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Influences of Different Stress Models on the Antioxidant Status and Lipid Peroxidation in Rat Erythrocytes

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The aim of this study was to investigate the influences of different stress models on the antioxidant status and lipid peroxidation (LPO) in erythrocytes of rats. Swiss-Albino female rats (3 months old) were used in this study. Rats were randomly divided into the following four groups; control group (C), cold stress group (CS), immobilization stress group (IS) and cold + immobilization stress group (CS + IS). Control group was kept in an animal laboratory (22 ± 2°C). Rats in CS group were placed in cold room (5°C) for 15 min/day for 15 days. Rats in IS group were immobilized for 180 min/day for 15 days. Rats in CS + IS group were exposed to both cold and immobilization stresses for 15 days. At the end of experimental periods, the activities of glucose-6-phosphate dehydrogenase (G-6-PD), Cu,Zn-superoxide dismutase (Cu,Zn-SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), and concentration of reduced glutathione (GSH) were measured. LPO was determined by measuring the contents of thiobarbituric acid-reactive substances (TBARS). Cu,Zn-SOD activity and TBARS concentration were increased after cold and immobilization stresses, but CAT and GSH-Px activities and GSH levels were decreased. Immobilization stress decreased the activity of G-6-PD. The activities of G-6-PD, CAT and GSH-Px, and the level of GSH were lower in CS + IS group than in the control group. Cu,Zn-SOD activity and TBARS levels were increased in CS + IS group when compared with the control group. From these findings, three stress models are thought to cause oxidative stress.

Keywords: Cold stress; Immobilization stress; Cold + immobilization stress; Antioxidant enzymes; Lipid peroxidation; Erythrocyte; Rat

INTRODUCTION

Stress is one of the major problems in modern societies. Under stress, the human organism redistributes its energy resources in the anticipation of an incoming threat. A number of epidemiological studies conducted during recent years have clearly demonstrated a link between stress and the development and course of many diseases. However, the biochemical bases for stress-induced effects are still mostly unknown.[1] One of the possible mechanisms might be the peroxidation of cell membrane lipids which is elicited by the production of reactive oxygen species (ROS). ROS can cause severe membrane injury by initiating lipid peroxidation.[2] ROS formed in cells include superoxide anion ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2), hydroperoxide, hydroxyl ($^{\bullet}OH$) and peroxyl ($ROO^{\bullet}$) radicals.[3] Antioxidant enzyme activities and low-molecular weight antioxidants are involved in protecting organisms from the deleterious effects of ROS. Cu,Zn-superoxide dismutase (Cu,Zn-SOD) converts $O_2^{-\bullet}$ to O_2 and H_2O_2, and catalase (CAT) and Se-dependent glutathione peroxidase (GSH-Px) remove H_2O_2. GSH-Px can also remove organic hydroperoxides.[4] Glucose-6-phosphate dehydrogenase (G-6-PD) catalyzes the reduction of nicotinamide adenine dinucleotide phosphate (oxidized) ($NADP^+$) to nicotinamide

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Groups	Experimental design
Control (C)	Rats were kept in an animal laboratory (22 ± 2°C)
Cold stress (CS)	Rats were placed in cold room (ambient temperature 5°C) for15 min/day for 15 days[6,7]
Immobilization stress (IS)	Rats were lightly anesthetized with ether and then immobilized by taping down all four limbs for 180 min once a day for 15 days at room temperature (22 ± 2°C)[8]
Cold + Immobilization stress (CS + IS)	Both cold and immobilization stresses were applied at the same time. Animals were exposed to cold stress at the last 15 min of immobilization stress

adenine dinucleotide phosphate (reduced) (NADPH). NADPH is used for the reduction of oxidized glutathione (GSSG) to the reduced glutathione (GSH).[5] In addition to being a mandatory substrate for GSH-Px activity, the tripeptide GSH is a hydroxyl radical and singlet oxygen scavenger and participates in a wide range of cellular functions.[4]

The effects of cold, immobilization and cold + immobilization stresses are controversial. The relationship between cold (or immobilization or cold + immobilization) stress and antioxidant status in erythrocyte has not been well examined. There are no reports concerning the influence of cold + immobilization stress on antioxidant status and lipid peroxidation. This is the first rat study to investigate the effect of cold + immobilization stress on erythrocyte antioxidant status and lipid peroxidation.

The aim of the present paper was to study the changes in the activity of the antioxidant enzymes (G-6-PD, Cu,Zn-SOD, CAT and GSH-Px) and in the concentrations of GSH and TBARS in the erythrocytes of rats after stress. We also compared the effects of different stress models on parameters above to each other. Three stress models were used: cold stress, immobilization stress and cold + immobilization stress.

