluctuation of environmental parameters such as temperature, salinity, O_2 level and to their physiological status, depending on the food availability (Viarengo *et al.*, 1991). Some studies have attempted to determine environmental influences on antioxidant enzyme activities in fishes. These include effects of feeding strategy on activation of SOD, CAT and GSH-Px (Heisinger and Dawson, 1983; Radi *et al.*, 1985). To the best of our knowledge, little if any research in the area of seasonal variation and temperature effects on oxidative stress and antioxidative response has been conducted.

Previous studies have shown increased lipid peroxidation in respiratory organs (gill, air sacs) (Parihar and Dubey, 1995) and liver (Parihar *et al.*, 1996) of *Heteropneustes fossilis* which would suggest changing pro-oxidant processes and a need for antioxidant responses to protect against increased ROS production. There is increasing evidence that gills of fish exhibit phagocytic response in warm water (Chilmonczyk and Monge, 1980). This is consistent with the fact that temperature exposure could increase the phagocytic recruitment and activation (Cheville, 1983; Halliwell, 1994a), which results in internalization of oxidases on the surface of the membranes (Robinson, 1978). Such cells utilize an NADPH oxidase enzyme system to generate directly O_2^- as part of their armoury (Babior, 1994):

$$2O_2 + NADPH \rightarrow 2O_2^- + NADP^+ + H^+$$

The involvement of active phagocytes in ROS production has been suggested in animal tissues (Halliwell, 1994a; Sen, 1995) including fish tissues (Higson and Jones, 1984; Secombes *et al.*, 1988). ROS are extremely reactive compounds that can interact with lipids, enzymes or DNA to produce harmful effects (Wiseman and Halliwell, 1996). The effectiveness of the antioxidant defense system in relation to the oxidative damage is of particular interest in the case of fish which show seasonal metabolic variations related to the fluctuation of environmental factors such as temperature. In order to clarify the relationship between short-term temperature responses and antioxidants in gill, a comprehensive study was performed in the present investigation. The activities of SOD, GSH-Px and GSH content were studied in gill of *Heteropneustes fossilis* exposed with elevated temperature from 25 to 37°C for the periods 1–4 h.

MATERIALS AND METHODS

Reagents

Glutathione reductase (EC 1.6.4.2), Trizma base, Trizma HCl, triton-X-100, 5,5′ dithiobis (2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Company Inc. U.S.A. Sodium dodecyl sulphate was obtained from BDH India, whereas GSH, pyrogallol and diethylene triamine pentaacetic acid (DTPA) were purchased from Loba Chemie, India. Ethylene-diaminetetraacetate (EDTA), reduced nicotinamide adenine dinucleotide phosphate (NADPH) and sodium azide were purchased from HiMedia Laboratories, India. All other chemicals were of analytical grade.

Animals and experimental design

Adult freshwater catfish *Heteropneustes fossilis* of mixed sex (average weight 42 ± 2.5 g and length 17 ± 1.3 cm) were caught in April at an unpolluted field site of Ujjain India, and acclimated for two weeks in a glass aquarium to laboratory conditions before the start of the experiment. The ambient temperature at which fish were collected was 24 ± 1°C. Animals were maintained at a water temperature of 25°C and a photoperiod of 12 h light: 12 h dark cycle. Fish were fed with a commercially available pellet containing dried proteins, carbohydrates, vitamin mixture and minerals every day. Four main groups of 40 animals each were used. The first group was kept as controls. The second, third and fourth groups were kept for temperature exposure.

Temperature exposure

Temperature was raised from 25°C (control) to 27, 32 and 37°C using a thermoregulated water heater. The animals were held during this process for periods of 1, 2, 3 and 4 h. The elevated temperature was attained to the required level within 10–15 min and then maintained for varying periods of 1–4 h.

Tissue homogenization

The animals were killed by a blow to the head, and the gill dissected out. Particular care was taken to minimize disturbance of fish in the other aquaria.

Immediately after harvest, all tissue samples were weighed, wrapped in aluminium foil and frozen. The preweighed tissue was homogenized at 4°C in a Polytron homogenizer at a speed of 13 000 rpm on the same day. The buffers for homogenization of tissue were: 50 mM Tris-HCl (pH 8.2) containing 1 mM DTPA for SOD activity assay; 50 mM K_2HPO_4/KH_2PO_4 buffer (pH 7.4) for GSH-Px activity assay and 0.1 M metaphosphoric acid containing 2 mM EDTA for GSH assay. The homogenates were kept frozen until they were analyzed.

