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Alterations in Hippocampal Antioxidant Enzyme Activities and Sympatho-Adrenomedullary System of Rats in Response to Different Stress Models

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Summary

The study deals with activity of three antioxidant enzymes, copper, zinc-superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), catalase (CAT) in hippocampus of rats, following the exposure to single chronic (individual housing or forced swimming) and acute (immobilization or cold) stress, as well as to combined chronic/acute stress. In addition, plasma noradrenaline (NA) and adrenaline (A) concentrations were measured in the same stress conditions, because their autooxidation can add to the oxidative stress. We observed that i) long-term social isolation and repeated forced swimming had minor effects on plasma catecholamines, but in the long-term pretreated groups, acute stressors caused profound elevation NA and A levels, ii) chronic stressors activate antioxidant enzymes, iii) acute stressors decrease catalase activity, their effects on CuZnSOD appear to be stressor-dependent, whereas MnSOD is not affected by acute stressors, and iv) pre-exposure to chronic stress affects the antioxidant-related effects of acute stressors, but this effect depends to a large extent on the type of the chronic stressor. Based on both metabolic and neuroendocrine data, long-term isolation appears to be a robust psychological stressor and to induce a "priming" effect specifically on the CuZnSOD and CAT activity.

Key words
Stress • Hippocampus • Antioxidant enzymes • Catecholamines

Introduction

In order to neutralize the effects of oxidative stress, the cell uses antioxidant enzymes such as copper, zinc-superoxide dismutase (CuZnSOD), manganese superoxide dismutase (MnSOD), catalase (CAT) and selenium-dependent glutathione peroxidase (Se-GSH-Px). Brain is the target for different stressors because of its high sensitivity to stress-induced degenerative conditions. It is well known that intensive stress response results in the production of ROS, i.e. superoxide anion radical ($O_2^{\cdot -}$), hydroxyl radical ($HO^{\cdot}$) and hydrogen peroxide (H_2O_2) that cause lipid peroxidation, especially in membranes and can play an important role in tissue

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injury (Kovacs *et al*. 1996). There are several studies that are related to the effects of stress on the antioxidant system and induction of lipid peroxidation in the brain after various stress exposure models (Liu *et al*. 1996).

Kaushik and Kaur (2003), showed that observed changes in the antioxidant defense system are tissue-specific, but it is evident that chronic exposure to cold leads to oxidative stress by displacing the prooxidant-antioxidant balance of this defense system by increasing the prooxidants while depleting the antioxidant capacities. The purpose of investigation by Somani *et al*. (1995) was to determine whether any alterations in antioxidant enzyme activities and levels of glutathione (GSH) in brain regions had occurred following exercise training. They concluded that exercise training altered SOD activity and the GSH to oxidized glutathione (GSSG) ratio differentially in different brain regions to cope with oxidative stress. On the other hand, Devi and Kiran (2004) suggested that integration of exercise training and vitamin E may emerge as a useful neuroprotector against the age-related decline in antioxidant enzymes and increased lipid peroxidation. Hippocampus and cerebral cortex which are the sites for memory and learning are benefited from training plus vitamin E even when initiated as late as in middle or old age. Radak *et al*. (2001) suggested that limb immobilization induces oxidative damage to the hippocampus, and this damage is associated with results in impairment of cognitive function. A single bout of exercise might have a capability to increase glutamate uptake and thus to eliminate the biochemical and cognitive stress caused by immobilization. Furthermore, Zaidi and Banu (2004) showed that immobilization of rats generated oxidative stress in the rat brain, by decreasing the activities of SOD, glutathione-S-transferase (GST), CAT and glutathione levels, while increasing the lipid peroxidation. Poststress vitamin E treatment was found more effective than vitamins A and C in enhancing the levels of glutathione and activities of SOD, GST and CAT and decreasing lipid peroxidation.

Activation of the sympatho-adrenal system represents one of the early responses to various stressors. Tajima *et al*. (1996) investigated the levels of glucose, adrenaline (A) and noradrenaline (NA) during immobilization stress in rat blood. Plasma level of A and NA significantly increased 30 min after the start of immobilization and remained at the elevated level during immobilization. The high turnover of catecholamines may participate in the increased production of reactive species and intensify the oxidative stress. Therefore, the important link between brain antioxidant enzymes and catecholamines were studied in parallel.