MATERIALS AND METHODS

Apparatus

Optical densities in the ultraviolet and visible region were measured with a Beckman 26 spectrophotometer.

Chemicals

D-Glucose-6-phosphate (disodium salt), β-nicotinamide adenine dinucleotide phosphate (β-NADP, sodium salt), (±)-epinephrine, glutathione reductase, glutathione (reduced form), β-nicotinamide adenine dinucleotide phosphate (reduced form) (β-NADPH, tetrasodium salt), 5,5′-dithio-bis(2-nitrobenzoic acid), 2-thiobarbituric acid and 1,1,3,3-tetraethoxypropane were obtained from Sigma (St. Louis, MO, USA). H_2O_2 and *t*-butyl hydroperoxide (70%, w/w) were obtained from Merck (Darmstadt, Germany).

Animals

Female Swiss-Albino rats (200–250 g) at 3 months age were used. Five animals were housed per cage, and animals were acclimatized to standard animal laboratory conditions (12:12-h light–dark cycle, temperature 22 ± 2°C and humidity 50 ± 5%). Rats were randomly divided into four treatment groups of 10 rats each.

The experiments were performed between 09:00 and 12:00 h in the morning. At the end of the experimental period, rats were deprived of food for 24 h and prepared for the experimental procedure under ether anesthesia.

Biochemical Assays

Heparinized blood samples were taken by cardiac puncture from each rat between 8:00 and 9:00 h in the morning. Some of the whole blood was used for the determination of GSH. Blood was centrifuged at 1500*g* for 10 min at 4°C to separate erythrocytes from plasma. Erythrocytes were washed three times with cold sodium chloride (0.15 M). G-6-PD activity was measured by the method of modified Zinkham.[9] Cu,Zn-SOD activity was measured as in Misra and Fridovich.[10] CAT activity was measured by the method of Aebi,[11] monitoring the dissapearance of H_2O_2 at 240 nm. The results were expressed as the rate constant (k) of a first order reaction per gram hemoglobin. GSH-Px activity against *t*-butyl hydroperoxide as substrate was measured with use of a coupled enzyme assay, monitoring the oxidation of NADPH at 340 nm.[12] All enzymatic activities were expressed per gram of hemoglobin at either 30°C (G-6-PD, Cu,Zn-SOD, CAT) or 37°C (GSH-Px). GSH concentration was assayed by the method of

TABLE I Antioxidant enzyme activities (G-6-PD, Cu,Zn-SOD, CAT and GSH-Px), GSH and TBARS values of erythrocytes in studied groups

Groups	n	G-6-PD (IU/g Hb)	Cu,Zn-SOD (U/g Hb)	CAT (k/g Hb)	GSH-Px (U/g Hb)	GSH (mg/g Hb)	TBARS (nmol/g Hb)
Control (C)	10	10.08 ± 1.11	1217.20 ± 70.20	314.67 ± 23.67	11.14 ± 0.96	4.07 ± 0.25	0.16 ± 0.01
Cold stress (CS)	10	9.90 ± 0.92	1418.74 ± 54.27	249.44 ± 11.38	8.54 ± 1.15	2.47 ± 1.15	0.22 ± 0.02
		a: n.s.	a: $p < 0.01$	a: $p < 0.01$	a: $p < 0.01$	a: $p < 0.01$	a: $p < 0.01$
Immobilization stress (IS)	10	8.70 ± 0.65	3004.17 ± 192.97	90.27 ± 11.07	6.20 ± 0.41	1.20 ± 0.10	0.24 ± 0.01
		b: $p < 0.05$	b: $p < 0.01$	b: $p < 0.01$	b: $p < 0.01$	b: $p < 0.01$	b: $p < 0.01$
		c: $p < 0.01$	c: $p < 0.01$	c: $p < 0.01$	c: $p < 0.01$	c: $p < 0.01$	c: $p < 0.05$
Cold + Immobilization stress (CS + IS)	10	8.62 ± 0.84	4437.95 ± 230.11	118.32 ± 4.25	4.41 ± 0.46	0.93 ± 0.07	0.28 ± 0.01
		d: $p < 0.05$	d: $p < 0.01$	d: $p < 0.01$	d: $p < 0.01$	d: $p < 0.01$	d: $p < 0.01$
		e: $p < 0.01$	e: $p < 0.01$	e: $p < 0.01$	e: $p < 0.01$	e: $p < 0.01$	e: $p < 0.01$
		f: n.s	f: $p < 0.01$	f: $p < 0.01$	f: $p < 0.01$	f: $p < 0.01$	f: $p < 0.01$

The values represent mean ± SEM of ten animals per group. Experimental procedures are described in the text. a: C vs CS; b: C vs IS; c: CS vs IS; d: C vs CS + IS; e: CS vs CS + IS; f: IS vs CS + IS; n.s: non significant.