SOD activity assay

SOD activity was assayed as described earlier (Parihar *et al.*, 1996). Briefly, the tissue homogenate (1:8 w/v) for SOD activity assay was treated with 1 ml of Triton X-100 (1%) for 30 min to ensure that full activity was released and then centrifuged at a speed of 16 000 × *g* at 4°C. The pellet was discarded and the supernatant was assayed for SOD activity by the method of Marklund and Marklund (1974), which involves the ability of enzyme to inhibit the autoxidation of pyrogallol. The assay system contained 1 mM DTPA, 50 mM Tris HCl buffer (pH 8.2) and tissue homogenate (0.5 ml). The assay mixture was transferred to a 3 ml cuvette and the reaction was initiated by the addition of freshly prepared 2.6 mM pyrogallol solution in 10 mM HCl. The enzyme kinetics was carried out at 420 nm for 10 min at room temperature (27°C) on a Perkin Elmer UV spectrophotometer. One unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation per 3 ml of assay mixture. Calculations were made per gram wet weight of tissue.

GSH-Px activity assay

GSH-Px activity was measured spectrophotometrically by a coupled enzyme procedure at 27°C, monitoring loss of NADPH at 340 nm as described by Lawrence and Burk (1976). The homogenate was centrifuged at 4°C at 500 *g* for 15 min and the resulting supernatant at 10 000 *g* for 20 min. The post mitochondrial supernatant (PMS) (the resulting supernatant at 10 000 *g*) was used for enzyme assay. The enzymatic reaction was conducted in 3 ml quartz cuvettes of 1 cm path length in a Perkin Elmer spectrophotometer. The reaction mixture contained 50 mM phosphate buffer (pH 7.4), 4 mM sodium azide, 1 mM EDTA, 4 mM reduced glutathione (GSH), 0.2 mM NADPH, 1 unit glutathione reductase, 0.1 ml of supernatant sample and 0.71 mM H_2O_2 as substrate. Reactions were initiated by the addition of H_2O_2. Direct proportionality was seen with GSH-Px enzyme activity over time and with sample concentration. Activities were estimated from the decrease of optical density at 340 nm due to NADPH oxidation between 2 and 4 min after the start of the reaction. The results are expressed as unit/g wet weight of tissue. One unit of the GPx is defined as the amount of the enzyme necessary to catalyze 1 nmol NADPH/min/g wet weight at 27°C.

GSH assay

The GSH was quantitated by the method of Jollow *et al.* (1974). Briefly, homogenate for GSH assay was centrifuged at 16 000 × *g* for 15 min at 4°C. The supernatant (0.5 ml) was added to 4 ml of ice cold 0.5 M solution of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) in 0.1 M phosphate buffer (pH 8). The optical density was read at 412 nm in a spectrophotometer (Perkin Elmer, U.S.A.). A calibration curve was prepared using GSH as a standard.

Statistical analysis

All values are expressed as mean ± SEM. Multiple group comparisons were made by using analysis of variance (ANOVA). Two way comparisons of data utilized student's *t*-test. The 0.05 level was selected as the point of minimal statistical significance in every comparison.

Results

In the gill, SOD activity was significantly ($p < 0.05$) increased in comparison to control at 32 and 37°C elevated temperature at various times (1–4 h) (Fig. 1), whereas no change was seen at 27°C. Extending the period of exposure to temperature increase from 1 to 4 h produced varied responses at the different elevated temperatures. SOD activity was significantly ($p < 0.05$) increased during the periods of 1 to 4 h at 32 and 37°C, whereas no change was

