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Marked changes in erythrocyte antioxidants and lipid peroxidation levels of rats exposed to acute, repeated and chronic restraint stress

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The aim of this study was to investigate the effects of acute, repeated and chronic restraint stress on the antioxidant status and lipid peroxidation. For this purpose, 48 male Wistar rats, aged three months were used in this study. Rats were separated into six groups as follows; control (C), acute stress (AS), restrained for 7 days (1 h/day) (RS), restrained for 21 days (1 h/day) (CS1), restrained for 28 days (1 h/day) (CS2) and restrained for 21 days (1 h/day) and allowed to recovery for 7 days (CS3). Copper, zinc-superoxide dismutase (Cu, Zn-SOD), catalase (CAT) and selenium-dependent glutathione peroxidase (Se-GSH-Px) activities, corticosterone, reduced glutathione (GSH) and thiobarbituric acid-reactive substances (TBARS) levels were measured in blood samples. Corticosterone levels of all groups were found to be elevated after stress compared to group C. Cu, Zn-SOD activity was lower in all stress groups than in group C. CAT and Se-GSH-Px activities were increased in all stress groups. All stress models decreased GSH levels except for the CS3 group. TBARS levels were higher in stress groups than in C group except for AS group. The highest corticosterone level, CAT and Se-GSH-Px activity and TBARS level were seen in group RS. The lowest Cu, Zn-SOD activity and GSH level were seen in group CS2. These results may have an important implication for impaired erythrocyte antioxidant enzyme activities and glutathione levels resulting from exposure to different stress models (acute, repeated and chronic restraint stress).

1. Introduction

Stress response is known as the disruption of homeostasis that is managed by complex formations of physiological and behavioural adaptive responses of the organism. Stress can be classified as acute, repeated and chronic, in respect to exposure times.

It is well known that stress contributes to the production of reactive oxygen species (ROS), e.g. hydrogen peroxide (H_2O_2), hydroxyl radical ($HO^{\bullet}$) and superoxide anion radical ($O_2^{-\bullet}$) that cause lipid peroxidation (LPO). High doses and/or inadequate removal of ROS result in oxidative stress which may cause severe metabolic malfunctions and damage to biological macromolecules (Fu et al. 1999). Superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) constitute the most important intracellular enzymatic antioxidant system. SOD is the antioxidant enzyme that catalyzes the dismutation of the highly reactive superoxide anion to molecular oxygen (O_2) and to the less reactive species H_2O_2. Peroxides can be destroyed by CAT or GSH-Px reactions. GSH-Px catalyses the reduction of hydroperoxides using GSH, thereby protecting mammalian cells against oxidative damage. Reduced glutathione (GSH) is a tripeptide whose antioxidant function is facilitated by the sulphydryl group of cysteine. GSH can react chemically with singlet oxygen ($^1O_2$), $O_2^{-\bullet}$ and $HO^{\bullet}$ and therefore serve directly as a free radical scavenger. GSH may stabilize membrane structures by removing acyl peroxides formed by LPO reactions (Siegers and Younes 1983). Although stress and its effects on organism have been investigated in several tissues, plasma and erythrocytes in earlier studies (Ohno et al. 1991; Shaheen et al. 1996; Sosnowski et al. 1993), most of the mechanisms are explained only by morphological studies. There is not any study about the comparison of different stress models (acute, repeated and chronic restraint stress).

In the present study, we compared the effects of acute, repeated and chronic restraint stress models on the erythrocyte antioxidant enzyme activities, reduced glutathione, plasma corticosterone levels and thiobarbituric acid-reactive substances (TBARS) levels as the lipid peroxidation marker.

