Arch. Biol. Sci., Belgrade, 66 (4), 1303-1310, 2014 DOI:10.2298/ABS1404303S

COLD STRESS-INDUCED LIPID PEROXIDATION AND NON-ENZYMATIC ANTIOXIDANT DEFENSE IN TISSUES OF THE COMMON INDIAN TOAD, *BUFO MELANOSTICTUS*

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Abstract - The aim of this study was to investigate the effect of exposure to short-term cold stress (30 min at 0-4^{0}C) on lipid peroxidation and the non-enzymatic antioxidant defense system in liver, kidney and brain tissues of an ectothermic animal, the common Indian toad, *Bufo melanostictus*. Cold stress resulted in a significant increase in lipid peroxidation in terms of TBARS formed in the liver, kidney and brain tissues, indicating increased generation of reactive oxygen species (ROS). Elevated levels of GSH measured in the liver, kidney and brain tissues of cold-stressed animals were probably due to an increased requirement for antioxidant defenses to meet the elevated peroxidative challenge. The observed decrease in ascorbic acid content may be due to its increased consumption and/or decreased synthesis following cold stress. Similarly, increased uric acid content in kidney and brain tissue following cold exposure and its decrease in liver tissue indicates that the effect of cold stress on the antioxidant system is both tissue-specific and dependent on the extent of cold exposure. Cold stress results in increased ROS production causing lipid peroxidation and compensatory alterations in the non-enzymatic antioxidant status in different tissues.

Key words: Cold stress; reactive oxygen species (ROS); lines of arrested growth (LAG); lipid peroxidation; GSH (reduced glutathione); uric acid; ascorbic acid.

INTRODUCTION

Exposure to stress alters homeostasis and leads to different physiological and behavioral adaptive responses in organisms. Acute stress is known to stimulate sympathetic activity and the hypothalamic-pituitary-adrenal (HPA) axis, causing the activation of enzymes responsible for the synthesis of catecholamine and neuropeptides (Nankova and Saban, 1999; Yuksel and Yurekli, 2003; Saito et al., 2005). Cold stress, which is characterized by increased metabolic rate, leads to an increased production of reactive oxygen species (ROS) which cause lipid peroxidation (Selman et al., 2000; Heise et al., 2003). Membrane injury following lipid peroxidation disrupts tissue integrity (Bagchi et al., 1999). Therefore, in order to convert ROS into less reactive species, organisms have evolved antioxidant enzymes (catalase, superoxide dismutase, glutathione peroxidase) and non-enzymatic antioxidants (reduced glutathione, ascorbic acid, uric acid, tocopherol) (Beckman and Ames, 1998; Leichtweis and Ji, 1997; Sohal et al., 1995). While certain aspects of antioxidants differ between species, they are present in all aerobic organisms (Beckman and Ames, 1998).

Oxidative stress occurs when the rate of ROS formation overwhelms the capacity of cellular antioxidant defenses so that net damage accumulates (Sies et al., 1983). Oxidative stress can occur under physiological situations such as ischemia/reperfusion and

exhaustive exercise (Halliwell and Gutteridge, 1989; Ji et al., 1992) as well as in response to extreme heat, cold or exposure to a variety of environmental pollutants (Kapoor et al., 1990; Hermes-Lima et al., 1991; Hausladen and Alscher, 1994).

Although cold stress induces a significant increase in the lipid peroxidation level in the liver, kidney and brain of rat (Sahin and Gumuslu, 2004 ; Kaushik and Kaur, 2003), it remains unchanged following cold stress (24 h. of freezing) in the freeze-tolerant wood frog *Rana sylvatica* (Joanisse and Storey, 1996). However, most studies related to the effect of cold stress on alterations in antioxidant activities are limited to endothermic animals such as mammals (Braja de Quiroga et al., 1991; Ohno et al., 1994; Teramoto et al., 1998; Rodojicic et al., 1999; Kaushik and Kaur, 2003; Selman et al., 2000; Sahin and Gumuslu, 2004; Yuksel and Asma 2006). This study was conducted to assess the effect of cold stress on lipid peroxidation and alterations in non-enzymatic antioxidant (glutathione, ascorbic acid and uric acid) levels in different tissues such as the liver, kidney and brain of an ectothermic animal model, i.e. the common Indian toad *Bufo melanostictus* (currently known as *Duttaphrynus melanostictus*, Frost et al., 2006). This study investigates whether an ectothermic animal displays changes in parameters of oxidative stress after exposure to cold stress.