We attempted to clarify the interaction of hippocampal antioxidant enzymes and peripheral changes of plasma catecholamines in different stress models. We investigated the activity of CuZnSOD, MnSOD and CAT in the hippocampus and the levels of adrenaline and noradrenaline in blood samples under various stress conditions. The end-point of this study was to observe differences in stress-induced responses, in terms of the antioxidant defense system and blood catecholamines, depending on combinations of stress conditions.

Methods

Animals and stress models

Experiments were performed in male Wistar rats, aged three months and weighing 330-400 g. They were housed in open colony cages (four per cage) under controlled conditions of temperature (21±2 ºC) and illumination (lights on between 07:00 and 19:00 h), and had free access to tap water and laboratory chow. The *Guiding Principles for the Care and Use of Animals* based upon Helsinki Declaration (1964) and *Protocol of the "Vinča" Institute on Care and Treatment of Laboratory Animals* were strictly followed.

The experiment had two parts. In part I, rats were exposed to the two types of acute stress by cold (COLD) or immobilization (IMMO) for 2 h, and to a chronic stress by individual housing (long-term isolation, LTI) for 21 days or long-term forced swimming (LTS) every day for 15 min in water heated to 32 °C during 21 days whereas untreated animals served as controls (C). In part II, rats previously exposed to either type of chronic stress were subjected to immobilization or cold for 2 h. Immobilization stress was induced as described by Kvetňanský and Mikulaj (1970). The animals exposed to cold were initially kept at ambient temperature and than carefully transferred into a cold chamber at 4 °C.

Blood sampling

On the day before blood sampling, a cannula was inserted into the tail artery under pentobarbital (40 mg/kg ip) anesthesia. This allowed the estimation of plasma catecholamines without additional stressing of the animals during manipulations. After the baseline blood collection, the rats were immobilized or exposed to cold stress, and the blood was collected again.

Preparation of hippocampal homogenates

Animals were sacrificed by decapitation with a guillotine (Harvard-Apparatus, USA), and hippocampi of all animals from each group were excised and kept frozen (–70 °C). After thawing (+4 °C), they were weighed and homogenized (1:6 w/v, Potter-Elvehjem teflon-glass homogenizer) in 0.25 M sucrose buffer containing 0.05 M Tris-HCl and 1 mM EDTA, pH 7.4. The homogenates were vortexed for 15 s three times, with intermittent cooling on ice, and left frozen at –70 ºC for 20 h in order to disrupt the membranes and release MnSOD from mitochondria into crude homogenates. They were defrosted at room temperature, vortexed 1 min and centrifuged (Eppendorf centrifuge 5417R, Eppendorf-Netheler-Hinz GmbH, Hamburg, Germany) at 9000 rpm for 15 min at 4 °C, and the supernatants were collected.

Catecholamine and enzyme activity measurements

Plasma catecholamines were measured by a standard radioenzymatic assay described previously by Peuler and Johnson (1977) and the values were expressed as pg/ml plasma. Catecholamines present in plasma aliquots were converted to their labeled O-methylated derivatives by S-(^{3}H) adenosylmethionine (Lacomed, Czech Republic) and the lyophilized catechol-O-methyl transferase isolated from the rat liver. The O-methylated derivatives of the amines were then extracted along with unlabeled carrier compounds.

SOD activity was measured by the method of Misra and Fridovich (1972), which is based on the inhibition of autooxidation of adrenaline to adrenochrome by SOD contained in the examined samples. According to the authors of the method, maximum inhibition caused by SOD is achieved at adrenaline concentration of 2.6×10^{-4} M. Elevation of the adrenaline concentration above 2.6×10^{-4} M does not influence the reaction rate. The reaction was perfomed in an incubation mixture containing 0.05 M Na_2CO_3, 0.1 M EDTA, 3×10^{-4} M adrenaline, pH 10.2, and monitored spectrophotometricaly at 480 nm and 26 ºC (Cecil CE 2040 spectrophotometer, Cecil Instruments Ltd., Cambridge, UK). After assaying the total SOD activity, the samples were treated with 4 mM KCN in order to inhibit CuZnSOD (Geller and Winge 1983), and subjected again to enzyme assay as described above. The values thus obtained and the differences between the two measurements were considered as MnSOD and CuZnSOD activities, respectively. The results were expressed as specific activity of the enzyme in units per mg protein (U/mg protein). One unit of SOD was defined as the amount of protein which causes 50 % inhibition of the conversion rate of adrenaline to adrenochrome between the 3rd and 4th minute under specified conditions.