2. Investigations and results

Plasma corticosterone levels, erythrocyte antioxidant enzyme activities (Cu, Zn-SOD, CAT and Se-GSH-Px), GSH and TBARS levels are shown in the Table. Plasma corticosterone levels of all experimental groups were significantly increased compared with the control group ($p < 0.05$). Corticosterone levels were higher in repeated and all chronic stress groups than in the AS group ($p < 0.001$). Plasma corticosterone level was lower in

Table: Effects of acute, repeated and chronic restraint stress models on the plasma corticosterone levels, erythrocyte antioxidant enzyme activities (Cu, Zn-SOD, CAT and Se-GSH-Px), GSH and TBARS levels of rats

Groups	n	Corticosterone (ng/ml)	Cu,Zn-SOD (U/g Hb)	CAT (k/g Hb)	Se-GSH-Px (U/g Hb)	GSH (mg/g Hb)	TBARS (nmol MDA/g Hb)
C	8	82.50 ± 5.29	7362.63 ± 135.47	137.38 ± 3.74	2.23 ± 0.11	3.34 ± 0.06	0.93 ± 0.01
AS	8	265.32 ± 28.05 a: $p < 0.05$	2382.25 ± 30.59 a: $p < 0.001$	171.88 ± 1.11 a: $p < 0.001$	6.38 ± 0.07 a: $p < 0.001$	1.40 ± 0.05 a: $p < 0.001$	0.97 ± 0.00 a: n.s
RS	8	1423.55 ± 34.30 b: $p < 0.001$ c: $p < 0.001$	2087.87 ± 22.99 b: $p < 0.001$ c: $p < 0.05$	223.37 ± 0.92 b: $p < 0.001$ c: $p < 0.001$	6.79 ± 0.11 b: $p < 0.001$ c: n.s	2.78 ± 0.07 b: $p < 0.001$ c: $p < 0.001$	2.44 ± 0.00 b: $p < 0.001$ c: $p < 0.001$
CS1	8	1234.77 ± 35.20 d: $p < 0.001$ e: $p < 0.001$ f: $p < 0.05$	2345.12 ± 53.14 d: $p < 0.001$ e: n.s f: n.s	188.25 ± 2.91 d: $p < 0.001$ e: $p < 0.05$ f: $p < 0.001$	4.99 ± 0.08 d: $p < 0.001$ e: $p < 0.001$ f: $p < 0.001$	1.17 ± 0.10 d: $p < 0.001$ e: n.s f: $p < 0.001$	1.38 ± 0.00 d: $p < 0.001$ e: $p < 0.001$ f: $p < 0.001$
CS2	8	1325.80 ± 43.21 g: $p < 0.001$ h: $p < 0.001$ i: n.s j: n.s	1637.62 ± 13.74 g: $p < 0.001$ h: $p < 0.001$ i: $p < 0.001$ j: $p < 0.001$	157.00 ± 3.44 g: $p < 0.001$ h: $p < 0.05$ i: $p < 0.001$ j: $p < 0.001$	5.52 ± 0.09 g: $p < 0.001$ h: $p < 0.001$ i: $p < 0.001$ j: $p < 0.05$	0.81 ± 0.06 g: $p < 0.001$ h: $p < 0.001$ i: $p < 0.001$ j: $p < 0.05$	1.48 ± 0.02 g: $p < 0.001$ h: $p < 0.001$ i: $p < 0.001$ j: $p < 0.001$
CS3	8	895.42 ± 36.21 k: $p < 0.001$ l: $p < 0.001$ m: $p < 0.001$ n: $p < 0.001$ o: $p < 0.001$	1862.75 ± 15.58 k: $p < 0.001$ l: $p < 0.001$ m: n.s n: $p < 0.001$ o: n.s	198.50 ± 2.28 k: $p < 0.001$ l: $p < 0.001$ m: $p < 0.001$ n: n.s o: $p < 0.001$	5.67 ± 0.12 k: $p < 0.001$ l: $p < 0.001$ m: $p < 0.001$ n: $p < 0.001$ o: n.s	4.56 ± 0.07 k: $p < 0.001$ l: $p < 0.001$ m: $p < 0.001$ n: $p < 0.001$ o: $p < 0.001$	1.29 ± 0.01 k: $p < 0.001$ l: $p < 0.001$ m: $p < 0.001$ n: $p < 0.001$ o: $p < 0.001$