MATERIALS AND METHODS:

Animals and experimental conditions

Twelve male common Indian toads, *Bufo melanostictus* (snout to vent length 8.0-8.3 cm and body weight 45-50 g), were collected from their natural habitat. They were found to have 3 LAGs (lines of arrested growth) in their long bones and phalanges and were considered to be from three to nearly four years old according to skeletochronology (Castanet 1994; Smirina 1994; Kumbar and Pancharatna 2001). These docile toads become fully mature at 2 years and have a maximum life span of 6 years in male individuals in the wild (Nayak and Dutta, 2007). The toads were maintained in a terrarium at room temperature (28±2°C) for 3 days (at daily 12 h light-dark intervals) on a diet of live earthworms and water *ad libitum*. The toads were randomly divided into two groups of 6 toads each to form the control group and the experimental (cold stress) group. Experimental animals were first put individually inside a pre-cooled (0-4°C) 500 ml wide mouth reagent bottle for 30 min. Since some toads did not survive continuous exposure to cold beyond 30 min, the maximum exposure time was limited to 30 min. The control group of animals was kept at room temperature and processed simultaneously.

Tissue preparation

Each specimen was killed by stunning its head and its snout-vent length and body weight were measured. Whole liver, kidney and brain were dissected out, cleaned of adherent tissues in ice-cold (4°C) amphibian Ringer's solution, weighed and processed immediately for different biochemical estimations.

Measurement of lipid peroxidation

The tissue lipid peroxidation (LPO) level was measured as thiobarbituric acid reactive substances (TBARS) formed using tissue homogenate and thiobarbituric acid (TBA) following the method of Sestin et al. (1991) with minor modification as suggested by Jena et al. (1991); 0.5 mL of 2.5% ice-cold aqueous tissue homogenate, 1.5 mL of 1% orthophosphoric acid and 0.5 mL of 0.6% TBA were mixed in an experimental hard glass test tube with stopper and heated in a water bath for 45 min. at 95°C. A control tube was also run simultaneously with 0.5ml distilled water instead of tissue homogenate. The mixtures were cooled to room temperature in running tap water. Three ml of chloroform and 1 ml of glacial acetic acid were added to both control and experimental tubes and their contents were centrifuged at 1 000 x g for 10 min. Extinction of the upper phase of the supernatant from the experimental tube containing TBARS was measured at 535 nm against the upper phase of the supernatant from the control. The amount of TBARS

formed was calculated from the extinction coefficient of 1.56 x 10^5 M^{-1} Cm^{-1} (Sinhuber et al., 1958) and was expressed as μ mol of TBARS formed per g of wet tissue.

Measurement of non-enzymatic antioxidants

Total glutathione

Reduced and oxidized glutathione (GSH and GSSG, respectively) were quantified by the method of Griffith (1980). Protein-free tissue extracts were prepared by homogenizing tissue samples in 5 volumes of ice-cold sulfosalicylic acid and then centrifuged at 10 000 x g for 15 min. The supernatant was collected and divided into two aliquots. One aliquot was used to measure total GSH equivalents (GSHeq = GSH + 2 GSSG) by following the rate of reduction of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) at 412 nm in an assay containing 0.2 mM NADPH, 0.6 mM DTNB, 5 mM EDTA, 125 mM sodium phosphate buffer (pH 7.5) and tissue extract in a final volume of 1 ml. After checking that the reaction rate was zero, the reaction was started by the addition of 0.5 U glutathione reductase (GR). The rate of the reaction is proportional to the concentration of GSH eq and was compared with a standard curve made with 0-6 μM GSH. The second aliquot was first treated with 170 mM 2-vinylpyridine for 1 h to derivatize GSH. The remaining GSSG was measured as above. Total GSH was calculated from the equation t GSH = GSH +2 GSSG and the result was expressed as μmol/g tissue wet weight.

Ascorbic acid

Ascorbic acid was extracted after homogenizing and centrifuging the tissue with 6% cold trichloroacetic acid (TCA). The TCA supernatant was used to estimate the ascorbic acid content following the method described by Roe (1954) with slight modification as suggested by Tewary and Pandey (1964). Ascorbic acid in the deproteinized tissue extract was oxidized to dehydroascorbic acid (DHAA), irreversibly transformed to 2,3-diketogulonic acid (DKA) and coupled with 2,4-dinitrophenylhydrazin to form a colored product with H_2SO_4. Its extinction was measured at 530 nm and the result was expressed as μg/g wet tissue.