Catalase activity was measured by the method of Beutler (1982). The method is based upon the rate of H_2O_2 degradation by CAT contained in the examined samples. The reaction was performed in an incubation mixture containing 1 M Tris-HCl, 5 mM EDTA, pH 8.0, and monitored spectrophotometricaly at 230 nm and 37 ºC. One unit of CAT was defined as the amount of protein which degrades 1 μmol H_2O_2/min under specified conditions.

Total protein concentration (mg/ml) was measured according to the method of Lowry *et al.* (1952).

Table 1. Antioxidant enzymes activity and catecholamines concentration of rats exposed to combined acute and chronic stress.

Acute stress	Chronic stress	MnSOD (U/mg prot)	CuZnSOD (U/mg prot)	CAT (U/mg prot)	NA (pg/ml)	A (pg/ml)
IMMO	LTI	9.22 ± 1.13	45.57 ± 4.83	5.76 ± 0.88	771.00 ± 30.32	1000.17 ± 36.82
	LTS	18.52 ± 1.11***	16.49 ± 1.84***	1.28 ± 0.16**	603.17 ± 24.43***	822.50 ± 32.30***
COLD	LTI	9.01 ± 0.66	32.93 ± 6.55	3.91 ± 0.46	627.83 ± 24.66$^{\#\#}$	45.83 ± 3.32$^{\#\#\#}$
	LTS	19.53 ± 1.82***	6.74 ± 1.07***	3.26 ± 1.09	536.17 ± 22.05	50.17 ± 3.11$^{\#\#\#}$

Values are means ± SEM, n=6; Two-way ANOVA: a significant ($p<0.001$) main effect of chronic stress was observed for MnSOD, CuZnSOD, CAT, NA, A; a significant ($p<0.05$) main effect of acute stress was observed for CuZnSOD and ($p<0.001$) for NA and A; a significant ($p<0.05$) interaction effect was observed for CAT and ($p=0.001$) for A; Symbols: ** $p<0.01$, *** $p<0.001$, significantly different from the LTI group; $^{\#\#}$ $p<0.01$, $^{\#\#\#}$ $p<0.001$ significantly different from the IMMO group.

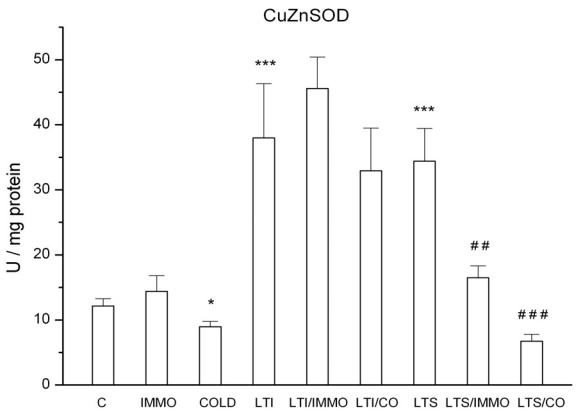

Fig. 1. The activity of CuZnSOD (U/mg protein) in hippocampus of rats exposed to immobilization (IMMO), cold (COLD), long-term isolation (LTI), long-term forced swimming (LTS), long-term isolation/immobilization (LTI/IMMO), long-term isolation/cold (LTI/CO), long-term forced swimming/immobilization (LTS/IMMO), long-term forced swimming/cold (LTS/CO). The values are means ± S.E.M. of 6 animals. Symbols: *p<0.05, ***p<0.001, when compared to C (*t*-test); # # p<0.01, # # #p<0.001, when compared to LTS (*t*-test).