Values represent mean ± SEM of eight rats per group. n denotes the number of rats in each group. The details of the experiments are described in the Experimental section. a: C vs AS, b: C vs RS, c: AS vs RS, d: C vs CS1, e: AS vs CS1, f: RS vs CS1, g: C vs CS2, h: AS vs CS2, i: RS vs CS2, j: CS1 vs CS2, k: C vs CS3, l: AS vs CS3, m: RS vs CS3, n: CS1 vs CS3, o: CS2 vs CS3, n.s non significant

group CS3 than in groups RS, CS1 and CS2 ($p < 0.001$), but higher than in groups C and AS ($p < 0.001$). The highest corticosterone level was seen in group RS.

Cu, Zn-SOD activities were found to be decreased in all stress groups compared to control ($p < 0.001$). Cu, Zn-SOD activity of group RS was lower than in group AS ($p < 0.05$). Cu, Zn-SOD activity in group CS3 was lower than in groups AS and CS1 ($p < 0.001$). Cu, Zn-SOD activity of group CS2 was lower than in the other groups studied ($p < 0.001$). The lowest Cu, Zn-SOD activity was seen in group CS2. CAT activities were found to be increased in all stress groups. CAT activity of group RS was higher than in group AS ($p < 0.001$). The most increase was seen in group RS while the lowest increase was found in group CS2.

Se-GSH-Px activities were found to be significantly elevated in all stressed groups ($p < 0.001$). Se-GSH-Px activities of groups AS and RS were higher than in all chronic stress groups ($p < 0.001$). Se-GSH-Px activity was higher in group CS2 than in group CS1 ($p < 0.05$). This enzyme activity was higher in group CS3 than in group CS1 ($p < 0.001$). The highest Se-GSH-Px activity was seen in group RS.

GSH levels were found to be significantly decreased in all stress groups ($p < 0.001$) except group CS3. The level of GSH in group AS was lower than in group RS ($p < 0.001$). GSH level of group CS1 was lower than in group RS ($p < 0.001$). GSH level was lower in group CS2 than in groups AS, RS and CS1 ($p < 0.05$). GSH level was higher in group CS3 than in groups C, AS, RS, CS1 and CS2 ($p < 0.001$). The lowest GSH level was found in group CS2.

TBARS levels were significantly higher in all stress groups than in group C ($p < 0.001$) except for group AS. TBARS level was higher in group RS than in group AS ($p < 0.001$). TBARS level of group CS1 was lower than in group RS while it was higher than in group AS ($p < 0.001$). TBARS level of group CS2 was higher than in groups AS and CS1, but lower than in group RS ($p < 0.001$). TBARS level of group CS3 was higher than in group AS, while it was lower than in groups RS, CS1 and CS2 ($p < 0.001$). The highest TBARS level was seen in group RS.

3. Discussion

In our study, an increment of plasma corticosterone levels were observed in all stress groups. This result shows that corticosterone is the best index of stress response. Our result is in agreement with previous studies (Marinesco et al. 1999; Kirby et al. 1997). No difference was observed between groups RS and CS2 with shows that restraint stress for 28 days has no additional effect on plasma corticosterone levels. This result supports earlier results expressing that adrenal cortex undergo the habituation in chronic stress (McEwen, 2001). We found that the plasma corticosterone levels in group CS3 were not decreased to the control values, although they were lower than in repeated and chronic stress groups. This result displays that a longer time is needed for this hormone to return to normal levels. It is known that a time of two weeks is needed to turn glucocorticoid receptors and mineralocorticoid receptors into the former condition (Bowman et al. 2001; Korte 2001). Because this situation affects the negative feedback mechanism, remaining of the hormone levels of the last group according to the control values is an expected result.