Uric acid

The uric acid content was estimated as described by Buchanan et al. (1965; 0.5 ml of 5% tissue homogenate prepared in ice-cold 50 mM phosphate buffer (pH 7.0) was deproteinized by adding 4 ml of H_2SO_4 and 0.5 ml of 5.6% sodium tungstate, following centrifugation at 1 000 x g for 10 min. The uric acid content in the 3 ml of deproteinized supernatant was estimated from the extinction measured at 720 nm of the product formed after adding 0.2 ml of phosphotungstic acid reagent (PTR) and 1 ml of 0.6N NaOH, using a standard curve. The values were expressed as μg/g wet tissue.

Statistical analysis

Data were expressed as the mean ± SEM. The parameters of the control and cold-stress groups were compared using the student's t-test. Differences were considered statistically significant when $P < 0.5$.

RESULTS

In this study, the effect of acute cold exposure (30 min at 0-4^{0}C) on lipid peroxidation and alterations in the concentration of small antioxidant molecules was studied in the liver, kidney and brain of the common Indian toad *Bufo melanostictus*.

Short-term acute cold exposure significantly increased (Fig. 1) the lipid peroxidation potential (TBARS formed) in all the tissues studied – liver ($P < 0.001$), kidney ($P < 0.001$) and brain ($P < 0.05$) – when compared to their respective control (Table 1).

Total glutathione equivalents significantly increased (Fig. 2) in the liver ($P < 0.001$), kidney ($P < 0.01$) and brain ($P < 0.05$) after acute cold stress in comparison with their respective control (Table 1). This increase was due to the increase in GSH content since increase in GSSG was not significant.

Table 1. Effect of cold stress (30 min at 0-4^{0}C) on lipid peroxidation (LPO) level, glutathione (GSH), ascorbic acid (ASA) and uric acid (UA) content of liver, kidney and brain tissue of the common Indian toad *Bufo melanostictus.*

		LPO (μ mole of TBARS/g tissue)	GSH (μ mole/g tissue)	ASA (μg/g tissue)	UA (μg/g tissue)
Liver	Control	0.206 ± 0.005	2.18 ± 0.069	185.66 ± 10.78	429.83 ± 9.04
	cold stress	0.364 ± 0.015***	2.96 ± 0.038***	175.66 ± 16.56	357.0 ± 17.79**
Kidney	Control	0.111 ± 0.002	0.87 ± 0.015	229.16 ± 21.74	445.83 ± 44.14
	cold stress	0.148 ± 0.006***	0.94 ± 0.013**	163.50 ± 18.20*	643.66 ± 59.03*
Brain	Control	0.385 ± 0.006	0.065 ± 0.0018	514.66 ± 3.57	207.5 ± 19.59
	cold stress	0.412 ± 0.007*	0.071 ± 0.0015*	383.16 ± 19.28***	400.33 ± 42.89**

Data are expressed as mean ± SEM, (n=6). The significant differences calculated from those in the control group are designated as *(p<0.05), **(P<0.01), ***(p<0.001)

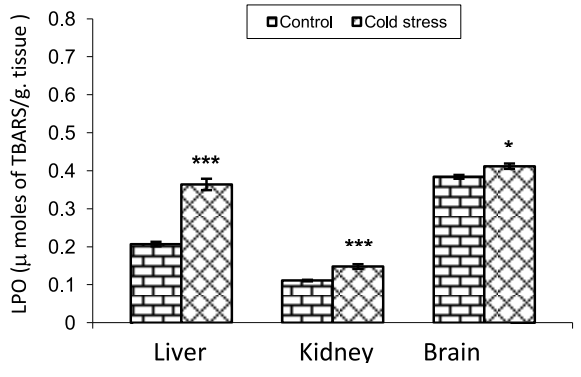

Fig. 1. Effect of cold stress (30 min at 0-4°C) on the LPO level of the liver, kidney and brain of male common Indian toad. Data are expressed as the mean ± SEM, (n=6). *(p<0.05); ***(p<0.001) different from control.