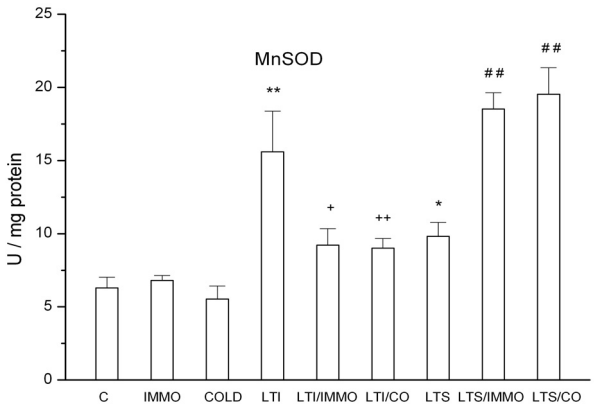

Fig. 2. The activity of MnSOD (U/mg protein) in hippocampus of rats exposed to immobilization (IMMO), cold (COLD), long-term isolation (LTI), long-term forced swimming (LTS), long-term isolation/immobilization (LTI/IMMO), long-term isolation/cold (LTI/CO), long-term forced swimming/immobilization (LTS/IMMO), long-term forced swimming/cold (LTS/CO). The values are means ± S.E.M. of 6 animals. Symbols: *p<0.05, **p<0.01, when compared to C (*t*-test); +p<0.05, ++p<0.01, when compared to LTI (*t*-test); # # p<0.01, when compared to LTS (*t*-test).

Data analysis

The results are reported as means ± S.E.M. Departures from normal distribution were determined by the Shapiro-Wilks test. Since the observed variables did not show significant departures from normal distribution, no data transformation was employed. *Part I.* Differences of antioxidant enzymes activity and catecholamines concentrations were analyzed by one-way ANOVA. To test the effects of acute stress (IMMO, COLD) and chronic stress (LTI, LTS) compared to untreated controls (C), as well as the effects of IMMO and COLD in comparison to LTI- and LTS-pretreated animals, the t-test was used. *Part II.* The effects of combined stress treatment were analyzed by two-way ANOVA to test for the two main effects (chronic and acute stress) and for the interaction between them. When a significant p-value was obtained, the Tukey HSD test was employed to determine differences between the groups. The level of statistical significance was set to 5 %.

Results

The hippocampal antioxidant enzyme activities and plasma catecholamine levels of all groups are shown in the Figures and Table 1. One-way ANOVA analysis revealed significant variations of CuZnSOD ($F_{8,45}$=10.77, p<0.001), MnSOD ($F_{8,45}$=15.74, p<0.001) and CAT ($F_{8,45}$=8.05, p<0.001) activity as well as catecholamine concentration (NA: $F_{8,45}$=106.13, p<0.001; A: $F_{8,45}$= 488.80, p<0.001) under the examined stress conditions.

Compared with the controls (Fig. 1), exposure to acute cold significantly decreased CuZnSOD activity (p<0.05, *t*-test), while both chronic stresses, LTI and LTS, profoundly elevated its activity (p<0.001, *t*-test). In comparison with both chronically pretreated groups, the effect of acute IMMO or COLD exposure led to a significant decrease of CuZnSOD activity (p<0.01 and p<0.001, respectively) only in the LTS-pretreated group. Two-way ANOVA analysis (Table 1) of combined stress treatment showed a significant main effect of chronic ($F_{1,20}$=43.14, p<0.001) and acute ($F_{1,20}$=7.08, p<0.05) stress on CuZnSOD activity. *Post-hoc* comparison revealed a significant decrease (p<0.001, Tukey test) of CuZnSOD activity when comparing LTS with LTI group.

Similarly to CuZnSOD, LTI and LTS significantly elevated the activity of MnSOD (Fig. 2) (p<0.01 and p<0.05, respectively, *t*-test). Both IMMO and COLD significantly suppressed MnSOD activity (p<0.05 and p<0.01, respectively) in the LTI-pretreated group, while marked increment was observed (p<0.01) in comparison with the LTS-pretreated group. Two-way ANOVA analysis (Table 1) of combined stress treatment showed a significant enhanced effect of chronic stress ($F_{1,20}$=62.77, p<0.001). Contrary to CuZnSOD, comparison of LTS vs. LTI group indicated a significant increase (p<0.001, Tukey test) of MnSOD activity.