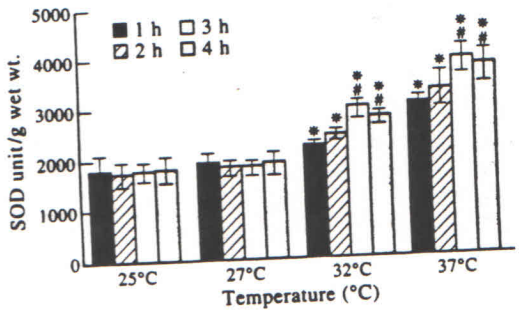

Fig. 1. Changes in gill superoxide dismutase (SOD) activity (unit/g wet weight) during exposure of *H. fossilis* to increased temperature from 25°C (control) to up to 37°C for varying periods (1–4 h). *$p < 0.05$ as compared to control (25°C); #$p < 0.05$ as compared to 1 h of each elevated temperature. Error bars indicated are SEM.

seen at 27°C. At the latter temperatures, maximum increase in SOD activity was achieved after the 3 h, with no significant ($p < 0.05$) difference existing between the results of 3 and 4 h.

In contrast, during temperature, exposure decrease in GSH-Px activity was observed at 32 (3 and 4 h) and 37°C (1–4 h), with no significant ($p < 0.05$) change at 27°C, in comparison to control (Fig. 2). However, increases were also observed significantly ($p < 0.05$) at 1 and 2 h at 32°C in comparison to control. Levels of GSH-Px activity were indicated to decrease when exposure times at 32 and 37°C were extended from 1 to 4 h, but with the exception of 3 and 4 h at 32°C and 4 h at 37°C, the changes were small and not statistically significant ($p < 0.05$).

The results for GSH content in gills after elevation of temperature from 25 (control) to 37°C are presented in Fig. 3. GSH content was slightly but significantly ($p < 0.05$) decreased at increasing temperature of 32 and 37°C, with no significant difference seen between 25 and 27°C. A comparison of GSH level at each temperature exposure (25–37°C) for various times (1–4 h) showed no significant ($p < 0.05$) change except a further reduction in its value was noted at 4 h in comparison to 1 h exposure at 37°C. The depleted levels of GSH at 32 and 37°C elevated temperatures were maintained more or less same for most of the times (1–4 h).

DISCUSSION

Data of the present experimental studies have demonstrated that after 32 and 37°C temperature exposure for varying periods (1–4 h), the SOD activity was increased concomitant with a decrease in GSH-Px activity and GSH content in gills. Low

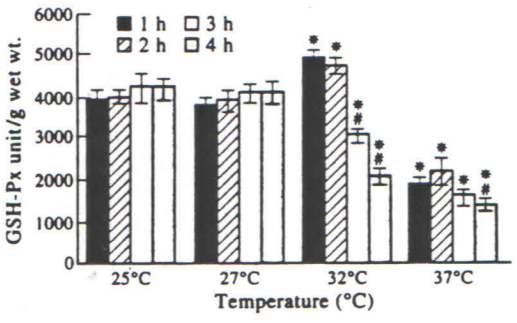

Fig. 2. Changes in gill glutathione peroxidase (GSH-Px) activity (unit/g wet weight) during exposure of *H. fossilis* to increased temperature from 25°C (control) to up to 37°C for varying periods (1–4 h). *$p < 0.05$ as compared to control (25°C); #$p < 0.05$ as compared to 1 h of each elevated temperature. Error bars indicated are SEM.

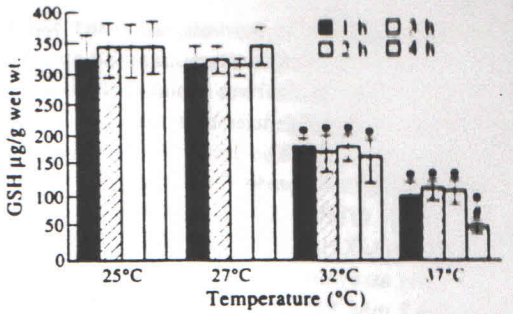

Fig. 3. Changes in gill reduced glutathione (GSH) content (µg/g wet weight) during exposure of *H. fossilis* to increased temperature from 25°C (control) to up to 37°C for varying periods (1–4 h). *$p < 0.05$ as compared to control (25°C). #$p < 0.05$ as compared to 1 h of each elevated temperature. Error bars indicated are SEM.