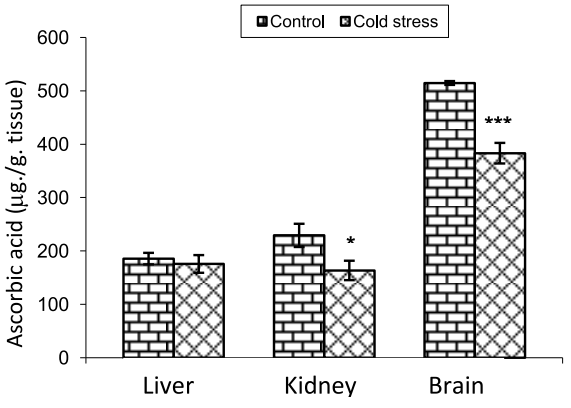

Fig. 3. Effect of cold stress (30 min at 0-4°C) on the ascorbic acid content in the liver, kidney and brain of the male common Indian toad. Data are expressed as the mean ± SEM, (n=6). * (P < 0.05), *** (P < 0.001) different from control.

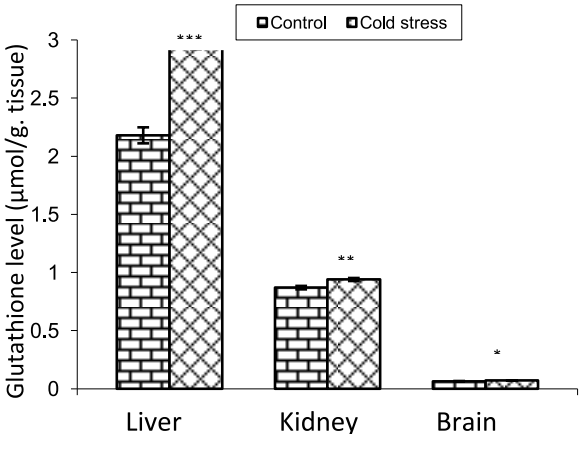

Fig. 2. Effect of cold stress (30 min at 0-4°C) on glutathione levels of the liver, kidney and brain of male common Indian toad. Data are expressed as the mean ± SEM, (n=6). ***(P<0.001),**(P <0.01),*(P<0.05) different from control.

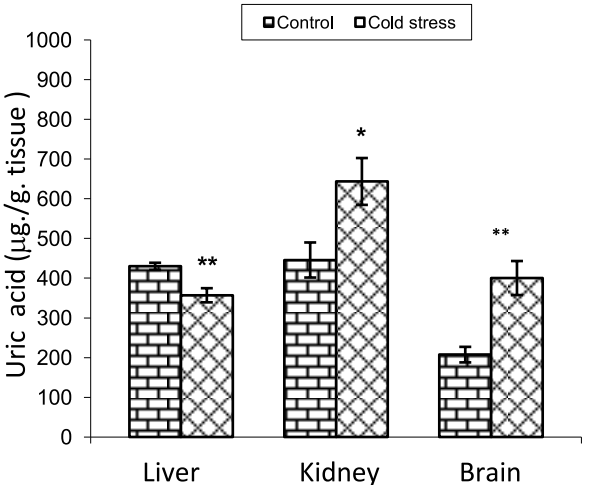

Fig. 4. Effect of cold stress (30 min at 0-4°C) on the uric acid content in the liver, kidney and brain of male common Indian toad. Data are expressed as mean ± SEM, (n=6). *(P < 0.05), **(P<0.01) different from control.

Short-term cold stress significantly decreased the ascorbic acid content in kidney ($P < 0.05$) and brain ($P < 0.001$) tissue. However, the decrease was not significant in liver tissue (Fig. 3) in comparison with its control (Table 1).

The uric acid content was found to be significantly increased in the kidney ($P < 0.05$) and brain ($P < 0.01$) tissues of cold-stressed animals, whereas in the liver tissue (Fig. 4) it decreased significantly ($P < 0.01$) with respect to the control (Table 1).

DISCUSSION

This study was conducted to investigate the effect of cold stress (30 min at 0-4°C) on lipid peroxidation and non-enzymatic antioxidant (GSH, ascorbic acid and uric acid) levels in the liver, kidney and brain tissues of the common Indian toad (*Bufo melanostictus*). Cold stress, which induces elevated thermogenesis by enhanced substrate combustion with increased oxygen consumption, is reported to produce ROS, which cause lipid peroxidation and play an important role in tissue injury (Barja de Quiroga et al., 1991; Kovacs et al., 1996).