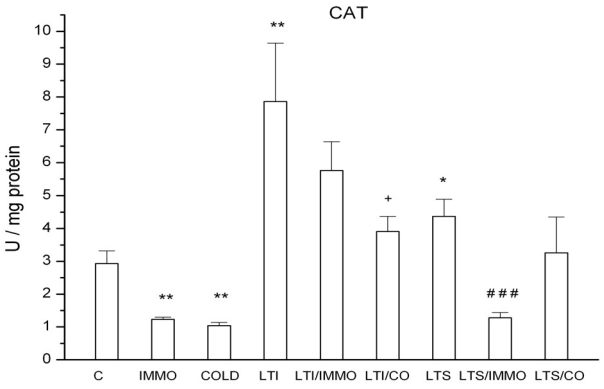

Fig. 3. The activity of CAT (U/mg protein) in hippocampus of rats exposed to immobilization (IMMO), cold (COLD), long-term isolation (LTI), long-term forced swimming (LTS), long-term isolation/immobilization (LTI/IMMO), long-term isolation/cold (LTI/CO), long-term forced swimming/immobilization (LTS/IMMO), long-term forced swimming/cold (LTS/CO). The values are means ± SEM of 6 animals. Symbols: *p<0.05, **p<0.01, when compared to C (*t*-test); $^{++}$p<0.01, when compared to LTI (*t*-test); $^{\#\#\#}$p<0.001, when compared to LTS (*t*-test).

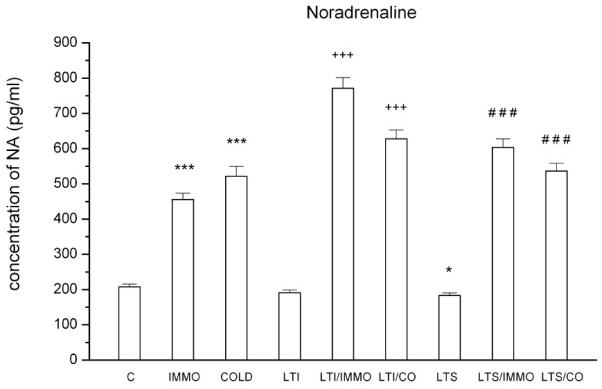

Fig. 4. Noradrenaline level (pg/ml) of rats exposed to immobilization (IMMO), cold (COLD), long-term isolation (LTI), long-term forced swimming (LTS), long-term isolation/immobilization (LTI/IMMO), long-term isolation/cold (LTI/CO), long-term forced swimming/immobilization (LTS/IMMO), long-term forced swimming/cold (LTS/CO). The values are means ± SEM of 6 animals. Symbols: *p<0.05, ***p<0.001, when compared to C (*t*-test); $^{+++}$p<0.001, when compared to LTI (*t*-test); $^{\#\#\#}$p<0.001, when compared to LTS (*t*-test).

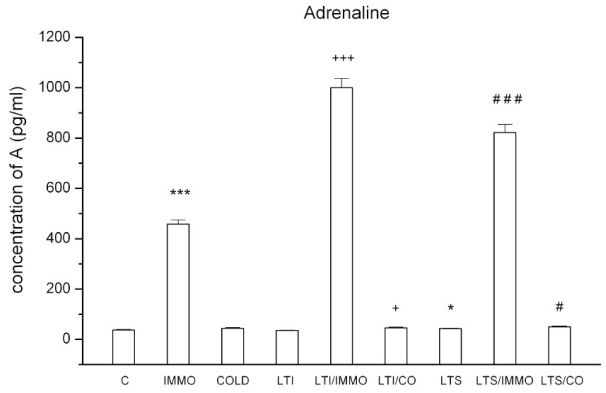

Fig. 5. Adrenaline level (pg/ml) of rats exposed to immobilization (IMMO), cold (COLD), long-term isolation (LTI), long-term forced swimming (LTS), long-term isolation/immobilization (LTI/IMMO), long-term isolation/cold (LTI/CO), long-term forced swimming/immobilization (LTS/IMMO), long-term forced swimming/cold (LTS/CO). The values are means ± SEM of 6 animals. Symbols: *p<0.05, ***p<0.001, when compared to C (*t*-test); $^{+}$p<0.05, $^{+++}$p<0.001, when compared to LTI (*t*-test); $^{\#}$p<0.05, $^{\#\#\#}$p<0.001, when compared to LTS (*t*-test).