Fairbanks and Klee.[13] The lipid peroxidation content of erythrocytes was determined by malondialdehyde production and assayed as TBARS by a spectrophotometric method of Stocks and Dormandy.[14] The amount of lipid peroxides was expressed as nmol malondialdehyde/g hemoglobin using 1,1,3,3,-tetraethoxypropane as standard. Hemoglobin concentrations were determined by the cyanmethemoglobin method.[13] Statistical evaluations were made by using Mann–Whitney U test. Statistical significance was accepted at $p < 0.05$. Results are expressed as means ± SEM.

RESULTS

The data obtained from all groups are shown in Table I.

Erythrocyte G-6-PD activity was not altered in CS group. G-6-PD activity in IS and CS + IS groups was significantly decreased compared to C group ($p < 0.05$). In IS and CS + IS groups, G-6-PD activities were significantly lower than those in CS group ($p < 0.01$). G-6-PD activity did not differ between CS + IS and IS groups.

Cu,Zn-SOD activity was significantly higher in CS, IS and CS + IS groups than in C group ($p < 0.01$). Cu,Zn-SOD activity was significantly higher in IS and CS + IS groups than in CS group ($p < 0.01$). Cu,Zn-SOD activity was significantly higher in CS + IS group than in IS group ($p < 0.01$).

CAT and GSH-Px activities and GSH concentration were significantly lower in CS group than in C group ($p < 0.01$). CAT and GSH-Px activities and GSH concentration were significantly lower in IS and CS + IS groups than in CS group ($p < 0.01$). CAT and GSH-Px activities and GSH concentration were significantly lower in CS + IS group than in IS group ($p < 0.01$).

TBARS levels were observed to be elevated in CS, IS and CS + IS groups in comparasion with C group ($p < 0.01$). TBARS concentration was significantly increased in IS and CS + IS groups than in CS group ($p < 0.01$). TBARS concentration was significantly increased in CS + IS group compared to IS group.

DISCUSSION

It is well known that intensive stress response can play an important role in tissue injury. The mechanisms involved in these tissue injuries are not definitely clear. One of the possible mechanisms might be the peroxidation of cell membrane lipids, which is elicited by production of ROS, e.g. hydrogen peroxide, hydroxyl radical, superoxide anion radical. It is well known that there are many enzymes which scavenge free radicals in erythrocytes such as

G-6-PD, Cu,Zn-SOD, CAT and GSH-Px.[15] GSH participates in the protection of sulphydryl group of cysteine in proteins and also in the protection of cells against oxidation by free radicals and ROS.[16]

The most important enzyme in the defense of the erythrocyte against oxidant attack is G-6-PD. In this study, mean G-6-PD activity which plays an important role in maintaining GSH levels in erythrocyte[17] was found to be unaffected by cold stress. But immobilization and cold + immobilization stresses induced a decrease in the activity of the same enzyme. However, no comparable report is available in the literature on the activity of G-6-PD affected by stress (cold, immobilization and cold + immobilization stresses) in the erythrocyte. The decrease in the activity of G-6-PD in IS and CS + IS groups may be due to the decrease of $NADP^+$. G-6-PD catalyzes the oxidation of glucose-6-phosphate (G-6-P) to 6-phosphogluconate with a concurrent conversion of $NADP^+$ to NADPH. The penthose phosphate pathway in erythrocyte provides NADPH for the reduction of GSSG to GSH, catalyzed by glutathione reductase (GR). In turn, GSH removes hydrogen peroxide from the erythrocyte in a reaction catalyzed by GSH-Px.[18] GSH production in IS and CS + IS groups may be decreased due to a significant decrease of NADPH in erythrocytes. If levels of NADPH are low in erythrocytes, as occurs when the activity of G-6-PD is low, then GSH is not regenerated in adequate amounts by the reaction of GR.[18]

All stress models elevated the activity of Cu,Zn-SOD. Cold exposure for 15 days resulted in very marked increase in Cu,Zn-SOD activity. This finding is in agreement with the report of the other authors.[16,19] Many contradictory results have been obtained in the alteration of Cu,Zn-SOD activity following immobilization stress. Cu,Zn-SOD activity has been reported to be decreased,[20] increased,[21,22] or unchanged.[23] In this study, a significant increase observed in Cu,Zn-SOD activity following all stress models which may constitute the protection against superoxide anion elevation. Because SOD catalyzes the decomposition of superoxide anions to hydrogen peroxide. This enzyme prevents the further generation of free radicals.[24]