Increased levels of TBARS, considered an indicator of lipid peroxidation, were found in the liver, kidney and brain of cold-exposed animals compared to their respective control. This is in agreement with the findings of previous studies (Gumuslu et al., 2002; Kaushik and Kaur 2003; Sahin and Gumuslu, 2004) in cold-exposed rats. The difference in lipid peroxidation levels among the tissues may be due to differences in mitochondrial biogenesis and the degree of pro-oxidant (ROS)-induced lipid peroxidation during cold stress. The presence of PUFA in brain tissue is highly susceptible to oxidative attack and may be the cause for the high lipid peroxidation level in it after cold acclimation (Barja de Quirga et al., 1991).

Cold exposure has been reported to upregulate the enzymatic antioxidant (catalase, glutathione peroxidase) activities in various tissues of the short-tailed field vole *Microtus agrestis* (Selman et al., 2000). In this study, increased levels of GSH measured in the liver, kidney and brain tissues of cold-stressed common Indian toads were probably the consequence of an increased need for antioxidant defense to meet the increased peroxidative challenge due to increased pro-oxidant activity. This observation is similar to those of earlier studies in endothermic mammalian models (Barja de Quiroga et al., 1991; Yuksel and Asma 2005). GSH plays a multifunctional role in antioxidant protection, maintaining other reductants and protein sulfhydryl groups in their reduced state, acting as a cofactor for a number of antioxidant enzymes and directly scavenging ROS and peroxides. Accordingly, the GSH level of cold-acclimated toads increased in the liver, kidney and brain tissues when compared to controls.

A somewhat different pattern was observed for ascorbic acid. Short-term cold stress significantly decreased ascorbic acid content in the kidney and brain tissues but in liver tissue, the decrease was not significant. Stress is always accompanied with an increased ascorbate demand because of its increased consumption and/or decreased synthesis and uptake (Banhegyi et al., 1998). Sayers and Sayers (1972) have shown the ascorbic acid concentration decrement under stress when adrenal cortical hormone activity is high. Ascorbic acid is a well-established antioxidant (Sies, et al., 1992; Chakrabarty et al., 1992; Ames et al., 1993; Hesekar, 1995; Chatterjee et al., 1995) and has been reported to act as a free radical trap (Sandnes, 1991) to counteract the damaging effects of ROS and lipid peroxides. Although the exposure of Wistar rats to cold stress did not change ascorbic acid concentration in the serum, adrenals and liver as compared to controls (Djordjevic et al., 2006), there was a significant decrease in ascorbic acid content in the kidney of *Calotes versicolour* (an ectothermic animal) at lower temperature (9^0C) (Padhi and Patnaik, 1978) and a significant decrease in ascorbic acid synthesis in the testes of toads at low temperature (Chowdhury and Mukharjee, 1976).

The antioxidant property of uric acid arises from its ability to scavenge the superoxide anion, peroxy radicals, and to bind traces of transition metal ions (Ames et al., 1986). Uric acid also maintains ascorbic

acid in its reduced form in biological fluids (Sevanian et al., 1985) and has a potential therapeutic role as an antioxidant (Glantzounis et al., 2005). In the present study, short-term cold stress significantly increased the uric acid content in the kidney and brain tissue, whereas the same parameter decreased in liver tissue. This supports the view that the effect of cold exposure on the antioxidant system is both tissue-specific (Buzgdzic et al., 1999) and highly dependent on the extent of cold exposure regime (Barja de Quiroga et al., 1991).

In conclusion, short-term cold stress increases peroxidative damage in liver, kidney and brain tissues of *Bufo melanostictus* resulting oxidative stress. Adaptation to this oxidative stress seems to involve an increase in non-enzymatic antioxidants, especially GSH, for scavenging lipid hydroperoxides. The decrease in ascorbic acid content in all the tissues studied and increase in uric acid content in kidney and brain tissues following cold stress show the tissue-specific response and dependency on the extent of cold exposure regime. The results of this study show that the response to cold stress by an ectothermic animal model (*Bufo melanostictus*) is similar to that in endotherms, as reported earlier (Yuksel and Asma 2005; Sahin and Gumuslu 2004; Barja de Quiroga et al., 1991).

Acknowledgments - This investigation was supported, in part, by a research project grant from the University Grant Commission, New Delhi, to D. D. Sahoo; we are grateful to the H.O.D, P.G Dept. of Life Sciences and Principal, S.K.C.G Autonomous College, Paralakhemundi, for providing laboratory facilities

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