Regarding CAT (Fig. 3), both IMMO and COLD induced significant decrease of activity (p<0.01, *t*-test) in comparison to the controls. Like CuZnSOD and MnSOD, elevated activity of CAT was recorded in rats chronically treated with LTI or LTS (p<0.01 and p<0.05, respectively). Exposure to COLD led to a significant decrease of CAT activity in the LTI-pre-treated group (p<0.01), but had no effect on the LTS group. On the other hand, exposure to IMMO induced significant decrease of CAT activity only in the LTS-pretreated group (p<0.001). Two-way ANOVA analysis (Table 1) showed a significant interaction effect of both stresses ($F_{1,20}$=6.72, p<0.05), and a significant effect of chronic stress ($F_{1,20}$=11.99, p<0.01). Furthermore, a significant decrease of CAT activity (p<0.01, Tukey test) was found only in one case of the LTS/IMMO vs. LTI/IMMO comparison.

Basal plasma NA and A levels (Figs. 4 and 5) did not significantly differ during the LTI stress, whereas NA level decreased (p<0.05, *t*-test), and A level increased (p<0.05, *t*-test) in the LTS group when compared with the controls. Acute IMMO induced significant increase of both NA and A (p<0.001), while acute COLD significantly increased only the NA level (p<0.001). In the LTI- and LTS-pretreated groups, stress by IMMO caused profound elevation of NA and A levels (p<0.001). Exposure to COLD also produced a high increase of NA levels in both groups (p<0.001), whereas less pronounced, but significant elevation of A level was recorded in LTI (p<0.05) and LTS (p<0.05) groups.

Two-way ANOVA analysis (Table 1) of combined stress showed a significant effect of chronic ($F_{1,20}$=25.79, p<0.001) and acute ($F_{1,20}$=16.92, p<0.001) stress on NA levels. A significant decrease of NA levels (p<0.001, Tukey test) was found in the case of the LTS/IMMO vs. LTI/IMMO comparison and in the case of the LTI/COLD vs. LTI/IMMO (p<0.01) comparison. Regarding adrenaline, a significant main effect of chronic stress ($F_{1,20}$=12.41, p<0.01), acute stress ($F_{1,20}$=1231.91, p<0.001), and interaction effect ($F_{1,20}$=13.69, p<0.001)

was observed. *Post-hoc* analysis revealed a significant decrease of A level ($p<0.001$, Tukey test) in the LTS/IMMO vs. LTI/IMMO comparison, and also a significant decrease in both, LTI/COLD vs. LTI/IMMO, and LTS/COLD vs. LTS/IMMO, comparisons ($p<0.001$, Tukey test).

Discussion

It is assumed that oxidative and sympatho-adrenal stress responses run in parallel and may be considered as two concomitant responses of the organism. Stress stimulates the sympathoadrenal system, causing activation of catecholamine biosynthetic enzymes (Mičutková *et al*. 2001). The effect of chronic variate stress on cerebral levels of monoamines was studied in rats by Gamaro *et al*. (2003). It has been reported that the production of radicals in the brain is due to catecholamine metabolism such as dopamine and noradrenaline (Venarucci *et al*. 1999), and elevated catecholamine levels may undergo autooxidation, in which electrons are generated that in turn can produce ROS (Carpagnano *et al*. 2003). In accordance with literary data, we supposed that in both LTI and LTS rats stress might elevate the rate of formation of $O_2^{\cdot-}$ and H_2O_2 in the hippocampus, and as a consequence, the main detoxifiers of free radicals, CuZnSOD, MnSOD and CAT, may work effectively to remove them. Therefore, the specific activity of these enzymes was significantly increased. Furthermore, Kaushik and Kaur (2003) found that chronic stress alters the prooxidant-antioxidant balance of the radical process in rat tissues. They showed that an increase in oxidative stress biomarkers, xanthine oxidase activity (XOD), malondialdehyde (MDA) and lipid peroxidation as products of oxidative stress might trigger secondary cell damages in response to chronic stress exposure.