Our data showed a decrease in CAT activity in cold exposure group. However, these findings contradict other authors who have reported increase in brown adipose tissue of rats after 75 and 105 days of cold exposure.[25] CAT activity in erythrocyte has been reported to be unchanged after 35, 75 and 105 days of cold exposure.[25] CAT activity was observed to be decreased in erythrocytes of rats after the immobilization stress. This result obtained for CAT activity in immobilization stress group is in contradiction with the report of Oishi *et al*.[22] Superoxide radicals are converted by the enzyme SOD to H_2O_2, which is broken down by CAT and GSH-Px.[15] However, this process can cause lipid peroxidation if H_2O_2 is not decomposed immediately. Significant decrease in CAT activity in response to stress can reduce the protection against lipid peroxidation.

Erythrocyte GSH-Px activity showed a marked decrease in all experimental groups. The results obtained for GSH-Px activity in CS group are in agreement with the report of Ohno *et al.*[19] but in contradiction with the results obtained by Siems *et al*.[26] Cold acclimation increased the activities of GSH-Px and the levels of LPO in rat brown adipose tissue.[27] The significant decrease observed in GSH-Px activity and GSH levels in response to all stress models can reduce the protection against lipid peroxidation. GSH-Px can interact directly with lipid peroxides and is sensitive to lower concentrations of H_2O_2.[17] A marked decrease in erythrocyte GSH-Px activity could be dependent on the depletion of erythrocyte GSH concentration in response to stress. Because GSH serves as a substrate for the enzyme GSH-Px, and it has been suggested that it is through the activity of this enzyme that GSH protects the red cell against oxidative damage.[28] This effect of stress on the GSH system was also shown in earlier studies.[16,19,25] It seems that stress not only produces oxidants but also impairs the enzyme system that is necessary for detoxification of H_2O_2 and organic hydroperoxides.

In addition to a direct antioxidant role for GSH, this thiol is involved as a substrate for GSH-Px and is thus needed for the detoxification of lipid peroxides by the enzyme.[27] There are limited reports concerning the influence of cold stress on GSH. Cold-restraint mice (8°C for between 0.5 h and about 3 h) showed depressed hepatic glutathione content.[29] Both cold-acclimated (5°C for 4 weeks) and cold-adapted (5°C, 40 successive generations)[19] rats showed significantly lower concentrations of erythrocyte glutathione than did control rats. These results agree with the result of the present study. In the present study, GSH levels were decreased in all experimental groups. However, these findings contradict with other authors who have reported increase in brown adipose tissue of cold-acclimated rats (6°C for 21 days).[27] Only one study has reported unchanged erythrocyte glutathione levels in acutely cold-exposed rats (−5°C for 1 h).[19] Our result may imply that stress induces a decrease in radical scavenging capacity. It is a fact that significant reduction of GSH observed in all stress groups support the notion that stress exerts its toxic effects by production of superoxide anions and H_2O_2. Another explanation for the decreased GSH level may be that a deficit in cystein, which is a key component in the synthesis of GSH, occured in the erythrocytes, as demonstrated by Lopez-Soriano and Alemany[30] in blood of rats cold-acclimated at 4°C

for 15 days. From these observations, it is postulated that GSH was used as a result of oxidative stress caused by cold or by immobilization stresses.

These discrepancies in antioxidant enzyme activities and GSH levels may be due to the differences in methodologies for erythrocyte preparations and enzyme determinations, experimental conditions used, differences in the strains, maintenance conditions of rats or differences in sex, species, stress models and age of animals studied.

There have been many reports of stress-induced lipid peroxidation in plasma[23] and in different tissues[20,22,31,32] of animals. These results agree with the result of this study. Our research showed that TBARS concentrations were elevated in erythrocytes of all stress groups. The increase observed in the TBARS concentrations of stress groups is a good indicator of increased LPO. Our data support the conclusion that, LPO occurs in erythrocytes following stress. This conclusion is consistent with many reports in the literature, at most, increase of TBARS. Our data clearly indicated that stress can induce LPO in erythrocytes, thus supporting the hypothesis of involvement of oxidants in stress.[23,33–35] From these findings, three stress models (cold, immobilization and cold + immobilization) are thought to be a kind of oxidative stress.

The results obtained from the present study indicate that three models (cold stress, immobilization stress and cold + immobilization stress) of stress enhance lipid peroxidation in the erythrocyte and influence the antioxidant enzymes of erythrocytes. According to our results, immobilization stress is more effective than cold stress on altering the antioxidant status and LPO but the most effective model is cold + immobilization stress. Further studies are needed to elucidate the mechanism accounting for these responses to different stress models.

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