In this study, all stress models decreased the activity of Cu, Zn-SOD. The most decrement was observed in group CS2. This result is in agreement with the studies of Bian

et al. (1997) and Fishman et al. (1987). The decrease in the activity of Cu, Zn-SOD in all stress groups may be due to an increase in $O_2^{-\bullet}$ and H_2O_2 concentrations. If $O_2^{-\bullet}$ concentration increases significantly, H_2O_2 formation also increases and can inhibit Cu, Zn-SOD activity by the product inhibition way. Decreased activity of Cu, Zn-SOD will reduce protection against free radicals.

CAT activities were found to be increased in all stress groups. This finding is in agreement with a study performed with immobilized rats (Oishi et al. 1999). CAT acts as an antioxidant enzyme in high concentrations of H_2O_2, while Se-GSH-Px acts in lower concentrations. Se-GSH-Px activities were found to be significantly increased in all stressed animals. This finding agrees with the report of Siems et al. (1999). It seems reasonable to ascribe the relatively high erythrocyte levels of CAT and Se-GSH-Px to a protective response against increasing concentrations of hydrogen peroxide. Both CAT and GSH-Px activities were higher in group RS than in the other stress groups. This finding shows that H_2O_2 concentration was higher in this group than in the other groups.

GSH levels were found to be decreased in all stress groups except for group CS3. The results obtained for GSH levels are in agreement with the results of Gumuslu et al. (2002) and Ohno et al. (1991). Only one study has reported unchanged erythrocyte glutathione levels in acutely cold-exposed (−5 °C for 1 h) rats. The decrease in the levels of GSH in all experimental groups may be due to an increase in Se-GSH-Px activity and radical concentrations. Because GSH reacts directly with radicals in nonenzymatic reactions and is also an electron donor in the reduction of peroxides catalyzed by Se-GSH-Px. The significant reduction of GSH observed in all stress groups supports the notion that stress exerts its toxic effects by production of superoxide anions and H_2O_2. The elevated level of GSH in group CS3 may be explained differently. It is well known that nitric oxide, a reactive and unstable gas, is one of the agents leading to lipid peroxidation. Concentrations of this radicalic gas rise in stress conditions and affect various cellular mechanisms (Houston et al. 1998). The existence of GSH provides another important antioxidant molecule called *S*-nitrosoglutathione (GSNO) which is formed by a reaction between GSH and NO (Chieueh 1999). The recovery period, as in group CS3, may decrease NO production and therefore the consumation of GSH for the formation of GSNO. Thus, GSH levels may be found to be increased in the recovery period after stress.

There have been many reports of stress-induced lipid peroxidation in plasma (Liu et al. 1994) and in different tissues (Oishi et al. 1999; Gumuslu et al. 2002; Seckin et al. 1997) of animals. These results agree with the result of this study. Our research showed that TBARS levels were elevated in erythrocytes of stressed animals (RS, CS1, CS2 and CS3). According to our results, repeated stress (RS) is more effective than the other stress models on altering TBARS levels. The increase observed in TBARS levels of stress groups is a good indicator of increased LPO. Our data support the conclusion that LPO occurs in erythrocytes after stress (Gumuslu et al. 2002). Our results clearly indicated that stress can induce lipid peroxidation in erythrocytes. From these findings, four stress models (RS, CS1, CS2 and CS3) are thought to be a kind of oxidative stress.

In conclusion, all stress models (acute, repeated and chronic restaint stress) increased activities of CAT and Se-GSH-Px and decreased the activity of Cu, Zn-SOD and the level of GSH. Repeated and chronic restraint stress models increased lipid peroxidation. Our results suggest that repeated stress for 7 days (RS) is more effective than the other stress models on altering erythrocyte enzyme activities of CAT and Se-GSH-Px and the level of LPO and level of plasma corticosterone. However, chronic restraint stress for 28 days (CS2) is more effective than the other stress models on altering Cu, Zn-SOD activity and GSH level. We think that the length of the exposure time to stress is an important factor for the free radical formation, antioxidant defense system and products of lipid peroxidation. These results may have an important implication for impaired erythrocyte antioxidant enzyme activities and glutathione levels resulting from exposure to different stress models (acute, repeated and chronic restraint stress).