degrees of temperature increase (25–27°C) for 1–4 h failed to produce any antioxidant response. In an earlier study, we demonstrated a temperature (25–37°C) and time (1–4 h) dependent increase in lipid peroxidation in respiratory organs (gill and air sacs) of *Heteropneustes fossilis* (Parihar and Dubey, 1995). The process of lipid peroxidation has been linked to production of O_2^- and tissue damage (Aitken and Fisher, 1994; Cheesman, 1982; Halliwell, 1994a; Halliwell and Chirico, 1993). Both prokaryotes and eukaryotes are able to upregulate dramatically their armoury of oxidant protection in response to an oxidative stress. Adaptive responses to oxidative stress has been shown to involve widespread alteration in gene expression (Davies, 1995). To adapt against an unfriendly ROS environment, living organisms produce a battery of antioxidative enzymes and low molecular weight scavengers (Halliwell *et al.*, 1995; Hassan *et al.*, 1995; Lemaire and Livingstone, 1993). SOD is the enzyme which inactivates the O_2^- (Ewing and Janero, 1995; Halliwell, 1994b; Sen, 1995), and an increase in its activity with exposure to temperature could be indicative of a need to protect against increased O_2^- production. Increase in SOD activity in response to ROS production was also reported in xenobiotics-induced fish (Di Giulio *et al.*, 1989; Winston and Di Giulio, 1991; Livingstone *et al.*, 1992, 1993). Many of these experimentally observed increases in SOD activity were transient and not large. Gills of various fish species have been shown previously to possess antioxidant enzyme activities including SOD, GSH-Px and CAT (Lemaire and Livingstone, 1993). Although in comparison to other respiratory organs, for example gas gland, the SOD level is lower in gills (Lemaire *et al.*, 1993), but the existence of the enzymatic antioxidant in control fish is probably one of the most convincing lines of evidence for the

presence of ROS *in vivo*. We have observed gills of *H. fossilis* showing enhanced activity of SOD with the increase in temperature at 32 and 37°C. At the latter temperatures, similar increase in SOD activity with the increase in exposure times (1–4 h) was also observed. The increases in gill SOD activity with exposure to increased temperature in the present study possibly indicate a role for scavenging O_2^-.

Reduced GSH and enzymes associated with its metabolism provide a major defense against ROS induced cellular damage (Grisham and McCord, 1986). GSH interacts with ROS and acts as a chain-breaking antioxidant (Cadenas, 1989). Two enzymes that are essential components of the effect of GSH on ROS are GSH-Px and glutathione-s-transferase (GST; EC 2.5.1.18). GSH-Px catalyzes the reduction of hydroperoxides (ROOH) to nonreactive products:

$$ROOH + 2GSH \xrightarrow{GSH-Px} ROH + GSSG + H_2O$$

This might be of particular importance in the reduction of lipid hydroperoxides thereby preventing the chain propagation reaction leading to the deleterious effects of lipid peroxidation (Keeling and Smith, 1982). GST catalyzes the reaction of GSH with electrophilic reagents (Smith *et al.*, 1983) such as those generated by microsomal metabolism of drugs and foreign chemicals. Some forms of GST have GSH-Px activity with respect to lipid hydroperoxides (Lawrence and Burk, 1976). GSH-Px and GST work in concert to counteract pro-oxidant processes (Saez *et al.*, 1990). Because GSH-Px is related to detoxification of ROS, in the present experiment the observed decrease in activity of GSH-Px may be due to an increased production of ROS generation. The impairment of GSH-Px may reflect the inability of mitochondria to maintain a normal functional state, thus favouring the development of peroxidative damage (Fridovich, 1986b). Fish gills have been observed to possess high content of GSH and associated enzymes but proportionally lower SOD activity (Lemaire and Livingstone, 1993; Lemaire *et al.*, 1993). A considerable decline in the gills GSH content during elevated temperature could be due to its utilization to challenge the prevailing oxidative stress. The gradual depletion of GSH would seem to enhance the risk of oxidative stress in the gills in view of the possible increased peroxidative burden produced by high-SOD state as has been obtained in the present study and increased lipid peroxidation as reported earlier (Parihar and Dubey, 1995).

In conclusion, the investigation demonstrates that elevated temperature alters both SOD and GSH-Px activities and GSH contents in gills. To an extent, SOD could be protective against loss of thiols by preventing their direct reaction with more toxic O_2^- derived products (Radi *et al.*, 1991) or with O_2^- itself (Munday and Winterbourn, 1989; Wefers and Sies, 1983).

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