On the other hand, the generalized decrease in CuZnSOD and CAT activities of acute cold stressed rats indicates a highly reduced enzyme capacity to scavenge ROS produced in the hippocampus in response to acute stress. The significant changes in CuZnSOD, but not in MnSOD, can be explained by the fact that mitochondrial MnSOD and cytosolic CuZnSOD use the same substrates for their action. Besides, the majority of metabolic processes is controlled by mitochondria in which mitochondrial MnSOD has a significant antioxidant enzyme capacity to remove free radicals (Radojičić *et al*. 1999).

It is interesting to note that acute stress conditions induced antioxidant enzymes only in animals pretreated with long-term stress similarly to the well known "priming" effects of estrogen, which appear to include progesterone modulation of cytoplasmic SOD activity in the rat brain (Blaustein and Feder 1979, Pajović *et al*. 1994). When all stress models were taken into consideration, the most pronounced "priming" effects of long-term isolation were on the CuZnSOD and CAT activity, whereas long-term swimming had a same effect on the MnSOD level.

From studies on the adaptation to stress, we know that a number of adaptive changes occur in the sympatho-adrenomedullary system, including increased synthesis and storage of catecholamines, increased basal levels of circulating catecholamines, and a decreased release of catecholamines into the circulation following exposure to chronic stress (Sanchez *et al*. 2003, Mičutková *et al*. 2003). Some mechanism to suppress peripheral changes under stressful conditions may act to offset central nervous system stimulation. Thus, these central cholinergic regulatory effects on adrenal secretion may require large and continuous increases of antioxidant activity (Finkelstein *et al*. 1985).

Our results show that long-term stressors with different characteristics induce different effects on hippocampal antioxidant enzyme activities and plasma catecholamines. Exposure of control, long-term isolation and long-term swimming rats to immobilization activated the sympatho-adrenal systems. The highest elevation of plasma catecholamines was seen in long-term isolation rats, a lesser degree in long-term swimming rats and least of all in control rats. We concluded that additional stressors such as immobilization or cold led to the most conspicuous activation of hippocampal CuZnSOD and CAT enzymes and sympatho-adrenomedullary system in the group exposed to long-term isolation. Based on these results, it may be concluded that long-term isolation acts as a robust psychosocial stressor and induces a priming effect specifically on the of CuZnSOD and CAT activity. Our results are consistent with the data of Endo and coworkers (Endo and Shiraki 2000, Endo *et al*. 2001) who found that long-term exposure to psychological, but not physical stress, caused a significant elevation of body temperature, probably due to an increase of sympathetic tone. The same authors also found that psychological stress could have a weaker influence as an acute stress, but in the case of repeated exposures, the effects of psychological stress could grow larger or persist for a longer period of time, as compared to physical stress.

Catecholamines such as dopamine, noradrenaline and adrenaline are synthesized from tyrosine and act as neurotransmitters in the peripheral and central nervous systems (Laverty 1978). Although catecholamines play a role in the control of emotions and movement, they can become central neurotoxins under certain conditions. Noh *et al*. (1999) suggested that catecholamines induce apoptosis at high doses but prevents free radical-mediated neurotoxicity as antioxidants without being coupled to the receptors. Thus, catecholamines at physiological doses exert a neuroprotective effect as potent antioxidants in addition to their well-documented role as neurotransmitters. This means that cooperative antioxidant effects of antioxidant enzymes and catecholamines represent a very effective defense mechanism through the oxidative stress response.

Finally, the results of our study suggest that different stress models exert a different degree of influences on antioxidant enzymes and plasma catecholamines. It provides us a rational look into this integrated dual mechanism through oxidative stress response which is involved in cellular defense against endogenous or exogenous oxidants. This may further trigger the introduction of antioxidative therapeutic strategies for the treatment of neurodegenerative diseases based on a high turnover of catecholamines and decreased antioxidative protection.

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