4. Experimental

4.1. Chemicals

All chemicals used in this study were from the Sigma Chemical Co. (St. Louis, MO, USA) and the Merck (Darmstadt, Germany).

4.2. Animals

In this study, 48 male Wistar rats (3 months old) weighing between 210–250 g were used. Four 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 six groups of 8 rats each as following:

1) Control group (C), 2) Acute stress (AS): Animals were exposed to a single 1 h restraint stress, 3) Repeated stress (RS): Animals were exposed to restraint stress for 1 h/day for 7 consecutive days, 4) Chronic stress 1 (CS1): Animals were restrained for 1 h/day for 21 consecutive days, 5) Chronic stress 2 (CS2): Animals were restrained for 1 h/day for 28 consecutive days and 6) Chronic stress 3 (CS3): Animals were restrained for 1 h/day for 21 consecutive days and animals were allowed to recovery for 7 days.

4.3. Stress model

Restraint stress was applied to the animals according to the method of Gamaro et al. (1999). Animals were placed in a 25 × 7 cm plastic bottle. The bottle was fixed with plaster tape on the outside so the animals were unable to move. There was a 1.5 cm hole at one end of the bottle for breathing.

4.4. Preparation of hemolysate

At the end of the experimental periods, rats were deprived of food for 24 h. Heparinized blood samples were taken by cardiac puncture from each rat between 9:00 and 10:00 h in the morning. Some of the whole blood was used for the determination of GSH and hemoglobin concentrations. The other part of the whole blood was centrifuged at 1500 × g for 10 min at 4 °C to separate erythrocytes from plasma. Plasma was used for the measurement of corticosterone levels. Then, erythrocytes were washed three times with an ice-cold isotonic sodium chloride solution. Hemolysis of washed cell suspension was achieved by mixing 1 volume with 9 volumes of cold distilled water. The hemolysate was used for the measurements of Cu, Zn-SOD, CAT and Se-GSH-Px activities and TBARS levels. Hemoglobin concentrations of all these parameters were measured in each prepared aliquat.

4.5. Determination of plasma corticosterone levels

The ImmuChem™ ^{125}I Corticosterone RIA kit (Inc., Costa Mesa, CA 92626) that is designed for use in laboratory mice and rats were used for measuring of plasma corticosterone levels in all experimental groups. The concentrations of plasma corticosterone were expressed as ng/ml using corticosterone standards prepared in different concentrations.

4.6. Enzyme assays

Cu, Zn-SOD activity was measured according to the method of Misra and Fridovich (1972). This method is based on the ability of Cu, Zn-SOD to inhibit the autooxidation of adrenalin to adrenochrome at alkaline pH. CAT activity was measured by the method of Aebi (1987) using hydrogen peroxide as substrate. The disappearance of H_2O_2 was followed spectrophotometrically at 240 nm. The results were expressed as the rate constant (k) of a first order reaction per gram hemoglobin. GSH-Px activity was

measured by the coupled method of Paglia and Valentine (1967) using t-butyl hydroperoxide as substrate. Hemoglobin concentrations were determined by the cyanmethemoglobin method (Fairbanks and Klee 1986). All enzymatic activities were expressed per gram of hemoglobin at either 30 °C (Cu, Zn-SOD and CAT) or 37 °C (Se-GSH-Px).

4.7. Determination of GSH levels

The level of GSH was assayed by the method of Fairbanks and Klee (1986).

4.8. Lipid peroxidation assay

The lipid peroxidation level of erythrocytes was determined by malondialdehyde (MDA) production and assayed as thiobarbituric acid-reactive substances (TBARS) by the method of Stocks and Dormandy (1984). The amount of lipid peroxides was expressed as nmol MDA/g hemoglobin using 1,1,3,3,-tetraethoxypropane as standard by measuring the absorbans at 532 nm.

4.9. Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Groups were compared among themselves using Mann-Whitney U test. P values less than 0.05 are considered statistically significant.

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